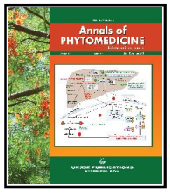
Annals of Phytomedicine 12(2): 1032-1038, 2023

DOI: http://dx.doi.org/10.54085/ap.2023.12.2.124

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Evaluation of the anxiolytic activity of ethanolic extract of *Trombidium grandissimum* Koch

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Article Info

Article history
Received 5 July 2023
Revised 22 August 2023
Accepted 23 August 2023
Published Online 30 December 2023

Keywords
Trombidium grandissimum Koch
Ethanol
GC-MS
Entomotherapy
Anxiolytic

Abstract

Examining the chemical profile and anxiolytic activities of the ethanolic extract of red velvet mite, *Trombidium grandissimum* Koch (EETG) is the objective of the current investigation. The GC-MS helped to figure out the six bioactive molecules that had been found in the EETG extract. A variety of tests were utilized to evaluate the anxiolytic action, including the light and dark test, open field test, elevated plus maze and hole board tests. The effectiveness of the extract was compared to the standard anxiolytic medication diazepam. Inquisitive behaviour was seen in all of the screening procedures with comparable dose levels in the albino rats used in this study. As a result, we conclude that the present findings unequivocally showed that the ethanolic extract of *T. grandissimum* has anxiolytic properties. This result is due to the extract's abundant amount of bioactive components. Through *in silico* analysis, isolation, and characterisation, future research will be required to pinpoint the chemical component responsible for the extract's observed biological effect.

1. Introduction

Entomotherapy is the practice of using insects and products generated from insects for therapeutic purposes. Utilizing insect-based medications ought to be considered from a cultural viewpoint because similar to cultural systems, medical systems are organized. The book Insectotheology discusses the idea that insects are there for human benefit (Costa-Neto, 2005). Terrestrial mite, *T. grandissimum* which is otherwise called a red velvet mite. It typically lives in the dirt or mulch and eats plant matter. It appears when it is raining. These mites live in the soil's surface debris. They have a lifespan of one to several years in the soil. They parasitically feed as larvae by attaching to a variety of arthropods. To extend sexual activity, the tribes of Chhattisgarh apply this oil to the male genitalia. It is used an hour before sexual activity. The oil lengthens the retention period, which delays ejaculation and boosts pleasure, among other benefits. For the treatment of paralysis, both internally and topically, the traditional healers of Chhattisgarh developed an oil known as Birbahutti or Ranikeeda oil (oil from *T. grandissimum*) (Oudhia, 2008). For a few weeks, the injured area is heated and rubbed frequently with the oil to obtain a full recovery from paralysis. The healers collect collected mite legs for internal usage and recommended the sufferers to ingest it. Generally speaking, one mite each day is advised. If they are hesitant, the healers will offer the patients betel vine (pan) leaves or banana fruit without disclosing that they contain mites. The red velvet mite produces oil that is effective in treating more than ten serious illnesses, such as paralysis, malaria, and urogenital disorders. Because they can raise feelings of sex, these mites are known as "Indian Viagra" (Annandale, 1906; Hill, 1905; Oudhia, 2002). Additionally, the advancement of sophisticated analytical methods as well as 21st century technology has greatly facilitated the identification and characterisation of natural compounds. Natural products are essential sources of novel medications (Srivani and Krishna Mohan, 2023). Several procedures must be used in order to identify the various components of complicated combina-tions of phytochemicals. Because it makes it possible to identify the precise natural components contained in an extract by correlating their mass spectra and respective retention periods, GC-MS serves as one of the most prevalent techniques for assessing phytochemical composition (Anand, 2015). Drug detection, investigation, analysis, investigation, and identification of unidentified compounds are among the uses of GC-MS (Chanchal and Deeplakshmi 2017). Considering the available information and folklore use of the *T. grandissimum* mite has not yet undergone biochemical and neuro-pharmacological investigation. Therefore, the current study's objective is to evaluate the antianxiety activity of EETG using a suitable animal model.

2. Materials and Methods

2.1 Insect collection

From November to January, fresh *T. grandissimum* mites were collected in the sandy soil of Parvathamalai highlands in Tamilnadu's Tiruvannamalai district. Dr M. Gabriel Paulraj of Loyola College, Entomology Research Institute (registered with DSIR), Chennai-60034, identified and authenticated the mites with a Voucher Specimen No: ESI/L/08-02-2020/01.

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2.2 Extraction

The mites were manually removed from the soil and placed in a collection jar and suspended in a sealed container in 95% ethanol. The mites in the container were removed, aseptically crushed using a mortar and pestle, and then kept in reflux for 2 h with 95% ethanol (Lighty George *et al*., 2010; Natarajan *et al*., 2019).

2.3 GC-MS analysis

The elite-5MS packed fused silica column, which is composed of 5% biphenyl and 95% dimethyl polysiloxane, was employed in the analysis. Helium served as the carrier gas during the separation process, flowing at a rate of 1 ml/min. The mass detector was run at the following parameters: 0.2 and 0.1 sec for each scan, pieces between 40 and 600 Da, and the transfer line at 230°C. The component spectra were contrasted with the component spectrum database kept in the GC-MS NIST (2008) library (Jeenu *et al*., 2021; Preety *et al*., 2020; Susmita *et al*., 2014).

2.4 Experimental animals

The Wistar albino rats that weighed 150-180 g were housed in a controlled environment for 12 h while receiving water and hygienic food. The research was carried out in a soundproof research lab. The Animal Ethics Committee's clearance was obtained by their approval number, KPCP/IAEC/2022/01.

2.5 Toxicological study

2.5.1 Acute oral toxicity

The OECD 423 guidelines, which require the use of only three animals, were approved for use in the acute oral toxicity investigation. Three of the test animals were weighed after being fasted for around 12 h. Every fasted animal received a 2000 mg/kg test dose of *T. grandissimum* ethanolic extract *via* oral gavages, determined by body weight. After dosage, Over the first 24 h, the animals were routinely and individually observed for behavioural abnormalities and general signs of toxicity, with particular focus on the initial 4 h. Following that, daily observation was carried out for a total of 14 days (Mrunali *et al*., 2022).

2.6 Antianxiety activity

2.6.1 Experimental design

A total of 24 albino rats of 6 rats each, were randomized, split into four groups, and they received the following treatments:

Table 1: Anxiolytic activity treatment protocol

S.No.	Group	Treatment
1	Control	Normal saline (5 ml/kg, p.o)
2	Standard	Diazepam (1 mg/kg, i.p)
3	EETG 200	EETG (200 mg/kg, p.o)
4	EETG 400	EETG (400 mg/kg, p.o)

2.6.2 Open field test

In this study, mobility, anxiety, and exploration were all simultaneously measured using the Open-Field test, which provides these measurements. Each rat was put into a 50 x 50 x 10 cm acrylic cage. There were 25 squares in the arena, with 16 spaces on each side of the boundaries and 9 squares inside the centre. Animals were put separately in one of the dark, sound-attenuated corner squares of the experiment room after an oral administration that lasted an hour. 5 min were spent counting the squares crossed, assisted rearings, and several rearings during this time (Kulkarni *et al*., 2008; Palla *et al*., 2022).

2.6.3 Light and dark test

The light and dark paradigm was used to assess rodents' innate fear of brightly illuminated areas. The box is 50 x 25 x 25 cm rectangular built that has two chambers (light and dark). Each rat was placed solely in the area within the bright and darkened box 60 min after dosing. The number of trips into the bright and dark chambers every step of the 5 min screening, the entire span of the period served in this bright segment, the assisted rearings and an apparent number of rearings, were all recorded (Barua *et al*., 2009; Bourin and Hascoet, 2003).

2.6.4 Elevated plus-maze test

There are two open arms, while the other two are closed and are made up of it, and all of them are stretched outward toward a single core station. The object was brought up 60 cm off the ground, and the arrangement with like arms positioned opposite one another, has the appearance of a plus sign. To prevent any potential cueing effects of scents left by earlier animals, the path was meticulously scrubbed with ethanol in water made from distillation in between trials. Rats were positioned on the platform in the centre, facing a closed arm. The trials lasted for 5 min. A trained observer who was ignorant of the treatment groups observed the rat's behaviour for 5 min and recorded it. Rats entered the open arms on average (a) how many times, and (b) some time on average. All four paws of the rat had to be in the arm for an arm entrance to be made. EETG extracts and diazepam were given 60 min before the test (Mahreen *et al.,* 2022; Palla *et al*., 2022).

2.6.5 Hole board test

Typically, it was believed that head dipping provided a level of investigation in the hole-board test that was not part of the movement. A rat pushing its snout into the hole would not be able to see the ground below. The tool was a 42 x 42 x 30 cm hardwood arena with 16 holes spaced uniformly. Each hole's centre was 10 cm space between the box's right next wall and the bottom was 15 cm above the ground. A rat was left in the hole board's middle for 5 min while it was free to investigate the tool. The number as well as the duration of head-dips during this time were noted (Pellow and File, 1984).

2.7 Statistical analysis

Mean ± SEM were used to express values. The mean parameters were assessed using Dunnett's test is used after a one-way ANOVA. $p<0.05$ and $p<0.01$ were utilized to validate the assessment, and whether the values were significant. Prism statistical software, 5.03 Version, was used to conduct the analysis.

3. Results

3.1 Colour

Fresh red velvet mite of *T. grandissimum*, was extracted using ethanol. It had a semisolid consistency and was a reddish-brown colour.

3.2 GC-MS analysis

The mite is abundant in bioactive chemicals, as shown by the GC-MS chromatogram spectra for EETG. The chromatogram revealed a total of 6 bioactive chemical compounds. The NIST library was used to predict the molecular weight, area percentage and retaining period of bioactive chemicals (Tables 2, 3, 4 and Figure 1). Six active compounds that could be distinguished as docosanoic acid ethyl ester, hexadecanoic acid ethyl ester, heneicosanoic acid methyl ester, 1-hexyl-2-nitro cyclohexane, 11-tricosene, and cis-9,10-epoxyoctadecan-1-ol were validated.

Table 2: Detection of an ethanolic extract of *T. grandissimum* by GC-MS

RT	Area	Chemical name	MF	MW
18.345	2.581	Docosanoic acid ethyl ester	$C_{24}H_{48}O_2$	368
18.935	1.732	Heneicosanoic acid methyl ester	$C_{22}H_{44}O_2$	340
19.045	11.697	Hexadecanoic acid ethyl ester	$C_{18}H_{36}O_2$	284
20.055	40.473	1-Hexyl-2-nitro cyclohexane	$C_{12}H_{23}O_2N$	213
21.686	40.853	11-Tricosene	$C_{23}H_{46}$	322
25.482	1.238	Cis 9,10 epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	284

Table 3: Chemical structure of bioactive components of ethanolic extract of *T. grandissimum*

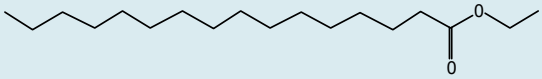
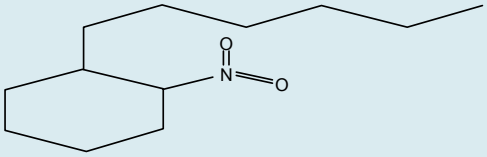
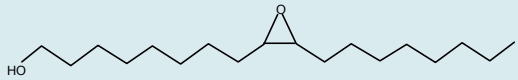
Name of the compound	Chemical structure
Docosanoic acid ethyl ester	
Heneicosanoic acid methyl ester	
Hexadecanoic acid ethyl ester	
1-Hexyl-2-nitro cyclohexane	
11-Tricosene	
Cis-9,10-epoxyoctadecan-1-ol	

Table 4: Biological activity of bioactive components of ethanolic extract of *T. grandissimum*

Compound name	Biological function
Docosanoic acid ethyl ester	Cosmetics, antimicrobial, antioxidant
Heneicosanoic acid methyl ester	Reports of no activity
Hexadecanoic acid ethyl ester	Antihyperlipidemic, nematicide, antioxidant, androgen antagonists, hemolytic, pesticide
1-Hexyl-2-nitro cyclohexane	Anti-inflammatory, analgesic and neuroactive
11-Tricosene	Reports of no activity
Cis-9,10-epoxyoctadecan-1-ol	Microbicide, antifungal, antioxidative, anti-inflammatory

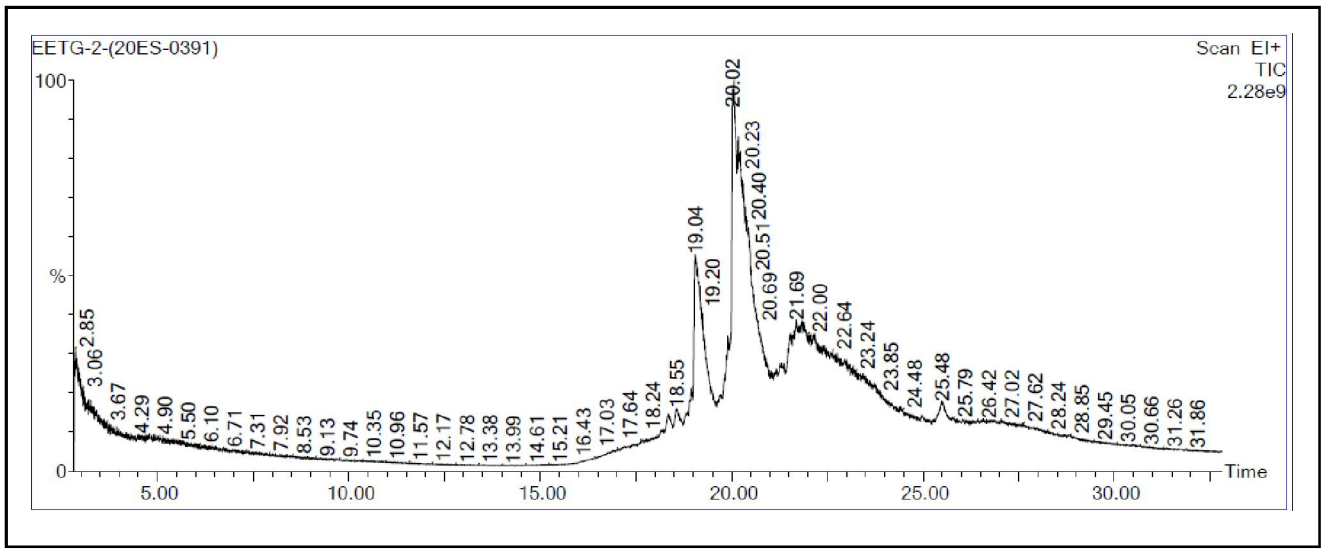


Figure 1: Peak area percentage of ethanolic extract of *T. grandissimum*.

3.3 Acute toxicity study

To determine the dose for the study, the acute oral toxicity of the ethanolic extract of *T. grandissimum* (EETG) was investigated. The extracts had no toxicity up to 2000 mg/kg. As a result, the effective dose was determined to be established as 200 and 400 mg/kg were 1/ 5^{th} as well as $1/10^{th}$ of the maximum tolerable dose, for assessing the activity.

3.4 Open field test

Figures 2, 3 and 4 show a substantial increase in squares crossed, rearings and assisted rearings, in comparison to control groups in the standard-treated rat. When compared to the EETG 400 and standard, the EETG 400 demonstrated a noticeable rise in squares crossed, rearings, and assisted rearings. In contrast, the EETG (200 mg/kg) treated group demonstrated less activity.

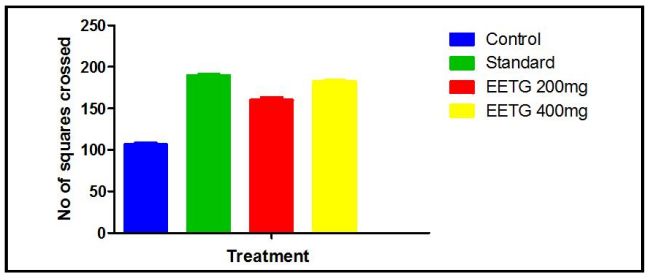


Figure 2: Effect of ethanolic extract of *T. grandissimum* on no of squares crossed in open field test model.

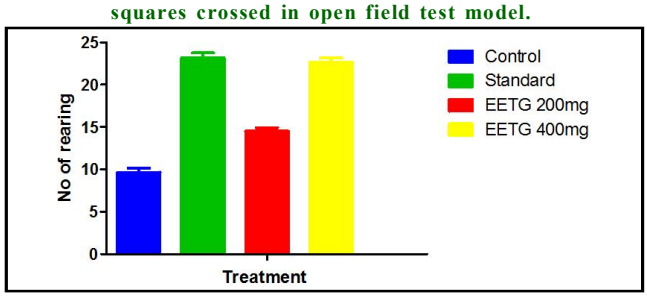


Figure 3: Effect of ethanolic extract of *T. grandissimum* on no of rearings in open field test model.

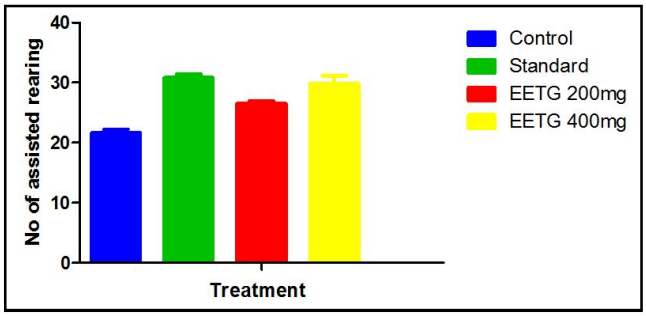


Figure 4: Effect of ethanolic extract of *T. grandissimum* on number of assisted rearings in open field test model.

3.5 Light and dark test

Figures 5, 6, and 7 demonstrate that the standard of care substantially boosted. The period occupied in the bright space and the entire amount of periods the rats crossed over into the dark space, while substantially lowering the period stayed in its dark space and the period of immobility. Additionally, the EETG extract (400 mg/kg) treated rats dramatically enhanced the period occupied in the bright chamber and the total quantity of instances they entered and exited the bright and dark spaces. There were substantially smaller moments spent in the pitch-black space and a shorter period of immobility in the EETG (200 mg/kg) in contrast to the control, even though that they were less active than the EETG (400 mg/kg) and standard.

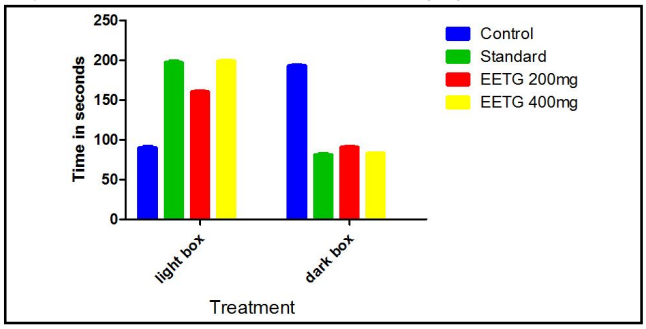


Figure 5: Effect of ethanolic extract of *T. grandissimum* on time spent in light and dark model.

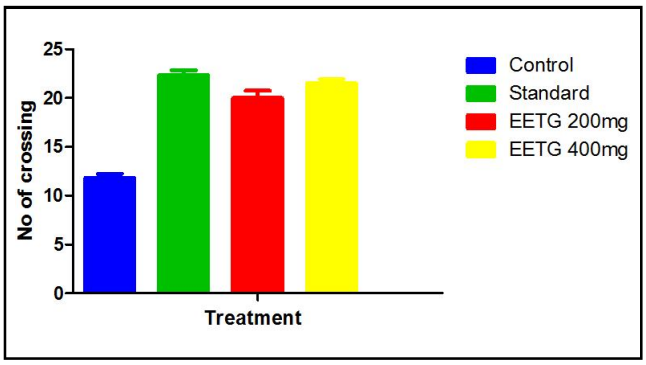


Figure 6: Effect of ethanolic extract of *T. grandissimum* on number of crossings in light and dark model.

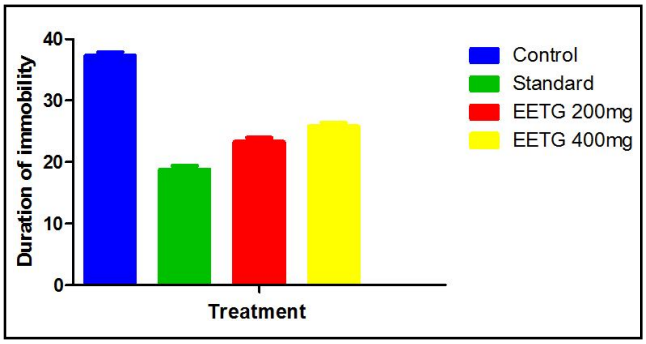


Figure 7: Effect of ethanolic extract of *T. grandissimum* on duration of immobility in light and dark model.

3.6 Elevated plus maze

Figures 8 and 9 depict that the standard treated group had notable a rise in the number of entrants and the length of the period they remained within open arms, as well as a fall in both of those variables in closed arms. Shared entry and duration in the open arm raised more significantly in the EETG extract-treated rats as contrasted with the control group, whereas the closed arm's input as well as the time shared dropped. But when EETG extract was used, the EETG (400 mg/kg) treated group outperformed EETG (200 mg/kg) in terms of activity.

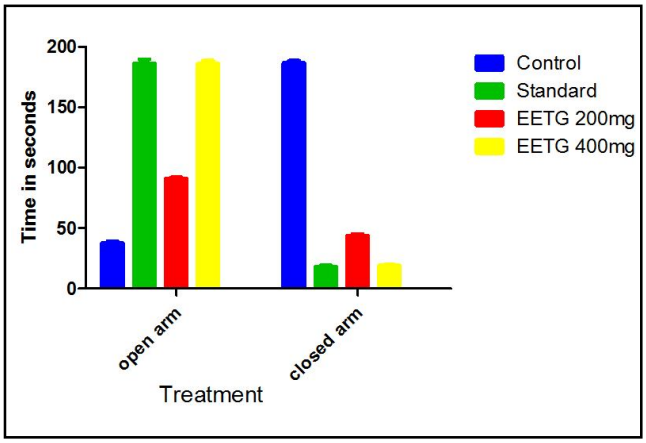


Figure 8: Effect of ethanolic extract of *T. grandissimum* on time spent in closed arm and open arm in elevated plus maze model.

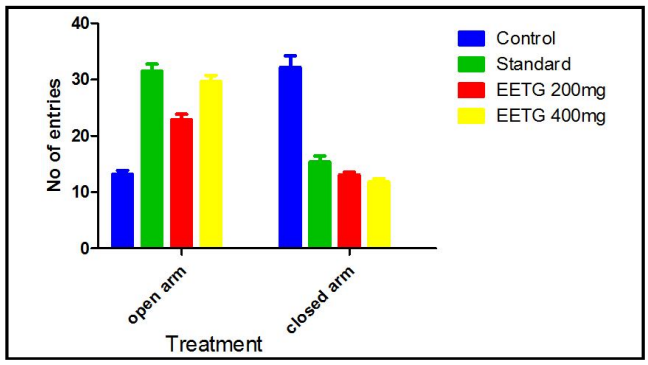


Figure 9: Effect of ethanolic extract of *T. grandissimum* on number of entries in closed arm and open arm in elevated plus maze model.

3.7 Hole board test

In terms of the duration of time the head dips, how often it dips, and the actual line crossings, Figures 10, 11, and 12 demonstrate a large disparity between the control and the standard group. The EETG (400 mg/kg) treated group, head dipping frequency, head dipping period, and line crossings all elevated considerably.

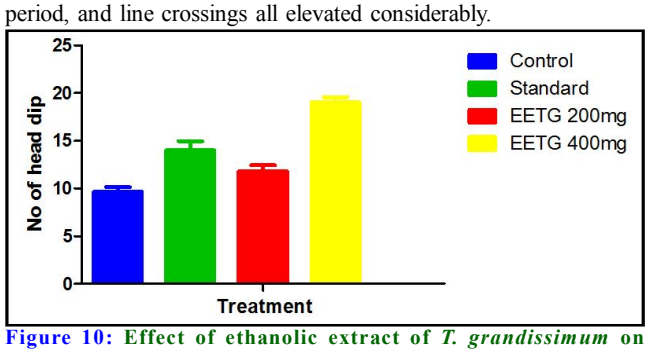


Figure 10: Effect of ethanolic extract of *T. grandissimum* on number of a head dip in hole board test model.

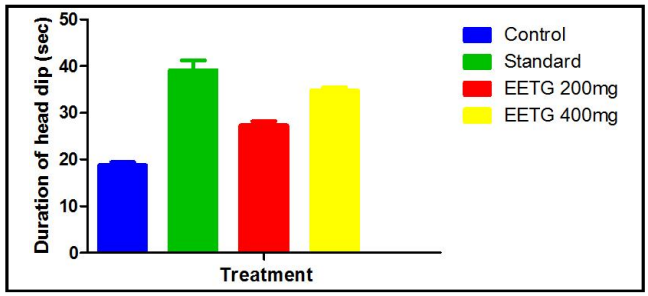


Figure 11: Effect of ethanolic extract of *T. grandissimum* on the duration of a head dip in hole board test model.

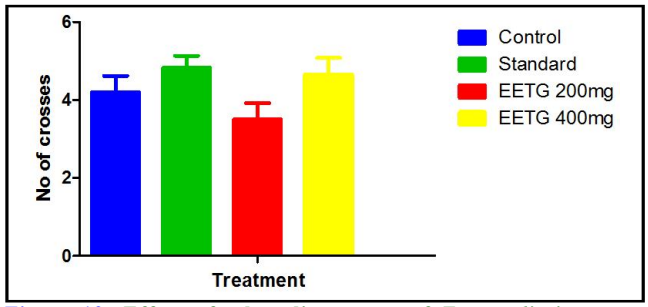


Figure 12: Effect of ethanolic extract of *T. grandissimum* on number of crosses in hole board test model.

4. Discussion

The biological activity of the bioactive components described for its pharmaceutical uses, such as 1-hexyl-2-nitrocyclohexane, has been mentioned for its anti-inflammatory, analgesic, and neuroactive (Thirumalaisamy *et al*., 2018; Bundy *et al*., 2019). Cis-9,10-epoxyoctadecan-1-ol is a microbicide, antifungal and antioxidant, and it treats inflammation (Chandralega and Ramdas, 2020). Hexadecanoic acid ethyl ester has been shown to have antihyperlipidemic, nematicide, antioxidant, androgen antagonists, haemolytic and pesticide (Mohamed *et al*., 2014; Elaiyaraja and Chandramohan, 2016). Cosmetics, antibacterial, and antioxidant applications for docosanoic acid ethyl ester have been documented (Sharad and Tanya Gupta, 2017; Jayanthi *et al*., 2020) (Table 4).

Anxiety is an awful feeling that emerges in anticipation of perceived dangers, which can be either internal or external, real or imagined (Moser, 2007). The anxiolytic efficacy of the EETG extract was attempted in many assessment models, such as open field test, light and dark, elevated plus maze and hole board in albino rats. When animals are taken from their place of residence and set up in an unknown setting, such as an open field test, they typically exhibit signs of anxiety and panic by ceasing all movement, ambulation, exploration, and freezing as a result of increased autonomic anxiety. Standard and EETG extract will lessen these occurrences. The extract and standard both considerably raised the quantity of rearing and facilitated rearing. The light-dark screening was developed to imitate the rat's naturalistic dislike of bright lights and their natural tendency to explore new environments and light when under mild stress. So, this test might help predict rat anxiolytic-like action. Migration is recognized as a measure of behavioural attempts due to habituation over a period a certain amount of time served in each area is said to be an indication of avoidance (Belzung *et al*,1987; Bourin and Hascoet, 2003). In this study, EETG and standard also had a substantial impact on how much time rats spent in the light box and how long they remained there. Rat psychomotor function and affective characteristics are assessed using the elevated plus maze instrument. Because they were anxious, the rats in the control group avoided the open arms in this model and they stayed the majority of their existence in closed arms. A significant percentage of the rat's period existed in the open arm in the conventional drug-treated group, and there was more accessibility to the open arms more prevalent than it is in the control. Animals given EETG extract showed identical activity to untreated animals. According to the hole-board hypothesis, an animal's enhanced head-dipping habit may be a manifestation of an anxiolytic condition (Barua *et al.,* 2009; Takeda *et al*., 1998). EETG considerably lengthened head dips and increased their frequency, with peaks at dosages of 400 and 200 mg/kg, implying an anxiolytic outcome. As was already mentioned, the bioactive component found in the extract is what gives EETG its pharmacological effects. The antioxidant properties of cis-9,10-epoxyoctadecan-1-ol, hexadecanoic acid ethyl ester, and docosanoic acid ethyl ester may be the cause of the extract's anxiolytic effects.

5. Conclusion

As a result, it is inferred that the calming effects of EETG are demonstrated by the study's results. This outcome is brought about by the extract's rich source of bioactive ingredients. Future research will be necessary to identify the chemical component causing the extract's reported biological action through *in silico* analysis, isolation, and characterization.

Acknowledgements

The extract was analyzed by the Chemistry Division's NMR and GC-MS Testing in the VIT-SIF Lab, SAS, for which the creators are quite grateful.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **G. Ragunath, D. Senthilkumar and N. Gnanasekar (2023). Evaluation of the anxiolytic activity of ethanolic extract of *Trombidium grandissimum* Koch. Ann. Phytomed., 12(2):1032-1038. http://dx.doi.org/10.54085/ap.2023.12.2.124.**