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Comprehensive Assessment of Anxiolytic Properties in 4-HPAA Derivatives: Bridging *in Vivo* Validation and Molecular Docking Analyses

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Abstract

Anxiety is a significant mental health issue that substantially affects an individual's quality of life. Feelings of uneasiness, irritability, and sleep disturbances characterize it. 4-Hydroxyphenyl acetic acid (4-HPAA) is identified in brain cells as a physiological byproduct of tyramine. This study hypothesizes that 4-HPAA may regulate anxiety due to its anxiolytic properties, acting as a modulator of the GABAergic system, which plays a crucial role in the pathophysiology of anxiety disorders. Our study aims to enhance the anxiolytic effects of 4-HPAA through chemical modification to improve its pharmacokinetic properties. Three derivatives, namely Isopropyl-4-hydroxy-[phenyl] acetate (IHPA), Isopropyl-4-hydroxy-[phenyl] acetate (MPAA), and 4-methoxyphenyl acetate (MPHA), have been synthesized from 4-HPAA. This assessment will use well-established animal models, specifically the Elevated Plus-Maze (EPM) and Zero Maze (EZM) tests, selected for their validity in replicating anxiety-like symptoms in animals. Chronic caffeine administration via drinking

water (0.3 g/l for 14 days) was employed to induce an anxiety state for testing purposes. IHPA and MPAA demonstrated significant anxiolyticactivity when tested in the EPM and EZM experiments. Molecular docking simulations using AutoDock Vina indicated that 4-HPAA derivatives had docking scores ranging from −5.8 to −4.8 kcal/mol, compared to the standard anxiolytic medication Diazepam, which scored −7.1 kcal/mol. These scores suggest a potential for 4-HPAA derivatives to interact effectively with the Gamma-aminobutyric acid (GABA_A) receptor. In conclusion, our *in vivo* and in silico analyses indicate a promising anxiolytic potential for 4-HPAA derivatives.

Keywords

Anxiolytics, 4-HPAA, Molecular Docking, Elevated Plus-Maze, Zero Maze Tests

1. Introduction

Anxiety, a widespread emotional condition, can lead to various psychological disorders if left untreated. Its origins involve genetic and epigenetic factors affecting neurotransmitter balance, particularly in the amygdala [1]. Dysfunctional brain circuit regulation results in autonomic hyperactivity and an overactive hypothalamic-pituitary-adrenal axis, both of which have been linked to anxiety and depression [2]. Neural circuits, including inhibitory GABAergic interneurons, play a significant role in anxiety symptoms [3]. Existing pharmacological treatments, such as benzodiazepines and antidepressants, are effective but pose risks of dependency and adverse effects. Therefore, innovative therapies with safer profiles are needed for improved anxiety management, driving advancements in pharmaceutical research and development. Identifying safe and effective therapeutic agents is crucial for enhancing mental health treatment [4].

Across the globe, individuals are increasingly turning to traditional medicine as a primary means of healthcare, with a particular emphasis on the utilization of herbal remedies [5]. The application of therapeutic herbs relies primarily on anecdotal evidence rather than contemporary scientific theory. While medicinal plants have the potential to treat a diverse array of illnesses, they are often subject to improper use or commercialization in the absence of adequate scientific investigation. Therefore, within the framework of present-day scientific inquiry, it is imperative to conduct comprehensive investigations into these botanical remedies. Several medicinal plants are now being used effectively in the treatment of various illnesses, including anxiety disorders. Clinical and experimental research has demonstrated the effectiveness of medicinal plants in treating psychological diseases [6]. GABAergic neurotransmitters are thought to play a role in the anxiolytic action of many therapeutic plants [7]. 4-hydroxyphenyl acetic acid [4-HPAA] is naturally found in some plants, such as mushrooms [8], cocoa beans [9], and olive oil [10]. It is also found in brain cells as a physiological product of

tyramine. [11] [12]. It has a significant role in the regulation of anxiety and depression [13]. According to previous research, there is a demonstrated association between reduced concentrations of 4-HPAA in the brain and the manifestation of depression and anxiety [14]. Therapeutically, 4-HPAA and its derivatives have the potential to modulate the GABAergic system, which is involved in the pathophysiology of anxiety disorders [15].

Despite the demonstrated effectiveness of 4-HPAA in treating anxiety, investigations in animal models have shown that it has poor blood-brain barrier permeability. Enhancing the pharmacokinetics of 4-HPAA requires implementing chemical modifications to its structure [16]. Therefore, the present study aims to examine the anxiolytic effects of 4-HPAA derivatives *in vivo*, employing various animal models and assessing them through the EZM, EPM, and motor coordination tests. This research also includes in-silico molecular modeling studies to support and substantiate the findings. Additionally, molecular docking simulations in this study provide further insight into the GABA_A facilitated anti-anxiety effects of the 4-HPAA derivatives.

2. Material and Methods

2.1. Animals Used

In the current investigation, BALB/C male mice, weighing between 25 and 30 grams, were used. Six mice were housed in each cage under standard laboratory lighting (12-hour light and dark cycles) and temperature controls. Water and food were always available. The studies were conducted between 9:00 a.m. and 3:00 p.m. Before the series of experimental trials, the animals were fasted for 12 hours (from food but not from drink). Mice were housed for at least 10 days before the first set of trials to ensure they had acclimated to the laboratory setting. The experimental protocols were developed according to ethical principles and guidelines and were approved by the European Committee Council Directive 14, 2021, with the approved protocol number SREC/21/12/018.

2.2. Chemical Agents

Diazepam vials, Isopropyl Myristate, Span 20, Iodomethane (CH3I), and Dimethylformamide (DMF) were purchased from Pro-Chem Industrial Sdn. Bhd. Sodium Hydroxide (NaOH) was obtained from Eastman Chemical. Isopropanol and Ethyl Acetate (EtOAc) were obtained from the Sancai Industry.

2.3. Induction of Anxiety

In this study, anxiety disorder induction was achieved through chronic caffeine administration. Caffeine can induce anxious behavior in humans and animal models by blocking adenosine A1 and A2 receptors, which are involved in anxiogenic effects [17]. Chronic caffeine administration via drinking water (0.3 g/L) for 14 days produced anxiogenic effects in the EPM and the EZM [18].

2.4. Evaluation of Anxiolytic Potential

2.4.1. Elevated Plus Maze Test [EPM]

The EPM consists of two enclosed arms (25 × 5 cm) with high surrounding walls (15 cm) and two open arms (25 × 5 cm) with 3 mm edges, elevated 50 cm above the floor. The central area (5 × 5 cm) is situated between the four arms. The test began when mice were placed individually in the central area, facing the closed arms. Mice behavior was recorded for 5 minutes using a video digital camera. The two primary parameters measured were the number of entries into the open arms (entries were counted when all four paws were in the open arms) and the time spent in the closed arms per second [19].

2.4.2. Elevated Zero Maze Test [EZM]

The EZM consists of a 6 cm wide ring with an outer diameter of 45 cm. It is divided into four equal quadrants with alternating closed and open sections, and the entire ring is elevated 50 cm above the floor. Mice behavior was recorded for 5 minutes to measure two key parameters: the time spent in the closed arms and the number of entries into the open arms [20].

2.4.3. Actophotometer Test [Activity Cage]

The actophotometer test monitors the locomotor behavior of mice using an activity cage equipped with a digital counter to measure horizontal locomotor activity. Each mouse was placed in the activity cage for 5 minutes. Mice were administered treatment via intraperitoneal injection, and the activity score was recorded after 1 hour [21].

2.5. Computational Studies

Computational investigations using molecular docking studies were conducted to probe the binding affinities of the human GABA_A receptor alpha1-beta2-gamma2 subtype. AutoDock Vina 1.1, integrated with PyRx software for its computational capabilities, was employed for these analyses. Protein structures were obtained from the Protein Data Bank (PDB) under accession code 6D6T and were refined by removing co-crystallized ligands and water molecules to ensure a clean base structure.

The chemical structure of the synthesized ligand was created using ChemDraw 20.0 and saved in MOL file format. Further refinement and optimization of the ligand structure were performed using Discovery Studio Visualizer BIOVIA, resulting in a modified structure saved in PDB format. Both the processed protein structures and the refined ligand were then prepared for molecular docking simulations, which were successfully conducted using AutoDock Vina [22].

The docking procedure involved selecting a specific grid box to guide the interactions. The results were thoroughly interpreted using Discovery Studio Visualizer, which provided insights into potential binding modes and interaction patterns within the human GABA_A receptor alpha1-beta2-gamma2 subtype. This comprehensive approach offered an extensive basis for understanding the fundamental molecular interactions [23].

2.6. 4-HPAA Derivative Synthesis

2.6.1. Synthesis of Isopropyl-4-Hydroxy-[Phenyl] Acetate IHPA

As shown in Figure 1, 4-HPAA [2.0 g, 0.013 mol] and 10 ml of isopropanol exposed to hydrochloric gas were taken in the round bottom flask. The solution was refluxed at 80˚C and heated with occasional stirring for 3 hours. The reactant was evaporated by reduced pressure, and then the residue was dissolved in 10 ml of ethyl acetate and washed by 30 ml of water three times. After the evaporation of ethyl acetate, the product was collected as oil 2.33 g yields 93% GC-MASS 194, v max [1750] cm^{-1}. H NMR [6H, d, CH_3], 2.6 [2H, s, CH_2], 4.3 [1H, s, CH], 5.5 [1H, m, OH]. 7 - 8 [4H, d, AR].

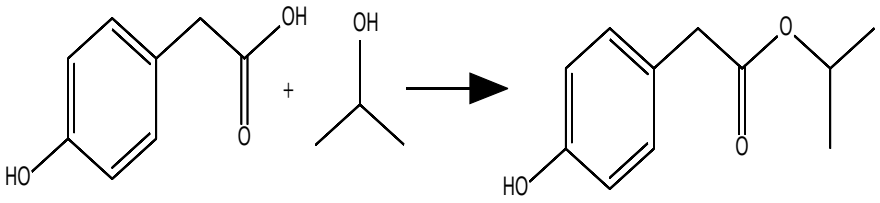

Figure 1. Reactionsynthesis of isopropyl-4-hydroxy-[phenyl] acetate.

2.6.2. Synthesis of Isopropyl 4-Methoxyphenylacetate MPHA

A mixture of CH3I [0.731 g 0.005 mole] and IHPA [1.0 g 0.005 mole] in DMF [5 ml] and NaOH solid [0.206 g 0.005 mole] was stirred at room temperature for 3 days. The reaction mixture was poured into ice-cold water [10 - 20 ml], then the ice was melted, the aqueous layer was removed using a separatory funnel, and the organic layer was dissolved in EtOAc and washed with 10 ml of distilled water three times. The organic layer was then treated with 20 ml of aqueous solution [10% NaOH] three times (Figure 2). Evaporate the organic layers to produce oil. 0.7 g yields 70%. GC MASS 208 v max 2840 cm^{-1}.H NMR, CD3CN, 1.2 [6H, s CH3], 2.6 [2H, s, CH2], 4.3 [1H, s, CH], 4.5 [3H, d, CH3], 7-8 [4H, m, AR].

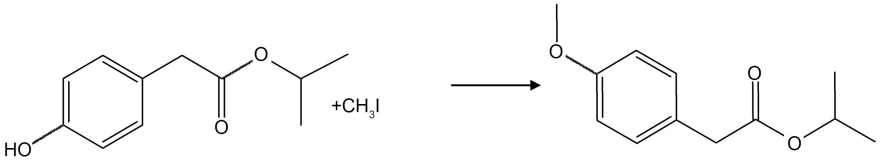

Figure 2. Reaction synthesis of isopropyl 4-methoxyphenylacetate.

2.6.3. Synthesis of 4-Methoxyphenylacetic Acid MPAA

As shown in Figure 3, MPHA [1 g, 0.004 mole] dissolved in 50 ml of 90% ETOH and NaOH [1.0 g 0.025 mole] were taken in a round bottom flask, and the reaction mixture was occasionally stirred for 3 hours at room temperature. Then ethanol evaporated, and dropwise, 40% HCl [0.5 - 1.0 ml] was added to form a precipitate at pH 3.4, then NaCl was added to obtain a saturated solution, and it was cooled in the fridge for 1 hour. The precipitate was filtered by filter paper and washed with 20 ml of water to obtain a 0.56 g yield. 70% m.p. 85˚C v max 1720 cm^{-1} GC MASS 166 HNMR, CD3CN, 3.5 [2H, S], 3.7 [3H, S, OCH3], 6.8 - 7.1 [4H, m, AR].

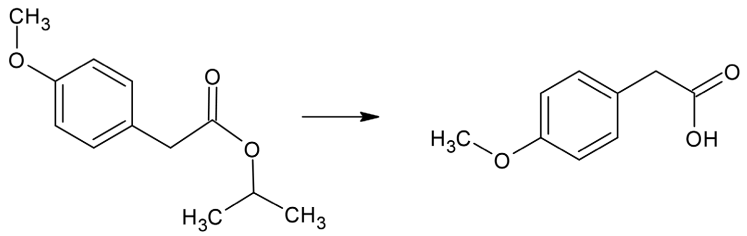

Figure 3. Reaction synthesis of Synthesis of 4-methoxyphenylacetic acid.

2.7. Preparation of 4-HPAA Derivatives for *in Vivo* Experiment

For anxiolytic evaluation tests, mice were distributed into seven groups [n = 6 per group] and were treated as follows:

1) Control group [vehicle]: Each mouse received the vehicle [Normal Saline, intraperitoneal (i.p.)]. Vehicle [Isopropyl Myristate, Normal Saline, and Span 20].

2) Positive control group: Each mouse received diazepam [1.5 mg/kg, i.p].

3) 4-HPAA group: Each mouse received 4-HPAA [0.5 mg/kg, i.p.] and was prepared with normal saline in the presence of sonication.

4) Isopropyl [4-hydroxyphenyl] acetate group: Each mouse received isopropyl [4-hydroxyphenyl] acetate at an equimolar dose to 4-HPAA [0.5 mg/kg, i.p.] and was prepared by 0.5 ml isopropyl myristate, 1 ml normal saline, and 6 drops span 20.

5) Isopropyl [4-Methoxyphenyl] acetate group: Each mouse received isopropyl [4-methoxyphenyl] acetate at an equimolar dose to 4-HPAA [0.5 mg/kg, i.p.] and was prepared by 0.5 ml isopropyl myristate, 1 ml normal saline, and 6 drops span 20.

6) 4-methoxyphenyl acetic acid group: Each mouse received 4-methoxyphenylacetic acid at an equimolar dose to 4-HPAA [0.5 mg/kg, i.p.] and was prepared with 0.5 ml isopropyl myristate, 1 ml normal saline, and 6 drops span 20.

After 1 hour of the dosage, all groups were subjected to the EPM, EZM, and actophotometer tests.

2.8. Statistical Analysis

The data will be analyzed using MS Excel 2007 and presented as the mean ± SD of three replicates. One-way analysis of variance (ANOVA) and Tukey tests were performed using the "StatPlus 2009 Professional" trial version software. A significant difference between groups was considered at a p-value less than 0.05.

3. Results

3.1. Elevated Plus Maze Test

Mice behaviors in the EPM apparatus are summarized in **Figure 4**. The results showed that treatment with diazepam led to a significant decrease in the time spent in the closed area ($p = 0.0273$) and a significant increase in the number of entries into the open areas ($p = 0.0223$) compared to the vehicle control (normal saline). Additionally, treatment with 4-HPAA did not result in a significant decrease

in the time spent in the closed areas or a significant increase in the number of entries into the open areas compared to the vehicle control (normal saline).

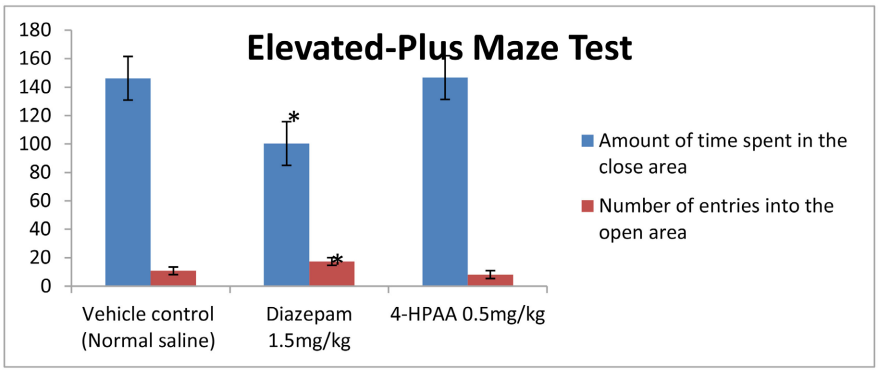

Figure 4. Amount of time spent in the close area and Number of entries into the open area of the Elevated-Plus Maze Test after administration of Vehicle control [Normal saline], Diazepam, and 4-HPAA. *Significant difference compared to Vehicle control [Normal Saline].

Figure 5 shows that diazepam caused a significant decrease in the average time spent in the closed area ($p < 0.05$) and a significant increase in the number of entries into the open areas ($p = 0.0001$) compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). IHPA significantly decreased the average time spent in the closed areas ($p < 0.05$) and increased the number of entries into the open areas ($p = 0.0021$) compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). Furthermore, MPHA significantly decreased the time spent in the closed areas ($p = 0.0002$) but did not significantly increase the number of entries into the open areas compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). In contrast, MPAA did not show a significant decrease in the time spent in the closed areas or a significant increase in the number of entries into the open areas compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20).

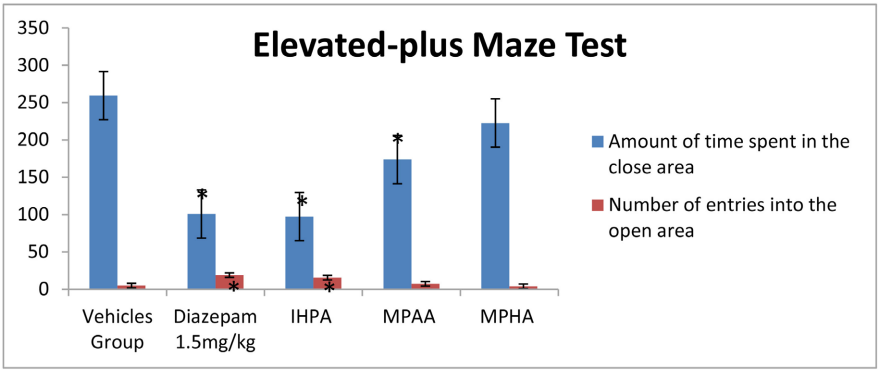

Figure 5. Amount of time spent in the close area and Number of entries into the open area of the Elevated-Plus Maze Test after administration of Vehicle control [Isopropyl myristate, Normal Saline, and Span 20], Diazepam, IHPA, MPHA, and MPAA. *Significant difference compared to Vehicle control [Isopropyl myristate, Normal Saline, and Span20].

3.2. Elevated Zero Maze Test

The behavioral responses of mice in the EZM test are systematically detailed in Figure 6. Administration of diazepam led to a statistically significant reduction in the mean duration spent in enclosed areas ($p < 0.05$) and a significant decrease in the frequency of entries into open areas ($p = 0.0025$) compared to the vehicle control (normal saline). In contrast, administration of 4-HPAA did not produce a statistically significant reduction in the duration spent in enclosed areas, nor did it increase the frequency of entries into open areas compared to the vehicle control (normal saline).

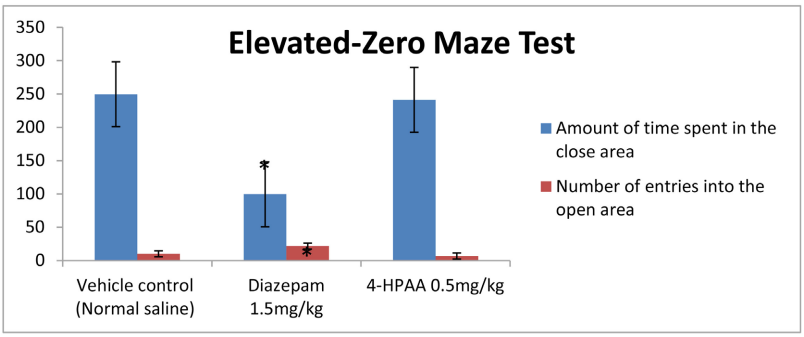

Figure 6. Amount of time spent in the close area and Number of entries in to the open area of the Elevated-Zero Maze Test after administration of Vehicle control [Normal saline], Diazepam, 4-HPAA. *Significant difference compared to Vehicle control [Normal Saline].

Figure 7 illustrates the anxiolytic effects of diazepam, showing a statistically significant reduction in the average duration spent in enclosed areas ($p < 0.05$) and a notable increase in the frequency of entries into open areas ($p < 0.000002$) compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). IHPA also demonstrated a statistically significant reduction in the average duration spent in enclosed areas ($p = 0.0004$) and a significant increase in the frequency of entries into open areas ($p = 0.0092$) compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). Similarly, MPHA showed a significant reduction in the average duration spent in enclosed areas ($p = 0.0024$) and an increase in the frequency of entries into open areas ($p = 0.0232$) compared to the vehicle controls.

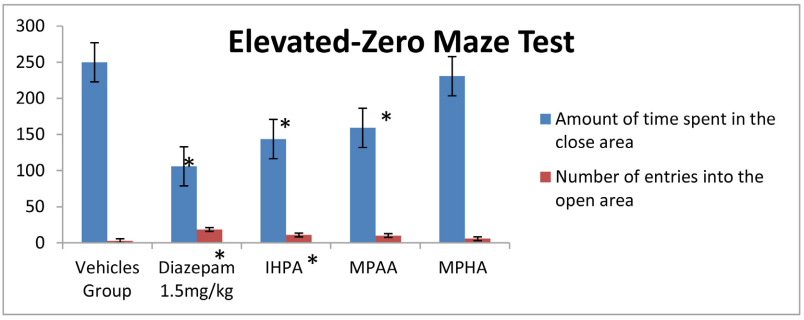

Figure 7. Amount of time spent in the close area and Number of entries into the open area of the Elevated-Zero Maze Test after administration of Vehicle control [Isopropyl myristate, Normal Saline, and Span 20], Diazepam, IHPA, MPHA, and MPAA. *Significant difference compared to Vehicle control [Isopropyl myristate, Normal Saline, and Span20].

Conversely, administration of MPAA did not result in a statistically significant reduction in the mean duration spent in enclosed areas or a significant increase in the frequency of entries into open areas compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20).

3.3. Actophotometer Test

Treatment of mice with diazepam at 1.5 mg/kg resulted in a significant increase in locomotor activity compared to the vehicle control (normal saline). In contrast, 4-HPAA did not show a significant increase in locomotor activity compared to the vehicle control (normal saline), as shown in Figure 8.

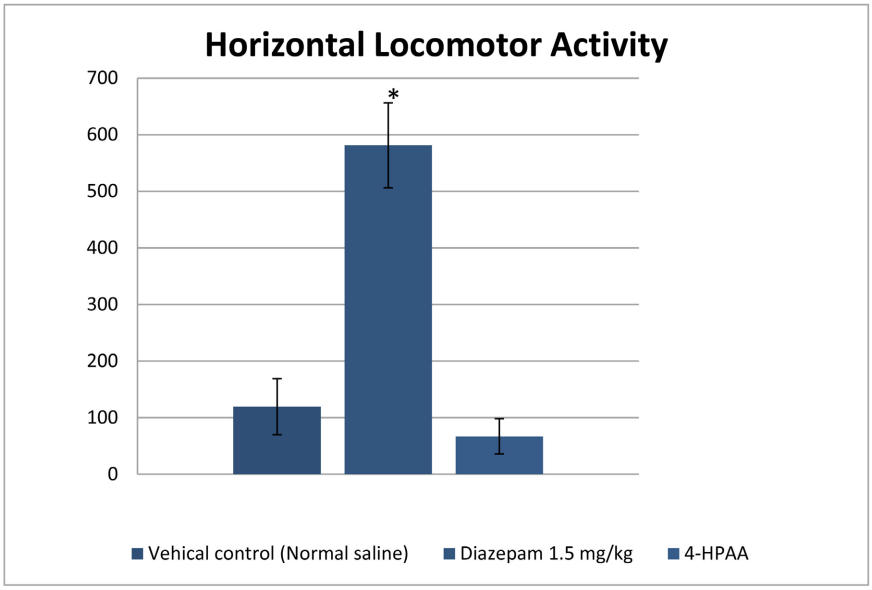

Figure 8. Horizontal Locomotor Activity of control [Normal saline], Diazepam, 4-HPAA. *Significant difference compared to Vehicle control [Normal Saline].

Figure 9 demonstrates that diazepam significantly increased locomotor activity ($p < 0.05$) compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). Additionally, IHPA did not cause a significant increase in locomotor activity compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). MPAA also did not significantly increase locomotor activity compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20). Similarly, treatment with MPHA did not result in a significant increase in locomotor activity compared to the vehicle controls (isopropyl myristate, normal saline, and Span 20).

3.4. Molecular Docking Analysis for Anxiolytic Activity

The docking analysis results for anxiolytic activity are presented in Table 1. In this study, the human GABA-A receptor alpha1-beta2-gamma2 (PDB ID: 6D6T) was used to perform the anxiolytic docking analysis. The ranking of the docking scores is as follows: diazepam > IHPA > MPAA > 4-HPAA > MPHA. The amino

acid residues THR111, PHE113, LEU143, LEU145, and ILE108 were involved in the interaction between MPAA and 6D6T, with a docking score of −5.3 kcal/mol.

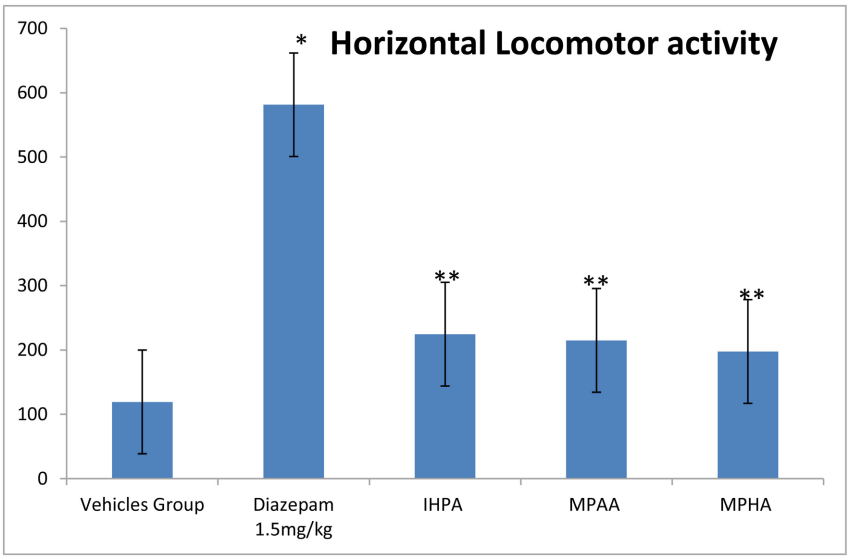

Figure 9. Horizontal Locomotor Activity of Vehicle control [Isopropyl myristate, Normal Saline, and Span 20], Diazepam 1.5 mg/kg, IHPA, MPHA, and MPAA. *Significant difference compared to Vehicle control [Isopropyl myristate, Normal Saline, and Span20]. ** significant difference compared to diazepam.

Table 1. Docking scores of the selected compounds with the Human GABA-A receptor alpha1-beta2-gamma2.

Chemical agents	Binding energy	Polar interaction	Nonpolar interaction
Diazepam	−7.1	THR111,	PHE113, ALA119, LEU145, ALA121, LEU143, ASP110
4-HPAA	−4.8	PHE113, PHE112 GLU80	LEU143, LEU145
IHPA	−5.8	PRO109, THR111	LEU143, LEU145, PHE113, ALA119, ILE147
MPAA	−5.3	THY111	PHE113, LEU145, TYR141, LEU143, ILE108
MPHA	−4.8	THR111	LEU145, PHE113

4. Discussion

Despite the availability of various therapeutic modalities for anxiety, achieving complete symptom eradication without adverse consequences remains elusive. Consequently, the therapeutic application of these drugs is constrained by side effects and poor pharmacokinetics. Additionally, the safety, efficacy, onset of action, duration of action, and side effects of existing pharmaceuticals are critical concerns, highlighting the need for new and improved drugs [24]. The therapy of disorders has shown promise through the utilization of herbal medicine, owing to its potential to treat a wide range of neurological conditions [7]. This study synthesizes 4-HPAA derivatives and analyzes their pharmacological effects on

reducing anxiety-like behaviors in mice, using several validated methodologies such as the EPM, EZM, and Actophotometer models. The literature provides extensive evidence suggesting that chemical modification strategies can enhance therapeutic action while reducing toxicity and adverse effects in both synthetic and phytochemical molecules. Additionally, the use of herbal medicine has shown promise in treating various neurological conditions, further supporting its potential for therapeutic applications [25].

In this study, the results were demonstrated using the EPM, a widely used model for behavioral assays, particularly for assessing anxiety-like behaviors. The two key indicators of an anxiety disorder measured during the five-minute test are the average time spent in the closed arms and the average number of entries into the open arms. One of the main challenges in EPM research is the phenomenon known as "one trial tolerance," where subjects may exhibit increased exploration time in the closed arms of the maze despite being administered anxiolytic treatments during their exposure to the EPM [19]. The average time spent in the closed arms and the average number of entries into the open arms in this study of 4-HPAA using the EPM did not show significant changes. This suggests that the mice treated with 4-HPAA did not exhibit any notable anxiolytic effects [13]. The current investigation utilized chemical modifications, specifically methylation and esterification techniques, to synthesize prodrugs.

Prodrugs are inactive substances that are chemically modified to become active within the body. The use of prodrug delivery methods aims to enhance effectiveness, optimize pharmacokinetics, and reduce toxicity [26]. Prodrugs are extensively used in drug development, especially for anxiolytic medications, to improve drug permeability. The esterification strategy is particularly effective for enhancing permeability across the blood-brain barrier. This approach increases lipophilicity and facilitates the conversion of the prodrug into its active form [27]. The suggestion involves converting the carboxylic acid moiety in 4-HPAA to an ester form, specifically an isopropyl ester, to enhance permeability. The current study synthesized IHPA and evaluated its effects on mice behavior in the EPM at a dose equivalent to 4-HPAA, administered intraperitoneally. The results demonstrated a significant anxiolytic effect, with a reduction in the average time spent in the closed arms and an increase in the number of entries into the open arms. These findings suggest that IHPA, with its ester group, may serve as a prodrug with improved pharmacokinetics, permeability, and activity. Previous studies also indicated that a single hydroxyl group on the benzene ring is more anxiolytic compared to having two or three hydroxyl groups [15]. In this study, the hydroxyl group in 4-HPAA was substituted with a methoxy group to explore its role. MPAA was synthesized and tested in the EPM, but the results showed no statistically significant changes in the average time spent in the closed arms or the number of entries into the open arms, indicating that MPAA lacks anxiolytic effects. Despite these findings, further investigation is needed to determine the necessity of the hydroxyl group.

The study also synthesized MPHA and assessed its effect on anxiety-like behavior using the EPM paradigm. MPHA showed a statistically significant decrease in the average time spent in the closed arms, but no significant increase in the number of entries into the open arms. This suggests that MPHA has an anxiolytic effect and that the hydroxyl group may not be essential for this activity, as indicated by the decreased time spent in the closed arms.

The EZM model, commonly used to evaluate anxiety-like behavior in mice, will be employed to further validate these findings.

The EZM and the EPM are similar tests; however, the EZM lacks a central square area, which can make it a more precise measure of anxiety-like behavior. The current study used the EZM to validate the findings obtained from the EPM, providing additional support for the results and ensuring their accuracy [28]. In this study, 4-HPAA was evaluated using the EZM, and the results showed no statistically significant differences in the average time spent in the closed arms or the average number of entries into the open arms, supporting the conclusion from the EPM that 4-HPAA does not exhibit an anxiolytic effect.

The evaluation of IHPA in the EZM demonstrated a significant anxiolytic effect, as evidenced by increased average time spent in the closed arms and increased entries into the open arms at equal molar concentrations after intraperitoneal administration. This confirms the anxiolytic effect observed in the EPM.

The study also investigated the effect of substituting the hydroxyl group with a methoxy group in MPAA. Testing MPAA in the EZM showed no significant changes in the average time spent in the closed arms or the number of entries into the open arms, indicating that MPAA does not have an anxiolytic effect. From a medicinal chemistry perspective, relying solely on MPAA findings may be inadequate due to potential interference from the carboxylic acid group in MPHA affecting blood-brain barrier absorption. Therefore, the synthesis and evaluation of MPHA in the EZM were undertaken.

The results for MPHA in the EZM revealed a significant reduction in the average time spent in the closed arms and an increase in the average number of entries into the open arms, indicating an anxiolytic effect and suggesting that the hydroxyl group may not be essential for this activity.

Tukey's multiple comparisons test revealed that diazepam significantly increased locomotor activity compared to the vehicle control group (Mean Diff. = −462.0, 95% CI of diff. = −578.2 to −345.8, $p < 0.0001$). This result aligns with existing literature indicating that diazepam can enhance locomotor activity at lower doses, an effect not observed in the normal control mice. In contrast, IHPA, MPAA, and MPHA showed increases in locomotor activity (Mean Diffs. = −105.3, −95.67, and −78.50, respectively) compared to the vehicle control, but these differences were not statistically significant ($p = 0.0891$, 0.1435, and 0.3025, respectively). These findings suggest that while diazepam markedly increases locomotor activity, the other compounds may potentially reduce side effects related to increased locomotion, offering a safer profile for therapeutic use

Molecular docking studies are commonly employed to predict ligand-target interactions and enhance our understanding of the biological activities of natural products. These studies provide insights into potential binding processes within protein binding pockets. By clarifying and validating biological investigations, molecular docking contributes to a deeper appreciation of the underlying mechanisms of action and potential efficacy of compounds [29]. To develop a deeper knowledge of the biological features of anxiolytic agents, a selection of three commonly used 4-HPAA compounds has been made for the aim of carrying out a docking study. These compounds were then docked against the human GABA-A receptor alpha1-beta2-gamma2 [PDB ID: 6D6T]. After interacting with the ligands through a series of linkages, they give docking scores between −4.8 and −7.14 kcal/mol. These findings indicate that interactions between phytoconstituents and target proteins play a significant role in anxiolytic activity. To evaluate the anxiolytic docking investigation, the phytoconstituents were molecularly docked with the potassium channel [PDB ID: 6D6T]. The range of the docking score has been demonstrated to range from −4.8 to −5.8 kcal/mol. In the context of anxiolytic docking research, several compounds, namely diazepam, IHPA, MPAA, 4-HPAA, and MPHA, showed significant docking scores when interacting with the Human GABA-A receptor alpha1-beta2-gamma2 [PDB ID: 6D6TAmong the tested derivatives, IHPA displayed the highest docking score when interacting with the GABA-A receptor alpha1-beta2-gamma2 (PDB ID: 6D6T). This suggests that IHPA has a strong binding affinity for the GABA-A receptor, which may correlate with its potential anxiolytic activity.

5. Conclusion

The present study provides compelling evidence that derivatives of 4-HPAA, namely IHPA and MPHA, offer significant therapeutic potential for the treatment of anxiety-related diseases. The results obtained from the *in vivo* study and the molecular docking analysis has indicated a probable involvement of GABA-A-mediated mechanisms in the anxiolytic effects. Further validation of the hypothesized mechanism of action of 4-HPAA requires an exhaustive investigation by implementing more comprehensive clinical studies

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Teleanu, R.I., Niculescu, A., Roza, E., Vladâcenco, O., Grumezescu, A.M. and Teleanu, D.M. (2022) Neurotransmitters—Key Factors in Neurological and Neurodegenerative Disorders of the Central Nervous System. *International Journal of Molecular Sciences*, **23**, Article No. 5954. https://doi.org/10.3390/ijms23115954

[2] Klimes-Dougan, B., Begnel, E., Almy, B., Thai, M., Schreiner, M.W. and Cullen, K.R. (2019) Hypothalamic-Pituitary-Adrenal Axis Dysregulation in Depressed Adolescents with Non-Suicidal Self-Injury. *Psychoneuroendocrinology*, **102**, 216-224. https://doi.org/10.1016/j.psyneuen.2018.11.004

[3] Thibaut, F. (2022) Anxiety Disorders: A Review of Current Literature. *Dialogues in Clinical Neuroscience*, **19**, 87-88.

[4] Garakani, A., Murrough, J.W., Freire, R.C., Thom, R.P., Larkin, K., Buono, F.D., *et al.* (2020) Pharmacotherapy of Anxiety Disorders: Current and Emerging Treatment Options. *Frontiers in Psychiatry*, **11**, Article 595584. https://doi.org/10.3389/fpsyt.2020.595584

[5] Chaachouay, N. and Zidane, L. (2024) Plant-Derived Natural Products: A Source for Drug Discovery and Development. *Drugs and Drug Candidates*, **3**, 184-207. https://doi.org/10.3390/ddc3010011

[6] Yao, J., Ju, B., Li, X., Ma, M., Li, L. and Zhang, Y. (2022) Clinical Study of Comprehensive TCM Therapy in the Treatment of Damp and Hot Stasis Erectile Dysfunction. *Medicine*, **101**, e30776. https://doi.org/10.1097/md.0000000000030776

[7] Sarris, J., Marx, W., Ashton, M.M., Ng, C.H., Galvao-Coelho, N., Ayati, Z., *et al.* (2021) Plant-Based Medicines (Phytoceuticals) in the Treatment of Psychiatric Disorders: A Meta-Review of Meta-Analyses of Randomized Controlled Trials [Les médicaments à base de plantes (phytoceutiques) dans le traitement des troubles psychiatriques: Une méta-revue des méta-analyses d'essais randomisés contrôlés]. *The Canadian Journal of Psychiatry*, **66**, 849-862. https://doi.org/10.1177/0706743720979917

[8] Promden, W., Viriyabancha, W., Monthakantirat, O., Umehara, K., Noguchi, H. and De-Eknamkul, W. (2018) Correlation between the Potency of Flavonoids on Mushroom Tyrosinase Inhibitory Activity and Melanin Synthesis in Melanocytes. *Molecules*, **23**, Article 1403. https://doi.org/10.3390/molecules23061403

[9] Chen, W., Zhang, L., Zhao, L., Yan, F., Zhu, X., Lu, Q., *et al.* (2021) Metabolomic Profiles of A-Type Procyanidin Dimer and Trimer with Gut Microbiota *in Vitro*. *Journal of Functional Foods*, **85**, Article ID: 104637. https://doi.org/10.1016/j.jff.2021.104637

[10] Kalka, J. and Drzymała, J. (2023) Vicia Faba Plant Suitability Assessment for Genotoxicity, Cytotoxicity, and Mutagenicity Testing of Pharmaceutical-Containing Wastewater. *Water*, **15**, Article No. 3044. https://doi.org/10.3390/w15173044

[11] Costa, V.M., Grando, L.G.R., Milandri, E., Nardi, J., Teixeira, P., Mladěnka, P., *et al.* (2022) Natural Sympathomimetic Drugs: From Pharmacology to Toxicology. *Biomolecules*, **12**, Article No. 1793. https://doi.org/10.3390/biom12121793

[12] Serra, S., Castagna, A., Marzorati, S. and Valentino, M. (2022) Biotransformation of the Proteogenic Amino Acids Phenylalanine, Tyrosine and Tryptophan by Yarrowia Species: An Application to the Preparative Synthesis of Natural Phenylacetic Acid.

Catalysts, **12**, Article No. 1638. https://doi.org/10.3390/catal12121638

[13] Alrumaihi, F., Almatroodi, S.A., Alharbi, H.O.A., Alwanian, W.M., Alharbi, F.A., Almatroudi, A., *et al.* (2024) Pharmacological Potential of Kaempferol, a Flavonoid in the Management of Pathogenesis via Modulation of Inflammation and Other Biological Activities. *Molecules*, **29**, Article No. 2007. https://doi.org/10.3390/molecules29092007

[14] Wu, X., Xu, J. and Wang, X. (2023) IDDF2023-ABS-0274 Bacteroides Vulgatus Affects Colitis and Depression-Like Behavior in Mice: Implications for the Gut-Brain Connection. *BMJ Publishing Group*, **72**. https://doi.org/10.1136/gutjnl-2023-iddf.111

[15] Vissiennon, C., Nieber, K., Kelber, O. and Butterweck, V. (2012) Route of Administration Determines the Anxiolytic Activity of the Flavonols Kaempferol, Quercetin and Myricetin—Are They Prodrugs? *The Journal of Nutritional Biochemistry*, **23**, 733-740. https://doi.org/10.1016/j.jnutbio.2011.03.017

[16] Moradi-Afrapoli, F., Oufir, M., Walter, F.R., Deli, M.A., Smiesko, M., Zabela, V., *et al.* (2016) Validation of UHPLC-MS/MS Methods for the Determination of Kaempferol and Its Metabolite 4-Hydroxyphenyl Acetic Acid, and Application to *in Vitro* Blood-Brain Barrier and Intestinal Drug Permeability Studies. *Journal of Pharmaceutical and Biomedical Analysis*, **128**, 264-274. https://doi.org/10.1016/j.jpba.2016.05.039

[17] Villanueva-García, D., Mota-Rojas, D., Miranda-Cortés, A., Mora-Medina, P., Hernández-Avalos, I., Casas-Alvarado, A., *et al.* (2020) Neurobehavioral and Neuroprotector Effects of Caffeine in Animal Models. *Journal of Animal Behaviour and Biometeorology*, **8**, 298-307. https://doi.org/10.31893/jabb.20039

[18] Liu, C., Wang, L., Zhang, C., Hu, Z., Tang, J., Xue, J., *et al.* (2024) Caffeine Intake and Anxiety: A Meta-analysis. *Frontiers in Psychology*, **15**, Article 1270246. https://doi.org/10.3389/fpsyg.2024.1270246

[19] Shoji, H. and Miyakawa, T. (2019) Age-Related Behavioral Changes from Young to Old Age in Male Mice of a C57BL/6J Strain Maintained under a Genetic Stability Program. *Neuropsychopharmacology Reports*, **39**, 100-118. https://doi.org/10.1002/npr2.12052

[20] Gaspar, L., Bartman, S., Coppotelli, G. and Ross, J.M. (2023) Effect of Apparatus Characteristics on Anxiety-Like Behavior in Young Adult and Old Mice of Both Sexes Assessed by the Elevated Plus Maze Assay. *Frontiers in Behavioral Neuroscience*, **17**, Article 1182661. https://doi.org/10.3389/fnbeh.2023.1182661

[21] Gangopadhyay, A., Malakar, J., Ghosh, A., Deb, J., Dey, S., Datta, S., *et al.* (2012) The Central Nervous System Activity of *Barleria prionitis* Linn. on the Locomotor Activity of Swiss Albino Mice Using Actophotometer. *International Journal of Pharmaceutical and Biological Archive*, **3**, 403-405.

[22] Pawar, R.P. and Rohane, S.H. (2021) Role of AutoDock Vina in PyRx Molecular Docking. *Asian Journal of Research in Chemistry*, **14**, 132-134.

[23] Zhang, C. and Du, W. (2024) The Mechanism of Celastrol in the Treatment of Metastatic Lung Adenocarcinoma Revealed by Network Pharmacology and Molecular Docking. *Journal of Biosciences and Medicines*, **12**, 275-285. https://doi.org/10.4236/jbm.2024.126023

[24] Carter, T. (2023) Understanding the Presentation and Treatment of Anxiety Disorders. *Nursing Standard*, **38**, 73-77. https://doi.org/10.7748/ns.2023.e12166

[25] Pandey, D.K., Devadoss, T., Modak, N. and Mahesh, R. (2016) Antidepressant & Anxiolytic Activities of N-[Pyridin-3-yl] Quinoxalin-2-Carboxamide: A Novel Serotonin Type 3 Receptor Antagonist in Behavioural Animal Models. *Indian Journal of*

Medical Research, **144**, 614-621.

[26] Li, J., Wang, Q., Xia, G., Adilijiang, N., Li, Y., Hou, Z., *et al.* (2023) Recent Advances in Targeted Drug Delivery Strategy for Enhancing Oncotherapy. *Pharmaceutics*, **15**, Article No. 2233. https://doi.org/10.3390/pharmaceutics15092233

[27] Ekhator, C., Qureshi, M.Q., Zuberi, A.W., Hussain, M., Sangroula, N., Yerra, S., *et al.* (2023) Advances and Opportunities in Nanoparticle Drug Delivery for Central Nervous System Disorders: A Review of Current Advances. *Cureus*, **15**, e44302. https://doi.org/10.7759/cureus.44302

[28] Tucker, L.B. and McCabe, J.T. (2017) Behavior of Male and Female C57BL/6J Mice Is More Consistent with Repeated Trials in the Elevated Zero Maze than in the Elevated Plus Maze. *Frontiers in Behavioral Neuroscience*, **11**, Article No. 13. https://doi.org/10.3389/fnbeh.2017.00013

[29] Young, T.A., George, M., Gugssa, A., Southerland, W.M., Fang, Y. and Lee, C.M. (2024) Molecular Docking Studies on Streptomycin Antileishmanial Activity. *Open Journal of Physical Chemistry*, **14**, 36-48. https://doi.org/10.4236/ojpc.2024.142003