Journal of Medicinal Natural Products

Scilight

Article

Check for updates

Behavioral Evidence of Anxiolytic Activity of Chloroform and Ethyl Acetate Fractions of *Aerva sanguinolenta* (L.) Blume

Joy Sarker [1,*,†], Khirul Islam Razu [2,†], Md. Rashedul Islam [2], and K. M. Khairul Alam [2]

[1] Department of Pharmacy, University of Rajshahi, Rajshahi 6205, Bangladesh
[2] Department of Pharmacy, Jahangirnagar University, Savar 1342, Bangladesh
* Correspondence: joyru.ac.bd@gmail.com
† These authors contributed equally to this work.
Received: 31 January 2026; Revised: 8 May 2026; Accepted: 14 May 2026; Published: 7 July 2026

Abstract: Anxiety disorders are among the most prevalent neuropsychiatric conditions, and current pharmacotherapies are often limited by adverse effects. The present study evaluated the anxiolytic-like activity of the chloroform (CHF) and ethyl acetate (EAF) fractions of *Aerva sanguinolenta* in Swiss albino mice. Acute oral toxicity studies demonstrated that both fractions were safe up to 4000 mg/kg. Anxiolytic activity was assessed using the hole-board test, open field test, and elevated plus maze test, with diazepam as the standard drug. Both CHF and EAF produced significant, dose-dependent anxiolytic-like effects across behavioral models, although their efficacy was lower than diazepam. These findings provide the first experimental evidence supporting the anxiolytic potential of *A. sanguinolenta* and justify further mechanistic investigations to identify the active constituents involved.

Keywords: *Aerva sanguinolenta*; anxiety; hole-board test; elevated plus maze; medicinal plants; anxiolytic activity

1. Introduction

Anxiety disorders represent one of the most prevalent neuropsychiatric conditions worldwide and constitute a major contributor to reduced quality of life and socioeconomic burden. Although benzodiazepines and related anxiolytic agents remain the mainstay of pharmacotherapy, their long-term use is frequently limited by adverse effects such as sedation, cognitive impairment, tolerance, and dependence [1,2]. These limitations have intensified interest in identifying safer, plant-derived alternatives with anxiolytic potential and improved tolerability profiles.

Medicinal plants have historically served as a cornerstone of drug discovery, particularly for disorders of the central nervous system [3,4]. A substantial proportion of the global population, especially in developing countries, continues to rely on plant-based remedies to manage neuropsychological conditions. The therapeutic efficacy of medicinal plants is largely attributed to structurally diverse secondary metabolites, including flavonoids, alkaloids, tannins, saponins, and glycosides, many of which are known to modulate neurotransmitter systems implicated in anxiety [5–7]. Notably, several phytoconstituents have demonstrated affinity for γ-aminobutyric acid ($GABA_A$) receptors and serotonergic pathways, which play central roles in the neurobiology of anxiety [8].

Oxidative stress has also emerged as a critical factor in the pathophysiology of anxiety disorders [9]. Chronic stress-induced overproduction of reactive oxygen species can disrupt neuronal homeostasis, impair synaptic plasticity, and exacerbate anxiety-like behaviors [10]. Consequently, plant extracts possessing both antioxidant and neuroactive properties may offer dual therapeutic advantages in anxiety management [11].

Aerva sanguinolenta (L.) Blume (family Amaranthaceae), locally known in Bangladesh as “Lal Bish Hori”, is traditionally used for the treatment of various ailments, including inflammatory conditions, urinary disorders, and skin diseases [12]. Preliminary pharmacological investigations have reported antioxidant and antimicrobial activities of this plant [13]. In our previous studies, we conducted a comprehensive phytochemical and biological evaluation of *A. sanguinolenta* extracts and demonstrated that the chloroform (CHF) and ethyl acetate (EAF) fractions exhibited significantly higher phenolic content and superior antioxidant and anticancer activities compared to the crude methanolic extract (CME) [14]. These findings suggest the enrichment of bioactive constituents within these semi-polar fractions that may exert neuromodulatory effects.

Despite its traditional use and emerging pharmacological relevance, no systematic *in vivo* investigation has been conducted to evaluate the anxiolytic potential of *A. sanguinolenta*. Moreover, the effects of its bioactive fractions on anxiety-related behavioral paradigms remain unexplored. Therefore, the present study was designed to evaluate the anxiolytic activity of the chloroform and ethyl acetate fractions of *A. sanguinolenta* using established behavioral models in Swiss albino mice, including the hole-board test, open field test, and elevated plus maze test. Acute oral toxicity assessment was performed to ensure safety and guide dose selection. Through this investigation, we aimed to provide experimental evidence supporting the potential of *A. sanguinolenta* as a natural source of anxiolytic agents and to establish a scientific basis for its traditional use.

2. Materials and Methods

2.1. Plant Collection and Identification

Flowering shrub species *Aerva sanguinolenta* was collected from the Rajshahi University Campus, Bangladesh (24°22′26″ N 88°36′04″ E) at an altitude of approximately 23 m above sea level in November-December 2019. Every kind of counterfeit was eliminated following plant collecting so that solely clean specimens of plants remained. A voucher specimen with the accession number 46770 was later supplied for future use when the dried sample was identified and submitted to the Bangladesh National Herbarium in Mirpur, Dhaka.

2.2. Extraction

The collected plant samples were shade-dried to ensure the active constituents were free from decomposition. Then, those were pulverized with a grinder to have a powdered form. An amount of 400 g of *A. sanguinolenta* powder was transferred to spotless glass containers with flat bottoms, immersed in 2 L of 100% methanol, followed by a 14-day period with intermittent stirring. After that, a rotary evaporator was used to obtain a gummy *A. sanguinolenta* methanolic extract of 21.98 g (yield = 5.495%).

2.3. Fractionation

The crude extract of *A. sanguinolenta* (21.98 g) was subjected to solvent–solvent partitioning based on increasing polarity. Briefly, 18.395 g of the extract was suspended in distilled water and successively partitioned with n-hexane, chloroform, and ethyl acetate using a separatory funnel. Each solvent extraction was repeated multiple times to ensure exhaustive partitioning. The organic layers were collected separately and concentrated under reduced pressure using a rotary evaporator to obtain the respective fractions. Among the obtained fractions, the chloroform (CHF) and ethyl acetate (EAF) fractions yielded 2.14 g and 0.985 g, respectively. These two fractions were selected for further pharmacological evaluation based on our previous study [14], in which they exhibited significantly higher total phenolic and flavonoid contents, along with superior antioxidant and cytotoxic activities compared to the n-hexane and aqueous fractions.

2.4. Preliminary Phytochemical Study

Preliminary phytochemical screening of the crude methanolic extract (CME) of *A. sanguinolenta* revealed the presence of several bioactive secondary metabolites, including flavonoids, alkaloids, phenolics, saponins, terpenoids, and glycosides [15]. These qualitative findings were further supported by instrumental analyses. GC–MS profiling identified multiple biologically active compounds [14], while HPLC analysis confirmed the presence of known polyphenolic constituents such as quercetin, kaempferol, and myricetin [16]. The enrichment of semi-polar compounds such as flavonoids and phenolics in the chloroform and ethyl acetate fractions may contribute to their observed pharmacological activities, including anxiolytic effects.

2.5. Animals

Healthy male Swiss albino mice (11–12 weeks old), weighing 30–35 g, were used for the experiments. The animals were obtained from the animal house of the Department of Pharmacy, University of Rajshahi, Bangladesh. The mice were housed under standard laboratory conditions, with a controlled temperature of 27 ± 2 °C, relative humidity of 55 ± 10%, and a 12 h light/dark cycle. They were provided with standard commercial rodent feed and had free access to water. All animals were acclimatized to the laboratory environment for at least one week before the experiments. All experimental procedures involving animals were conducted in accordance with internationally accepted guidelines for the care and use of laboratory animals. The study protocol was reviewed

and approved by the Institutional Animal Ethics Committee of the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh on December 2019 (Approval No: 72 (23)/320/IAMEBBC/IBSc).

2.6. Acute Toxicity Study

Acute toxicity research was conducted in accordance with the OECD (Organisation for Economic Co-operation and Development) recommendations No. 425. CHF and EAF were separately delivered orally in dosages of 100, 200, 400, 800, 1000, and 2000 mg/kg to male mice. The percentage mortality was recorded throughout 24 h. During the first hour following sample administration, the mice were monitored for any gross behavioral change and the parameters assessed, including grooming, hyperactivity, drowsiness, convulsions, breathing, lack of righting reflex, urination, salivation, and defecation [17].

2.7. Drugs

The standard drug diazepam was purchased from Incepta Pharmaceuticals Limited, Bangladesh, and sodium carboxy methyl cellulose (Na-CMC) was obtained from CDH-Laboratory Reagent Pvt. Ltd., New Delhi, India.

2.8. Evaluation of Anxiolytic Activity

2.8.1. Experimental Design and Treatment Protocol

The animals were randomly divided into six groups (n = 5 per group):

- Group I (Control): received vehicle (Na-CMC, 1%, p.o.)
- Group II (Standard): received diazepam (1 mg/kg, p.o.)
- Group III (CHF-200): received chloroform fraction (200 mg/kg, p.o.)
- Group IV (CHF-400): received chloroform fraction (400 mg/kg, p.o.)
- Group V (EAF-200): received ethyl acetate fraction (200 mg/kg, p.o.)
- Group VI (EAF-400): received ethyl acetate fraction (400 mg/kg, p.o.)

All treatments were administered orally using an intragastric feeding needle. Behavioral assessments were conducted 30 min after treatment administration. The same grouping and dosing regimen were followed for all behavioral experiments.

Hole-Board Test

The hole-board test was performed to evaluate exploratory behavior and anxiety-like responses in mice, following a previously described method with slight modifications [18]. Thirty minutes after treatment, each mouse was individually placed at the center of the hole-board apparatus and allowed to explore freely for 5 min. The number of head-dipping events (insertion of the head into the holes) was recorded as an index of exploratory activity. A decrease in the number of head dips was considered indicative of anxiolytic-like activity. The percentage inhibition of head-dipping behavior was calculated relative to the control group. The apparatus was cleaned with 70% ethanol between trials to eliminate olfactory cues.

Open Field Test

The open field test was conducted to evaluate locomotor and exploratory behavior in a novel environment [19]. The apparatus consisted of a square arena (60 cm × 60 cm × 60 cm) divided into nine equal sections. Thirty minutes after treatment, each mouse was placed gently at the center of the arena and allowed to explore freely for 5 min. The number of squares crossed and entries into the central zone were recorded as indices of exploratory behavior. An increase in locomotor activity and central zone exploration was considered indicative of anxiolytic-like activity. The arena was cleaned with 70% ethanol between trials to avoid olfactory bias.

Elevated Plus Maze Test

The elevated plus maze test was performed to assess anxiety-like behavior using a standard method with minor modifications [20]. The apparatus consisted of two open arms and two closed arms elevated above the floor. Thirty minutes after treatment, each mouse was placed individually at the center of the maze, facing an open arm, and allowed to explore for 5 min. The number of entries into the open arms and the time spent in open and closed arms were recorded. An arm entry was defined as the placement of all four paws into an arm. An increase in open-arm entries and time spent in open arms was considered indicative of anxiolytic-like activity. The maze was cleaned with 70% ethanol between trials.

2.9. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons using SPSS software (version 25, IBM, Chicago, IL, USA). A p-value of <0.05 was considered statistically significant. Graphs were generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Phytochemical Screening

Preliminary phytochemical analysis of the crude methanolic extract (CME) of *A. sanguinolenta* revealed the presence of several bioactive secondary metabolites (Table 1). The extract showed a high abundance (+++) of flavonoids and phenolic compounds, moderate amounts (++) of glycosides, and mild presence (+) of saponins, terpenoids, and alkaloids. However, tannins and proteins were not detected in the extract.

Table 1. Qualitative tests of crude methanolic extract of *A. sanguinolenta*.

Phyto-Constituents	Saponins	Tannins	Flavonoids	Terpenoid	Glycoside	Protein	Phenolics	Alkaloid
CME	+	-	+++	+	++	-	+++	+

(-) = Not present; (+) = Present in mild amount; (++) = Present in moderate amount; (+++) = Present in significant amount. Here, CME = Crude methanolic extract.

3.2. Acute Oral Toxicity Studies and Dose Selection

Acute oral toxicity evaluation demonstrated that the chloroform (CHF) and ethyl acetate (EAF) fractions were well tolerated at doses up to 4000 mg/kg. No signs of toxicity or mortality were observed following oral administration of *A. sanguinolenta* extracts at this dose level. Based on these findings, the extracts were considered safe and unclassified under acute toxicity criteria. Consequently, experimental doses of 200 and 400 mg/kg, corresponding to one-tenth of the maximum tolerated dose, were selected for subsequent studies.

3.3. The Anxiolytic Effect of A. sanguinolenta Fractions in the Hole-Board Test

The anxiolytic activity of the chloroform (CHF) and ethyl acetate (EAF) fractions of *A. sanguinolenta* was evaluated using the hole-board test by measuring the number of head-dipping events in Swiss albino mice. As presented in Table 2, the control group exhibited a high frequency of head dipping, whereas diazepam (1 mg/kg) significantly reduced head-dipping events compared with the control group ($p < 0.05$). Oral administration of both CHF and EAF resulted in a dose-dependent decrease in the number of head dips. At 200 mg/kg, CHF and EAF produced a significant reduction in head-dipping frequency compared with the control group ($p < 0.05$). A more pronounced effect was observed at 400 mg/kg, where both fractions significantly suppressed head-dipping events relative to the control and low-dose treatment groups ($p < 0.05$). However, the reduction in head dipping produced by the extract-treated groups remained less pronounced than that observed with diazepam. The percentage inhibition of head-dipping events further confirmed the anxiolytic-like effect of CHF and EAF, with higher inhibition observed at the 400 mg/kg dose (Figure 1).

Table 2. Effects of *A. sanguinolenta* fractions on exploratory behavior in the hole-board test conducted on Swiss albino mice.

Treatment Group	Dose (mg/kg)	Average No. of Head-Dipping
Control (Na-CMC)	-	$60.0 \pm 1.30^{\theta,\blacktriangle,\Delta,\omega,\psi}$
Standard (diazepam)	1	$13.5 \pm 0.62^{*,\blacktriangle,\Delta,\omega,\psi}$
CHF	200	$43.6 \pm 1.44^{*,\theta,\Delta,\omega,\psi}$
CHF	400	$21.9 \pm 1.04^{*,\theta,\blacktriangle,\omega}$
EAF	200	$32.2 \pm 0.93^{*,\theta,\blacktriangle,\Delta}$
EAF	400	$23.4 \pm 0.62^{*,\theta,\blacktriangle,\omega,\psi}$

Note: CHF: chloroform fraction; EAF: ethyl acetate fraction. Statistics represent averages across five repetitions ± SEM; * $p < 0.05$ vs. control; $^{\theta}$ $p < 0.05$ vs. diazepam 1 mg/kg; $^{\blacktriangle}$ $p < 0.05$ vs. chloroform fraction 200 mg/kg; $^{\Delta}$ $p < 0.05$ vs. chloroform fraction 400 mg/kg; $^{\omega}$ $p < 0.05$ vs. ethyl acetate fraction 200 mg/kg; $^{\psi}$ $p < 0.05$ vs. ethyl acetate fraction 400 mg/kg (Dunnett's t test).

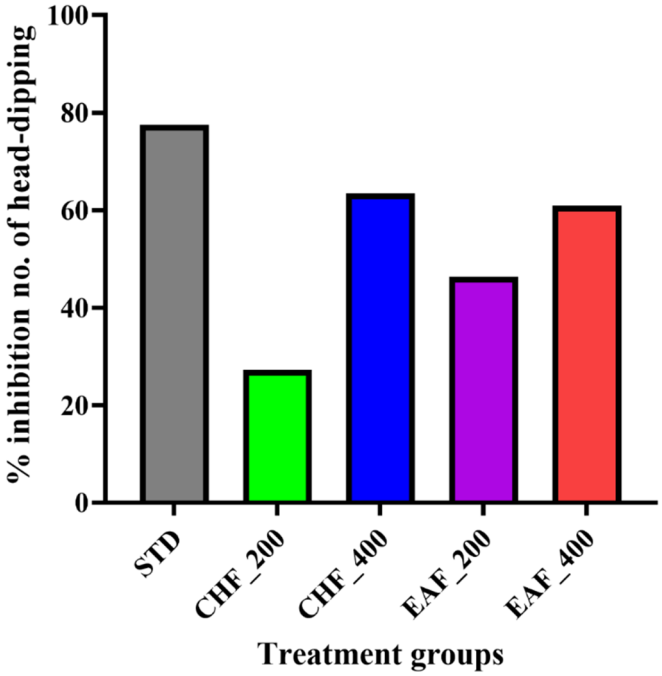

Figure 1. The percent of head dipping inhibition in the hole-board test conducted on Swiss albino mice. Here, STD = Standard (Diazepam); CHF = Chloroform fraction; EAF = Ethyl acetate fraction.

3.4. The Anxiolytic Effect of A. sanguinolenta Fractions in the Open Field Test

The effects of the chloroform (CHF) and ethyl acetate (EAF) fractions of *A. sanguinolenta* on locomotor and exploratory activity were evaluated using the open field test. As shown in Figure 2, treatment with both CHF and EAF increased the number of squares crossed compared with the control group. This increase was dose-dependent and statistically significant at higher doses ($p < 0.01$). However, the effect remained less pronounced than that observed with diazepam. The enhanced locomotor activity suggests increased exploratory behavior in the treated animals.

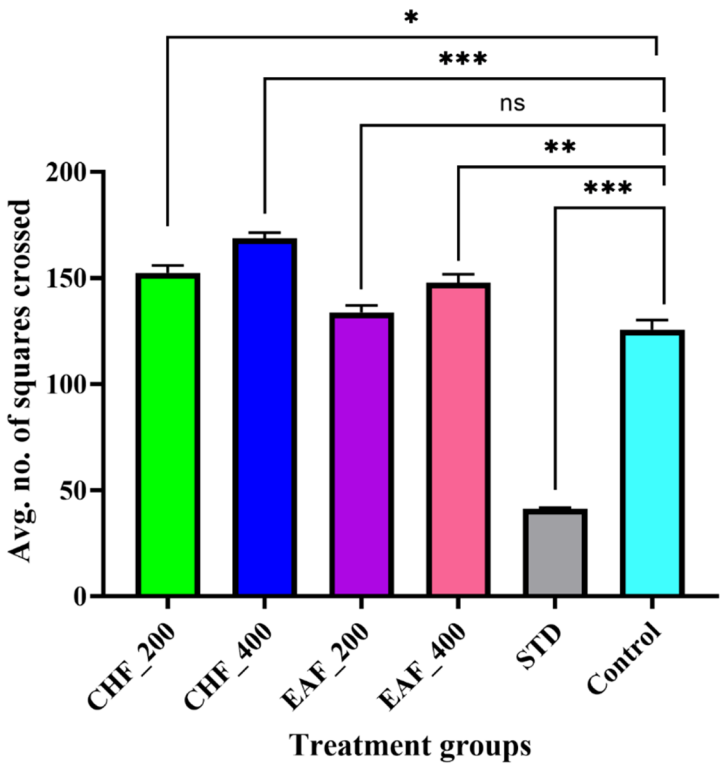

Figure 2. Effect of *A. sanguinolenta* fractions on the number of squares crossed in the open field test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant, as compared to control group.

3.5. Anxiolytic Effect of A. sanguinolenta Fractions in the Elevated Plus Maze Test

The anxiolytic activity of the CHF and EAF fractions was further assessed using the elevated plus maze test by recording the number of open-arm entries and the time spent in the closed arms. The results are summarized in Table 3. Diazepam (1 mg/kg) produced a significant alteration in behavioral parameters compared with the control group ($p < 0.05$). Treatment with CHF and EAF at doses of 200 and 400 mg/kg significantly affected open-arm exploration and closed-arm occupancy in a dose-dependent manner. At 400 mg/kg, both fractions significantly modified the number of open-arm entries compared with the control group ($p < 0.05$), while lower doses showed comparatively weaker effects. Similarly, the time spent in the closed arms was significantly altered in the extract-treated groups, with a more pronounced effect observed at the higher dose.

Overall, the behavioral changes induced by CHF and EAF in the elevated plus maze test were statistically significant when compared with the control group, though their effects were less pronounced than those produced by the standard anxiolytic drug diazepam.

Table 3. Effects of *A. sanguinolenta* fractions on anxiolytic activity on Swiss albino mice in the elevated plus-maze test.

Treatment Group	Dose (mg/kg)	Avg. No of Open Arm Entries	Avg. Time Spent in Closed Arm (Sec)
Control (Na-CMC)	-	$14.1 \pm 0.98^{\theta,\blacktriangle,\Delta,\omega,\psi}$	$141.5 \pm 5.36^{\psi}$
Standard (diazepam)	1	$3.1 \pm 0.42^{*,\blacktriangle,\omega}$	225.7 ± 10.95
CHF	200	$5.7 \pm 0.48^{*,\theta,\Delta,\psi}$	$172.4 \pm 7.93^{\psi}$
CHF	400	$3.2 \pm 0.37^{*,\blacktriangle,\omega}$	214.5 ± 12.75
EAF	200	$5.5 \pm 0.32^{*,\theta,\Delta,\psi}$	175.6 ± 15.44
EAF	400	$3.4 \pm 0.36^{*,\blacktriangle,\omega}$	$245.0 \pm 13.96^{*\blacktriangle}$

Note: CHF: chloroform fraction; EAF: ethyl acetate fraction. Statistics represent averages across five repetitions ± SEM; * $p < 0.05$ vs. control; $^{\theta}$ $p < 0.05$ vs. diazepam 1 mg/kg; $^{\blacktriangle}$ $p < 0.05$ vs. chloroform fraction 200 mg/kg; $^{\Delta}$ $p < 0.05$ vs. chloroform fraction 400 mg/kg; $^{\omega}$ $p < 0.05$ vs. ethyl acetate fraction 200 mg/kg; $^{\psi}$ $p < 0.05$ vs. ethyl acetate fraction 400 mg/kg (Dunnett's *t* test).

4. Discussion

Anxiety is a conserved behavioral response that enables organisms to adapt to novel or potentially threatening environments; however, persistent or exaggerated anxiety represents a pathological condition that adversely affects quality of life [21]. Current pharmacological management relies predominantly on benzodiazepines and related agents, which, despite their efficacy, are associated with sedation, tolerance, and dependence following prolonged use [1,2]. These limitations have driven sustained interest in identifying plant-derived anxiolytic agents with improved safety profiles.

In the present study, the anxiolytic potential of the chloroform (CHF) and ethyl acetate (EAF) fractions of *Aerva sanguinolenta* was evaluated using validated behavioral models in mice. Acute oral toxicity testing demonstrated that both fractions were well tolerated at doses up to 4000 mg/kg, indicating a wide margin of safety and supporting their suitability for behavioral assessment.

The hole-board test is a well-established paradigm for assessing exploratory behavior and emotional reactivity in rodents exposed to a novel environment [22]. In this model, alterations in head-dipping behavior reflect changes in the animal's emotional state. In the present study, treatment with CHF and EAF produced a significant, dose-dependent reduction in head-dipping frequency compared with the control group, indicating anxiolytic-like activity. Similar findings have been reported for several medicinal plants rich in flavonoids and phenolic compounds. For example, extracts of *Passiflora incarnata*, *Valeriana officinalis*, and *Bacopa monnieri* have demonstrated anxiolytic effects in the hole-board test through modulation of central neurotransmitter systems, particularly the GABAergic pathway [23–25]. The comparable behavioral responses observed in the present study suggest that the bioactive constituents of *A. sanguinolenta* may exert similar neuropharmacological effects.

The elevated plus maze is considered one of the most reliable experimental models for evaluating anxiolytic activity in rodents [26]. In this study, treatment with CHF and EAF increased exploratory behavior in the open arms, indicating reduced anxiety-like responses. Comparable effects have been reported for several phytochemical-rich medicinal plants, including *Matricaria chamomilla*, *Piper methysticum*, and *Ginkgo biloba*, which have shown anxiolytic properties in elevated plus maze studies [27–29]. Flavonoids and phenolic compounds identified in *A. sanguinolenta* may contribute to the observed effects, as many flavonoids are known to interact with $GABA_A$ receptor complexes like benzodiazepines.

The open field test is commonly used to evaluate locomotor and exploratory activity associated with emotional behavior [30]. The observed changes in locomotor activity following treatment with CHF and EAF are consistent with reports of other plant-derived anxiolytic agents evaluated using the same model. Previous studies on extracts of *Centella asiatica*, *Withania somnifera*, and *Melissa officinalis* demonstrated altered exploratory

behavior and reduced anxiety-like responses in rodents [31–33]. Since locomotor suppression may also indicate sedative activity, the open field findings in the present study were interpreted alongside the results of the hole-board and elevated plus maze tests, which collectively support anxiolytic-like activity of the tested fractions.

The anxiolytic-like effects observed in the present study may be attributed to the presence of flavonoids, phenolics, and other secondary metabolites identified during preliminary phytochemical screening. Several flavonoids, including quercetin and kaempferol, have previously been reported to possess neuroactive properties through modulation of GABAergic and serotonergic neurotransmission. In addition, the antioxidant activity of these compounds may contribute to neuroprotection and stabilization of neuronal signaling associated with anxiety-related behavior.

Despite these promising findings, the present study has certain limitations. The precise molecular mechanisms underlying the anxiolytic-like effects of *A. sanguinolenta* were not investigated, and receptor-level interactions were not evaluated. Furthermore, biochemical markers of oxidative stress and neurotransmitter modulation were not assessed. Future studies involving receptor-binding assays, neurotransmitter quantification, and isolation of active constituents are warranted to elucidate the mechanisms responsible for the observed behavioral effects.

Overall, the findings of this study provide the first experimental evidence supporting the anxiolytic potential of *A. sanguinolenta* and justify further pharmacological and mechanistic investigations to validate its therapeutic relevance.

5. Conclusions

The chloroform and ethyl acetate fractions of *Aerva sanguinolenta* demonstrated significant anxiolytic-like effects in validated behavioral models in mice and were well tolerated in acute toxicity studies. Although their effects were less pronounced than diazepam, the findings indicate central nervous system–mediated activity, particularly at higher doses. These results provide the first experimental evidence supporting the anxiolytic potential of *A. sanguinolenta*. Further studies are required to elucidate the underlying mechanisms and identify the active constituents responsible for these effects.

Author Contributions: J.S.: conceptualization, visualization, methodology, formal analysis, writing original manuscript, correspondence; K.I.R.: methodology, review & edit manuscript; M.R.I.: software, review & edit manuscript; K.M.K.A.: validation, fund acquisition, review & edit manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All methods of animal experiments were implemented following proper rules and directives of the institution, along with ethical authorization granted from the Institute of Biological Sciences (IBSc), Rajshahi University, Bangladesh (license no: 72 (23)/320/IAMEBBC/IBSc).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Acknowledgments: The authors wish to thank the Faculty of Science for providing a research grant (2048/5/52/R.U./Science-48/2023-24) and the Department of Pharmacy, University of Rajshahi, for their kind support in the progress of the research.

Conflicts of Interest: The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies: During the preparation of this work, the authors used ChatGPT to enhance readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the final manuscript.

Abbreviations

CME	crude methanolic extract
CHF	chloroform fraction
EAF	ethyl acetate fraction
Na-CMC	sodium carboxymethyl cellulose
SEM	standard error of the mean
OECD	Organisation for Economic Co-operation and Development
CNS	central nervous system
ANOVA	analysis of variance

References

1. Stevens, J.C.; Pollack, M.H. Benzodiazepines in clinical practice: Consideration of their long-term use and alternative agents. *J. Clin. Psychiatry* **2005**, *66*, 21–27.

2. Sanabria, E.; Cuenca, R.E.; Esteso, M.Á.; et al. Benzodiazepines: Their use either as essential medicines or as toxic substances. *Toxics* **2021**, *9*, 25. https://doi.org/10.3390/toxics9020025.
3. Chaachouay, N.; Zidane, L. Plant-derived natural products: A source for drug discovery and development. *Drugs Drug Candidates* **2024**, *3*, 184–207. https://doi.org/10.3390/ddc3010011.
4. Ratheesh, G.; Tian, L.; Venugopal, J.R.; et al. Role of medicinal plants in neurodegenerative diseases. *Biomanufacturing Rev.* **2017**, *2*, 2. https://doi.org/10.1007/s40898-017-0004-7.
5. Singla, R.; Kamboj, S.; Duvey, B.K.; et al. Flavonoids and anxiety: Decoding their role in brain function and pathophysiology. *Med. Drug Discov.* **2025**, *27*, 100214. https://doi.org/10.1016/j.medidd.2025.100214.
6. Fedotova, J.; Kubatka, P.; Büsselberg, D.; et al. Therapeutical strategies for anxiety and anxiety-like disorders using plant-derived natural compounds and plant extracts. *Biomed. Pharmacother.* **2017**, *95*, 437–446. https://doi.org/10.1016/j.biopha.2017.08.107.
7. García Ríos, R.; Mora-Perez, A.; Ramos-Molina, A.; et al. Neuropharmacology of secondary metabolites from plants with anxiolytic and antidepressant properties. In *Behavioral Pharmacology—From Basic to Clinical Research*; IntechOpen: Rijeka, Croatia, 2020; ISBN 978-1-78985-679-8.
8. Alramadhan, E.; Hanna, M.S.; Goldstein, T.A.; et al. Dietary and botanical anxiolytics. *Med. Sci. Monit.* **2012**, *18*, RA40–RA48. https://doi.org/10.12659/MSM.882608.
9. Fedoce, A.G.; Ferreira, F.; Bota, R.G.; et al. The role of oxidative stress in anxiety disorder: Cause or consequence? *Free Radic. Res.* **2018**, *52*, 737–750. https://doi.org/10.1080/10715762.2018.1475733.
10. Trofin, D.-M.; Sardaru, D.-P.; Trofin, D.; et al. Oxidative stress in brain function. *Antioxidants* **2025**, *14*, 297. https://doi.org/10.3390/antiox14030297.
11. Tkaczenko, H.; Buyun, L.; Kołodziejska, R.; et al. Neuroactive phytochemicals as multi-target modulators of mental health and cognitive function: An integrative review. *Int. J. Mol. Sci.* **2025**, *26*, 8907. https://doi.org/10.3390/ijms26188907.
12. Sarker, J.; Ali, M.R.; Khan, M.A.; et al. The plant *Aerva sanguinolenta*: A review on traditional uses, phytoconstituents and pharmacological activities. *Pharmacogn. Rev.* **2019**, *13*, 89–92. https://doi.org/10.5530/phrev.2019.2.9.
13. Maqbool, M.; Muhammad, A.; Muhammad, I.; et al. Investigation of antimicrobial and antioxidant activity of *Aerva sanguinolenta* (L.) Blume from district Bhimber (AJK), Pakistan. *Biosci. Res.* **2021**, *18*, 640–652.
14. Sarker, J.; Reza, A.S.M.A.; Ashraful, A.B.M.; et al. *In vitro* antioxidant and p53-targeted anticancer activity of *Aerva sanguinolenta* (L.) Blume across different human cancer cell lines and Ehrlich ascites carcinoma model. *J. Chem. Health Risks* **2026**, *16*, 1292–1316.
15. Sarker, J.; Nawaz, S.M.B.; Yeasmin, M.N.; et al. Phytochemical profile, acute toxicity, and antinociceptive potential of methanolic extract of *Aerva sanguinolenta* (L.) Blume in Swiss albino mice. *J. Chem. Health Risks* **2026**, *16*, 3431–3442.
16. Sarker, J.; Ashraful, A.B.M.; Hossain, M.S.; et al. Identification and *in silico* evaluation of polyphenolic compounds from *Aerva sanguinolenta* (L.) Blume for anticancer potential. *Nat. Prod. Res.* **2025**, 1–5. https://doi.org/10.1080/14786419.2025.2598825.
17. Vogel, H.G.; Vogel, W.H.; Schölkens, B.A.; et al. (Eds.) *Drug Discovery and Evaluation*; Springer: Berlin/Heidelberg, Germany, 2002.
18. Islam, A.; Hussain, M.S.; Sen, N.; et al. Investigation of *in vitro* thrombolytic and anti-helminthic activity and *in vivo* anxiolytic and antidepressant potentiality with phytochemical nature of methanolic extract of *Leucas lavandulifolia*. *Sustain. Chem. Pharm.* **2017**, *6*, 61–66. https://doi.org/10.1016/j.scp.2017.09.001.
19. Walsh, R.N.; Cummins, R.A. The open-field test: A critical review. *Psychol. Bull.* **1976**, *83*, 482–504.
20. Pellow, S.; File, S.E. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol. Biochem. Behav.* **1986**, *24*, 525–529. https://doi.org/10.1016/0091-3057(86)90552-6.
21. Kenwood, M.M.; Kalin, N.H.; Barbas, H. The prefrontal cortex, pathological anxiety, and anxiety disorders. *Neuropsychopharmacology* **2022**, *47*, 260–275. https://doi.org/10.1038/s41386-021-01109-z.
22. d'Isa, R.; Comi, G.; Leocani, L. The 4-hole-board test for assessment of long-term spatial memory in mice. *Curr. Protoc.* **2021**, *1*, e228. https://doi.org/10.1002/cpz1.228.
23. Dhawan, K.; Kumar, S.; Sharma, A. Anxiolytic activity of aerial and underground parts of *Passiflora incarnata*. *Fitoterapia* **2001**, *72*, 922–926. https://doi.org/10.1016/s0367-326x(01)00322-7.
24. Hattesohl, M.; Feistel, B.; Sievers, H.; et al. Extracts of *Valeriana officinalis* L. s.l. show anxiolytic and antidepressant effects but neither sedative nor myorelaxant properties. *Phytomedicine* **2008**, *15*, 2–15. https://doi.org/10.1016/j.phymed.2007.11.027.
25. Bhattacharya, S.K.; Ghosal, S. Anxiolytic activity of a standardized extract of *Bacopa monniera*: An experimental study. *Phytomedicine* **1998**, *5*, 77–82. https://doi.org/10.1016/S0944-7113(98)80001-9.
26. Walf, A.A.; Frye, C.A. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* **2007**, *2*, 322–328. https://doi.org/10.1038/nprot.2007.44.

27. Amsterdam, J.D.; Li, Y.; Soeller, I.; et al. A randomized, double-blind, placebo-controlled trial of oral *Matricaria recutita* (chamomile) extract therapy for generalized anxiety disorder. *J. Clin. Psychopharmacol.* **2009**, *29*, 378–382. https://doi.org/10.1097/JCP.0b013e3181ac935c.
28. Rex, A.; Morgenstern, E.; Fink, H. Anxiolytic-like effects of kava-kava in the elevated plus maze test—A comparison with diazepam. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2002**, *26*, 855–860. https://doi.org/10.1016/S0278-5846(01)00330-X.
29. Woelk, H.; Arnoldt, K.H.; Kieser, M.; et al. *Ginkgo biloba* special extract EGb 761 in generalized anxiety disorder and adjustment disorder with anxious mood: A randomized, double-blind, placebo-controlled trial. *J. Psychiatr. Res.* **2007**, *41*, 472–480. https://doi.org/10.1016/j.jpsychires.2006.05.004.
30. Kraeuter, A.-K.; Guest, P.C.; Sarnyai, Z. The open field test for measuring locomotor activity and anxiety-like behavior. *Methods Mol. Biol.* **2019**, *1916*, 99–103. https://doi.org/10.1007/978-1-4939-8994-2_9.
31. Wijeweera, P.; Arnason, J.T.; Koszycki, D.; et al. Evaluation of anxiolytic properties of gotukola—(*Centella asiatica*) extracts and asiaticoside in rat behavioral models. *Phytomedicine* **2006**, *13*, 668–676. https://doi.org/10.1016/j.phymed.2006.01.011.
32. Bhattacharya, S.K.; Satyan, K.S.; Shibnath Ghosal, S. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian J. Exp. Biol.* **1997**, *35*, 236–239.
33. Taiwo, A.E.; Leite, F.B.; Lucena, G.M.; et al. Anxiolytic and antidepressant-like effects of *Melissa officinalis* (lemon balm) extract in rats: Influence of administration and gender. *Indian J. Pharmacol.* **2012**, *44*, 189–192. https://doi.org/10.4103/0253-7613.93846.