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Research article

Investigating anxiolytic effect of intranasal micro emulsion in experimental animal models

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Abstract

This investigation aimed to assess the anxiolytic potential of an intranasal micro emulsion formulation in an experimental model utilizing Swiss Albino Mice. The evaluation of anti-anxiety activity was conducted through the Elevated plus Maze and Open Field tests. In the Elevated plus Maze, parameters such as the total count of arm entries and the duration spent in both open and enclosed arms were meticulously recorded. Similarly, the Open Field tests involved the documentation of entries in the central region, the time spent in the central region, entries into the peripheral zone, and time spent in the peripheral zone. The experimental subjects received treatment with the intranasal micro emulsion formulation at a dosage of 50mg/kg. The results from the Elevated plus Maze exhibited a noteworthy ($p<0.01$**) augmentation in the quantity of entries and the duration of sojourn within the open arm. Parallelly, the Open Field tests revealed a significant ($p<0.01$**) escalation in the count of squares traversed and the frequency of crossings. This investigation unequivocally indicated that the intranasal micro emulsion formulation elicited a pronounced anti-anxiety effect in the experimental animal model. This research contributes to the expanding body of knowledge on the potential therapeutic efficacy of intranasal micro emulsion formulations in addressing anxiety-related behaviors, thereby paving the way for further investigations and potential clinical applications.

Introduction

Anxiety disorders are a group of mental health conditions characterized by excessive worry, fear, or apprehension. They can significantly impair daily functioning and quality of life. Common types include generalized anxiety disorder (GAD), panic disorder, social anxiety disorder, and specific phobias. The prevalence of anxiety disorders is substantial on a global scale. According to the World Health Organization (WHO), anxiety disorders affect around 3.8% of the world's population, making them one of the most prevalent mental health conditions. However, regional variations exist, with higher rates often reported in developed countries. Additionally, women are generally more likely than men to experience anxiety disorders. Generalized Anxiety Disorder (GAD) involves persistent and excessive worry about various aspects of life. It affects approximately 6% of the global population, according to data from the Anxiety and Depression Association of America (ADAA). Panic disorder is characterized by recurrent, unexpected panic attacks. The global prevalence is estimated at around 2.7%, as reported by the WHO. Social anxiety disorder involves intense fear of social situations. It affects approximately 7% of the global population, according to the ADAA. Specific phobias are characterized by irrational fears of specific objects or situations. These are among the most common anxiety disorders, with a global prevalence of around 12.5%, according to a study published

in the Journal of Psychiatric Research. The impact of anxiety disorders extends beyond individual suffering, contributing to substantial economic costs and healthcare utilization. Effective treatments, including psychotherapy and medication, exist, emphasizing the importance of early detection and intervention [1-3]. One-eighth of the world's population suffers from anxiety disorders, which are among the most prevalent mental, emotional, and behavioral issues. As a result, anxiety disorders are a major topic of interest for psychopharmacology research. There are many different ideas on the etiology of anxiety disorders, including behavioral, cognitive, genetic, psychodynamic, psychoanalytic, and biological ones [4]. Anxiety disorders are a diverse set of disorders that most likely do not share a common cause. Compared to other frequently occurring chronic illnesses, it is believed to have a substantially earlier age of beginning and to have increased prevalence in recent cohorts in many countries [5]. Anxiety disorders affect performance on a wide range of tasks and are linked to high rates of symptoms that are not explained by medicine, higher healthcare usage, strong and independent associations with chronic illnesses, bad quality of life, and disability [6, 7]. A variety of medications have been used throughout history to treat anxiety pharmacologically. Barbiturates were the first class of medications to be produced, and although they were quite effective, they also had a limited therapeutic index and could cause respiratory arrest [8]. The benzodiazepines were developed as a safer alternative to barbiturates, however, their beneficial effects are overshadowed by the emergence of physical and psychological dependence and withdrawal reactions [9, 10]. Other drugs used for treatment of anxiety having unfavorable side-effect profiles include buspirone [11, 12], antidepressants [13-15] and beta-blockers. The side effects of the medications that are already on the market cause most patients taking anxiolytics to stop their treatment before they fully recover [16]. Furthermore, in controlled investigations, one-third of the patients do not respond to any drug [17]. Therefore, the creation of novel anxiolytic agents is desperately needed. Medicinal plant research has advanced globally in the quest for novel therapeutic items to treat neurological illnesses, showcasing the pharmacological efficacy of several plant species in a range of animal models [18].

Therefore, the objective of the present work was to analyse the possible anxiolytic effects of the intranasal micro emulsion formulation in mice using the elevated plus-maze and open field tests as animal models of anxiety.

Materials and Method

Materials and reagents

Catechin and epigallocatechin gallate were acquired from YUCCA Enterprises, located in Mumbai, Maharashtra. The aromatic resin, Oudh, was sourced from K.K. Dawasaz, while the reference standard drug, clonazepam, was obtained from Abbott India.

Extraction of aromatic resin

A quantity of 100 grams of aromatic resin underwent maceration at ambient temperature for a duration of 12 hours using a 30% ethanol solution. Subsequently, the macerated extract was subjected to evaporation under room temperature conditions following filtration through filter paper (Whatman International, Maidstone, UK).

Composition and preparation of intranasal micro emulsion

In a suitable mixing apparatus, a homogeneous blend was prepared by combining equivalent volumes of aromatic resin extract with Catechin and Epigallocatechin gallate for the formulation of an intranasal micro emulsion. The composition was enriched with Nigella sativa oil at a concentration of 9%, and Smix Twin 80, along with methanol as the surfactant and co-surfactant, maintained at a ratio of 12:4, respectively. The resulting mixture was subjected to magnetic stirring at ambient temperature until the aromatic resin extract was completely dissolved. Subsequently, a carefully controlled addition of water was initiated drop-wise into the stirring mixture until a visually clear and transparent micro emulsion was achieved. The attainment of clarity and transparency in the formulation signifies the successful formation of the micro emulsion, indicative of the proper incorporation and dispersion of the components. This method ensures the uniformity and stability of the intranasal micro emulsion, potentially enhancing its efficacy and applicability in relevant pharmaceutical or therapeutic contexts.

Animals

Either sex of Swiss albino mice, weighing between 20 and 30 grams, were procured from Wockhardt Ltd, Aurangabad. The mice were maintained under standard laboratory conditions with a temperature of 25 ± 2°C, relative humidity ranging from 45 to 55 percent, and a consistent environmental regimen of 12 hours light and 12 hours dark cycles. During housing, the animals were provided unrestricted access to a standard diet and water. To ensure adaptation to the laboratory setting, the animals were acclimatized for two hours prior to the commencement of the experiments. The experimental procedures conducted on the mice were in accordance with the ethical standards set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Y.B. Chavan College of Pharmacy, Aurangabad (IAEC/844/ac/04/CPCSEA). The details of the experimental protocol are outlined in Table 1. This rigorous adherence to ethical guidelines and standardization of environmental conditions is crucial in maintaining the welfare of the experimental animals and ensuring the

reliability and validity of the scientific research conducted in accordance with the principles outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Table 1. Experimental protocol.

Group	Treatment
A	Normal control group p.o. (Vehicle, 0.1ml)
B	Clonazepam p.o. 1mg/kg
C	Treatment A (Intranasal Micro emulsion formulation, 50mg/kg)

Acute toxicity study

A study on acute toxicity was conducted in accordance with internationally recognized protocols outlined in the Organisation for Economic Co-operation and Development (OECD) guidelines 425 (OECD, 2001). Overnight fasted and healthy mice (n = 5) were subjected to intranasal administration of a micro emulsion, with doses up to 2000 mg/kg of body weight. The subjects were continuously observed for a duration of 4 hours, followed by additional observations at the 24-hour mark for any signs of abnormality or mortality. The results indicated the safety of the intranasal micro emulsion at the tested dose level of 2000 mg/kg. Consequently, a dose of 50 mg/kg was selected for subsequent experimentation in the context of anti-anxiety studies [20].

Animal model for anxiety

Elevated plus maze

The experimental apparatus utilized in this study was the elevated plus maze, comprising two open arms (16 x 5 cm) and two closed arms (16 x 5 x 12 cm) with an open roof, forming a plus-shaped structure elevated 25 cm above the floor. This apparatus was employed to assess anxiolytic behavior in animals. Prior to the experiment, the animals underwent an 18-hour fasting period. The administration of doses was timed such that each mouse was exposed to the plus maze 45 minutes post-dose administration. For the 5-minute experimental duration, each animal was centrally positioned on the elevated plus maze, oriented with its head facing the open arms. Behavioral observations were made, including a) the animal's preference for initial entry into the open/closed arm, b) the number of entries into the open arm, and c) the average time spent by the animal in the open arm. The metric used to quantify antianxiety activity was the average time spent by the animals in the open arms of the Elevated plus Maze (EPM). Throughout the experiment, socialization among animals was permitted, and meticulous measures were implemented to prevent any external stimuli from disturbing the subjects [21].

Open field test

Each subject was confined within a transparent acrylic enclosure with dimensions measuring 50 × 50 × 10 cm. The open-field arena was partitioned into a grid of 25 squares, consisting of 9 inner squares positioned centrally and 16 squares distributed peripherally along the enclosure walls. Following an oral administration period involving the application of vehicle, clonazepam and intranasal micro emulsion treatment, individual animals were situated within one of the corner squares. Subsequently, the occurrences of entries into the central zone, time spent in the central zone, entries into the peripheral zone, and time spent in the peripheral zone were meticulously observed over a designated duration of five minutes [22].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 9.5.1 (Graph Pad Software, San Diego, CA, USA). The results represent means ± standard error (SE). Differences between groups were evaluated using ANOVA (one-way) test followed by Dunnett's multiple comparison test and a p-value $< 0.05^{*}$ was considered statistically significant, p-value $<0.01^{**}$ was regarded as statistically highly significant and p-value $<0.001^{***}$ was extremely significant and ns was non-significant.

Results

Acute toxicity study

The Intranasal Micro emulsion formulation showed no behavioral changes nor mortality at dose 2000 mg/kg, as shown in (Table 2).

Anti-anxiety activity models

Elevated plus maze

Figure 1 A-D illustrates a comparative analysis of the anxiolytic efficacy of intranasal micro emulsion (Group C) in comparison to oral administration of the test substance (Group B), standard drug clonazepam, and a control group (Group A) in mice, as evaluated through the elevated plus maze test. The results (Table 3) indicate a statistically significant increase ($p < 0.01^{**}$) in the number of entries into the open arms and the time spent in the open arms for both the intranasal micro emulsion (50 mg/kg) and clonazepam (p.o. 1 mg/kg) treatment groups. Conversely, a significant decrease ($p < 0.01^{**}$) is observed in the number of entries into the closed arms and the time spent in the closed arms for the aforementioned treatment groups.

This experimental outcome suggests a notable anxiolytic effect associated with the intranasal micro emulsion, as evidenced by the augmented exploratory behavior in the open arms of the elevated plus maze. The anxiolytic impact is comparable to that of clonazepam, a standard anxiolytic drug, as indicated by the analogous behavioral responses in the experimental paradigm. The statistical significance of these observations underscores the robustness and reliability of the findings. It is imperative to note that these results contribute to the understanding of the potential therapeutic implications of intranasal micro emulsion in the

management of anxiety-related conditions, presenting a novel avenue for further investigation and development of alternative anxiolytic interventions.

Open field test

Figure 2 A-D depict the comparative evaluation of the anxiolytic efficacy of intranasal micro emulsion (Group C), oral administration of vehicle (Group B), and the reference anxiolytic clonazepam with control (Group A) using the open field test paradigm in mice. An intranasal micro emulsion dosage of 50 mg/kg shown a statistically significant increase ($p < 0.01$**) (table 4) was observed in the number of entries into the central zone, accompanied by a corresponding elevation in the time spent within the central zone. Conversely, a marked decrease ($p < 0.01$**) was noted in both the number of entries into the peripheral zone and the time spent therein. The administration of clonazepam at an oral dose of 1 mg/kg also demonstrated a highly significant increase ($p < 0.001$***) in the number of entries into the central zone, the percentage of time spent within the central zone, and the overall percentage of time spent in the central zone. Concurrently, a substantial reduction in both the entries and time spent in the peripheral zone was observed. These findings suggest that intranasal micro emulsion, as well as oral clonazepam, exhibit anxiolytic effects in mice, as evidenced by increased exploration and time spent in the central zone along with a concomitant decrease in activity within the peripheral zone. The observed behavioral changes underscore the potential anxiolytic properties of the administered substances.

Table 2. Acute toxicity study of intranasal micro emulsion formulation.

Sr. No	Response	Head		Body		Tail	
		Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent
3	Restlessness	Absent	Absent	Absent	Absent	Absent	Absent
4	Touch response	Absent	Absent	Absent	Absent	Absent	Absent
5	Touch response	Normal	Normal	Normal	Normal	Normal	Normal
6	Pain response	Present	Absent	Present	Absent	Present	Absent
7	Tremors	Absent	Absent	Absent	Absent	Absent	Absent
8	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent
9	Righting reflux	Normal	Normal	Normal	Normal	Normal	Normal
10	Gripping strength	Normal	Normal	Normal	Normal	Normal	Normal

Table 3. Effect of Intranasal microemulsion on EPM test.

Group	Open Arm: entries		Open Arm: time (s)		Closed Arm: entries		Closed Arm: time (s)	
	SD	SE	SD	SE	SD	SE	SD	SE
Control	±0.95	±0.47	±27.77	±13.88	±3.5	±1.75	±27.77	±13.88
Standard	±2.21	±1.10***	±41.71	±20.85***	±2.16	±1.08***	±41.71	±20.85***
Treatment A	±2.88	±1.44**	±44.17	±22.08**	±0.95	±0.47***	±44.17	±22.08**

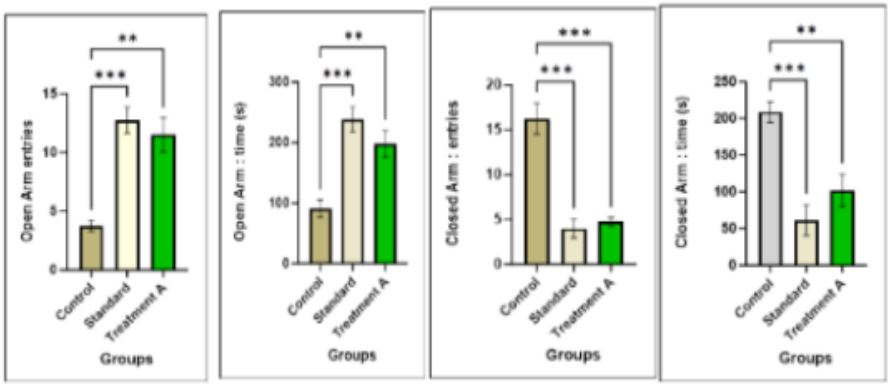

Figure. 1 (A) The open arm entries in a 5 min session of EPM was recorded. (B) The open arm time in a 5 min session of EPM was recorded. (C) The close arm entries in a 5 min session of EPM was recorded. (D) The close arm time in a 5 min session of EPM was recorded. Data represent means ± SE (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns- non-significant vs. the normal control group.

Table 4. Effect of Intranasal microemulsion on OFT test.

Group	Centre: entries		Centre: time (s)		Peripheral Zone: entries		Peripheral Zone: time (s)	
	SD	SE	SD	SE	SD	SE	SD	SE
Control	±3.10	±1.55	±32.94	±16.47	±15.14	±7.57	±32.94	±16.47
Standard	±12.89	±6.44***	±23.14	±11.57****	±5.43	±2.71**	±23.14	±11.57****
Treatment A	±6.27	±3.13*	±22.84	±11.42***	±3.20	±1.60***	±22.84	±11.42***

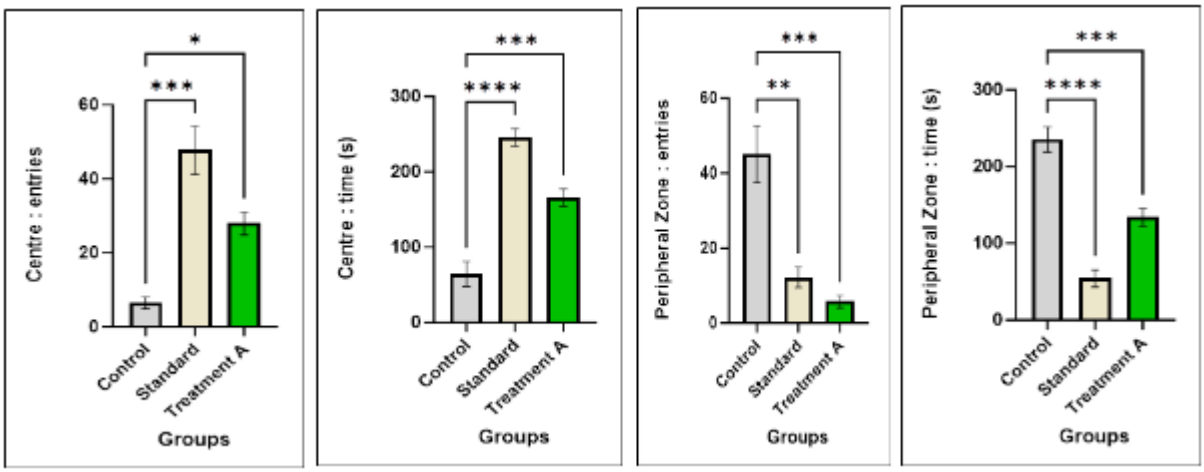

Figure. 2 (A) The center entries in a 5 min session of OFT was recorded. (B) The center time in a 5 min session of OPT was recorded. (C) The peripheral zone entries in a 5 min session of OPT was recorded. (D) The peripheral zone time in a 5 min session of OFT was recorded. Data represent means ± SE (n = 5). * p < 0.05, ** p < 0.01, *** p < 0.001, ns- non-significant vs. the normal control group.

Discussion

The field of psychiatric research in humans has prominently focused on anxiety, a condition afflicting approximately one-eighth of the global population [23, 24]. Presently, there is a rising trend in utilizing herbal remedies and dietary supplements enriched with flavonoids and vitamin C for managing mild to moderate anxiety disorders and depression [25]. However, there exists a critical need for further scientific investigation and studies to comprehensively assess the pharmacological and toxicological implications of plant-derived interventions. Such research endeavors hold promise for the identification of novel pharmaceutical agents derived from herbal sources, thereby potentially endorsing the utilization of traditional remedies in clinical settings. Anxiety disorders are intricately associated with GABAergic and serotonergic mechanisms, with the involvement of adrenergic and dopaminergic pathways also documented. Over the last four decades, benzodiazepines (BZA) have been extensively employed to address various forms of anxiety. Nevertheless, due to the undesirable adverse reactions associated with BZA, alternative therapeutic approaches with more favorable side effect profiles have been developed. Medicinal plants represent a valuable reservoir for the discovery of novel remedies to address the complexities of anxiety disorders, presenting an avenue for the exploration of alternative and potentially more tolerable treatment modalities.

The investigation encompassed an assessment of the acute toxicity of intranasal micro-emulsion, with the determination of the LD50 values resulting in an observed value of 2000 mg/kg. The Elevated plus Maze, acknowledged as a reliable animal model for anxiety, leverages innate stimuli such as the apprehension induced by a novel, brightly illuminated open space and the fear associated with balancing on a relatively narrow elevated platform [26]. Furthermore, it is established that anxiolytic agents augment both the frequency of entries and the duration spent in the open arms of the Elevated plus Maze [27]. In the current study, the intranasal micro-emulsion formulation exhibited a statistically significant ($p < 0.01$**) increase in entry frequency and time spent in the open arms, coupled with a concurrent decrease in entry frequency and time spent in the closed arms. The open field test was employed to gauge the emotional state of the subjects. This model delves into anxiety-related behavior predicated on the innate aversion of animals to an exposed environment. Consequently, animals transitioning from their accustomed enclosures to an unfamiliar setting manifest signs of distress and apprehension, as reflected by alterations in various parameters. Mice subjected to the intranasal micro-emulsion formulation demonstrated a noteworthy ($p < 0.01$**) elevation in the temporal and spatial metrics associated with central zones, alongside a reduction in the count of entries and time spent in peripheral zones. The outcomes derived from behavioral assessments underscore the discernible potential of the intranasal micro-emulsion formulation as a

promising candidate for further development as an anti-anxiety formulation.

Conclusion

A research investigation was undertaken to assess the anxiolytic properties of an intranasal micro emulsion formulation. The acute toxicity examination of the intranasal micro emulsion formulation demonstrated its safety profile up to a dose of 2000 mg/kg body weight. Evaluation through behavioral tests, namely the Elevated plus Maze test and the Open Field test, revealed that the intranasal micro emulsion formulation elicited substantial anti-anxiety effects comparable to those observed in the control group. The findings suggest that the developed intranasal micro emulsion may hold promise as a viable anxiolytic intervention, warranting further exploration in clinical settings.

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Consent for publication

All authors agreed with the content and that all gave explicit consent to submit and publish.

Competing interests

The authors declare no competing interests.

Author contribution

All authors have carefully reviewed and given their approval for final version of the manuscript. The contributions of Ansari Vikhar Danish Ahmad in design of pharmacological study, *in vivo* experiment and statistical analysis. Dr. Subur. W. Khan provided guidance and supervision throughout the research work.

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Data availability

All data generated or analyzed during this study are included in this published article.

Abbreviation

EPM: Elevated Plus Maze, OFT: Open Field Test, IAEC: Institutional Animal Ethics Committee, OECD: Organization for Economic Cooperation and Development, ANOVA: Analysis of variance.

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