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RESEARCH

Combining anxiolytic activity of thymol and *trans*-ferulic acid: a potential GABAergic intervention

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Abstract

Trans-ferulic acid (TFA), a phenolic acid abundant in fruits and cereals, and thymol (THY), a monoterpenoid phenol from thyme oils, are bioactive natural compounds with notable neuropharmacological potential. This study evaluated the combined anxiolytic effects of THY and TFA in Swiss albino mice, focusing on GABAergic mechanisms and pharmacokinetic profiles through in vivo and in silico methods. Mice received vehicle, diazepam (DZP, 2 mg/kg), TFA (50 mg/kg), THY (50 mg/kg), or their combination (THY + TFA; 50 + 50 mg/kg, p.o.). Behavioral assessments in open-field, swing, hole-cross, and light–dark tests indicated that the combined treatment produced more pronounced effects, markedly reducing locomotor and exploratory activity (e.g., square crosses: 25.8 ± 3.7 vs. 94.6 ± 6.2 in control; hole crosses: 3.4 ± 1.8 vs. 15.6 ± 1.9) and increasing time in the light chamber, consistent with anxiolytic-like activity comparable to DZP. Molecular docking targeted gamma-aminobutyric acid A ($GABA_A$) receptor subunits (α2, α3), and ADME/toxicity profiles were analyzed in silico. Docking analysis revealed moderate binding affinities (BAs) to $GABA_A$ receptor subunits AF-P26048-F1 (α2 subunit) (− 5.8 kcal/mol) and 8G4X (α3 subunit) (− 5.3 kcal/mol) for TFA and α2 (− 4.7 kcal/mol) and α3 (− 5.6 kcal/mol) for THY, relative to DZP (− 6.9 kcal/mol). These findings suggest that the THY + TFA combination elicits enhanced GABAergic modulation, offering a potential natural therapeutic strategy for anxiety management with reduced toxicity risk. However, formal interaction analyses (e.g., isobologram or two-way ANOVA) are warranted to confirm the combining effect.

Keywords Anxiolytic · Thymol · *Trans*-ferulic acid · $GABA_A$ · Combining effect · In vivo

Introduction

Anxiety disorders are among the most prevalent mental health conditions globally, characterized by excessive fear, apprehension, and physiological hyperarousal that interfere with daily functioning (Stein et al. 2021; Haque 2021; Lin et al. 2021). Common symptoms include restlessness, muscle tension, palpitations, and difficulty concentrating, reflecting both psychological and somatic manifestations (Hacke et al. 2020). Prolonged anxiety impairs quality of life and increases the risk of comorbid conditions, including depression and cardiovascular disease (Veskovic et al. 2023). According to the World Health Organization (WHO), anxiety disorders affect approximately 301 million people worldwide, making them a major contributor to the global burden of mental illness (Depressive disorder (depression) (who.int); https://www.who.int/newsroom/fact-sheets/detail/mental-disorders, accessed on 20 April 2023).

GABA serves as the principal inhibitory neurotransmitter in the central nervous system (CNS), maintaining the balance between neuronal excitation and inhibition (Ströhle et al. 2018). Reduced GABAergic transmission has been implicated in the pathogenesis of anxiety and related mood disorders (Cutler et al. 2023). GABA receptors have three subtypes: $GABA_A$, $GABA_B$, and $GABA_C$ (Zizzo et al. 2022). The $GABA_A$ receptor, a ligand-gated chloride ion channel, plays a crucial role in mediating anxiolytic and sedative actions, with the α2 and α3 subunits primarily responsible for anxiolytic activity (Ghit et al. 2021; Qian et al. 2023).

Benzodiazepines such as DZP and alprazolam are well-established $GABA_A$ receptor modulators and remain the standard pharmacotherapy for anxiety (Hozack et al. 2022). When overdosed, they often only show mild to severe toxicity signs (Bhuia et al. 2023b). However, chronic use often

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leads to tolerance, dependence, sedation, cognitive impairment, and withdrawal symptoms (Ritvo et al. 2023; Magro et al. 2016; Islam et al. 2022). These limitations highlight the need for safer, naturally derived GABAergic agents with comparable efficacy but fewer adverse effects.

Natural product therapeutics are bioactive compounds derived from natural sources used in the treatment and prevention of diseases (Al Hasan et al. 2025; Rakib et al. 2025a). They hold pharmacological importance due to their diverse chemical structures and potent activities, making them valuable leads in drug discovery (Sharma et al. 2025; Rakib et al. 2025b). On this occasion, complementary medicines based on naturally occurring bioactive substances might be a hopeful option to manage anxiety and anxiety disorders (Bhuia et al. 2023b; Islam et al. 2022). Furthermore, such undesirable side effects justify the search for new agents from plant-based materials with better efficacy and fewer side effects in the treatment of anxiety (Fedotova et al. 2017).

Trans-ferulic acid (TFA) (trans-4-hydroxy-3-methoxycinnamic acid) is one of the most prevalent phenolic acids in plants and a stereoisomer of ferulic acid (FA), which is extensively dispensed in nature and is present in high concentrations in foods and fruits, such as apples, bananas, coffee seeds, eggplant, oranges, peanuts, pineapples, rice, tomatoes, and wheat as well as in seeds and cell walls of commelinid plants (such as cereals, oats, and pineapple) (Ogbuta et al. 2023; Rezaeiroshan et al. 2022). TFA has several pharmacological activities, including antiallergic, anticancer, antidiabetic, antifungal, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, hepatoprotective, and neuroprotective (Alzheimer's disease) as well as uses in skin disease (Stompor-Gorący and Machaczka 2021; Kim and Park 2019). FA is effective against several neuroprotective illnesses such as anxiety, depression, epilepsy, insomnia, neuropathic pain, neurotoxicity, psychosis, and Parkinson's disease (Lorigooini et al. 2021; Nagarajan et al. 2015; Chen et al. 2015; Singh et al. 2017; Deng et al. 2018; Dhaliwal et al. 2020; Xu et al. 2016; Salau et al. 2020). Numerous investigations have demonstrated that FA acts as an efficient anxiolytic via a variety of receptor interaction mechanisms, such as 5-HT_{1A}, NMDA receptors, and $GABA_A$ (Sborgi et al. 2021; Lorigooini et al. 2021). Therefore, TFA is a potential phytochemical for developing and designing as an anxiolytic agent.

Thymol (THY) 2-isopropyl-5-methylphenol is a naturally occurring phenolic monoterpene chemical that is found in the oils of thyme and plants such as Thymus species (Akbor et al. 2023; Salehi et al. 2018). Because of the therapeutic benefits of THY, it has a wide range of functional possibilities in the medical area (Akbor et al. 2023). THY possesses analgesic, anti-cancer, antifungal, antihyperlipidemic, anti-hyperglycemic, anti-inflammatory, antimicrobial, antioxidant, antiseptic, antiviral, carminative, and diaphoretic activity (Alagawany et al. 2021; Aljelehawy et al. 2023). THY acts as a $GABA_A$ receptor agonist/modulator (Nagoor Meeran et al. 2017). Recent studies have demonstrated that THY has anxiolytic activity in albino mice (Bhandari and Kabra 2014).

While both compounds have been independently studied, no previous research has investigated their combined effects on anxiety-related behaviors or their potential interaction at the $GABA_A$ receptor level using in vivo and in silico approaches. Furthermore, there is limited comparative evidence integrating behavioral outcomes with docking-based receptor binding, ADME profiling, and toxicity prediction of these phytochemicals.

Therefore, this study aimed to evaluate the combined anxiolytic potential of THY and TFA in Swiss albino mice and to explore their possible GABAergic mechanisms through molecular docking, pharmacokinetic, and toxicity analyses. This integrated approach may clarify whether their co-administration yields enhanced or complementary anxiolytic effects, offering a foundation for developing safer, plant-based alternatives to conventional anxiolytics.

Materials and methods

In vivo (animal) study

Chemicals and reagents

THY (CAS No. 89–83-8, purity: 98.5%) and TFA (CAS No. 537–98-4, purity: 99%) were purchased from Sigma-Aldrich (Germany), while diazepam (DZP) and water for injection (WFI) were purchased from Square and Opsonin Pharmaceuticals Ltd., Bangladesh, respectively. Tween-80 (polyoxyethylene sorbitan monooleate; analytical grade) used as a vehicle and solubilizing agent was purchased from Merck (India). All reagents and chemicals were of analytical grade and used without further purification.

Experimental animals

For the current investigation, male Swiss albino mice weighing 24–30 g were kept in the Pharmacology Lab of Gopalganj Science and Technology University (GSTU), Gopalganj, after being acquired from the animal house of Jahangirnagar University, Bangladesh. Ad libitum access to normal food and water was granted to the animals. Before the test started, they were maintained at 27 ± 2 °C in a controlled lighting environment with a 12-h dark/light cycle. After behavioral testing, animals were observed for 18 h to monitor any acute signs of toxicity or mortality following oral administration. This observation period was adopted

based on previously published behavioral pharmacology studies evaluating anxiolytic and CNS-active agents in mice (Islam et al. 2014; Bhuia et al. 2023b), where short-term post-treatment monitoring was sufficient to detect immediate adverse effects.

However, since the present work was designed as a behavioral efficacy study rather than a full toxicological assessment, extended observation for several days, as recommended by OECD guidelines (e.g., OECD 423/425 for acute oral toxicity), was not implemented. Future investigations involving repeated dosing or dose–response experiments will incorporate OECD-compliant toxicity monitoring periods to comprehensively assess safety outcomes.

The experimental procedures were reviewed and approved by the Department of Pharmacy and the Ethical Committee of Gopalganj Science and Technology University (#gstu16-11/22). All animal experiments were conducted in strict accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and in compliance with the International Council for Laboratory Animal Science (ICLAS) and National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Every effort was made to minimize animal suffering and reduce the number of animals used.

Study design

Before the test, the animals involved in the research were fasted for six hours. A total of 30 Swiss albino mice were used in this study ($n = 6$ per group, five groups in total). The group size was based on established precedents from similar behavioral studies rather than a formal power calculation. To ensure objectivity, the study employed a blinded design where one researcher handled group allocation and treatment administration, while another, unaware of the group identities, conducted the behavioral assessments. All treatments, including both test samples and control drugs, were administered in doses adjusted to each mouse's individual body weight.

THY and TFA were all dissolved in the vehicle (NC). The NC (10 ml/kg) was administered to the first group. Group two was treated with DZP. The selection of a single dose (50 mg/kg) for THY and TFA was based on previously published studies that reported significant neuropharmacological or GABAergic effects at this dose level (Sancheti et al. 2014; Bhuia et al. 2024). The primary objective of this preliminary study was to evaluate possible combined interactions between THY and TFA rather than to establish a full dose-response relationship. Future studies will include multiple doses to characterize the pharmacodynamic profile in detail. The animals were given treatments 30 min before the studies. All the treatments were given orally (Table 1).

Table 1 Study groups, compositions, and treatments

Treatment group	Description	Dose (p.o.)
NC	Vehicle (0.05% Tween-80 + 0.9% NaCl solution)	10 ml/kg
DZP	DZP	2 mg/kg
TFA-50	TFA	50 mg/kg
THY-50	THY	50 mg/kg
THY50 + TFA-50	THY + TFA	(50 + 50) mg/kg

NC negative control (vehicle: 0.05% Tween-80 + 0.9% NaCl solution, 10 ml/kg); ***DZP*** diazepam (2 mg/kg); ***TFA-50*** trans-ferulic acid (50 mg/kg); ***THY-50*** thymol (50 mg/kg)

Open-field test

Locomotor and exploratory activities were evaluated in the open-field test following the procedure of (Tan et al. 2011). Each mouse was individually placed in a square arena (30 × 30 × 30 cm) for 5 min. The number of square crossings, rearings, and grooming episodes were recorded as indicators of exploratory behavior and anxiety-like activity.

Swing test

The swing test was used to assess general locomotor activity and central nervous system excitability (Islam et al. 2014). Mice were placed in the swing apparatus for 5 min, and the number of head swings was counted. A reduction in swings indicates sedative or anxiolytic-like effects.

Hole-cross test

The hole-cross test was used to further evaluate exploratory behavior (de Almeida et al. 2012). Each mouse was placed in a two-compartment box separated by a partition containing a central hole (3 cm diameter). The number of crossings through the hole within 5 min was recorded, reflecting locomotor and anxiety-related activity.

Light–dark test

Anxiety-like behavior was also assessed using the light–dark transition test (Subhan et al. 2008). Mice were placed in the light chamber of a two-compartment box (one illuminated, one dark). The time spent in the dark box (seconds) was recorded over 5 min. Increased time in the dark chamber indicates anxiolytic-like behavior.

Statistical analysis

Using GraphPad Prism software (version 9.5), one-way analysis of variance (ANOVA) was utilized to examine the data after the Tukey's post hoc test, taking into account

95% confidence intervals at $p < 0.05$. Data normality was assessed using the Shapiro–Wilk test before analysis. The mean ± standard error of the mean (SEM) was used to display the values. The control (vehicle) group's results were contrasted with those of the test and/or standard groups.

In silico studies

Selection and preparation of macromolecule

We selected the $GABA_A$ receptor α2 and α3 subunits for molecular docking. The α2 subunit structure (AlphaFold ID: AF-P26048-F1, UniProt: P26048) was obtained from the AlphaFold Protein Structure Database (https://alphafold.com/), which provides predicted three-dimensional models generated by deep-learning algorithms rather than experimentally solved crystal structures. α3 (PDB: 8G4X (Chain G [auth C])) subunits, which were gathered from the Protein Structure Database (AlphaFold) and Protein Data Bank (RCSB PDB) (https://www.rcsb.org/). The only organization in the world to assemble and make available to the public the three-dimensional (3D) structures of biological macromolecules and their complexes is the Protein Data Bank (PDB). For biological research, it is a state-of-the-art online resource (Islam et al. 2025a; Al Hasan et al. 2025b). The grid box's center was set to $-16.60 \times 1.47 \times 20.64$ Å for α2 and $145.14 \times 162.03 \times 126.56$ Å for α3 along the X, Y, and Z-axes to accelerate the docking process. Procedures intended to eliminate docking interference after collection reduced the number of receptors. PyMOL version 1.7.4.5 was used to enhance the macromolecules by removing water molecules, amino acid residues, and unnecessary heteroatoms (Ahammed et al. 2025). The GROMOS96 43 B1 force field was then modified using the Swiss-PDB Viewer program, reducing the protein structures' energy consumption (Jahan et al. 2025; Bhuia et al. 2023a).

Ligand preparation

Prior to docking, the Swiss-PDB Viewer software version 4.1.0 was used to minimize the crystal structure's energy usage (Al Hasan et al. 2024). Moreover, the chemical formulas of THY (PubChem ID: 6989), TFA (PubChem ID: 445,858), and standard drug DZP (PubChem ID: 3016) have all been provided. The Chem3D Pro Pro21.0 program was used to optimize each ligand's internal energies (PerkinElmer Informatics, Inc.) (Ahmmed et al. 2025; Ghosh et al. 2025). The 2D structures of the compounds are given in Fig. 1.

Docking protocol

Docking research simulation is a computerized drug design technique used in drug discovery. The pharmacodynamic properties of an active drug (ligand) are assessed using the PyRx v0.8 virtual analytic approach, which involves examining and positioning molecules at particular binding sites (Fahmi et al. 2025). The degree of binding to a target molecule's catalytic site is shown by the docking data. BIOVIA Discovery Studio v21.1.0 and PyMol v1.7.4.5 Edu are utilized to examine the characteristics of the ligands in the initial target protein grids for these active binding regions of the target protein (Islam et al. 2024, 2025b).

ADME and toxicity prediction

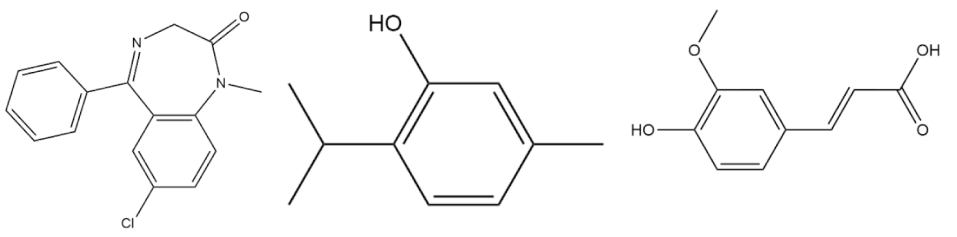
Effective candidates' physicochemical properties, including water solubility, lipid affinity, and pharmacokinetics, were analyzed using the SwissADME online platform (http://www.swissadme.ch/) (Daina et al. 2017). Pharmacokinetic activities were assessed using the pkCSM online application, and THY and TFA's toxicity properties were tested using the ProTox-II site, which is accessible to the public (https://tox-new.charite.de/protox_II/) (Ahmed and Alkali 2019).

Results

In vivo investigation

Open-field test

Figure 2 describes the results from the open-field test. The animals in the control group showed the highest numbers of grooming (NG), rearing (NR), and square crosses (NSC), all of which were indicative of typical exploratory activity. In



Diazepam | Thymol | Trans-ferulic acid

Fig. 1 The 2D structures of diazepam, thymol, and trans-ferulic acid

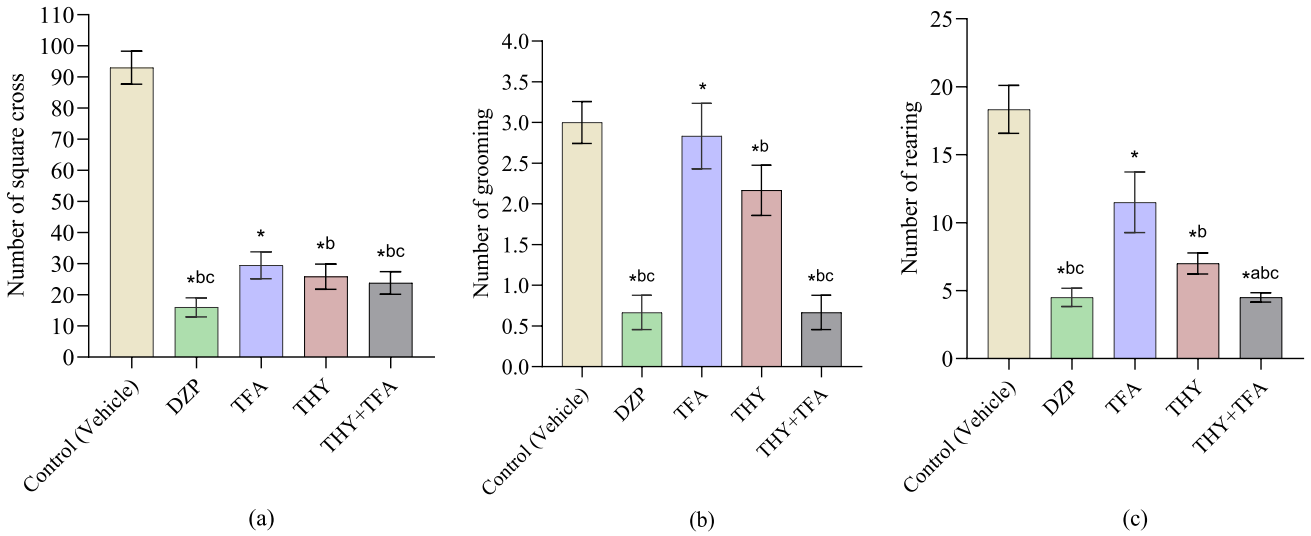


Fig. 2 **a** Number of square cross (NSC), **b** number of grooming (NG), and **c** rearing (NR) observed in test and control groups. [Values are the mean ± standard error of the mean (SEM); one-way ANOVA and Tukey *post hoc* test with multiple comparisons at 95% confidence intervals; $p < 0.05$ when *compared to the control (vehicle) group; [a]compared to the DZP;[b]compared to the TFA; [c]compared to the THY; DZP: diazepam (2 mg/kg); TFA: trans-ferulic acid (50 mg/kg); THY: thymol (50 mg/kg).]

contrast, treatment with DZP significantly ($p < 0.05$) reduced these parameters, exerting anxiolytic effects. The TFA group exhibited a moderate reduction in locomotor and exploratory activity compared to the control group significantly. Similarly, THY treatment significantly decreased NSC and NG, suggesting a partial reduction in exploratory behavior. The combination group (THY + TFA) showed further suppression of locomotor activity, with a marked decrease in NR and NG, similar to the DZP group. The number of square crosses, rearing, and grooming by the animals treated with test samples and control is described in Table 1.

Swing test

In comparison to the control group, all treatment groups experienced a significant ($p < 0.05$) decrease in the number of head swings (NHC). DZP had the lowest NHC, while the control (vehicle) group had the highest. With a more noticeable effect in the combination group (THY + TFA), TFA and THY also showed a significant drop in NHC (Fig. 3).

Hole-cross test

All treated groups had considerably ($p < 0.05$) fewer crossings (NS) than the control group, indicating a decrease in exploratory activity. The DZP group had the lowest NS values (2.40 ± 0.67), while the control group had the highest. Both TFA and THY showed intermediate decreases, and locomotor activity was further suppressed by the combined treatment (THY + TFA) (Fig. 3).

Light–dark test

Anxiolytic-like behavior was assessed by measuring the time spent in the dark box. The control group spent 117.00 ± 2.95 s, while DZP treatment significantly increased dark box duration, ($p < 0.05$), indicating reduced exploratory activity. Similarly, TFA (128.80 ± 2.85 s) and THY increased dark box preference compared to the control. The highest time was observed in the THY + TFA group (Fig. 3). Tables 2 and 3 describes the effects of test samples and controls in Swiss mice (hole cross test, swing test, and light–dark test).

In silico investigation

Molecular docking

For the 8G4X (α3 subunit of $GABA_A$) receptor, DZP exhibited the highest BA (− 6.9 kcal/mol) and formed two hydrogen bonds (HBs) with ASN C:112 and LEU C:110, along with additional interactions with ASN C:140, LEU C:142, and ARG C:156. TFA showed a moderate BA (− 5.3 kcal/mol) with two HBs involving ARG C:91 and ASN C:140, as well as non-bonded interactions with PHE C:89. THY had a slightly higher BA (− 5.6 kcal/mol) than TFA but did not form any HBs. However, it interacted with PHE C:89, ARG C:156, and LEU C:142.

For the AF-P26048-F1 (α2 subunit of $GABA_A$) receptor, DZP again exhibited the strongest BA (− 6.9 kcal/mol) and formed a single HB with SER A:421, while also interacting with residues PHE A:425, PHE A:323, LEU A:428, MET

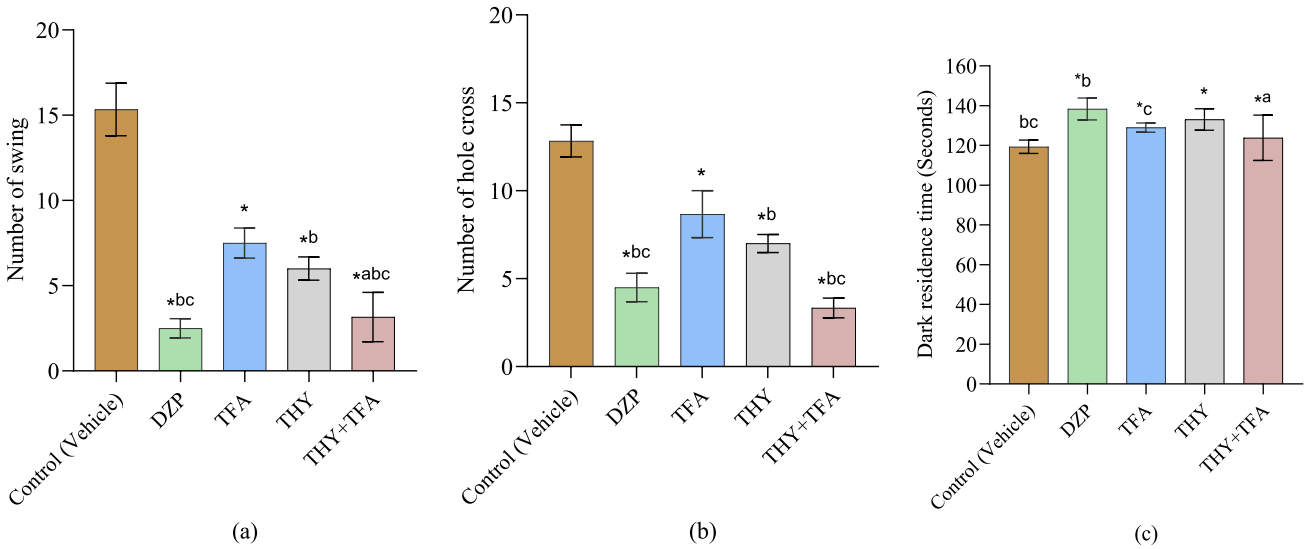


Fig. 3 **a** Number of hole cross (NHC), **b** number of swing (NS), **c** dark residence time (DRT) observed in test and/control groups. [Values are the mean ± standard error of the mean (SEM); One-way ANOVA and Tukey post hoc test with multiple comparisons at 95% confidence intervals; $p < 0.05$ when *compared to the control (vehicle) group; [a]compared to the DZP;[b]compared to the TFA; [c]compared to the THY; DZP: diazepam (2 mg/kg); TFA: trans-ferulic acid (50 mg/kg); THY: thymol (50 mg/kg).]

Table 2 Number of square crosses, rearing, and grooming by the animals treated with test samples and control

Treatment groups	NSC	NR	NG
Control (Vehicle)*	94.60 ± 6.21	18.80 ± 2.08	3.00 ± 0.32
DZP[a]	17.60 ± 3.22^{*bc}	4.60 ± 0.81^{*bc}	0.60 ± 0.24^{*bc}
TFA[b]	33.00 ± 3.11*	13.00 ± 2.02*	2.60 ± 0.40*
THY[c]	27.80 ± 4.35^{*b}	7.40 ± 0.81^{*b}	2.20 ± 0.37^{*b}
THY + TFA	25.80 ± 3.74^{*bc}	4.40 ± 0.39^{*abc}	0.60 ± 0.24^{*bc}

NSC number of square crosses, *NR* number of rearing, *NG* number of grooming, *Control* (vehicle): 0.05% Tween-80 + 0.9% NaCl solution, *DZP* diazepam (2 mg/kg), *TFA* trans-ferulic acid (50 mg/kg), *THY* thymol (50 mg/kg)

Table 3 Effects of test sample and controls in Swiss mice (hole cross test, swing test, and light–dark test)

Treatment groups	NHC	NS	DRT (s)
Control (vehicle)*	12.80 ± 1.11	15.60 ± 1.86	117.00 ± 2.95^{bc}
DZP[a]	4.40 ± 0.98^{*bc}	2.40 ± 0.67^{*bc}	143.00 ± 3.55^{*b}
TFA[b]	9.60 ± 1.17*	8.00 ± 0.89*	128.80 ± 2.85^{*c}
THY[c]	7.20 ± 0.58^{*b}	6.00 ± 0.84^{*b}	130.64 ± 5.89*
THY + TFA	2.80 ± 0.19^{*abc}	3.40 ± 1.75^{*bc}	134.60 ± 4.69^{*a}

NHC number of hole cross, *NS* number of stretches, time spent in dark box (sec), *Control* (vehicle): 0.05% Tween-80 + 0.9% NaCl solution, *DZP* diazepam (2 mg/kg), *TFA* trans-ferulic acid (50 mg/kg), *THY* thymol (50 mg/kg)

A:420, and VAL A:424. TFA showed a BA of − 5.8 kcal/mol and established four HBs with SER A:179, ARG A:443, ASP A:1172, and GLU A:171, indicating a stable binding interaction. THY displayed the weakest BA (− 4.7 kcal/mol), forming only one HB with ILE A:65, along with additional interactions with LYS A:98 and PRO A:59. Docking analysis revealed that THY and TFA exhibited moderate binding affinities to the $GABA_A$ receptor subunits (α2 and α3), suggesting plausible ligand–receptor interactions that may contribute to their observed anxiolytic effects. Table 4 summarizes the BA, HBs, and other bonds engaging with their amino acid residues, alongside the conformation of the 3D and 2D structures of the non-bond interactions shown in Fig. 4.

Pharmacokinetic and drug-likeness properties

The ADME analysis of THY, TFA, and DZP highlights significant differences in their pharmacokinetic properties. Physicochemical properties show that THY and TFA have a lower molecular weight and number of heavy atoms compared to the standard DZP. All three compounds comply with Lipinski's rule. TFA has the highest bioavailability score of 0.85, while THY and the standard DZP have a score of 0.55, suggesting favorable oral bioavailability. Lipophilicity analysis reveals that THY and DZP are more lipophilic

Table 4 The binding affinities between the chosen ligands diazepam, trans-ferulic acid, and thymol and $GABA_A$ receptor subunits α3 (8G4X) and α2 (AF-P26048-F1), as well as the quantity, length, and kind of HBs and other bonds interacting with the amino acid residues

Ligands	Receptors (PDB ID and AlphaFold ID)	BA (kcal/mol)	No of HB	Amino acid (AA) residues	
				HBs	Others
DZP	8G4X (α3 subunit of $GABA_A$)	−6.9	2	ASN C: 112 (1.94) LEU C:110 (3.46)	ASN C:140 LEU C:142 ARG C:156
TFA		−5.3	2	ARG C: 91 (2.26) ASN C: 140 (2.26)	PHE C: 89
THY		−5.6	–	–	PHE C: 89 ARG C:156 LEU C:142
DZP	AF-P26048-F1 (α2 subunit of $GABA_A$)	−6.9	1	SER A: 421 (2.92)	PHE A: 425 PHE A: 323 LEU A: 428 MET A: 420 VAL A: 424
TFA		−5.8	4	SER A: 179 (2.19) ARG A: 443 (2.51) ASP A: 172 (2.84) GLU A: 171 (3.57)	VAL A: 446
THY		−4.7	1	ILE A: 65 (2.25)	LYS A: 98 LYS A: 98 PRO A: 59

BA binding affinity, *DZP* diazepam, *HBs*, *TFA* trans-ferulic acid, *THY* thymol

than TFA, which correlates with their respective absorption profiles. All three compounds are water soluble. In terms of intestinal absorption, DZP shows the highest intestinal absorption (97.42%), followed by TFA (93.685%) and THY (90.843%). Skin permeability values indicate that TFA has the lowest dermal absorption potential (−2.72). DZP (0.331) and THY (0.407) have better BBB permeability than TFA (−0.239), making them more likely to penetrate the CNS. CNS permeability of TFA is also the lowest. DZP is metabolized by CYP3A4, whereas THY and TFA do not interact with major CYP substrates. TFA has the highest clearance with a value of 0.623 ml/min/kg, while DZP is the only renal OCT2 substrate among the three compounds. Pharmacokinetic and drug-likeness evaluations are shown in Table 5.

Toxicity prediction

Based on the toxicity prediction data in Table 5, THY, TFA, and DZP exhibit distinct toxicity profiles. TFA is not neurotoxic, although THY and the standard drug DZP are. TFA exhibits immunotoxicity, whereas THY and DZP do not. TFA exhibits clinical toxicity, indicating possible negative consequences upon administration; however, THY does not. Only DZP exhibits cytotoxicity, suggesting a greater risk of cellular damage in comparison to THY and TFA. All three substances, however, did not show hepatotoxic, mutagenic, cardiotoxic, and carcinogenic properties, indicating a low risk in these domains. THY and TFA are classified as having reduced toxicity in class 4, while DZP is classified as having a higher toxicity risk in class 2. These results demonstrate that THY and TFA have a safer profile than DZP.

Discussion

The open field test is a widely recognized behavioral paradigm for assessing anxiety and exploratory behavior in rodents (Kraeuter et al. 2019). In our study, the control group exhibited normal exploratory behavior with the highest NSC, NR, and NG, indicating typical activity. Treatment with DZP significantly decreased these parameters, confirming its anxiolytic effects. The TFA-treated group showed a moderate reduction in locomotor and exploratory activities, while THY produced a similar partial reduction, suggesting anxiolytic-like properties in both cases. The combined administration of THY and TFA produced a more pronounced reduction in exploratory behavior, comparable to DZP, indicating a potential combined anxiolytic effect.

In the swing test, which assesses general locomotor activity and CNS excitability (Islam et al. 2014), DZP resulted in the lowest NHC, while the control group displayed the highest. Both TFA and THY caused a significant reduction in NHC ($p < 0.05$), with the combination showing a further decrease, implying synergistic activity. The hole-cross test,

which evaluates exploratory behavior (de Almeida et al. 2012), showed a similar trend. DZP significantly reduced the number of crossings (NS), while TFA and THY produced moderate decreases; the combination group again displayed the greatest reduction, suggesting enhanced anxiolytic efficacy.

However, in the light–dark test, one of the most validated models for distinguishing anxiety-related from sedative behavior (Bourin and Hascoët 2003; Arrant et al. 2013), an increase in time spent in the dark compartment generally indicates enhanced anxiety or reduced exploratory drive, rather than anxiolysis. In this study, DZP treatment increased dark residence time, consistent with its sedative and CNS-depressant profile. Both TFA and THY also increased the duration in the dark box compared to the control, and the THY + TFA combination produced the longest dark residence time. These outcomes therefore likely reflect a predominance of sedative or CNS-suppressive effects rather than a clear anxiolytic action. Taken together, the behavioral results suggest that THY and TFA, alone or in combination, modulate central nervous system activity. As animal locomotor activity is commonly used to estimate central nervous system excitability, a reduction in movement may reflect sedative, calming, antidepressant, or anxiolytic effects (Aziz et al. 2016). Therefore, future studies incorporating sedation control assays (e.g., rotarod or grip-strength tests) are needed to more clearly distinguish anxiolytic activity from motor suppression.

The current findings align well with prior investigations highlighting the GABAergic modulatory roles of both compounds. THY, a phenolic monoterpene found in *Thymus species*, has been reported to possess significant anxiolytic and sedative effects via $GABA_A$ receptor modulation (Bhandari and Kabra 2014; Akbor et al. 2023; Nagoor Meeran et al. 2017). It has also demonstrated a broad pharmacological spectrum, including antioxidants, antimicrobial, antihyperlipidemic, and neuroprotective activities (Alagawany et al. 2021; Aljelehawy et al. 2023). The observed anxiolytic

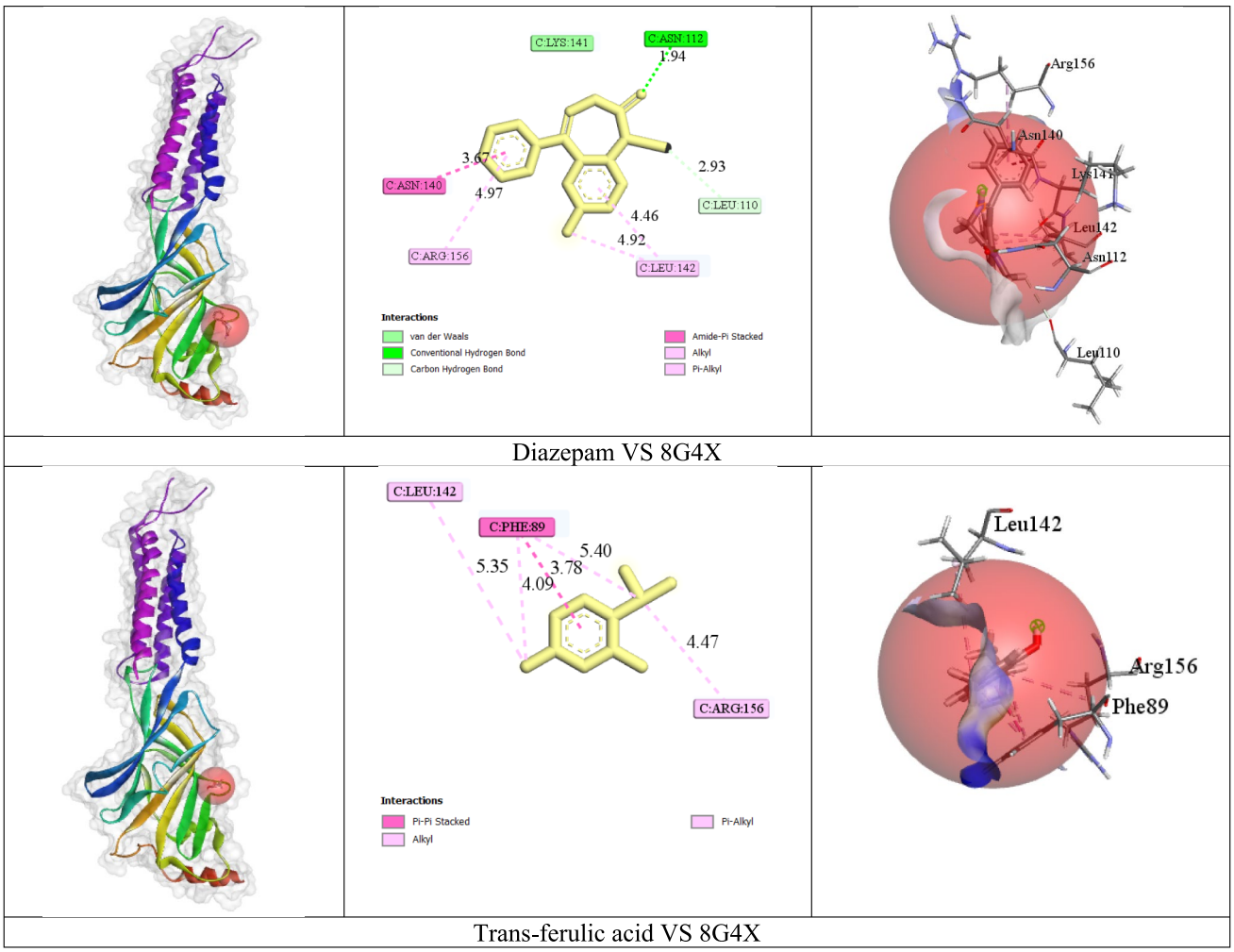


Fig. 4 The 2D and 3D non-bond interaction of diazepam, thymol, and trans-ferulic acid with the selected macromolecule

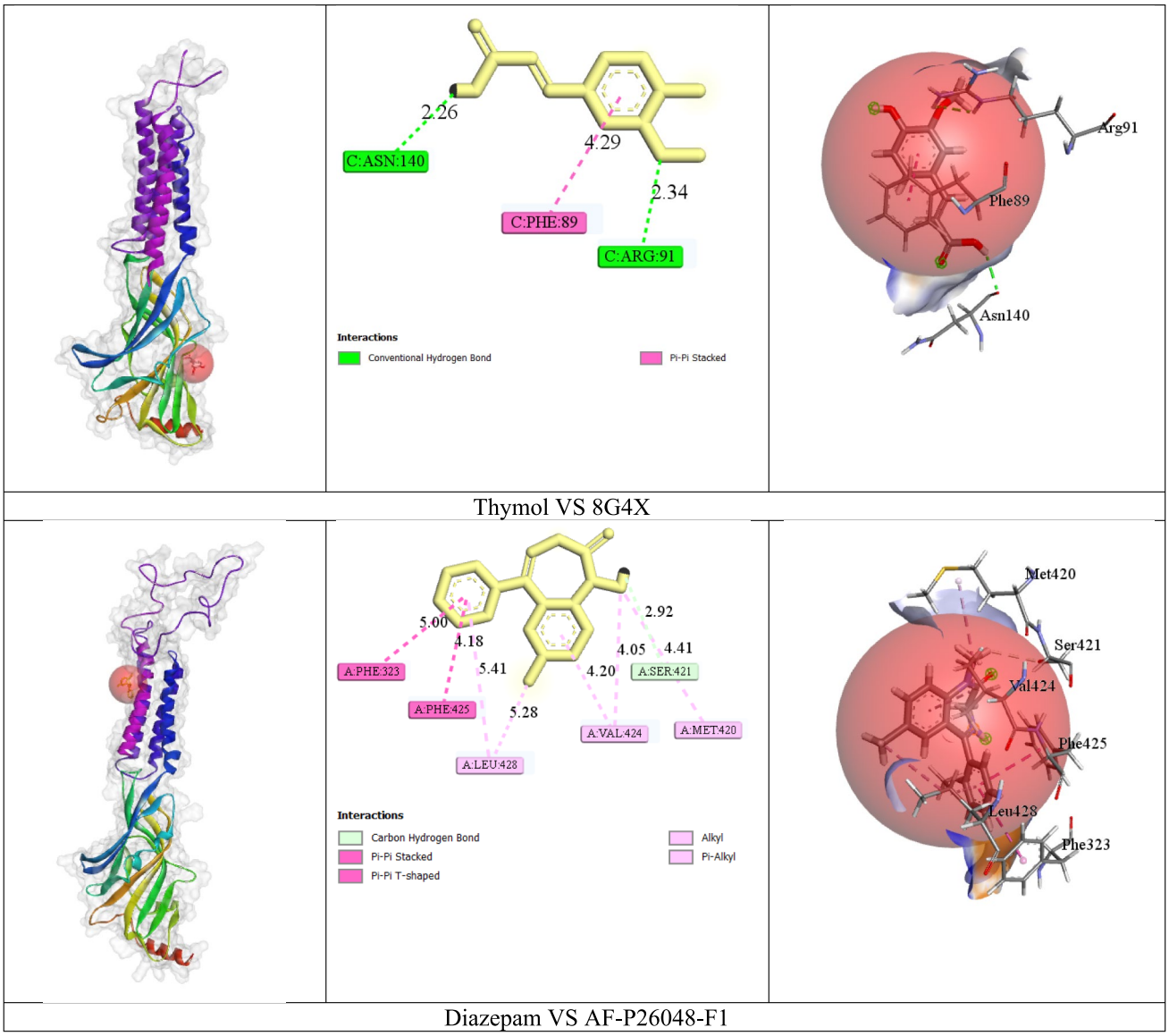


Fig. 4 (continued)

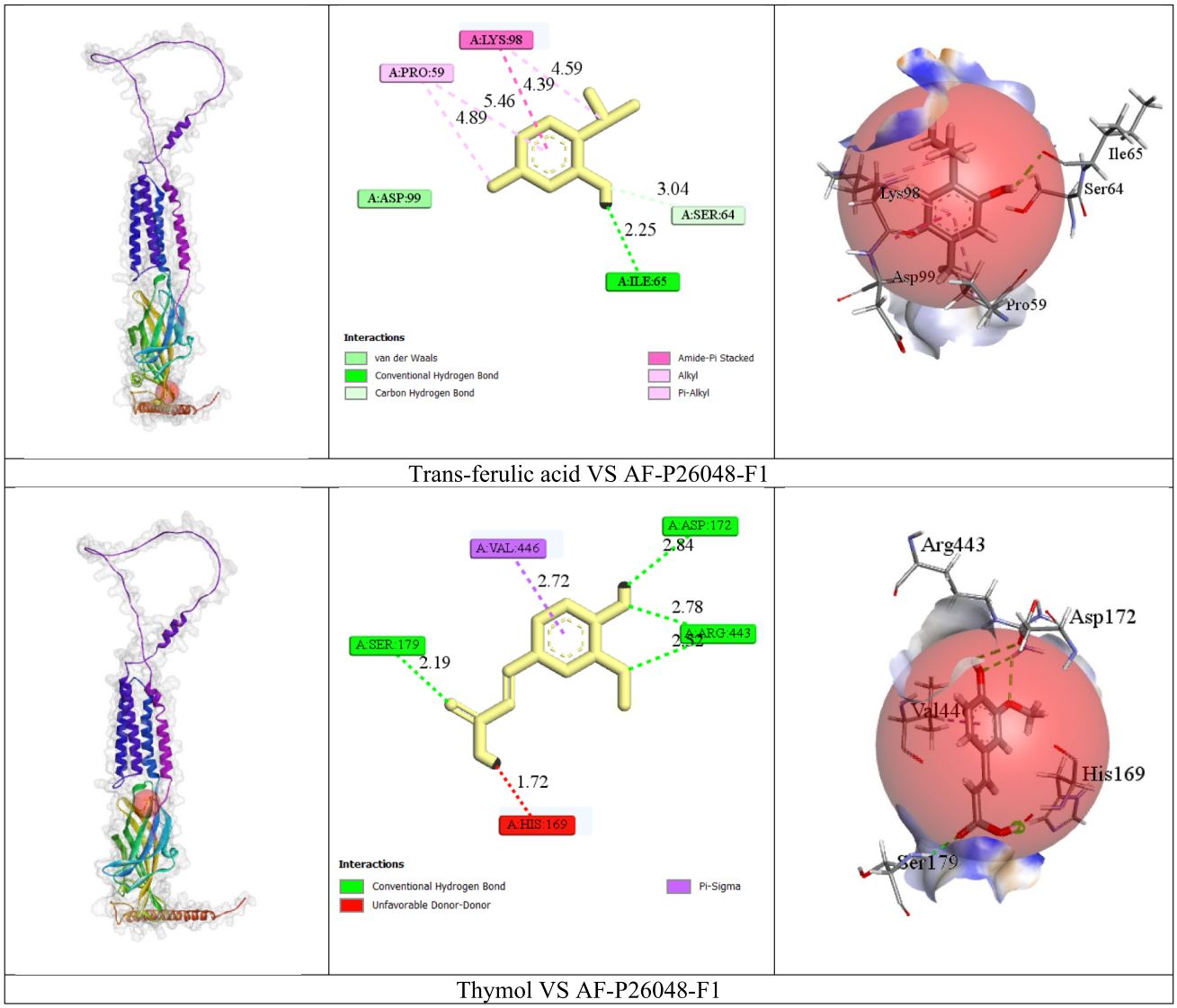


Fig. 4 (continued)

action of THY in this study is consistent with its previously established CNS-depressant and GABAergic characteristics.

Similarly, TFA, a phenolic acid abundant in cereals, fruits, and vegetables, exhibits multiple pharmacological activities such as antidiabetic, hepatoprotective, neuroprotective, and anxiolytic properties (Stompor-Gorący and Machaczka 2021; Kim and Park 2019; Lorigooini et al. 2021; Bhuia et al. 2023b). Numerous studies have revealed that ferulic acid (FA), the structural analog of TFA, exerts anxiolytic-like effects through modulation of serotonergic (5-HT1A), NMDA, and GABAergic receptors (Nagarajan et al. 2015; Singh et al. 2017; Chen et al. 2015; Deng et al. 2018; Dhaliwal et al. 2020; Xu et al. 2016; Salau et al. 2020; Sborgi et al. 2021). Thus, the current findings reinforce earlier evidence suggesting that TFA acts as a promising neuroactive phytochemical with anxiolytic potential mediated via the GABAergic system.

The enhanced behavioral effects of the THY + TFA combination resemble those observed in other synergistic phytochemical studies targeting $GABA_A$ receptors, such as citronellal (Islam et al. 2024), linalool-sclareol co-treatment (Islam et al. 2025b), and sesamol-thymol co-administration (Akbor et al. 2023). Furthermore, Al Hasan et al. (2025b) reported that tangeretin potentiated the sedative activity of DZP through $GABA_A$ receptor interactions. These parallels suggest that combined modulation of $GABA_A$ receptor subunits by multiple phytochemicals can enhance anxiolytic efficacy while minimizing sedative risk.

In contrast to benzodiazepines, which act as potent $GABA_A$ receptor agonists but are associated with tolerance, dependence, and neurotoxicity (Hozack et al. 2022; Ritvo et al. 2023; Magro et al. 2016), both THY and TFA displayed

Table 5 Pharmacokinetic and drug-likeness evaluations along with toxicity prediction of diazepam, trans-ferulic acid, and thymol

Properties	Factors/Parameters	Thymol	Trans-ferulic acid	Diazepam
Physicochemical Properties	Formula	$C_{10}H_{14}O$	$C_{10}H_{10}O_4$	$C_{16}H_{13}ClN_2O$
	Molecular weight (g/mol)	150.22	194.18	284.74
	Number of heavy atoms	11	14	20
	Number of aromatic heavy atoms	6	6	12
	Number of H-bond donors	1	2	0
	Number of H-bond acceptors	1	4	2
	Molar refractivity	48.01	51.63	87.95
Lipophilicity	Log Po/w (XLOGP3)	3.30	1.51	2.99
Drug-likeness	Lipinski	Yes; 0 violation	Yes;0 violation	Yes; 0 violation
	Bioavailability score	0.55	0.85	0.55
Water Solubility	Log S (ESOL)	− 3.19	− 2.11	− 3.87
	Class	Soluble	Soluble	Soluble
Absorption	Caco2 permeability (log Papp in 10^{-6} cm/s)	1.606	0.176	1.554
	Intestinal absorption (human) numeric (%Absorbed)	90.843	93.685	97.42
	Skin permeability (log Kp cm/h)	− 1.62	− 2.72	− 2.205
	P-glycoprotein I inhibitor	No	No	No
	P-glycoprotein II inhibitor	No	No	No
Distribution	BBB permeability (log BB)	0.407	− 0.239	0.331
	CNS permeability (log PS)	− 1.664	− 2.612	− 1.397
	VDss (human) (log L/kg)	0.512	− 1.367	0.365
Metabolism	CYP2D6 substrate	No	No	No
	CYP3A4 substrate	No	No	Yes
	CYP2D6 inhibitor	No	No	No
	CYP3A4 inhibitor	No	No	No
Excretion	Total clearance (log ml/min/kg)	0.211	0.623	0.294
	Renal OCT2 substrate	No	No	Yes
Toxicity	Hepatotoxicity	Inactive	Inactive	Inactive
	Neurotoxicity	Active	Inactive	Active
	Immunotoxicity	Inactive	Active	Inactive
	Mutagenicity	Inactive	Inactive	Inactive
	Cardiotoxicity	Inactive	Inactive	Inactive
	Carcinogenicity	Inactive	Inactive	Inactive
	Clinical toxicity	Inactive	Active	Active
	Cytotoxicity	Inactive	Inactive	Active
	Toxicity class	4	4	2

DZP diazepam, *TFA* trans-ferulic acid, *THY* thymol, *BBB* blood–brain barrier, *CNS* central nervous system

favorable toxicity profiles. Neither compound exhibited hepatotoxicity, mutagenicity, cardiotoxicity, nor carcinogenicity. TFA showed mild immunotoxicity, and THY displayed limited neurotoxicity, while DZP exhibited broader toxicity, consistent with prior reports (Bhuia et al. 2023b). These results are comparable with natural agents such as quercetin and citronellal that interact with GABAA receptors while exhibiting low toxicity (Islam et al. 2022, 2024). Therefore, the present findings not only corroborate existing literature but also extend it by providing novel evidence of combining anxiolytic effects between THY and TFA, supported by behavioral, molecular, and computational data.

Molecular docking provides insight into ligand–receptor interactions that underlie observed pharmacological effects. The $GABA_A$ receptor is the principal mediator of inhibitory neurotransmission in the brain, with its α2 and α3 subunits playing key roles in anxiolysis (Ghit et al. 2021; Bryson et al. 2023; Qian et al. 2023). Docking analysis showed that DZP, as expected, exhibited the strongest binding affinity (− 6.9 kcal/mol) with multiple hydrogen bonds and hydrophobic interactions. TFA displayed moderate affinity (− 5.8 kcal/mol for α2; − 5.3 kcal/mol for α3) and formed several stabilizing hydrogen bonds (SER A:179, ARG A:443, ASP A:172,

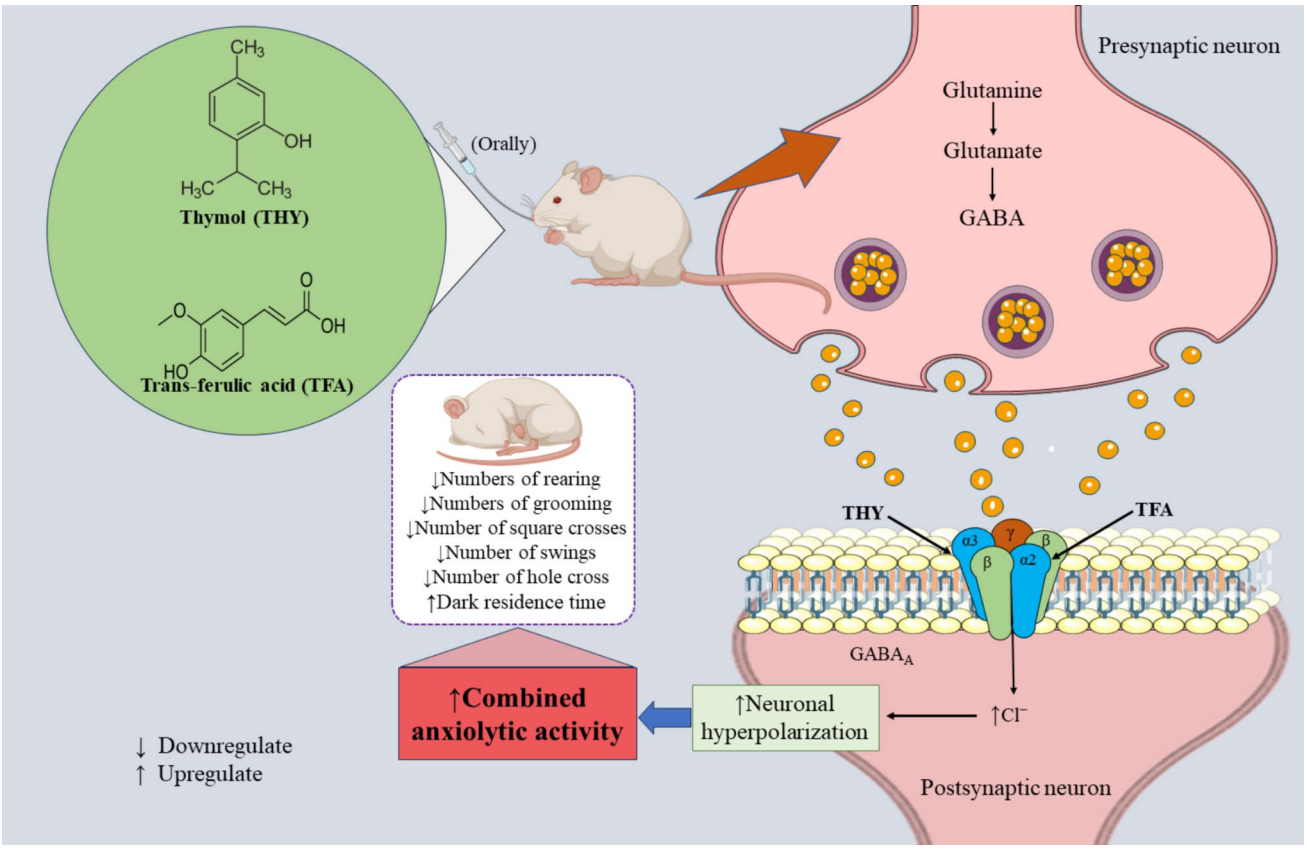


Fig. 5 The anxiolytic effects of trans-ferulic and thymol. [Thymol and trans-ferulic acid enhance gamma-aminobutyric acid (GABA) activity at $GABA_A$ receptors. This increases chloride ion (Cl^-) influx into the postsynaptic neuron, causing neuronal hyperpolarization. The resulting effect is a reduction in anxiety-related behaviors, indicating combining anxiolytic activity.]

GLU A:171), while THY exhibited slightly weaker affinity (− 4.7 kcal/mol for α2; − 5.6 kcal/mol for α3), primarily via hydrophobic interactions with residues such as PHE C:89, ARG C:156, and LEU C:142.

These interactions suggest that TFA provides stable hydrogen bonding within the receptor pocket, enhancing binding stability, while THY's hydrophobic interactions may facilitate allosteric modulation of receptor activity. The combination of both molecules may thus exert a combining effect on $GABA_A$ receptor modulation, leading to enhanced chloride influx, neuronal hyperpolarization, and reduced excitability, culminating in anxiolytic behavior (Fig. 5).

In addition, pharmacokinetic data support this mechanism: THY exhibited greater BBB permeability (log BB = 0.407), ensuring effective CNS access, whereas TFA showed higher oral bioavailability (0.85) and metabolic stability. The complementary pharmacokinetic properties of the two compounds likely enhance their combined neuropharmacological efficacy. The absence of CYP inhibition and low predicted toxicity further indicate that this pairing could provide a safe therapeutic alternative to conventional benzodiazepines.

Despite the encouraging results, several limitations should be acknowledged. The behavioral tests employed primarily measured locomotor and exploratory activities, which may not fully distinguish between anxiolytic and sedative effects (Aziz et al. 2016). Therefore, the claim of anxiolysis should be interpreted cautiously, as the observed behavioral suppression could partly reflect sedation rather than a specific anxiolytic response. Sedation-control assays such as rotarod or grip-strength tests were not performed, which may limit interpretation. The use of a single dose (50 mg/kg) for both THY and TFA restricts the assessment of dose–response relationships and pharmacodynamic variability. The exclusive use of male Swiss albino mice also limits generalization to other sexes or strains. The molecular docking utilized an AlphaFold-predicted model for the α2 subunit, which, while accurate, may lack the precision of experimentally resolved structures. Furthermore, in silico predictions from SwissADME, pkCSM, and ProTox-II provide only preliminary evidence of safety and pharmacokinetic suitability; these computational results require empirical validation through chronic and acute toxicity assays following OECD 423/425 guidelines.

Finally, this study focused on acute behavioral effects rather than long-term neuroadaptive changes or pharmacological tolerance, which will be necessary to establish translational therapeutic relevance. This study provides the first comprehensive evidence that the co-administration of THY and TFA produces a combined anxiolytic effect by modulating the GABAergic system, positioning these plant-based compounds as promising, safer candidates for anxiety treatment compared to benzodiazepines, due to their favorable safety and pharmacokinetic profiles. To advance these findings, future research must quantitatively confirm the combination through isobolographic and dose–response analyses, incorporate sedation-control assays to rule out motor-impairing effects, and validate the proposed mechanism using molecular dynamics and binding studies. Furthermore, establishing their clinical applicability and long-term safety through chronic toxicity studies and OECD-compliant trials is essential for developing THY and TFA into viable next-generation nutraceutical or phytomedicine solutions for anxiety disorders.

Conclusion

The present study demonstrates that THY and TFA possess measurable anxiolytic-like effects in mice, and their co-administration enhances these effects compared to individual treatments. Behavioral observations across the open-field, swing, hole-cross, and light–dark tests suggest that this combination modulates anxiety-related responses, possibly through interaction with GABAergic pathways. The molecular docking results further indicate that both compounds exhibit moderate binding affinities toward the α2 and α3 subunits of the $GABA_A$ receptor, supporting a potential mechanistic basis for the observed in vivo effects. In addition, the pharmacokinetic and toxicity predictions indicate favorable absorption, blood–brain barrier permeability, and safety margins for both phytochemicals. However, these findings should be interpreted with caution. The behavioral models employed do not entirely distinguish anxiolytic from sedative effects, and the absence of dose–response assessments, chronic dosing evaluations, and sex-based comparisons limits the generalizability of the results. Moreover, the in silico analyses, while supportive, remain preliminary and require validation through molecular dynamics simulations, receptor-binding assays, and electrophysiological studies. The AlphaFold-predicted receptor models and computational toxicity estimates provide useful guidance but cannot substitute for experimental verification. Overall, this study provides initial evidence that THY and TFA may act as complementary GABAergic modulators with anxiolytic-like potential. Nonetheless, further investigations incorporating detailed dose–response experiments, chronic administration protocols, sedation-control tests, mechanistic analyses, and validation in diverse animal models are essential before any translational or clinical inferences can be drawn. The results presented here should therefore be regarded as a preliminary but encouraging step toward understanding the neuropharmacological potential of these two naturally occurring compounds.

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Author contributions All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas that is, revising or critically reviewing the article; giving final approval of the version to be published; agreeing on the journal to which the article has been submitted; and confirming to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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