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Preclinical Insights into ANXO-125 as a Promising Anxiolytic Herbal Formulation

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Abstract

Background: Anxiety disorders are among the most prevalent mental health conditions worldwide, affecting millions of individuals and significantly impairing quality of life. Although conventional anxiolytic medications are effective, their long-term use is often associated with adverse effects such as sedation, cognitive impairment, dependence, and other systemic complications. This has increased interest in safer, plant-based therapeutic alternatives. ANXO-125, a novel polyherbal formulation containing *Centella asiatica* L. (CA), *Nardostachys jatamansi DC*, *Tinospora cordifolia* (Willd.) Hook, *Terminalia chebula* Retz, and *Trilokya Vijaya Vati*, has been developed based on their documented neuroprotective, antioxidant, adaptogenic, and anxiolytic properties. The present study was undertaken to evaluate the anxiolytic potential of ANXO-125 in experimental animal models and to explore its suitability as a natural therapeutic option for anxiety disorders. **Aim:** The anxiolytic effect of the test formulation, ANXO-125 containing a combination of different herbs, was investigated in this study. **Methods:** Female Swiss Albino mice, divided into five groups were examined for toxicity by the acute oral toxicity test, whereas the changes in their anxiety-like behaviours were studied through the Elevated plus maze, social interaction and marble burying test. At the end of these three experiments, mice were sacrificed and their brain homogenates examined for neurotransmitter release. **Results:** For two out of the three behavioural tests, ANXO-125 at higher doses was observed to exhibit anxiolytic effects, comparable to the standard, Diazepam. In the final behavioural test, where observations were taken for a longer period of 30 minutes, the lower and higher doses were inferred to be strong anxiolytics, similar to the standard. With the estimation of neurotransmitter release, ANXo-125 was confirmed as herbal anxiolytic agent. **Conclusion:** The study concludes that ANXO-125 has the capability of presenting anxiolytic effects owing to its variable herbal constituents, is safe when administered orally and has the potential of a prospective therapeutic formulation to relieve anxiety in patients.

Major Findings: ANXO-125 demonstrated significant dose-dependent anxiolytic activity in mice, with higher doses producing effects comparable to Diazepam across behavioural models. The formulation significantly elevated brain serotonin levels, suggesting a serotonin-mediated mechanism underlying its anxiolytic action. These findings establish ANXO-125 as a promising novel polyherbal anxiolytic candidate and support its further evaluation in chronic studies and clinical trials.

Keywords: Anxiety, Anxiolytic, ANXO-125, Herbal, Pre-clinical, SDG 3, Serotonin

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1. Introduction

Common psychological disorders such as anxiety and depression often co-exist to impact the lives of people. Frequently triggered by stressful life events in biologically and psychologically vulnerable individuals, these conditions are characterised by inappropriate activation of negative emotions like fear and sadness.

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Specifically, anxiety is a complicated response to real or imagined dangers that includes cognitive, bodily, and behavioural changes. It can generate an adrenaline surge, resulting in anxiety behaviours known as the fight-or-flight response. Anxiety symptoms can sometimes last longer or be more severe than the original stressor. Physical symptoms such as high blood pressure and nausea may also occur. These reactions progress from worry to an anxiety disorder, which can interfere with everyday functioning[1,2].

Anxiety disorders have emerged as a major global health problem, with a prevalence rate of 4.05% and an estimated 301 million persons affected globally. This number shows a significant rise of 55% between 1990 and 2022, highlighting the growing demand for efficient treatment plans[3]. There have been many reports pertaining to behavioural investigations of synthetic drugs in mice models. For example, synthetic cannabinoids and Δ9-THC were found to decrease anxiety levels in mice, although with varied effects among different compounds. In another anxiety study in C57BL/6J mice, Chlordiazepoxide failed to produce an anxiolytic (anxiety-reducing) effect while the drugs Picrotoxine and Methylphenidate demonstrated anxiogenic (anxiety-promoting) effects[4,5].

Despite the availability of extremely effective medications for anxiety disorders, only around one-quarter of those in need receive any treatment. Lack of knowledge that this is a curable health problem, insufficient investment in mental health services, a scarcity of skilled health care practitioners, and societal stigma are all barriers to care[6,7]. Moreover, the Synthetic drugs used for anxiety and depression have been popular with significant side effects. Synthetic cannabinoids, often used recreationally, can cause severe acute reactions including respiratory difficulties, hypertension, psychosis, and even death. Consumers have reported intense adverse effects during intoxication, hangover symptoms, and withdrawal issues after long-term use. Synthetic antidepressants may cause gastrointestinal disturbances, sexual dysfunction, insomnia, and weight gain, with rarer but serious effects like serotonin syndrome and cardiac complications. Other than these, common anxiolytics such as Benzodiazepines can lead to sedation, cognitive impairment, and dependence[7,8]. Amidst this milieu, there is a growing desire for natural therapies, driven by a trend towards holistic and integrative approaches to health.

This study investigates a novel polyherbal tablet, ANXO-125, which is developed by Sudhatatva Pharmacy, Dr. D. Y. Patil College of Ayurved and Research, Pune. The composition of this formulation incorporates *C. asiatica* L, *N. jatamansi* DC, *T. cordifolia* (Willd.) Hook, *T. chebula* Retz, and *T. Vijaya Vati*. These components were chosen precisely for their ability to target distinct anxiety circuits, providing a holistic influence on the diseases[9-14].

The anxiolytic effects of *C. asiatica* L. (CA) are attributed to its active compounds madecassoside and asiaticoside. It is known to exhibit multiple neuroprotective mechanisms. It significantly improves anti-anxiety behaviours, attenuates oxidative damage, and reduces neuroinflammation markers, effects which are mediated through nitric oxide modulatory mechanisms and anti-stress pathways. CA also demonstrated anxiolytic effects in chronically stressed mice, improving behavioural markers and normalising serum corticosterone levels[15,16]. Similarly, the anxiolytic effects of *N. jatamansi* DC are partly attributed to its antioxidant properties and ability to mitigate oxidative stress markers in the brain. Further, it also modulates Monoamine Oxidase (MAO) enzymes and interacts with GABA-B receptors to exercise its antidepressant-like properties[17,18]. *T. cordifolia* (Willd.) Hook demonstrates antidepressant effects by modulating neurotransmitters like norepinephrine, serotonin, and dopamine, while also showing GABA-B receptor antagonism and antioxidant effects contributing to improved cognition and memory[19]. The role of *T. chebula* Retz extracts in anxiety has been evidenced in mice, particularly mediated by the monominergic pathways[20]. *T. Vijaya Vati* exerts its effects through the endocannabinoid system owing to one of its constituents, *Cannabis sativa*[21]. By using a combination of these herbs in a standardised tablet form, the potential for adverse reactions or toxicity is minimised, which is essential when conducting preclinical studies in animal models.

The demand for natural remedies for anxiety and related conditions is a significant motivating factor for this research. The major goal of this study is to assess the anxiolytic potential of this herbal preparation, therefore offering new insights into the treatment of

anxiety disorders and adding to the larger discussion of natural therapeutic possibilities. This research intends to bridge the gap between traditional wisdom and current scientific confirmation, perhaps providing an innovative, effective, and natural answer to a worldwide health crisis.

2. Materials and Methods

2.1 Experimental Animals

Female Swiss Albino mice (*Mus musculus sp.*) aging 10-12 weeks and weighing 20-30 g, were housed in an animal facility in opaque polypropylene cages (28 × 21 × 14 cm) under controlled conditions: temperature 20+3°C and relative humidity 30-70%. These mice underwent 12-h light and 12-h dark cycles, which were manually controlled. The study was performed as per the recommendations set forth in the Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines for Laboratory Animal Facility, updated in 2022. The floor of the experimental room was cleaned and mopped twice with a disinfectant solution every day to ensure that the animals were not exposed to any contaminants. The feed and drinking water provided to the mice were contaminant-free and provided ad libitum. The procedures and protocols used in the study were approved by the CCSEA as per Proposal no: DYPIPSR/IAEC/Nov/23-24/P-10 in an IAEC meeting dated 30/11/2023.

2.2 Standard and Test Drug preparation

Diazepam (Batch No. VLB22003; Exp. May 2025; Manufactured by Abbott Healthcare Pvt. Ltd.) was used as the Standard drug. To prepare the suspension, 2.5 mg of Diazepam was dissolved in 1 ml of Carboxymethylcellulose (CMC) 0.5% w/v, to be administered to an animal. For the administration of the Test (Anxo-125) drug, the ANXO-125 tablet was crushed to powder and dissolved in 0.5% CMC solution to make the formulation suspension. The amount of drug powder was taken to make suspensions of 50 mg/ml, 100 mg/ml, and 200 mg/ml for dosages of 50, 100 and 200 mg/kg.

2.3 Experimental Design

In this pre-clinical study, five experimental groups, namely, normal control (G1) receiving no treatment, standard group (G2) receiving Diazepam, test groups receiving ANXO-125 at concentrations of 50 mg/kg (G3), 100 mg/kg (G4) and 200 mg/kg (G5), consisting of 8 mice each, were formed (Table 1). A total of 49 animals were used in the study. All the animals were randomly allocated to the groups to ensure that the study is unbiased, reliable and reproducible. The drug was orally administered directly into the gastrointestinal tract using an oral gauge 20G, 30 min before the start of the behavioural tests. Mice were acclimatised for seven days and observed for general health before initiation of experimentation. An acute oral toxicity study was done to determine the safety profile of the test drug. The mice were administered the respective doses mentioned in Table 1, and after 30 min of dosing, various evaluation tests (Elevated Plus Maze, Social interaction and Marble burying test) were commenced to assess the anxiolytic effects of the herbal formulation. After the anxiolytic studies, all the animals were sacrificed to evaluate the neurotransmitter (Serotonin) level in the brain homogenate samples of the mice. Observations were recorded by personnel blinded to the experimental design.

2.4 Acute Oral Toxicity Study

OECD TG 423 guidelines were followed for the acute oral toxicity study of the ANXO-125 formulation[22]. Female Wistar rats were divided into two groups: A normal control group (n=3) receiving the vehicle (1 mL, p.o.) and a test group (n=6) administered the formulation at a limit dose of 2000 mg/kg orally. Post-administration, animals were observed individually, focusing on the first 30 min to 4 h, followed by periodic checks for 24 h and daily for 14 days. On day 15, a gross necropsy was performed to examine vital organs for pathological changes.

Table 1. Experimental design and allocation of animals in the study

Groups	Treatment	Dose	No. of Animals
G1	N Control	P.O. (per os)	8
G2	Diazepam	2.5 mg/kgp.o.	8
G3	ANXO-125	50 mg/kgp.o.	8
G4	ANXO-125	100 mg/kgp.o.	8
G5	ANXO-125	200mg/kgp.o.	8

2.5 Measurement of Behavioural Effects

The behavioural parameters indicating the anxiolytic effects of ANXO-125 were assessed using the Elevated Plus Maze, Social interaction and the Marble burying tests.

The *Elevated Plus Maze (EPM) test* relies on the fact that mice are naturally averse to high and open spaces as well as on their usual, spontaneous behaviour of exploring new surroundings. The test includes an apparatus consisting of a centre area with open and closed arms, crossed in the middle perpendicular to one another. 7 mice from each group were allowed to move freely across all the arms. Each animal was placed on the centre facing open arm and then observed for 5 min to record the number of open arm entries and the time spent in both open arms to evaluate open space-induced anxiety in the mice[23].

The *Social Interaction Test* evaluates the anxiolytic effects of a drug in rodents, typically using mice or rats. It involves pairing two mice, one treated with the drug and the other with a placebo. After drug administration, both animals were placed in a controlled testing environment. For this test, a plastic apparatus (60 cm × 40 cm × 20 cm) divided into 3 chambers (20 cm × 40 cm × 20 cm) by 2 transparent walls was used to assess social interaction. A covered doorway was used to access each of the three chambers. Social interaction behaviours, such as grooming and sniffing, were monitored for 5 min. An increase in social interactions in the drug-treated group suggests anxiolytic effects, indicating reduced anxiety. Interaction with the placebo was recorded when the experimental mouse was in close proximity (~1 cm), nose-oriented towards the placebo mouse[24].

The *Marble Burying Test* assesses anxiety-like behaviour in rodents by placing them in a cage with marbles. The animals were placed individually and in a corner of a husk-filled cage for 30 min with 15 marbles arranged on the surface. The number of marbles that were at least two-thirds buried with husk during a set time indicated repetitive, compulsive-like behaviour, indicating anxiety[25].

2.6 Measurement of Neurotransmitter Levels

Since the neurotransmitter Serotonin is greatly involved in mood regulation and anxiety, its levels in brain homogenate samples were also recorded towards the end of the study. ST GENLISA™ ELISA kit manufactured by KRISHGEN BioSystems (Mumbai, Maharashtra, India) was utilised, and the evaluation was done as per the manufacturer's protocol. 50 µl each of prepared standard and diluents was added to the respective standard wells and the blank wells, while the test sample wells received 40 µl of the samples. Further, 10 µl of Biotinylated Serotonin antibody was added to these sample wells. 50 µl Streptavidin: Horseradish Peroxidase (HRP) was added to all the wells, and the entire plate was incubated at 37°C for 1 h. After thorough aspiration and washing of the wells, 100 µl 3,3′,5,5′-Tetramethylbenzidine (TMB), which is the substrate for the HRP, was added with further incubation at 37°C for 10 min. Finally, a stop solution was added to prevent saturation in the plates, and the absorbance in the samples was measured at 450 nm.

2.7 Statistical Analyses

R software version 4.1.1/Graph Pad Prism 9.3.1 (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. All data were expressed as mean ± Standard Error of the Mean (SEM). For comparison between groups, One-way ANOVA was performed followed by Tukey's post hoc multiple comparison tests to determine the statistical significance. pvalue≤0.05 was considered statistically significant. No animals, experimental units, or data points were excluded from any of the analyses.

3. Results

3.1 Acute Oral Toxicity of ANXO-125

At a single dose of 2000 mg/kg, ANXO-125 was observed to cause no mortality. No significant observational difference was found in the skin, fur, eyes, salivation, and mucous membrane. The grooming behaviour was also observed in the normal control group, which may be attributed to handling stress or may be the natural behaviour of animals. Sedation was observed, suggesting that the formulation affects the Central Nervous System (CNS). The daily food and water consumption was found to be normal. Altogether, these observations suggest that ANXO-125 is non-toxic and that the oral dose of 2000 mg/kg was

safe. The neuro-behavioural changes observed over a period of 4 h are included in the following Table 2.

3.2 Anxiolytic Effects of ANXO-125

3.2.1 Observations from the Elevated Plus Maze test

The G2, G4 and G5, Diazepam, ANXO (100 mg/kg), and ANXO (200 mg/kg) groups, respectively, showed a highly significant increase in the number of open arm entries compared to the Normal Control group ($p<0.0001$), with no significant difference amongst these three groups. No such increase was observed in the G3 or the ANXO (50 mg/kg) group. In fact, fewer open arm entries were reported upon ANXO (50 mg/kg) treatment than those observed in the Diazepam group. These results indicate that higher doses of the test drug ANXO-125 (100 mg/kg and 200 mg/kg), but not 50 mg/kg, efficiently display a strong anxiolytic effect comparable to the standard Diazepam (Figure 1). Similarly, the Diazepam, ANXO (100 mg/kg), and ANXO (200 mg/kg) groups showed a highly significant increase in the time spent in the open arms compared to the Normal Control group G1. The ANXO (50 mg/kg) group also showed a significant increase in the time spent in the open arms compared to the Normal Control group but with a less stringent significance level ($p<0.01$). No significant difference was observed in the time spent in the open arms between the Diazepam group and any of the other treatment groups (ANXO 50 mg/kg, 100 mg/kg, and 200 mg/kg). These results further evidence the anxiolytic effects of ANXO-125, similar to the standard anxiolytic, Diazepam (Figure 1).

3.2.2 Observations from the Social Interaction Test

The time spent by the mice interacting with each other as a measure of reduction of anxiety was tested in this test. The time spent by the Diazepam, ANXO (100 mg/kg), and ANXO (200 mg/kg) groups in social interaction was significantly compared to the Normal

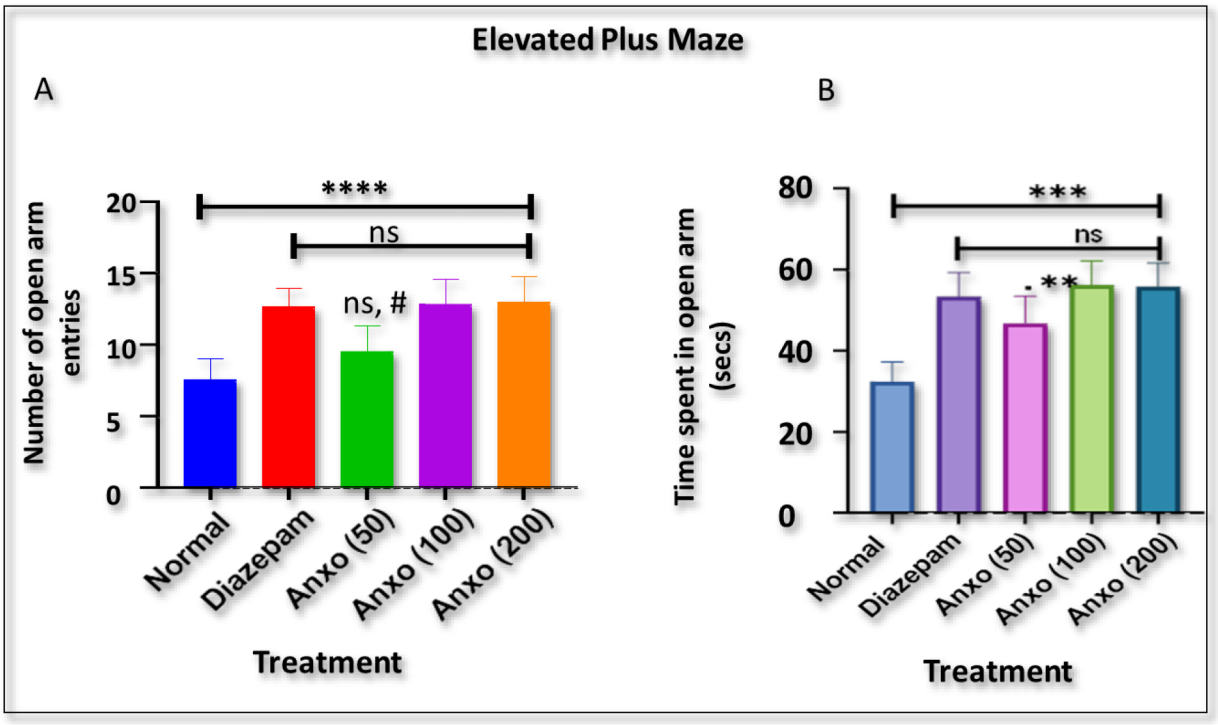


Figure 1. Effect of ANXO-125 on the number of open-arm entries and the time spent in open arms in the Elevated Plus Maze behavioural test. **(A).** A significant difference was observed in the number of open-arm entries among the groups (F-value = 15.47, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: Non-Significant). **(B).** A significant difference was observed in the time spent in the open arms among the groups (F-value = 14.61, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: Non-Significant). Both (A) and (B) show that ANXO-125 at higher concentrations of 100 and 200 mg/kg shows an anxiolytic effect similar to Diazepam, reducing anxiety considerably as compared to the Normal control group.

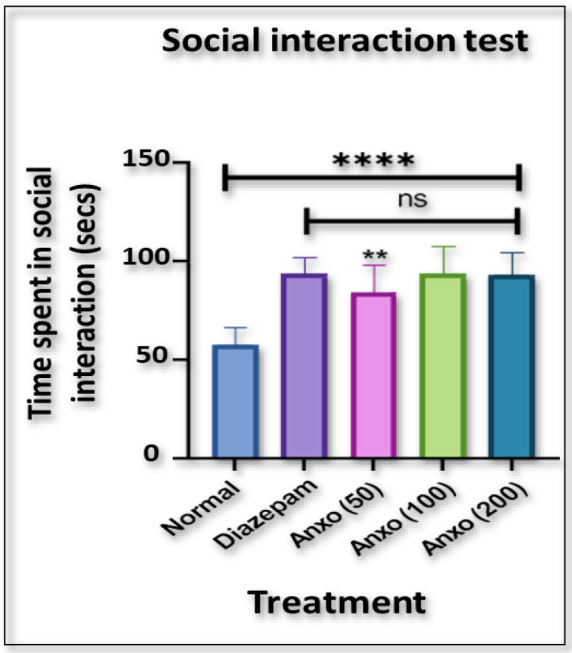
Control group (p<0.0001). While the social interaction was found to be elevated upon ANXO (50 mg/kg) administration, this elevation was not significant compared to the normal group and was not comparable to the standard. Therefore, the anxiolytic effect of ANXO-125 was further confirmed with the test drug considerably raising the time spent in social interaction (Figure 2).

3.3 Observations from the Marble Burying Test

The marble burying test was carried out for 30 min, after which the number of marbles buried by each animal in each group was noted. Marble burying is indicative of obsessive, compulsive behaviour and is a mark of anxiety. It was determined that upon completion of the test, all the doses of ANXO-125 (50, 100 and 200 mg/kg) were capable of reducing this compulsive behaviour in mice, similar to the standard drug, diazepam. This was evident from the fact that the number of buried marbles was remarkably less in the ANXO-125-treated groups as compared to the normal group with no treatment (Figure 3). No significant difference was observed in the number of buried marbles between the Diazepam group and any of the other treatment groups (ANXO 50 mg/kg, 100 mg/kg, and 200 mg/kg). Therefore, all the 3 behavioural tests confirm the anxiolytic effect of the test Ayurvedic drug, ANXO-125, in Swiss Albino mice.

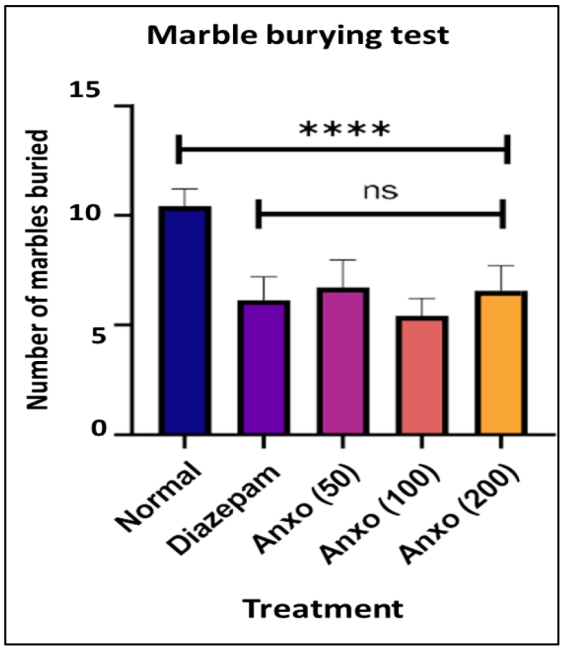
3.4 Determination of Serotonin Levels in Mice

No significant difference was observed in serotonin levels between the Diazepam and ANXO (50 mg/kg) groups. However, the ANXO (100 mg/kg) and ANXO (200 mg/kg) groups showed a significantly increased level of serotonin compared to the Diazepam group (p<0.001) (Figure 4). The significant increase in serotonin levels observed in the ANXO (100 mg/kg) and ANXO (200 mg/kg) groups can be attributed to



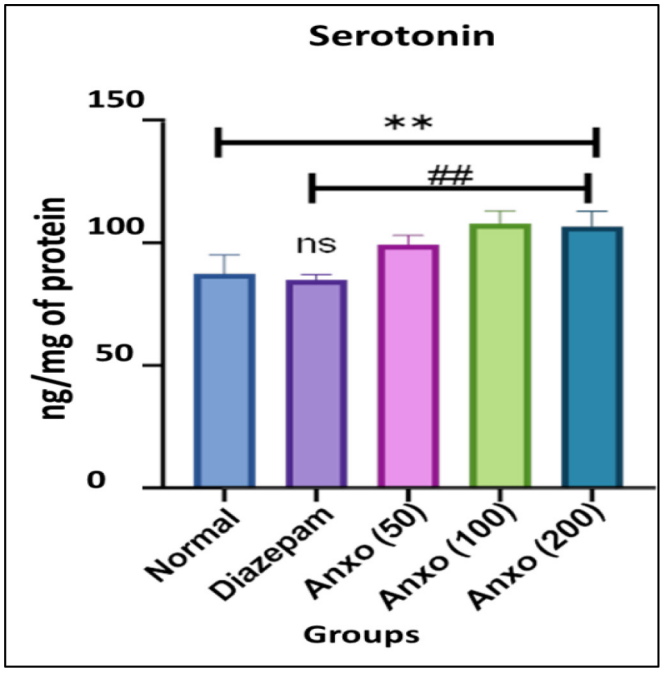
A significant difference was observed in social interaction times among the groups (F-value = 12.77, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: Non-Significant). Groups included: Normal (untreated control), Diazepam (2.5 mg/kg, standard anxiolytic), and ANXO-125 at doses of 50, 100, and 200 mg/kg, respectively.

Figure 2. Effect of ANXO-125 on the time spent in social interaction between the mice in the social interaction test.



A significant difference was observed in the marble-burying behaviour among the groups (F-value = 25.40, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: Non-Significant). 3 groups, each treated with different concentrations of ANXO-125 (50 mg/kg, 100 mg/kg and 200 mg/kg), showed statistically significant anxiolytic effects compared to the normal group receiving neither the standard treatment nor the test treatment. All ANXO-treated groups showed similar anxiety-reducing activity as the standard drug, Diazepam, as indicated by the lack of significant difference between these groups.

Figure 3. Effect of ANXO-125 on the marble burying behaviour by mice.



The serotonin protein levels in the brain are expressed in ng/mg. A significant difference was observed in serotonin levels among the groups (F-value = 11.89, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ##p<0.01, ns- non-significant). **p<0.01 is used to represent statistically significant data upon comparison of the treatment groups with the normal group, and ##p<0.01 represents the same for comparison of the test treatment with the standard treatment. The number of * and # denote the extent of significance of the results.

Figure 4. Effect of Anxo-125 Tab on Serotonin level in brain homogenate sample of mice.

the polyherbal composition of the ANXO tablets. The differential effect on serotonin levels points towards the distinct mechanisms through which ANXO and Diazepam exert their anxiolytic effects, with ANXO's polyherbal components potentially offering a multifaceted approach to anxiety reduction.

4. Discussion

Our study successfully establishes a safety profile for the test drug ANXO-125 along with its anti-anxiety efficacy through behavioural tests and indicates a serotonin-dependent activity through biochemical assay for neurotransmitter release to accomplish the anxiolytic properties in mice.

An acute oral toxicity study established that ANXO at a high dose of 2000 mg/kg did not produce any mortality or significant adverse effects on parameters such as skin, fur, eyes, salivation, and mucous membranes. This suggests that ANXO-125 may be safely administered at a 2000 mg/kg dose. Our results align with several studies that have investigated the toxicity profiles of various natural and anxiolytic compounds at high doses in mice. Natural products *Strychnos potatorum* L. f. seed extract and *Celastrus paniculatus* seed oil also demonstrated no toxic effects at 2000 mg/kg in mouse models[26,27]. Another anxiolytic, novel benzodiazepine derivative JM-20, has also been reported to have a safe profile at this high concentration[28]. Our findings clearly demonstrate that the herbal ANXO-125 tablets are non-toxic and safe for oral use even at this high dosage, offering a reassuring safety profile for this natural anxiolytic formulation to support further pharmacological studies.

There are numerous reports on the effects of various anxiolytics on rodent anxiety-like behaviours across different behavioural tests. Diazepam, considered a standard anxiolytic, has been shown to reduce anxiety through increased open arm time in both male and female rats in the Elevated Plus Maze test (EPM) in a dose-dependent manner[29]. Diazepam also reduced marble burying activity in mice, suggesting the marble burying test is a suitable test for testing anxiolytic compounds[30]. Carvacryl acetate, a natural product affecting GABAergic signalling, exhibits anxiolytic effects in rodent models, observed using the EPM and marble burying test[31]. In contrast, activation of 5-HT1A receptors in the baso-lateral amygdala produces anxiogenic effects in the social interaction test[32]. These findings highlight the importance of using multiple behavioural tests to assess and confirm the anxiolytic efficacy in rodent models, as we have done in our study. Where the anxiolytic effect of a lower dose of ANXO-125 (50 mg/kg) was not observed in EPM and social interaction tests, it was recognised in the marble burying test, which was observed for a longer time interval of 30 min, indicating its potent anxiolytic properties across a range of doses.

Low levels of Serotonin and suppressed serotonergic activity in the brain have been incriminated in depression and anxiety. Antidepressants have therefore been developed that directly or indirectly enhance the long-term serotonergic activity in patients[32-34]. Complying with this approach, biochemical analysis revealed a significant increase in serotonin levels in the brain homogenates of mice treated with 100 mg/

kg and 200 mg/kg doses of ANXO-125. Interestingly, Diazepam did not significantly alter serotonin levels, aligning with its primary action on the GABAergic system[35]. This differential effect on serotonin highlights the distinctive mechanism of ANXO-125, potentially offering a multifaceted approach to anxiety reduction through the modulation of multiple neurotransmitter systems.

These findings are consistent with earlier studies on polyherbal formulations, showing that herbs like *Ashwagandha* (*Withania somnifera*), *Brahmi* (*Bacopa monnieri*), and *Jatamansi* (*N. jatamansi*) possess anxiolytic properties by modulating neurotransmitter levels, including serotonin and GABA[36,37]. Specifically, the constituents of ANXO-125 are attributable to the multifaceted mechanisms of anxiolytic actions. While *C. asiatica*, *N. jatamansi* and *T. chebula* are known to exert their anxiolytic effects through GABA neurotransmission modulation, *C. sativa* constituents are known to interact with the 5-HT1A receptor of the serotonin neurotransmission pathway. Moreover, *T. cordifolia* components can affect both the GABA and Serotonin cascades[38-40].

The novelty of the study lies in the formulation itself, which combines the efficiencies of different herbal anxiolytics. Such combinatorial approaches have recently gained popularity owing to the challenges pertaining to toxicity. A study combining Diosgenin and silymarin at lower doses (50 mg/kg each) demonstrated significant anxiolytic effects compared to higher individual doses (100 mg/kg), suggesting synergistic action[41]. Similarly, resveratrol (50 mg/kg) combined with rufinamide (50 mg/kg) showed exceptional anxiolytic effects in animal models, outperforming resveratrol alone. Molecular docking studies indicated that resveratrol and rufinamide may inhibit GABA reuptake, increasing synaptic GABA levels to showcase their synergistic potential[42]. These findings highlight the potential of combining natural compounds at lower doses to achieve enhanced anxiolytic effects, underscoring the synergistic potentialities of combination therapies.

The current study proposes that the specific combination of these herbs in ANXO-125 may be responsible for its significant anxiolytic effects, particularly at higher doses. All in all, the newly formulated polyherbal tablet ANXO-125 demonstrates significant anxiolytic activity in mice, comparable to Diazepam, across various behavioural models. The study indicates that ANXO-125 is effective at 100 mg/kg and 200 mg/kg, with potential advantages in enhancing serotonin levels. These findings underscore the therapeutic potential of polyherbal formulations in managing anxiety.

Although our study successfully demonstrates the anxiolytic effects of the non-toxic ANXO-125 formulation, we realise that it still faces the challenges of clinical studies. Given that the toxicity of the test drug has already been assessed at a much higher dose than that used in the treatment, it is a fact that not all pre-clinical results get translated into clinical trials. It would thus be advantageous if the efficacy of the formulation could be enhanced while lowering its dose. Recent research has explored nano-formulations of natural products to enhance the anxiolytic effects of the formulations. Nano- and microemulsions have demonstrated improved solubility, brain bioavailability, and therapeutic efficacy for antidepressant and anxiolytic drugs, with some formulations increasing drug strength up to 20,270-fold compared to aqueous solubility[43]. Nano-encapsulation of Withaferin-A using poly-(lactic acid) has shown potential for increased bioavailability and water solubility[44]. Various phytochemicals, including curcumin, berberine, and resveratrol, have been nanosized to increase their solubility, bioavailability, and efficacy in managing metabolic syndrome, which is often associated with anxiety[45]. These studies highlight the potential of nanoformulations in remarkably enhancing the anxiolytic effects of the natural products remarkably, a prospective research avenue for our ANXO-125 formulation.

5. Conclusion

The present study successfully demonstrated the significant anxiolytic activity of the newly formulated polyherbal tablet, ANXO-125, in mice using various behavioural models. ANXO-125, at higher doses, exhibited effects comparable to the standard anxiolytic drug Diazepam, indicating its robust potential as an anxiolytic agent. Additionally, the ability of ANXO-125 to significantly increase serotonin levels in brain homogenates at these doses suggests a

serotonin-dependent mechanism of action similar to other reported anxiolytic Ayurvedic herbs, *Withania somnifera* L Dunal and *Bacopa monnieri* L. While the results of this study are promising, it is important to note that the evaluation was limited to specific behavioural models and acute administration in mice. Future studies should explore the efficacy of ANXO in chronic stress models to better understand its potential for long-term use. Additionally, clinical trials will be essential to confirm the anxiolytic effects observed in preclinical models and to evaluate the safety and efficacy of ANXO-125 in a clinical setting. With respect to the clinical studies, development of nano-formulations of this test drug might prove to be advantageous as this further decreases the dosage to enhance the anxiolytic effects.

Conclusively, these findings support the therapeutic potential of ANXO-125 as a novel anxiolytic agent, pave the way for future research and development aimed at addressing anxiety disorders through a multifaceted herbal approach and provide a foundation for future research and clinical development of ANXO-125 as a novel anxiolytic agent.

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Author Contribution

Conceptualisation – ST, SJ Data curation – ST, Formal analysis – ST, Investigation – MM, ST, Resources – SJ; Methodology – ST, Supervision – SJ Project administration – ST, Validation –ST; Visualisation – SJ; Writing - original draft – ST, SJ and Writing - review and editing – ST. All authors have read and agreed to the published version of the manuscript.