Evaluation of Anxiolytic Activity of Ethanolic Extract of *Thymus Vulgaris* Leaves in Rats Using Open Field Test (OFT)

***Modupe Iretiola Builders*[1*]*, Rachel Abimbola Oyefeso*[1]*, Samuel Otokpa Ede*[3]*, Simeon Oyepata Joseph* [4]*, Peter Uduak Ise*[1]**

[1]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Bingham University, Karu, Nasarawa State, Nigeria*

[2]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Jos, Plateau State, Nigeria*

[3]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Federal University, Oye-Ekiti, Ekiti State, Nigeria*

Corresponding Author
Email Id: modupebuilders@yahoo.com

ABSTRACT

Thymus vulgaris is a medicinal plant and aromatic plant, which is popularly known as thyme.It is a common flowering plant belonging to the family of lamiaceae, the phytochemical analysis of the plant indicated the presence of polyphenols, essential oils, tannins, saponins and flavonoids. The ethinomedicinal uses of T. vulgaris include anti-anxiety, sedative, hypnotic, antihelminthic, expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antioxidant, antivirotic, carminative, and diaphoretic effects. Anxiety is a mental disorder characterized by a continuous state of restlessness in patients, these psychiatric ailments are difficult to treat and also overlap with mental disorders such as panic attacks and depression. The present anxiolytics have serious adverse effects due to narrow therapeutic index, thus, there is need for novel drugs in form of plants which are cheaper, more accessible and more affordable. The aim of the study was to evaluate the anxiolytic activity of the T. vulgaris leaves by Open Field Test (OFT). The anxiolytic activity of T.vulgaris L was evaluated using three different doses (50mg/kg, 100mg/kg and 200mg/kg body weight of the extract), 5mg/kg diazepam was also used a reference drug . The result showed dose-dependent reduction of anxiety activities OFT test, further study should be carried out to determine the exact mechanism of action responsible for the anxiolytic activity.

Keywords: *Anxioltylic Activity, Thymus Vulgaris Leaves, Open Field Test.*

INTRODUCTION

Anxiety is a mental disorder, which affects the state of mind. It is classified into simple anxiety, an anxiety which is lasts for a short period of time and simple to treat while the chronic anxiety lasts for a prolong period of time and very difficult to treat [1]. This psychiatric disorder is the most rampant mental disorders in the world and is associated mainly with a deep-seated and highly unpleasant feeling of apprehension and uneasiness [2].

The symptoms of this deliberating diseases are headache, fever, tachycardia, restlessness perspiration, tightness in the chest, and mild stomach discomfort depending on the type of anxiety. Some chronic disorders such as diabetes mellitus, depression, psychosis, hereditary diseases, environmental factors drug abuse and miscellaneous conditions including stress are manifested as stress [3].

Benzodiazepines are the most commonly prescribed drugs for treating anxiety, however benzodiazepines have narrow therapeutic margin which is associated serious unwanted side effects. Thus, there is need for new therapeutic alternative drug with fewer undesirable effects
[4].

The open field test (OFT) is one of the most widely used model in animal behavioral studies, OFT was introduced as a tool for measuring the emotional conditions of rats. It is a model that reflects different aspects of anxiety like behavior [5]. Research has shown that emotionality is not one-dimensional and has the ability to vary along several axes in the multidimensional space. This means that different kind of environments. For example, open spaces, illuminated or elevated areas, will give different kind of behavioral responses. Instead of using batteries of tests to analyze the disorder, only one test is used to determine the behavioral response [6].

A number of essential conventional and etiological parameters can be collected and analyzed during the performance of the OFT. These data allow to measure behaviors ranging from overall locomotor activity to anxiety-related emotional behaviors [7]. However, the use of OFT has some limitations. One of shortcoming is the wide range of statistic variables that can be manipulated during any testing session. Examples include time, lighting conditions and novel object inclusion. Variability in experimental protocol setup and design, which are important to support a broad-spectrum of applications, can make it difficult to compare studies. When subject variability, such as background or transgenic mouse lines and drug treatments are included, the difficulty in test comparisons can increase even more. Despite the issues, the OFT remains one of the most widely applied techniques in rodent behavioral research [8]. The OFT is illustrated in Figure 1a below.

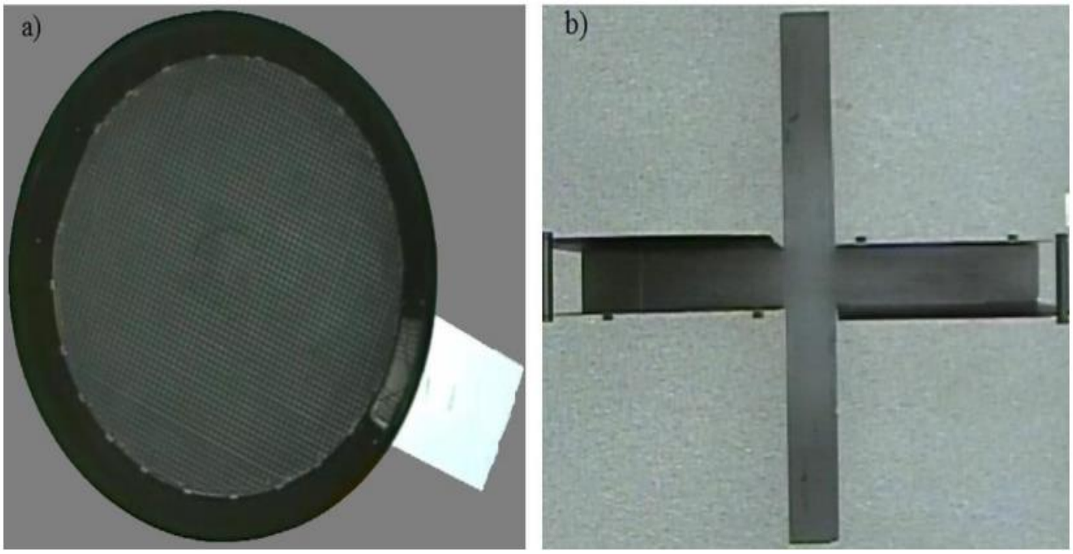

Fig. 1. A) The open field test with a start box attached and b) the elevated plus maze

T. vulgaris L. is an aromatic and medicinal plant, commonly known as thyme. *T. vulgaris* is a perennial, straight growing plant which measures up 10–30 cm in height with woody base. Leaves are small, opposite, greyish green coloured, oblong–lanceolate to linear, and are gland-dotted. They are measured up to 5–10 mm long and 0.8–2.5 mm wide with recurved margins. Flowers are light violet in colour, two-lipped, and possess a hairy glandular calyx. They measure up to 5 mm long with leaf-like bracts in loose whorls arranged in axillary clusters on the branchlets or in terminal oval or rounded heads as indicated in Figure 2 [9].

Fig. 2. A) Plant b) Flower C) Leaves, d) Seeds of Thymus vulgaris L.

The bioactive components of *T.vulgaris* are essential oils, phenolic compounds, tannins, saponins and flavonoids , which also serve as antioxidants. Pharmacologically, *T. vulgaris* are used for treating cardiovascular disease such as hypertension, respiratory diseases such as colds, asthma, inflammatory disorders including pains, rheumatism, infections, wounds, cancer and osteoporosis in ethanomedicinal world [10]. In food industry, *T.vulgaris* is used as food preservative and food flavourant and also as disinfectant as well as sanitizer [11].

The research on the evaluation of the anxiolytic activity of *T.Vulgaris* is scanty, therefore, in this study, ethanolic extract of *T. vulgaris* leaves was used to analyze rat anxiety and emotionality in OFT.

EXPERIMENTAL ANIMALS

Male Wistar rats, weighing 230-250 g, were allowed to acclimatized for 72 hours prior to the test. They were housed in groups of six per cage under a 12:12 dark/light cycle (lights on at 07:00 AM) at 22 ± 2^0C and given free access to food and water. Rats were randomly assigned to different treatment groups (n=5). Animals were tested under the same experimental conditions. All experiments were carried out in a quiet room under controlled light conditions between 11:00 AM and 3:00 PM. Behavioral observations were conducted in quiet rooms at the same period of the day to reduce the confounding influence of diurnal variation on spontaneous behavior. Each animal was tested only once [12].

ETHICAL APPROVAL

All research and animal care procedures were approved by the Bingham University Research Ethics Committee and were performed in accordance with international standards of animal welfare recommended by the Society for Neuroscience . The minimum number of animals and the minimum duration of observation required to obtain consistent data were used [13, 14].

Grouping of Animals and Dosing

Thirty (30) female wistar albino rats of 230-250g were randomly divided into five different groups of six rats in each group. The negative control (NC) group was given distilled water (GROUP 1). Treatment groups II, III and IV were given T.*vulgaris* L ethanolic extract 50mg/kg, 100mg/kg and 200mg/kg respectively. Group V was treated with 5mg/kg diazepam.

Collection, Preparation and Extraction of Plant Material

Leaves of *T. vulgaris* were collected in spring. The plant material was dried at 40^0C with air circulation, ground, and extracted with ethanol by percolation at room temperature. The extract was then taken to the laboratory for the process of evaporation. The evaporation process involved complete removal of ethanol and water used for the extraction. The extracts were dried at 40^0C under vacuum and finally freeze dried. Pharmacological assays were carried out with aqueous suspensions of the dried extract. The doses were expressed as milligrams of dried extract per kilogram of rat body weight. The extracts were dissolved in their solvents prior to each individual experiment.

Open Field Test (OFT)

The Open field was fabricated to contain a black stainless-steel circle, diameter 90 cm, with 35 cm high walls and a stainless-steel wire-mesh floor, 10 mm between the bars as indicated in Figure 1a. The possibility to attach a start-box at the wall was used for one experimental group, which also enabled measures of shelter seeking in the start box. In this group the rat was inserted in the start box. For the group without start box the rat was placed at the same spot, nose pointing to the wall, when starting each session. Each trial lasted 10 minutes and the maze was cleaned with 10 % ethanol solution between the trials. The lightning conditions (1x) were 100 in the center [15].

STATISTICAL ANALYSIS

Results were expressed as mean standard error of the mean. The difference between the mean was determined by one-way analysis of variance (ANOVA). In all cases, differences were considered significant if less than 0.05 ($P<0.05$) and highly significant if greater than ($P> 0.05$).

RESULTS AND DISCUSSION

Table 1: Anxiolytic Activities Of Ethanolic Extract of T.*Vulgaris* Leaves in Rats by Open Field Test (OPT)Control Group

Weight of rats(kg)	Line crossing frequency	Defecation	Centre square entry	Rearing	Time it stays at the center
231.50	31 ± 0.01	1 ± 0.41	2 ± 2.11	4 ± 0.63	18secs ± 1.70
196.60	15± 0.12	1 ± 1.00	3 ± 1.12	8 ± 0.97	16secs ± 0.07
195.00	5 ± 1.02	0 ± 1.11	0± 1.44	11± 2.00	20secs ± 0.47
206.70	6 ± 0.87	5± 0.66	0 ± 0.40	14 ± 0.54	16secs ± 1.22
153.00	22 ± 0.00	3± 2.01	0 ± 0.76	13 ± 0.33	18secs ± 2.16

191.80	6 ± 1.00	3± 0.52	1 ± 0.05	11 ± 1.12	17secs ± 1.00

GROUP 1

Weight of rats(kg)	Line crossing frequency	Defecation	Centre square entry	Rearing	Time it stays at the center
156.50	5 ± 0.51**	0 ± 0.01	1 ± 1.62	3 ± 0.03**	22secs ± 0.96
202.40	12 ± 0.11*	3 ± 0.50	0 ± 0.22	6 ± 0.10*	26secs ± 0.69
188.80	13 ± 1.00*	0 ± 1.00	1 ± 1.22	7 ± 1.00*	28secs ± 1.12*
190.50	6 ± 2.16**	3 ± 0.68	1 ± 0.67	6 ± 2.00*	24secs ± 2.00
186.30	4 ± 0.02**	4 ± 1.23	1 ± 0.72	2 ± 1.11**	28secs ± 1.00*
93.20	3 ± 2.00**	0 ± 2.03	0 ± 0.25	2 ± 0.11**	17secs ± 0.08

GROUP 2

Weight of rats(kg)	Line crossing frequency	Defecation	Centre square entry	Rearing	Time it stays at the center
198.50	6 ± 0.45**	0 ± 0.01	1 ± 1.00	8± 0.06*	30secs ± 0.03*
202.40	6 ± 0.41**	1 ± 2.00	2 ± 2.12	10 ± 0.64	38secs ± 0.31**
209.80	8 ± 1.40**	3 ± 2.02	0± 0.05	11± 0.40	40secs ± 1.13**
190.50	9 ± 0.01**	1 ± 0.02	3± 0.51	10 ± 0.32	36secs ± 1.00**
210.50	10 ± 1.07*	0 ± 0.00	0± 1.09	3 ± 2.11**	35secs ± 1.11**
168.30	11 ± 0.23*	0± 0.83	0 ± 1.21	8 ± 1.22*	34secs ± 0.11**

GROUP 3

Weight of rats(kg)	Line crossing frequency	Defecation	Centre square entry	Rearing	Time it stays at the center
214.60	3 ± 0.10**	0 ± 0.00	1 ± 1.00	5± 0.20**	48secs ± 0.12**
148.60	16 ± 2.00*	0 ± 0.15	2 ± 1.10	8 ± 1.00**	46secs ± 1.00**
209.80	5 ± 1.42**	0 ± 0.20	1 ± 1.11	4± 1.21**	50secs ± 1.42**
192.20	5 ± 0.40**	2 ± 1.00	0 ± 0.10	5 ± 1.11**	46secs ± 1.21**
177.30	8 ± 0.70**	0 ± 0.27	0 ± 0.21	9 ± 0.90*	48secs ± 0.84**
167.50	13 ± 0.23*	0 ± 0.20	2 ± 0.20	6 ± 0.66**	57secs ± 0.43**

GROUP 4

Weight of rats(kg)	Line crossing frequency	Defecation	Centre square entry	Rearing	Time it stays at the center
214.60	3 ± 0.11**	0 ± 0.00	1 ± 1.00	5 ± 1.14**	48secs ± 0.10**
148.50	15 ± 1.02*	0 ± 0.12	2 ± 2.00	7 ± 1.00**	45secs ± 1.00**
210.00	5± 0.72**	0 ± 0.21	1 ± 1.08	4 ± 0.01**	48secs ± 2.42**
191.90	4± 0.61**	2 ± 1.20	0 ± 0.01	4 ± 0.04**	46secs ± 2.12**
177.30	8± 0.88**	0 ± 0.02	0 ± 0.05	9 ± 1.01*	48secs ± 1.12**
168.10	14± 1.21*	0 ± 0.20	2 ± 2.02	6 ± 1.12**	5secs ± 0.05

Values are represented as mean ± S.E. M, n=6, *P<0.05, significantly, *P>0.05, highly significant different from control (Distilled water).

Anxiolytic Activities of Ethanolic Extract of *T.Vulgaris* Leaves in Rats by Open Field Test (OFT)

The ethanolic extract of *T.vulgaris* leaves exhibited both significant ($P< 0.05$) and highly significant ($P> 0.05$) dose-dependent anxiolytic activities by reducing line crossing frequency, rearing and the time the rats spent at the center.

Several medicinal plants have been reported to have anxiolytic effect in different part of the world, the documentation on the evaluation of the anxiolytic activities of *T.vulgaris* leaves is limited [10]. Therefore, OFT was used to assess the anxiolytic activities of ethanolic extract of *T.vulgaris* leaves in rats. In this test, five parameters (line crossing frequency, defecation, centre square entry, rearing and the time the animals spent at the center) were used to investigate the anti-anxiety effects of *T.vulgaris* leaves.

Locomotor activity of the test animal is important prior to analysis of open field data or any animal behavioral maze. When comparing different groups of rats or different effects of drug treatments, the ambulatory ability of the rat is essential. If locomotor ability is compromised due to treatment effects, then measuring activities that rely on the ability of the animal to move is confounded. The first step in this study was to compare the total movements between the groups (control group and extract treated groups) [16]. There was no much difference in total distance travelled by either rats throughout the 10mins time frame of the experiment. In this study, the movement of the animals was equal allowing direct use of unadjusted open field derived data to further investigate anxiolytic parameters [16]. In this research, the treated animals displayed significantly and highly significantly reduction of line crossing frequency comparable to the control group.

Several studies have shown that highly emotional animals exhibit increased defecation because there is a strong correlation between defecation and anxiety, this corroborates the total number of fecal pellets that reduced during the study. Furthermore, current findings show that defecations may indeed be a useful indicator of emotional anxiety-related behaviors in relatively short test periods as performed here as opposed to long observations (30 min) where differences in responses are less clear [17].

Rearing behavior consists of subject animals standing on both hind paws in a vertical upright position. It is considered an exploratory behavior and has been used as a measure of anxiety in OFT. However, there is no clear indication that rearing is either anxiolytic or anxiogenic. Some studies indicate increased rearing is in concordance with increased anxiety levels in rats while others which postulated decreased rearing behavior is indicative of increased anxiety. In our study, significantly dose-dependent reduction and highly significantly dose-dependent reduction of rearing were observed comparable to the control group [18].

Rearing could be used to discriminate anxiety-linked behaviors from simple ambulatory behavior. It is being proposed that measuring anxiety in rodents is much more complicated than using a single parameter in a single maze environment. Therefore, using multiple measures in a single test can strengthen evaluation of results [19].

The treated animals spent more time at the center comparable to the control group in both significant dose-dependent and highly significant dose-dependent manner, therefore the ethanolic extract of *T.vulgaris* leaves exhibited dose-dependent reduction of anxiety. This test can also be used to test other behaviors such as novel object recognition and memory, depending on the type of memory being analyzed, time in the field with a novel object can vary from 5 min to 24 hr. The anxiety-related behaviors measured are the cumulation of several behavioral underlying processes. Thus, once a response is detectably measured, it is often necessary to investigate that response further to identify a specific defect [20].

Factors such as food or water deprivation, lighting during the testing, odor, temperature, humidity, noise and human activity can affect the sensitivity of OFT. Therefore, the test was performed in a temperature and humidity controlled room with non-direct lighting at the same time each day [21].

The phytochemical composition of *T.vulgaris* leaves includes essential oil, polyphenols, tannins, saponins and flavonoids, these antioxidants may be responsible for the anxiolytic activities of the T.vulgaris leaves. The imbalance between oxidation and the antioxidant defense system have been attributed to cause depression and anxiety according enormous research conducted to indicate multiple neural substrates and mechanisms of anxiety and depression. Some studies have further demonstrated the role of oxidative stress in anxiety of rodents. Furthermore, it has been reported that two drugs that induce oxidative stress, buthionine-(S,R)-sulfoximine and xanthine plus xanthine oxidase(X+XO) cause increased anxiety-like behavior in rats [21].

CONCLUSION

The ethanolic extract of *T.vulgaris* leaves was evaluated to be effective in the treatment of simple anxiety in OFT. Further study should be carried out to isolate and characterize the active compounds responsible for this anxiolytic activity.

ACKNOWLEDGEMENT

The authors are grateful to both technical and non-technical staff for their co-operation and support

REFERENCES

1) Horvath P, Zuckerman M. Sensation seeking, risk appraisal, and risky behavior. Personal. Individ. Differ. 1993; 14: 41–11p.
2) Montgomery KC. The relation between fear induced by novel stimulation and exploratory behavior. J. Comp. Physiol. Psychol. 1995; 48: 254–6p.
3) Roman E, Stewart RB, Bertholomey ML et al. Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. Addict. Biol. 2012; 17: 33-13p.
4) Pellow S, Chopin P, File SE et al. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods. 1985; 14: 149–18p.
5) Meyerson B, Jurek B, Roman E. A Rank-Order Procedure Applied to an Ethoexperimental Behavior Model—The Multivariate Concentric Square FieldTM (MCSF) Test. J. Behav. Brain Sci. 2013; 3: 350–11p.
6) Ramos A. Animal models of anxiety: do I need multiple tests? Trends Pharmacol. Sci. 2008; 29: 493–5p.
7) Roman E, Karlsson O. Increased anxiety-like behavior but no cognitive impairments in adult rats exposed to constant light conditions during perinatal development. Ups. J. Med. Sci. 2013; 118: 222-5p.

8) Roman E, Stewart RB, Bertholomey ML, et al. Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. Addict. Biol. 2012; 17: 33-13p.
9) Basch E, Ulbricht C, Hammerness P et al. Thyme [*Thymus Vulgaris*. L], Thymol. Journal of herbal pharmacotherapy. 2004; 4[1]: 49-18p.
10) Builders MI, Oyefeso RA, Ede SO, et al. Evaluation of anxiolytic activity of ethanolic extract of *Thymus vulgaris* leaves using Elevated Plus Maze (EPM) test. J.Adv.Pharmacol. 2023; 3 (1): 11-7p.
11) Ali Esmail Al-Snafi. Medicinal plants with central nervous effects [part 2]: plant-based review. IOSR Journal of Pharmacy. 2016; 6 [8]: 52-23p.
12) Arrant AE, Schramm-Sapyta NL, Kuhn CM et al. Use of the light/dark test for anxiety in adult and adolescent male rats. Behav. Brain Res. 2013; 256: 119–8p.
13) Deacon RMJ. Housing, husbandry and handling of rodents for experiments. Nat. Protoc. 2006; 1: 936–10p.
14) Burn CC. 2008. What is it like to be a rat? Rat sensory perception and its implications for experimental design and rat welfare. Applied Animal Behaviour Science. 2008; 112: 1-31p.
15) Ekmark-Lewén, S, Lewén A, Meyerson BJ, et al. The multivariate concentric square field test reveals behavioral profiles of risk taking, exploration, and cognitive impairment in mice subjected to traumatic brain injury. J. Neurotrauma. 2010; 27: 1643–12p.
16) Fonken LK, Finy MS, Walton JC, et al. Influence of light at night on murine anxiety- and depressive-like responses. Behav. Brain Res. 2009; 205: 349–5p.
17) Hall CS. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 1934; 18: 385–18p.
18) Doremus TL, Brunell SC, Varlinskaya EI, et al. Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. Pharmacol. Biochem. Behav. 2003; 75: 411–7p.
19) Palm S, Daoura L, Roman E, et al. Effects of rearing conditions on behaviour and endogenous opioids in rats with alcohol access during adolescence. PloS One 2013; 8: e76591.
20) Montgomery KC. “Spontaneous alternation” as a function of time between trials and amount of work. J. Exp. Psychol. 1951; 42: 82–13p.
21) Manou I, Bouillard L, Devleeschouwer MJ, et al. Evaluation of the preservative properties of Thymus vulgaris essential oil in topically applied formulations under a challenge test. J Appl Microbiol 1998;84[3]:368-8p.