

Preclinical Investigation of the Anxiolytic Effects of *Pimpinella Anisum* Seeds Extract in a Rat Model of Anxiety

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(Submitted: 11 March 2026 – Revised version received: 13 April 2026 – Accepted: 16 May 2026 – Published online: 26 June 2026)

Abstract

Objective: The aim of this study is to compare the anxiolytic effects of *Pimpinella anisum* extract with diazepam in a rat model of anxiety induced by caffeine and chronic unpredictable stress (CUS).

Method: A total of 36 rats were randomly divided into six groups, including control, induced anxiety, diazepam-treated, and three plant-treated groups receiving different doses of *Pimpinella anisum* extract (250, 350, and 500 mg/kg). Anxiety-like behaviors were evaluated using the Elevated plus Maze, light/dark exploration and Open Field Test. Additionally, brain tissue biomarkers including serotonin, tumor necrosis factor- α , interleukin-1 β , total antioxidant capacity, and nuclear factor erythroid 2-related factor 2 were assessed in brain tissue using ELISA techniques.

Results: The *Pimpinella anisum* extract treated groups exhibited results comparable to those of diazepam with a significant increase in time spent in the open arms, center of field and light chamber as compared to corresponding control group ($P < 0.05$). Additionally, all tested doses of extract treatment also showed comparable results to those of diazepam. *Pimpinella anisum* extract showed a dose-dependent effects, with higher doses approaching the efficacy of diazepam, the findings were significant ($P < 0.05$) for the 250 mg/kg and 350 mg/kg in most behavioral tests and biomarkers levels in brain tissue, for the 500 mg/kg the results were significant ($P < 0.05$) in most behavioral tests and biomarkers levels in brain tissue.

Conclusion: *Pimpinella anisum* showed anxiolytic effects with significant modulation in the anti-inflammatory, antioxidant biomarkers, neurotransmitter levels and behavioral assessment tests in a comparable manner to diazepam. In addition *Pimpinella anisum* extract anti-oxidant potential evidenced through the levels of NRF2 and total anti-oxidant capacity.

Keywords: Anxiety, *Pimpinella anisum*, diazepam, EPS, TNF- α , serotonin, Nrf2, IL-1 β

Introduction

Anxiety disorders are primarily manifested as persistent or excessive feelings of fear, uncertainty, and discomfort, often associated with a wide range of physical and psychological symptoms.¹ These disorders develop during childhood and early adulthood may recur throughout life, greatly affecting one's performance in daily activities, relationships, academic achievement, and quality of life.² Anxiety may contribute to years of disability, especially in the Middle Eastern region, where anxiety disorders are one of the major contributors to disability.² Anxiety has diverse etiological factors, such as genetics, environment and neurotransmitter systems dysfunction. Neurochemical imbalance plays a central role in the pathophysiology of anxiety. In particular, many neurotransmitters are involved like γ -amino butyric acid (GABA), serotonin, glutamate, noradrenaline systems and alterations in serotonergic signaling can contribute to the development of anxiety-related behaviors.³ In addition, neuroendocrine dysfunction, particularly hyper activation of the hypothalamic–pituitary–adrenal (HPA) axis, and elevated inflammatory biomarkers such as TNF- α and IL-1 β further contribute to anxiety pathogenesis.⁴ Pharmacological therapy remains to be a crucial treatment for anxiety disorders. Drug groups that can be included in pharmacotherapy for anxiety disorders include serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine inhibitors (SNRIs) and benzodiazepines like diazepam. The major mechanism by which diazepam produces its anxiolytic action is via enhancement of GABAergic neurotransmission through binding of GABA-A receptors in the CNS.⁵ Limitations such as adverse effects, tolerance, and dependence, especially in long-term benzodiazepine use are

associated with Pharmacological agents.⁵ In the in recent years there had been growing interest in herbal and plant-based therapies as potential alternatives to conventional pharmacological treatments.⁶ *Pimpinella anisum*, commonly known as anise, is a medicinal plant widely used in traditional medicine for various therapeutic indications, including neurological and psychological disorders.⁷ The plant contains bioactive compounds, particularly trans-anethole and cis-anethole, which have been linked to neuroprotective, anti-inflammatory, and antioxidant effects.⁸ Previous studies showed that the effects of *Pimpinella anisum* were mediated through modulation of the GABAergic system, reduction of oxidative stress, and reduction of pro-inflammatory cytokines such as TNF- α and IL-1 β .⁹ These mechanisms align with the recent understanding of anxiety pathophysiology, supporting the potential role of this plant as a therapeutic agent. This study was designed to evaluate and compare the anxiolytic effects of *Pimpinella anisum* extract with diazepam in a rat model of anxiety induced by caffeine and chronic unpredictable stress. The study further aimed to assess behavioral changes and investigate the underlying neurobiological mechanisms through the measurement of relevant brain tissue neurotransmitters, anti-inflammatory, antioxidant biomarkers.

Methods

Ethical Approvals

This study adheres with essential 10 ARRIVE guidelines 2.0 of laboratory animals use regarding study design, sample size, inclusion and exclusion criteria, randomization and blinding, procedures, statistical method and study results.

Highlighting the significance of transparency and reproducibility in animal research. The ethical approval from the scientific research ethics committee in the university of Babylon/ faculty of medicine was obtained with a written consent from the committee (Ref. NO. 4-48. 11/6/2025).

Sample Size Selection

This study was designed to detect large and various biological effects due to the behavioral and biomarkers differences in anxiety metrics. Resource equation was used to calculate the power of detection which was slightly >20 . For standard sensitivity power analysis ($1 - \beta = 0.80$) and ($\alpha = 0.05$) and One-Way ANOVA, 36 total animals with 6 groups establish an efficient power of detection especially with anxiety related biomarkers and behavioral tests that yield large and distinct results. These calculations were considered to maintain reduced animal size and avoid underpowered detection.

Animals' Preparation

Thirty-six Swiss albino male rats (200–250 g) were obtained from the animal research center in the university of Baghdad / Faculty of Veterinary Medicine and housed in the animal research center of the University of Babylon/Faculty of Medicine. The rats were acclimatized for 14 days under standard laboratory conditions, such as a controlled temperature of 25°C, normal humidity, and a 12 hour light/dark cycle. The animals were housed in poly propylene cages with metal mesh covers, ad libitum. Sawdust bedding was used and replaced daily to maintain hygienic conditions.¹⁰

Preparation of *Pimpinella anisum* Seeds Extract

The seeds of *Pimpinella anisum* were obtained from local markets and authenticated by the University of Babylon/ Faculty of Science/ Department of Medicinal Plants (6609- July 12, 2025). Extraction of the active compounds was performed using the Soxhlet extraction method. A hydro alcoholic solvent (70% ethanol) was used due to its effectiveness in extracting both polar and moderately non-polar phytochemicals, particularly flavonoids and phenolic compounds. The extraction process was conducted for 6 hours at a temperature of 60°C. The obtained extract was then dried in the oven at 40–44°C for 36 hours, and stored under 2–8°C until use.¹¹ Using the Soxhlet extraction method with 70% ethanol the extraction yield value was 21.3% calculated by taking the percentage of dried extract compared to the starting weight of grinded *Pimpinella anisum* seeds.

Phytochemical Characterization of the Extract

HPLC and GC-mass tests were performed in the ministry of science and technology labs (scientific research committee) to determine the phytochemical composition of the seeds. The composition of the *Pimpinella anisum* seeds showed a 41.2% of trans-p-Anethole, 28.86% of Anethole isomer cis-p-Anethole and 1.78% of Esragole (methyl chavicol) with a retention time 9.535 min, 9.745 min and 8.133 min respectively.

Preparation of Drugs

Caffeine and diazepam were obtained from Macklin company/ China and prepared according to their manufacture instructions and dosing were performed according to animals weight.

Experimental Protocol

This study was designed as experimental, randomized, controlled in vivo study conducted on rats. A total of 36 rats were randomly allocated into six experimental groups. The experimental model was based on the induction of anxiety using a combination of chronic unpredictable stress and pharmacological induction with caffeine.³ The doses of the *Pimpinella anisum* extract selected according to the previous studies considering pharmacological and safety profile of the plant with three doses 250 mg/kg, 350 mg/kg and 500 mg/kg to evaluate possible dose- dependent response.^{12–14}

As illustrated in Figure 1, the animals were divided into six groups in a random manner as follows:

Group 1 (Control): Received D.W IP. Without any treatment in order to act as baseline.

Group 2 (Induced Anxiety): The experimental rats in this group induced anxiety by couple of CUS for 14 days and Caffeine (25 mg/kg) I.P for 7 days.^{3,15}

Group 3 (Diazepam-treated): The experimental rats in this group induced anxiety by couple of CUS for 14 days and Caffeine (25 mg/kg) I.P for 7 days. for two weeks and then treated with diazepam (1 mg/kg, IP.) for 12 days.¹⁶

Group 4: The experimental rats in this group induced anxiety by couple of CUS for 14 days and Caffeine (25 mg/kg) I.P for 7 days. for two weeks and then treated with *Pimpinella anisum* extract (250 mg/kg PO.) for 12 days.^{6,14}

Group 5: The experimental rats in this group induced anxiety by couple of CUS for 14 days and Caffeine (25 mg/kg) I.P for 7 days. For two weeks and then treated with *Pimpinella anisum* extract (350 mg/kg PO.) for 12 days.¹³

Group 6: The experimental rats in this group induced anxiety by couple of CUS for 14 days and Caffeine (25 mg/kg) I.P for 7 days. For two weeks and then treated with *Pimpinella anisum* extract (500 mg/kg PO.) for 12 days.¹⁷

Animal Randomization

The animals were randomized by using tail tags, all animals were numbered and divided into six groups each one had six rats in a random manner using simple lottery randomization. Randomization was maintained throughout all behavioral tests by flipping the tail tags and writing symbols on the back of tail tags in a random manner to give each animal a new unknown identity during tests.

Animal Masking Procedure

Single blind evaluated by other investigator who is unaware about grouping of animals and each animal was coded.

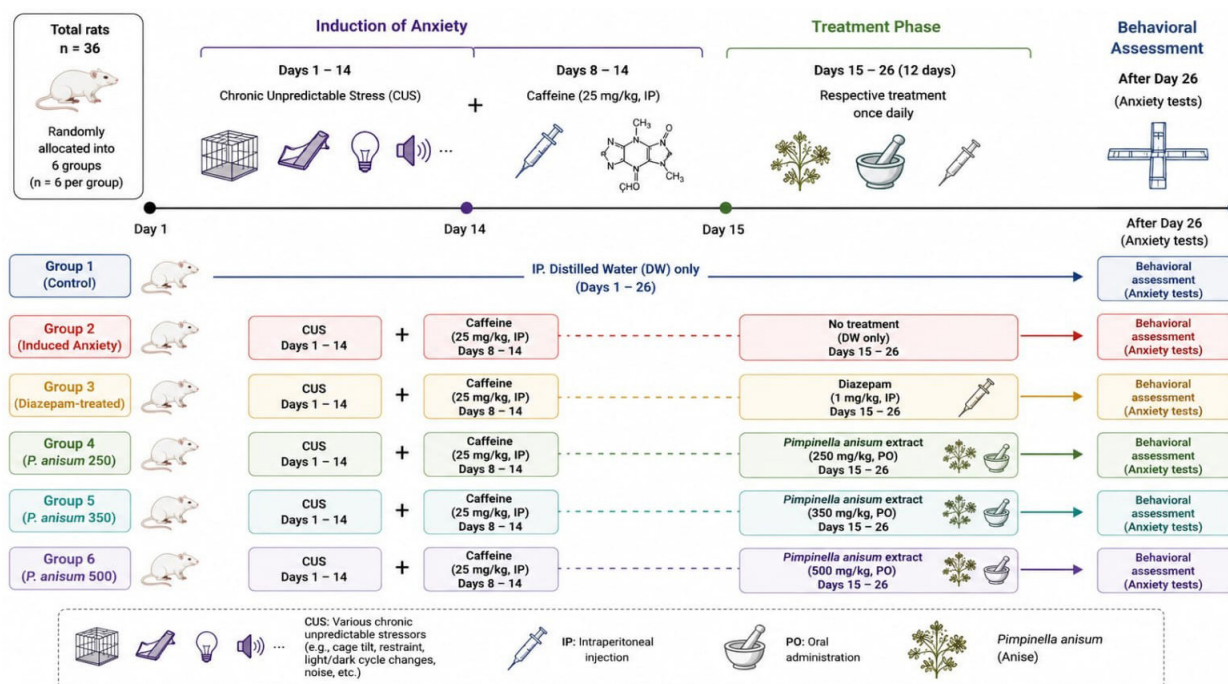


Fig. 1 Schematic representation of experimental protocol.

Induction of Anxiety

Anxiety was induced using a combination of chronic unpredictable stress (CUS) and caffeine administration. The model was selected due to its established effectiveness in producing persistent anxiety-like behaviors in animal models.³ The stressors included heat exposure, cage shaking, water splash, tilting of cages, noise, and disturbance of the sleep cycle.¹⁸ These stressors were applied randomly and unpredictably to prevent adaptation. The stress protocol was continued for 14 days. In addition, caffeine was administered at a dose of 25 mg/kg for 7 days to enhance the induction of anxiety-like behavior, based on previously established experimental models.¹⁵

Behavioral Assessment

Behavioral evaluation of anxiety-like activity was performed using three established tests:

Elevated plus maze

The Elevated plus Maze test consisted of four arms (two closed and two open), each 50 cm in length and elevated 50 cm above the ground. Anxious animals usually tend to spend more time in closed arms, while less anxious animals spend more time in open arms. Time spent and number of entries into each arm was recorded for each animal.¹⁹

Open field test

The open field test consisted of a square arena divided into equal sections. Anxiety-like behavior was assessed by measuring loco motor activity, line crossing, and time spent in the center versus the periphery. Less anxious animals tend to explore the central area more frequently.²⁰

Light/Dark box test

This test consisted of two compartments: one illuminated and one dark. Anxious animals tend to remain in the dark compartment, whereas reduced anxiety is associated with increased exploration of the illuminated area.²¹

Isolation of brain tissue samples

At the end of the experimental period and following behavioral assessment, the animals were euthanized by administration of ketamine 80 mg/kg I.P and xylazine 10 mg/kg I.P simultaneously followed by cervical dislocation and decapitation. Brain tissues were collected, and the amygdala region which is located deep in the temporal lobe bordered by the hippocampus²² was dissected and washed with PBS due to its critical role in anxiety regulation.²³ The samples were preserved in deep freeze until biochemical analysis was performed.

Biomarkers of study

Biochemical assessment was performed using ELISA kits obtained from sunlong/china to quantify the levels of key biomarkers, which includes: TNF- α CAT.NO: SL0722Ra, IL-1 β CAT.NO: SL0402Ra, Serotonin CAT.NO: SL1046Ra, NRF2 CAT.NO: SL0985Ra, Total antioxidant capacity (TAC) CAT.NO: AK0455, The ELISA was performed according to manufacturer catalogues.

Statistical analysis

Data analysis and graphical presentation were executed using Graphpad prism for Microsoft Windows. Results are showcased as (means $\pm$ SD). Data distribution was assessed using the Shapiro-Wilk test. One-way ANOVA for normally

distributed data, followed by Tukey's HSD post hoc test was implemented.

Results

Behavioral Assessment

As illustrated in Figures 2–4, the induction of anxiety resulted in clear highly significant ($P < 0.01$) behavioral alterations compared to the control group. Animals in the induced anxiety group demonstrated increased anxiety-like behavior, evidenced by highly significant ($P < 0.01$) reduced exploration of open arms in the EPM, decreased time spent in the center in the OFT, and increased preference for the dark compartment in the L/D box test. Diazepam treatment significantly improved behavioral parameters, with animals presenting increased exploration time of open arms, higher loco motor activity, and reduced anxiety-like behavior. Treatment with *Pimpinella anisum* extract showed an anxiolytic effect. Lower doses (250 mg/kg) showed improvement, while higher doses (350 and 500 mg/kg) produced more pronounced behavioral changes, approaching the effects observed with diazepam (Tables 1–3).

Tumor Necrosis Factor-Alpha (TNF- α)

Analysis of TNF- α level revealed as it shown in Figure 5, a significant increase in the induced anxiety group compared to the control, indicating activation of inflammatory pathways associated with anxiety. Treatment with diazepam resulted in a significant reduction in TNF- α level. Similarly,

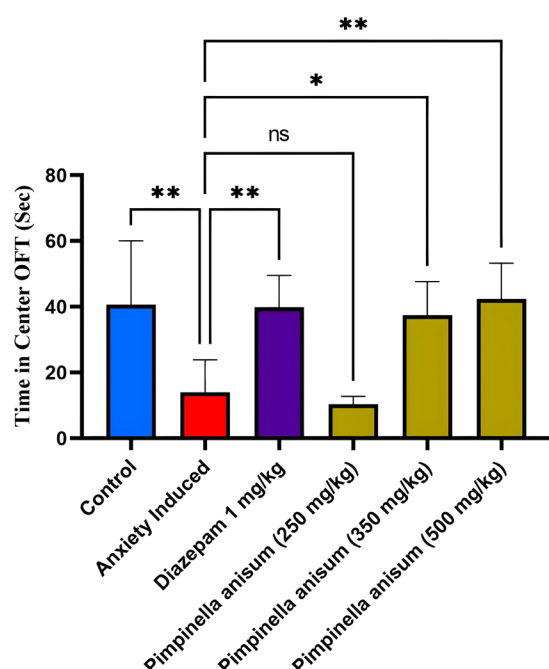


Fig. 2 Effects of diazepam and *Pimpinella anisum* seeds extract on time spent in central zone through OFT. The time was recorded and exhibited in seconds. Data presented as (means $\pm$ SD) $F(5, 30) = 9.502, P < 0.0001, n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

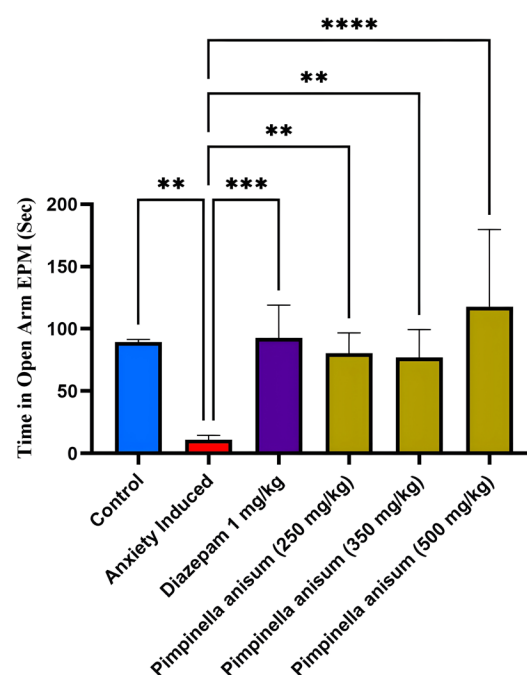


Fig. 3 Effects of diazepam and *Pimpinella anisum* seeds extract on time spent in open arms through EPM test. The time was recorded and exhibited in seconds. Data presented as (means $\pm$ SD) $F(5, 30) = 8.692, P < 0.0001, n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

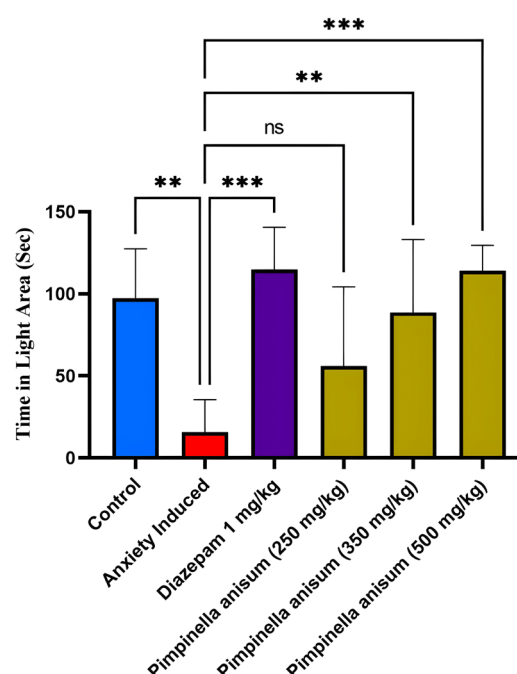


Fig. 4 Effects of diazepam and *Pimpinella anisum* seeds extract on time spent in light area through L/D test. The time was recorded and exhibited in seconds. Data presented as (means $\pm$ SD) $F(5, 29) = 8.158, P < 0.0001, n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

Table 1. Mean and standard deviation of open field test for the experimental groups

Group	1	2	3	4	5	6
Mean	40.67	14	39.83	10.33	37.5	42.33
Std. deviation	19.45	9.899	9.766	2.422	10.17	10.98

Table 2. Mean and standard deviation of elevated plus maze test for the experimental groups

Group	1	2	3	4	5	6
Mean	89.33	11	92.67	80.17	77	117.8
Std. deviation	2.066	3.578	26.4	16.57	22.36	61.91

Table 3. Mean and standard deviation of light/dark exploration test for the experimental groups

Group	1	2	3	4	5	6
Mean	97.4	15.83	114.8	56	88.67	114.2
Std. deviation	30.02	19.68	25.77	48.29	44.41	15.38

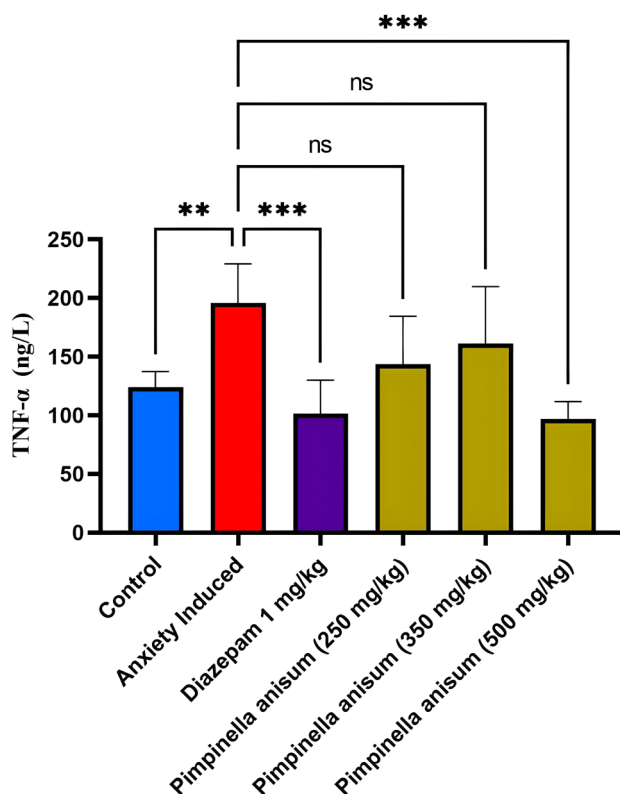


Fig. 5 Effects of diazepam and *Pimpinella anisum* seeds extract on brain tissue TNF- α . Results were measured by ELISA in ng/ml. Data presented as (means $\pm$ SD) $F(5, 30) = 8.060, P < 0.0001, n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

Pimpinella anisum extract demonstrated a reduction in TNF- α , with the highest dose (500 mg/kg) producing the most notable decrease, approaching normal levels (Table 4).

Serotonin Levels

As explained in Figure 6, Serotonin levels were altered in response to anxiety induction and treatment. Treatment with diazepam normalized serotonin levels toward control values. Administration of *Pimpinella anisum* extract resulted in modulation of serotonin levels, with higher doses producing more balanced normalization (Table 5).

Interleukin-1 Beta (IL-1 β)

IL-1 β levels were significantly elevated in the induced anxiety group compared to the control group as illustrated in Figure 7.

Table 4. Mean and standard deviation of TNF- α for the experimental groups

Group	1	2	3	4	5	6
Mean	124	195.7	101.5	143.7	161.3	97.01
Std. deviation	13.27	33.6	28.64	40.65	48.52	14.78

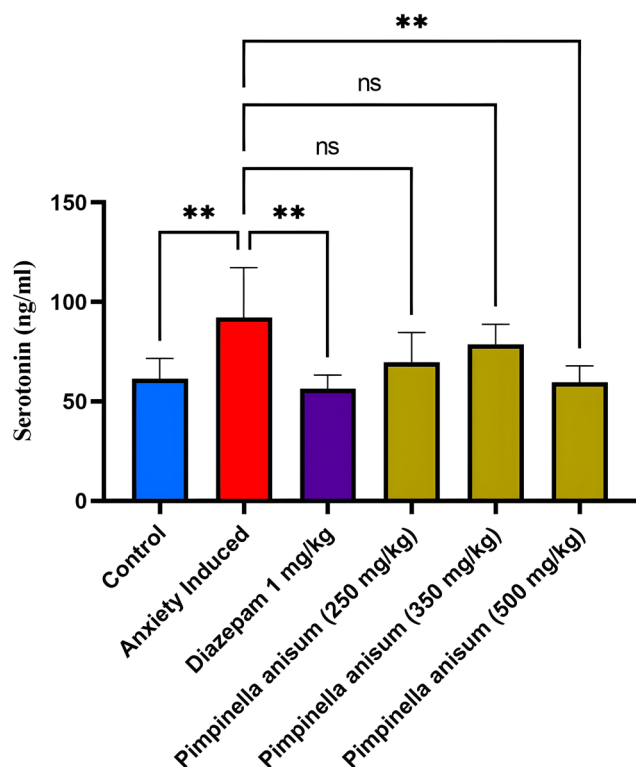


Fig. 6 Effects of diazepam and *Pimpinella anisum* seeds extract on brain tissue serotonin. Results were measured by ELISA in ng/ml. Data presented as (means $\pm$ SD) $F(5, 30) = 5.723, P = 0.0008, n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

Treatment with diazepam significantly reduced IL-1 β levels. *Pimpinella anisum* extract also reduced IL-1 β with higher doses producing anti-inflammatory effects similar to those with diazepam (Table 6).

Nrf2 and Total Antioxidant Capacity

Oxidative stress markers showed significant alterations following anxiety induction as they are shown in Figures 8 and 9. NRF2 levels and total antioxidant capacity were elevated in the induced anxiety group. Treatment with diazepam significantly

Table 5. Mean and standard deviation of serotonin for the experimental groups

Group	1	2	3	4	5	6
Mean	61.45	92.15	56.27	69.76	78.8	59.69
Std. deviation	10.2	25.13	6.978	14.95	10.06	8.232

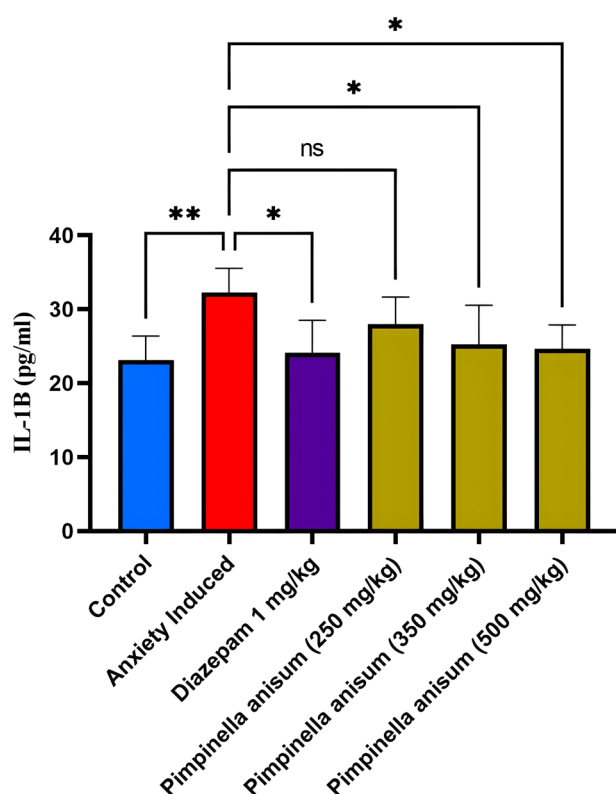


Fig. 7 Effects of diazepam and *Pimpinella anisum* seeds extract on brain tissue IL-1 β . Results were measured by ELISA in pg/ml. Data presented as (means $\pm$ SD) $F(5, 30) = 4.457, P = 0.0037$. $n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

Table 6. Mean and standard deviation of IL-1 β for the experimental groups

Group	1	2	3	4	5	6
Mean	23.14	32.29	24.15	27.97	25.26	24.64
Std. deviation	3.226	3.252	4.351	3.683	5.299	3.25

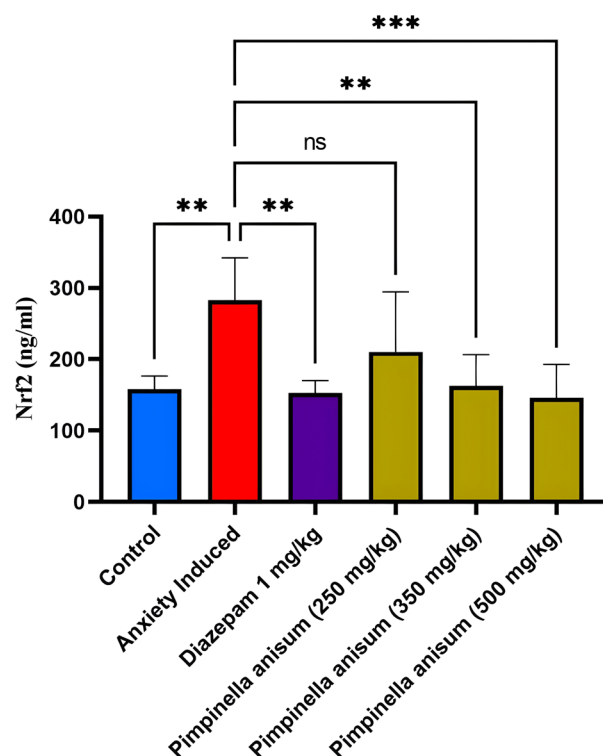


Fig. 8 Effects of diazepam and *Pimpinella anisum* seeds extract on brain tissue NRF2. Results were measured by ELISA in pg/ml. Data presented as (means $\pm$ SD) $F(5, 30) = 6.610, P = 0.0003$. $n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

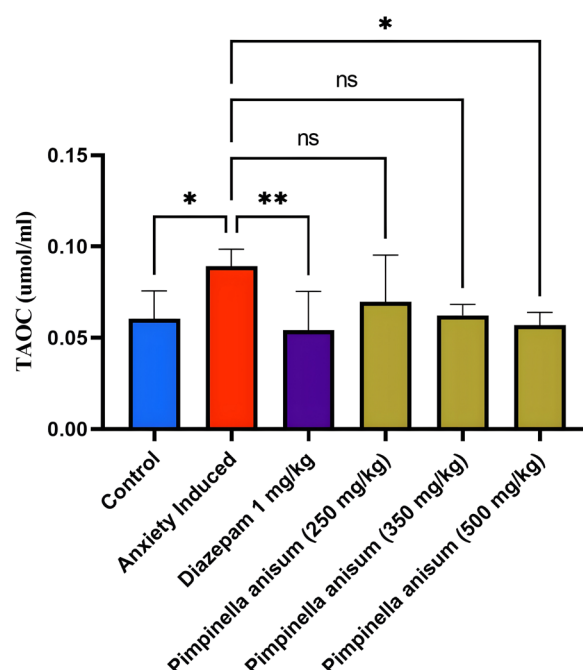


Fig. 9 Effects of diazepam and *Pimpinella anisum* seeds extract on brain tissue TAO. Results were measured by ELISA in $\mu\text{mol}/\text{ml}$. Data presented as (means $\pm$ SD) $F(5, 30) = 3.876, P = 0.0079$. $n = 6$ per group. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$) and ns ($P > 0.05$). Data were analyzed using one-way ANOVA and Tukey's post hoc multiple comparison test.

improved antioxidant status. Notably, *Pimpinella anisum* extract demonstrated a marked decrease in NRF2 levels and total antioxidant capacity, particularly at higher dose (Tables 7 and 8).

Discussion

The study shows that *Pimpinella anisum* extract exhibits significant anxiolytic effects in rat model of anxiety induced by chronic unpredictable stress and caffeine.^{12,15} Behavioral tests and biochemical levels support the capability of *Pimpinella anisum* as a natural therapeutic agent. The increased time in open arms in the EPM and enhanced loco motor activity in the OFT indicate reduced fear and improved behavior.¹⁹ The *Pimpinella anisum* treated groups findings are comparable to those observed with diazepam treated groups. As it was demonstrated in the previous studies one of the findings of this study is the significant role of inflammation in anxiety as observed with the elevated levels of pro inflammatory biomarkers in the brain tissue samples.^{16,24,25} TNF- α and IL-1 β elevated levels in the induction group confirm the involvement of pro-inflammatory cytokines in anxiety pathophysiology.²⁶ *Pimpinella anisum* extract administration in different doses led to a significant decrease in these inflammatory biomarkers. The mechanism involved in this process is related to bioactive substances like trans-and cis-anethole that act on immune responses by modulating pro-inflammatory cytokines.⁸ As demonstrated in the GC mass performed on the seeds of *Pimpinella anisum*, anethole isomers were making up to 71% of the seeds. In addition to its anti-inflammatory effects, *Pimpinella anisum* demonstrated significant antioxidant activity, as evidenced by normalization of NRF2 expression and total antioxidant capacity toward physiological control values, suggesting attenuation of oxidative stress and restoration of redox balance.⁹ NRF2 acts as an important regulator in cellular protection against oxidative stress,²⁷ and its up regulation indicates a defensive response to avoid neuronal injury. The elevation in levels of Nrf2 in the anxiety induction group and their reduction in the treated groups suggest that oxidative stress is an important element of anxiety disorders and that antioxidants could be effective treatments.²⁷ Oxidative stress and oxidative biomarkers have been studied deeply in the recent years, and as it was demonstrated in previous studies, oxidative stress is an etiological factor in the pathogenesis on anxiety.^{28,29} NRF2 regulates the activation of expression of cytoprotective

genes. NRF2 existence in cells mainly regulated by four factors, one of them is KEAP1 which binds NRF2 and degrade it to regulate the levels of NRF2 in cells, ROS that are abundant in increased oxidative stress react with cysteine found in KEAP1 and renders it inactive allowing the NRF2 bypass the regulatory action of KEAP1 and results in increased levels of NRF2 which in turn increases the expression of cytoprotective genes which counteract ROS that contributed in anxiety.³⁰ Total anti-oxidant capacity measurement relays on the ability of enzymes and components of the sample to neutralize oxidants, and the results are directly proportional related to the levels of antioxidants in samples.³¹ Adding this to the findings and interpretation of NRF2 levels, highlighting the anti-oxidative potential of *Pimpinella anisum* extract. In this study Serotonin levels were elevated in the induction group. While this finding may appear paradoxical, it can be explained by knowing the role of serotonin in different regions of the brain and understanding that different serotonin receptors act in complex manner.³² Increased 5HT-3A serotonin receptor gene expression has been related to anxiety as a driver, while 5-HT4 receptors act to reduce and modulates anxiety.³³ As demonstrated in the recent studies that an acute elevation in the level of serotonin especially in the amygdala, hippocampus and bed nucleus of stria terminalis can induce anxiety.^{32,34,35} Paradoxically elevated serotonin levels in the dorsal raphe nucleus contribute to the development of anxiety related behaviors in response to induction.³⁶ The normalization of serotonin levels after treating the groups with diazepam and in dose dependent manner with *Pimpinella anisum* extract indicates that both diazepam and *Pimpinella anisum* have regained neurochemical balance. This finding shows that the plant seed extract may act as an alternative or adjunct to pharmacological treatments. The anxiolytic effects of *Pimpinella anisum* appear to be mediated through multiple mechanisms, including modulation of neurotransmitters, reduction of inflammation, and enhancement of antioxidant defenses. These combined actions provide a comprehensive approach to anxiety management.

Conclusion

Pimpinella anisum has significant anxiolytic activity in animal models of anxiety caused by chronic unpredictable stress and caffeine. The findings showed that the plant extract successfully improved behavioral activity and affected important biomarkers related to anxiety. In particular, the anxiolytic effect of the plant seed extract was found to be dose-dependent since a higher dose of it caused better results comparable to the action of diazepam. The mechanisms involved in the anxiolytic action of *Pimpinella anisum* are multiple and include the reduction in inflammatory biomarkers, such as TNF- α and IL-1 β , activation of the antioxidant response by NRF2, up regulation and alteration of serotonin levels. Also, due to its anti-inflammatory and antioxidant activity, the plant has additional beneficial potentials comparing to the other

Table 7. Mean and standared deviation of Nrf2 for the experimental groups

Group	1	2	3	4	5	6
Mean	158.3	283	152.8	210.5	162.6	146.1
Std. deviation	18.15	59.15	17.34	83.9	43.75	46.75

Table 8. Mean and standared deviation of total antioxidant capacity for the experimental groups

Group	1	2	3	4	5	6
Mean	0.06048	0.08917	0.0542	0.0697	0.06232	0.05692
Std. deviation	0.01525	0.00942	0.02124	0.0256	0.006038	0.006985

pharmacologic agents. Anxiolytic properties of *Pimpinella anisum* extract through modulation of the oxidative stress and ROS have been witnessed by the levels of NRF2 and total anti-oxidant capacity. Additional researches are needed to study long-term safety and clinical utility of the plant extracts.

Study Limitations

The sample size was limited which can reduce the generalizability and statistical role of the findings additionally the duration of the induction and treatment was short to evaluate the long term effects of the plant extract and the study included only male rats which may affect the findings of this study. Not all anxiety related biomarkers were evaluated in this study and the absence of molecular analyses like gene expression affects the power of study findings.

AI Use Statement

Language and grammar editing tools were used exclusively for linguistic improvement. The authors independently verified all scientific content in the paper and take full responsibility for the manuscript.

Conflict of Interest

The authors declare that there are no competing financial and personal interests that could influence the work reported in this paper.

Funding

No funding was received. ■

References

- Penninx BW, Pine DS, Holmes EA, Reif A. Anxiety disorders. *Lancet*. 2021 Mar 6;397(10277):914–27.
- Aletaha R, Kolahi AA, Mousavi Z, Sullman MJ, Safiri S. Anxiety disorders in the Middle East and North Africa region; 1990 to 2021. *Acta neuropsychiatrica*. 2025 Jan 1:1–17.
- Zhao H, Zhou M, Liu Y, Jiang J, Wang Y. Recent advances in anxiety disorders: focus on animal models and pathological mechanisms. *Animal models and experimental medicine*. 2023 Dec 1;6(6):461–75.
- Davies C, Appiah-Kusi E, Wilson R, Blest-Hopley G, Bossong MG, Valmaggia L. Altered relationship between cortisol response to social stress and mediotemporal function during fear processing in people at clinical high risk for psychosis: a preliminary report. *European archives of psychiatry and clinical neuroscience*. 2021 Dec 1;272(3):461–75.
- Edinoff AN, Nix CA, Hollier J, Sagrera CE, Delacroix BM, Abubakar T. Benzodiazepines: uses, dangers, and clinical considerations. *Neurology international*. 2021 Nov 10;13(4):594–607.
- Mustaqeem S, Rauf A, Rehman S. Therapeutic potential of *Pimpinella anisum* L. (*Anisoon*): a comprehensive review of potent herbal drug in traditional medicine. *International journal of unani and integrative medicine*. 2024 May 1;8(3):105–16.
- Sun W, Shahrajabian MH, Cheng Q. Anise (*Pimpinella anisum* L.), a dominant spice and traditional medicinal herb for both food and medicinal purposes. *Cogent biology*. 2019 Jan 1;5(1):1673688.
- Giordano M, Petropoulos SA, Kyriacou MC, Graziani G, Zarrelli A, Roupheal Y. Nutritive and phytochemical composition of aromatic microgreen herbs and spices belonging to the Apiaceae family. *Plants*. 2022 Nov 11;11(22):3057.
- Khodadadian R, Balali-Dehkordi S. A comprehensive review of the neurological effects of anethole. *IBRO neuroscience reports*. 2024 Dec 1;18:50–62.
- Voipio HM, Tsai PP, Brandstetter H, Gyger M, Hackbarth H, Hansen AK. Housing and care of laboratory animals. *Cost manual of laboratory animal care and use refinement, reduction, and research*. 2016 Apr 19:29–73.
- Leal PF, Almeida TS, Prado GH, Prado JM, Meireles MA. Extraction kinetics and anethole content of fennel (*Foeniculum vulgare*) and anise seed (*Pimpinella anisum*) extracts obtained by Soxhlet, ultrasound, percolation, centrifugation, and steam distillation. *Separation science and technology*. 2011 Aug 1;46(11):1848–56.
- Vecchio MG, Gulati A, Minto C, Lorenzoni G. *Pimpinella anisum* and *Illicium verum*: the multifaceted role of anise plants. *The open agriculture journal*. 2016 Oct 14;10(1):81–6.
- Abdollahi Fard M, Shojaii A. Efficacy of Iranian traditional medicine in the treatment of epilepsy. *BioMed research international*. 2013 Jul 25;2013:692751.
- Niksokhan M, Hedarieh N, Najafifard M, Najafifard M. Effect of hydro-alcoholic extract of *Pimpinella anisum* seed on anxiety in male rat. *Journal of Gorgan University of Medical Sciences*. 2014 Jul 10;16(2):28–33.
- Khurana K, Bansal N. Lacidipine attenuates caffeine-induced anxiety-like symptoms in mice: role of calcium-induced oxido-nitrosative stress. *Pharmacological reports*. 2019 Dec 1;71(6):1264–72.
- Al-Abbasi FA. Diazepam ameliorates altered proinflammatory and cardiac markers in stress exposed rats. *Saudi journal of biological sciences*. 2021 Jan 1;28(1):566–71.
- Gamberini MT, Rodrigues DS, Rodrigues D, Pontes VB. Effects of the aqueous extract of *Pimpinella anisum* L. seeds on exploratory activity and emotional behavior in rats using the open field and elevated plus maze tests. *Journal of ethnopharmacology*. 2015 Jun 20;168:45–9.
- Lezak KR, Missig G, Carlezon WA. Behavioral methods to study anxiety in rodents. *Dialogues in clinical neuroscience*. 2017 Jun 1;19(2):181–91.
- Bath KG, Chuang J, Spencer-Segal JL, Amso D, Altemus M, McEwen BS. EPM. *Biological psychiatry*. 2012 Sep 15;72(6):499–504.
- Ferreira J. Factors contributing to the open-field test variability. *Laboratory animals*. 2025 Apr 1;54(2):135–6.
- Es-Safi I, Mechchate H, Amaghnouje A, Elbouzidi A, Bouhrim M, Bencheikh N. Assessment of antidepressant-like, anxiolytic effects and impact on memory of *Pimpinella anisum* L. total extract on Swiss albino mice. *Plants*. 2021 Aug 4;10(8):1573.
- Mohammadi S, Haghir H, Fazeli AR, Vafaei A. Analysis of amygdala nucleus in the rat brain: a review study. *Electronic physician*. 2013 Nov 1;5(4):639–42.
- Schatz KC, Agajanyov M, Varlinskaya EI, Diaz MR. The central amygdala corticotropin releasing factor system regulates anxiety-like behavior in an age- and sex-dependent manner in rats. *Addiction neuroscience*. 2026 Mar 1;19:100257.
- Shalaginova IG, Tuchina OP, Turkin AV, Vylegzhanina AE, Nagumanova AN, Zachepilo TG. The effect of long-term emotional and painful stress on the expression of proinflammatory cytokine genes in rats with high and low excitability of the nervous system. *Journal of evolutionary biochemistry and physiology*. 2023 Apr 1;59(2):642–52.
- Luo Y, Jiang N, Zhang Y, Zhao Y, Chen F, Li X. Chronic unpredictable mild stress induces anxiety-like behavior in female C57BL/6N mice, accompanied by alterations in inflammation and the kynurenine pathway of tryptophan metabolism. *Frontiers in neuroscience*. 2025 Jan 22;19:1556744.
- Hole C, Dhamsania A, Brown C, Ryznar R. Immune dysregulation in depression and anxiety: a review of the immune response in disease and treatment. *Cells*. 2025 Apr 17;14(8):607.
- Bhandari R, Kaur J, Kaur S, Kuhad A. The Nrf2 pathway in psychiatric disorders: pathophysiological role and potential targeting. *Expert opinion on therapeutic targets*. 2021 Feb 1;25(2):115–39.
- Smaga I, Niedzielska E, Gawlik M. Oxidative stress as an etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism. *Pharmacological reports*. 2015 Aug 1;67(4):569–80.

29. Lv H, Zhu C, Wu R, Ni H, Lian J, Xu Y. Chronic mild stress induced anxiety-like behaviors can be attenuated by inhibition of NOX2-derived oxidative stress. *Journal of psychiatric research*. 2019 Jul 1;114:55–66.
30. He F, Ru X, Wen T. NRF2, a transcription factor for stress response and beyond. *International journal of molecular sciences*. 2020 Jul 6;21(13):4777.
31. Silvestrini A, Meucci E, Ricerca BM, Mancini A. Total antioxidant capacity: biochemical aspects and clinical significance. *International journal of molecular sciences*. 2023 Jul 1;24(13):10978.
32. Tseilikman VE, Tseilikman OB, Karpenko MN, Traktirov DS, Obukhova DA, Shatilov VA. Unraveling the serotonergic mechanism of stress-related anxiety: focus on co-treatment with resveratrol and selective serotonin reuptake inhibitors. *Biomedicines*. 2024 Nov 1;12(11):2455.
33. Lin J, Liu W, Guan J, Cui J, Shi R, Wang L. Latest updates on the serotonergic system in depression and anxiety. *Frontiers in synaptic neuroscience*. 2023 Jan 9;15:1124112.
34. Chin PW, Augustine GJ. Serotonergic input into the cerebellar cortex modulates anxiety-like behavior. *Journal of neuroscience*. 2025 May 15;45(20):4112–25.
35. Frick A, Åhs F, Engman J, Jonasson M, Alaie I, Björkstrand J. Serotonin synthesis and reuptake in social anxiety disorder: a positron emission tomography study. *JAMA psychiatry*. 2015 Aug 1;72(8):794–802.
36. Marcinkiewicz CA, Mazzone CM, D'Agostino G, Halladay LR, Hardaway JA, Diberto JF. Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala. *Nature*. 2016 Sep 1;537(7618):97–101.

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