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Research Article

Anxiolytic activity of black pepper (*Piper nigrum* L.) fruit extract*: in vivo* and *in silico* study

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ABSTRACT

Black pepper (*Piper nigrum* L.) contains piperine, which exhibits several pharmacological activities, including anxiolytic and antidepressant effects. This study aimed to determine the anxiolytic activity of black pepper extract (BPE) *in vivo* and *in silico*. Fifty mice were prepared for the anxiolytic activities test using the mouse rotarod and forced swimming test (FST). The volatile compounds of BPE were analysed with GC-MS and then tested for anxiolytic activity in silico. BPE at 100 and 200 mg/kg BW reduced rotarod fall time and increased %MPE, producing values of 61 ± 26.578 and 51.67 ± 29.155 at 120 minutes, which were comparable to alprazolam (61.35 ± 20.394; p=1.000 and p=0.602). In the FST, BPE at both doses significantly increased immobility time (166 ± 7.616 and 177.8 ± 21.218 s) compared to the control group (100 ± 23.270 s), with p = 0.002 and p < 0.001. Apart from piperine, Germacrene D, (+)-delta-Cadinene, 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]- in BPE has been proven to have potential anxiolytic activity based on in silico tests. In conclusion, BPE, with its various secondary metabolite contents, could be a promising candidate for further development as an anxiolytic agent.

Keywords:
Black pepper extract; Piperine; Anxiolytic; *In vivo*; *In silico*

1. INTRODUCTION

Anxiety disorder is a condition in which a person experiences persistent and severe anxiety symptoms and has irrational fears that can significantly interfere with normal daily functioning [1]. The worldwide prevalence of anxiety disorders has increased, especially during the COVID-19 pandemic. In 2019, the prevalence of anxiety disorders increased by 11.2%, and in 2020, there was an increase of 25.6% [2]. In Indonesia, there is no precise data regarding the prevalence of anxiety disorders. According to the 2018 Basic Health Research, 9.8% of Indonesians aged 15 years and over suffer from mental and emotional disorders, and around 24 million Indonesians experience depression [3].

Benzodiazepines are anxiolytic agents used in the treatment of anxiety disorders to treat acute anxiety symptoms quickly. Side effects of benzodiazepines include ataxia, vertigo, dizziness, fatigue, impaired motor coordination, confusion, disorientation, and anterograde amnesia. In addition, long-term use may lead to dependence in most patients [4]. The side effects of conventional drugs for anxiety disorders encourage more people to use herbal medicine as an alternative treatment. People assume that herbal medicines are safe and cause fewer side effects [5].

Black pepper (*Piper nigrum* L.) is a plant from the Piperaceae family. It is one type of medicinal plant found in tropical and subtropical regions, including Indonesia [6]. The plant has long been valued in traditional medicine, where it has been used for its antiseptic, antispasmodic, analgesic, anti-inflammatory, antipyretic, and aphrodisiac properties, as well as for the management of rheumatism and various other health conditions [7].

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Black pepper contains various secondary metabolites, including alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, anthraquinones, and essential oils [8]. The primary active compound in black pepper is piperine, an alkaloid often used in cooking and medicine [9]. Piperine exhibits several pharmacological activities, including anxiolytic, antidepressant, anti-inflammatory, and analgesic [10].

Previous research has shown that piperine exhibits anxiolytic activity in mice using the elevated plus-maze (EPM) method and the light/dark box test [11]. Another study demonstrated that the methanol extract of black pepper fruit has anxiolytic and antidepressant activity in Alzheimer's model mice using the EPM and FST methods [12]. Meanwhile, no study has been done on the anxiolytic activity of black pepper ethanol extract. The study aimed to determine the anxiolytic activity of black pepper extract (BPE) in mice using the rotarod and FST methods. A forward pharmacology approach, incorporating in silico tests, was also employed to elucidate the alleged mechanism of action of the active compounds in the extract.

2. MATERIALS AND METHODS

2.1. Materials

Simplicia powder of black pepper fruit (*Piper nigrum* L.), derived from Materia Medica Batu; Hexane (Sigma), ethanol 96%, sodium-CMC, and aqua-destilled were purchased from Brataco, and alprazolam tablets produced by PT Mersifarma were used as a positive control.

2.2. Methods

2.2.1. Black pepper extract preparation

The powder (*Piper nigrum* L.) was macerated for 3 × 24 hours with 96% ethanol at a 1:10 ratio. Then, the macerate was separated by filtration, and re-maceration was carried out with a volume of solvent equal to half the amount of solvent in the first extract. The macerate was collected and evaporated at 40°C with a rotating evaporator before being concentrated in an oven at 40-50°C. The crude extract is weighed to determine the yield, then stored at a temperature of 2-8°C before use.

2.2.2. In vivo anxiolytic activities

Fifty male Balb/c mice used in this research were 2-3 months old, 20-30 grams in body weight, and healthy. The mice were divided into two groups for the test methods (rotarod and FST) in equal numbers. In each technique, mice were divided randomly into five groups (5 animals per group), which were: negative control group given 1% sodium-CMC treatment; positive control group treated with Alprazolam 1 mg/kg per oral; then three groups treated with black pepper extract (BPE) p.o in different doses: 50 mg/kg BW, 100 mg/kg BW, and 200 mg/kg BW. The protocol was approved by the Ethical Committee of Medical Research, Dental Faculty, University of Jember (No.1978/UN25.8/KEPK/DL/2023).

A. Mice rotarod test

The anxiolytic activity was determined using a mouse rotarod device (Ugo Basile). Before testing, mice were trained for at least three consecutive days to survive on a rotating bar for 180 seconds. A total of 25 mice that had met the requirements were randomly divided into five groups as previously described. Testing was carried out 24 hours after the last training session. On the testing day, mice were treated according to their respective groups. Anxiolytic effect was observed by placing the mice on a rotarod at a constant speed of 40 rpm. Testing was conducted at 0, 60, 90, 120, 150, and 180 minutes after administration. Determination of the time for mice to fall from the rotating rod was carried out with a maximum time limit of 60 seconds [13]. The percentage of maximum possible effect (% MPE) was calculated using the following formula from the data on the time of falling mice [14].

$$\%\text{MPE} = \frac{(\text{Pre Treatment} - \text{Post Treatment})}{\text{Pre Treatment}} \times 100\%$$

B. Force swim test (FST)

The test was carried out using a transparent cylindrical glass container with a diameter (d) of 20 cm and a height (h) of 20 cm. The compartment was filled with water at 25°C and adjusted to the water depth according to the mice's size, so the hind legs of the mice could not touch the bottom of the cylinders. Anxiolytic effects were observed 30 minutes after BPE administration. One testing session was conducted for 6 minutes and divided into a pre-test (the first 2 minutes) and a test (the last 4 minutes). Immobility time was recorded [15].

C. Data analysis

For the *in vivo* test, the MPE percentage and the mean immobility time of the mice were analyzed using the One-Way Analysis of Variance (ANOVA) test, followed by Tukey's post hoc test. P-value <0.05 indicates a significant difference.

2.2.3. GC-MS Metabolite Profiling

A. GC-MS preparation

Homogenization was performed using a vortex for 5 minutes, followed by centrifugation for 1 minute at 6000 rpm and room temperature. The supernatant was injected into the GC-MS/MS.

B. GC-MS condition analysis

GCMS-TQ8030 Shimadzu with detector MS/MS mode Q3, Column SH-5MS w/5m Integrated Guard (length = 30 m; ID = 0,32 mm; thickness = 0,25 µm) (Shimadzu), carrier gas Helium – linear velocity, oven temperature 60°C injection temperature 240°C interface temperature 240°C, GC Column oven temp 60°C, injection temperature 240°C, MS condition ion source 200°C, Interface Temp 240°C solvent cut time 2.8 min.

2.2.4. In Silico Study

This research was conducted on a PC equipped with 20GB of RAM and an Intel Core i5-10400 CPU, running the Windows 11 operating system. Pyrx, Biovia (Discovery Studio 2021 client), and the Protein Data Bank (PDB) were used to source human receptors. PDB-formatted secondary metabolites derived from *Piper nigrum* were also obtained from the PubChem website.

A. Preparation of ligand compounds

To determine the chemical's structure, the PubChem software, accessible at www.pubchem.ncbi.nlm.nih.gov, was utilized. After obtaining the Simplified Molecular Input Line Entry System (SMILES) for the P. *nigrum* compound, a PASS test was carried out on the www.swisstargetprediction.ch website. Additionally, the SMILES is entered to conduct a pre-Swiss Adsorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET) test on the www.swissadme.ch/ website.

B. Molecular docking

The protein structure and its native ligand were retrieved from the Protein Data Bank (PDB). Using PyMol, the ligand was removed from the protein structure, retaining polar hydrogens and applying Kollman charges. Docking simulations were performed with AutoDock Vina, integrated into PyRx 0.9.9. The grid was configured with a completeness value of 8 and dimensions of 50 × 50 × 50, centered at coordinates x = -45.00, y = -35.00, z = -10.00. RMSD values below 2 Å were deemed valid for docking result.

3. RESULTS

3.1. *In vivo* anxiolytic activity

3.1.1. Rotarod method

The anxiolytic effect of BPE on the rotarod method is shown in Figure 1. Fall time remained unchanged in the sodium-CMC group but decreased in the alprazolam group. BPE at 50 mg/kg BW produced a slight reduction, whereas the 100 and 200 mg/kg BW doses resulted in more pronounced decreases in fall time.

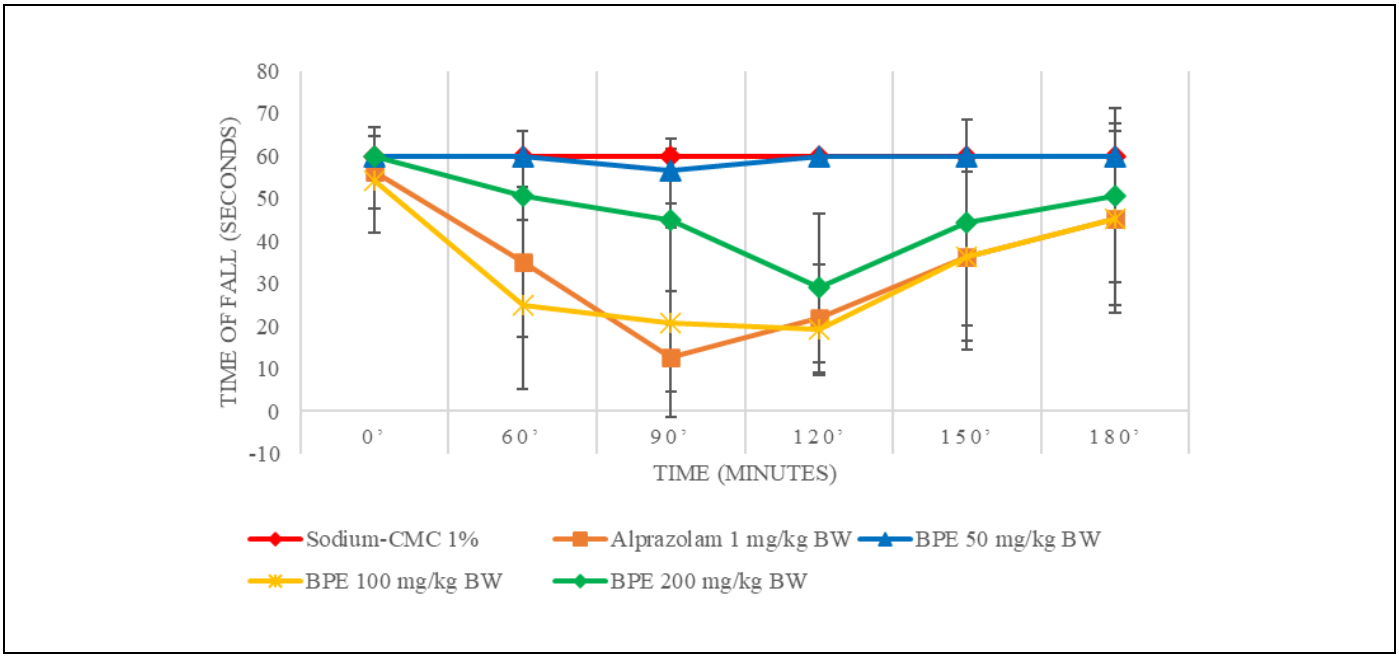

Figure 1. Mean of mice falling time in the rotarod test after the treatment. n = 5, BPE = Black Pepper Extract

Table 1. Mean percentage MPE in all treatment groups

Groups	Mean MPE (% ± SD)		
	60’	90’	120’
Sodium-CMC 1%	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
ALP 1 mg/kg BW	39.33 ± 26.383^{b}	78.54 ± 12.418^{b}	61.35 ± 20.394^{b}
BPE 50 mg/kg BW	0 ± 0^{a}	$5.67 \pm 12.671^{a,c}$	0 ± 0^{a}
BPE 100 mg/kg BW	53.08 ± 32.871^{b}	$61.25 \pm 36.669^{b,c}$	61 ± 26.578^{b}
BPE 200 mg/kg BW	$15.33 \pm 25.315^{a,b}$	$25 \pm 27.988^{a,c}$	51.67 ± 29.155^{b}

Data were analyzed using the Kruskal-Wallis test and followed by the Mann-Whitney test (n=5). Different superscript letters indicate significant differences between groups ($p < 0.05$). ALP: Alprazolam; BPE: Black Pepper Extract.

The MPE percentage is the most significant effect that can be achieved under specific experimental conditions, as presented in Table 1. The negative control showed no increase in % MPE, while alprazolam produced a marked elevation, peaking at 90 minutes. BPE at 50 mg/kg BW increased % MPE at 90 minutes; the 100 mg/kg BW dose increased %MPE at 90 and 120 minutes; and the 200 mg/kg BW dose showed an increase only at 120 minutes.

Analysis of % MPE revealed significant differences between the negative and positive control groups at 60, 90, and 120 minutes. BPE at 100 mg/kg BW differed significantly from the negative control at all time points ($p = 0.018$; 0.019; 0.005). BPE at 200 mg/kg BW showed no significant difference at 60 ($p = 0.136$) and 90 minutes ($p = 0.054$), but differed significantly at 120 minutes ($p = 0.019$). Neither BPE 100 nor BPE 200 mg/kg BW differed significantly from the positive control ($p = 0.002$ and $p > 0.001$).

3.1.2. Force swim test (FST) method

Immobility time in the FST reflects the anxiolytic effect of BPE, as summarized in Table 2. The negative control showed the lowest values, while the positive control showed the highest. BPE produced a dose-dependent increase in immobility time. Statistical analysis indicated that BPE at 100 and 200 mg/kg BW significantly increased immobility time compared with the negative control ($p = 0.002$ and $p<0.001$). Still, neither dose differed significantly from the positive control ($p = 0.368$ and $p = 0.827$)

Based on *in vivo* test results, it is known that BPE 100 and 200 mg/kg BW can significantly impact the anxiolytic effect. These compounds can shorten the rotarod fall time and increase % MPE, equivalent to alprazolam, as well as the immobility time of mice using the FST method.

3.2. Phytochemical compounds analysis

Fifty compounds were identified from an 11.7% yield of BPE. The chromatogram of *Piper nigrum* essential oil is presented in Figure 2, while the chemical constituents with their retention time (RT) and peak area are presented in Table 3.

3.3. *In silico* test

3.3.1 Swiss target prediction and ADMET test

A comprehensive literature review was conducted to identify secondary metabolites present in *Piper nigrum*. The selected compounds were subjected to SwissTargetPrediction to determine their potential interactions with anxiolytic-related targets, particularly the cannabinoid receptor 2 (CB2), serotonin transporter (SERT), monoamine oxidase B (MAO-B), and butyrylcholinesterase (Bu-ChE). Based on the prediction scores and relevance to anxiolytic pathways, ten major compounds were shortlisted for further analysis, with piperine included as the reference compound (Table 4). Each compound was subsequently evaluated using ADMET analysis, which included

Table 2. Data on the mean immobility time in all treatment groups

Groups	Mean Immobility Time (seconds ± SD)
Sodium-CMC 1%	100 ± 23.270^{a}
ALP 1 mg/kg BW	193.2 ± 26.948^{b}
BPE 50 mg/kg BW	$134.8 \pm 29.79^{a,c}$
BPE 100 mg/kg BW	$166 \pm 7.62^{b,c}$
BPE 200 mg/kg BW	$177.8 \pm 21.22^{b,c}$

Data were analyzed using the One-Way ANOVA test and continued with Tukey's Post Hoc test (n=5). Different superscript letters indicate significant differences between groups ($p < 0.05$). ALP: Alprazolam; BPE: Black Pepper Extract.

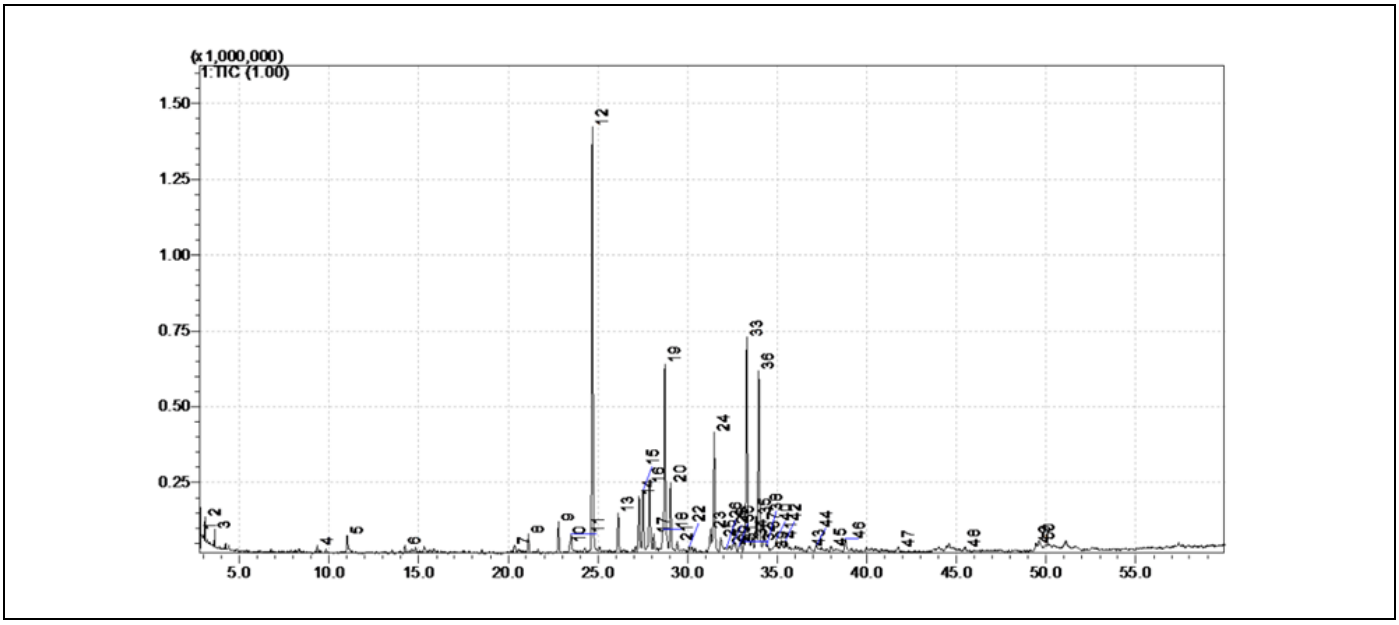

Figure 2. GC-MS chromatogram of black *Piper nigrum* L.

the Lipinski rule of five parameters: molecular weight ≤ 500, molecular Log P ≤ 4.15, N or O ≤ 10, and NH or OH ≤ 5.

3.3.2 Molecular docking analysis

Docking analysis against the CB2 receptor showed that germacrene D exhibited the strongest binding affinity (–5.9 kcal/mol). This compound interacted with key binding residues also observed in the reference ligand, including Phe 1103, Asp 1069, Ala 1072, Ala 1073, Gly 1029, and Val 1102 (Figure 3), suggesting a potentially similar binding mode. In silico analysis revealed that piperine and germacrene D are located within a conserved binding pocket primarily defined by Phe A:1103, Asp A:1069, and Ala A:1073. Both ligands displayed comparable interaction patterns, with Phe A:1103 functioning as the significant anchoring residue through stable π-π-alkyl and π–π stacking interactions, indicating an aromatic-driven stabilisation mechanism. Asp A:1069 contributed additional stability via van der Waals forces; furthermore, Ala A:1073 and Ala A:1072 formed a compact hydrophobic subpocket that accommodated

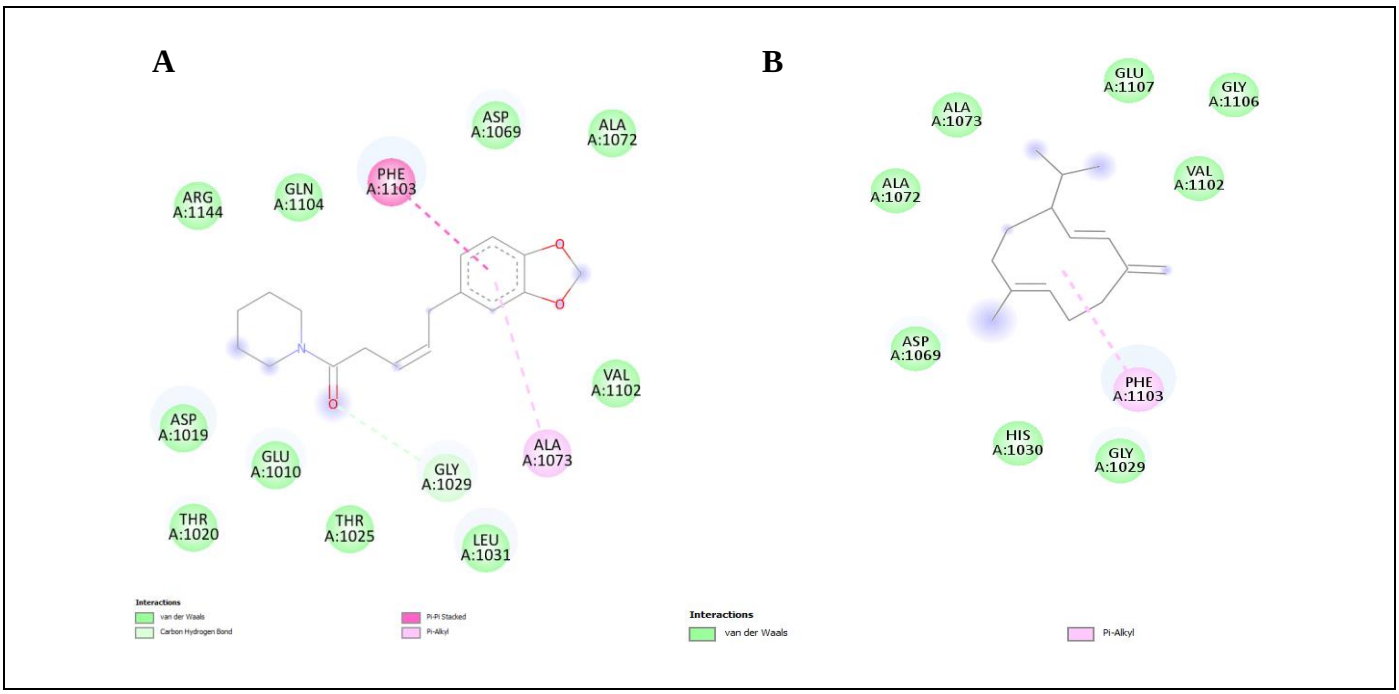

Figure 3. Molecular docking visualisation 5ZTY with piperine (A) and Germacrene D (B)

Table 3. Chemical constituents of Black Pepper in GC-MS analysis

Peak	Rt (min)	Peak area	% Peak area	Peak Intensity	% Peak intensity	Chemical
1	2.823	77938	0.25	47358	0.77	Hexane
2	3.079	142857	0.45	80078	1.31	Benzene, methyl-
3	3.616	101387	0.32	58663	0.96	Hexane, 2,3,3-trimethyl-
4	9.35	82241	0.26	21602	0.35	Nonane, 5-(2-methyl propyl)-
5	11.017	217909	0.69	50632	0.83	Linalool
6	14.235	101446	0.32	22731	0.37	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
7	20.347	118990	0.38	22165	0.36	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-
8	21.116	294509	0.93	62084	1.01	Delta-elemene
9	22.8	431175	1.36	97794	1.59	Alpha-copaene
10	23.42	89057	0.28	24737	0.4	Alpha-cubebene
11	23.507	264109	0.83	56634	0.92	Beta-elemene
12	**24.684**	**6729921**	**21.25**	**1397612**	**22.79**	**Trans-caryophyllene**
13	26.126	570763	1.8	123940	2.02	Alpha-humulene
14	**27.294**	**932806**	**2.95**	**181076**	**2.95**	**Germacrene-D**
15	**27.512**	**1013578**	**3.2**	**198762**	**3.24**	**Beta-selinene**
16	**27.877**	**1202858**	**3.8**	**212750**	**3.47**	**Alloaromadendrene**
17	28.11	245419	0.77	51263	0.84	Naphthalene, 1,2,4A,5,6,8A-Hexahydro-4,7- Dimethyl-1-(1-Methyleethyl)-, [1S-(1.Alpha,4A.Alpha.,8A.Alpha.)] -
18	28.616	261931	0.83	67075	1.09	Phenol, 2,6-bis(1,1-dimethyl ethyl)-4-methyl-
19	**28.728**	**3324602**	**10.5**	**612224**	**9.98**	**Torreyol**
20	**29.051**	**1049203**	**3.31**	**217594**	**3.55**	**Delta-cadinene**
21	29.424	140504	0.44	29647	0.48	Alpha-copaene
22	30.11	73567	0.23	16478	0.27	Elemol
23	31.268	328816	1.04	68776	1.12	1H-Cycloprop[E]Azulen-7-ol, Decahydro-1,1,7- Trimethyl-4-Methylene-
24	**31.479**	**2238535**	**7.07**	**387764**	**6.32**	**(-)-Caryophyllene oxide**
25	31.85	260808	0.82	46244	0.75	Naphthalene, 1,2,3,4,4a,5,6,8a-octahedron-7-methyl-4-methylene-1-(1-methyl ethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-
26	32.164	105446	0.33	11983	0.2	Humulen-(v1)
27	32.338	148192	0.47	18816	0.31	Valencene
28	32.531	158613	0.5	26358	0.43	Humulene oxide
29	32.649	174346	0.55	29017	0.47	Beta-guaiene
30	32.868	83409	0.26	17138	0.28	1,4-Methanoazulen-7(1H)-one, octahydro- 4,8,8,9-tetramethyl-, (+)-
31	32.97	171600	0.54	32378	0.53	Epiglobulol
32	33.16	145233	0.46	32773	0.53	Beta-panasinsene
33	**33.302**	**3992836**	**12.61**	**708683**	**11.56**	**1H-Cycloprop[E]Azulen-7-ol, Decahydro- 1,1,7-Trimet-4-Methylene-**
34	33.588	356611	1.13	49617	0.81	Aromadendrenepopoxide-(I)
35	**33.809**	**648450**	**2.05**	**118538**	**1.93**	**T-muurolol**
36	**33.967**	**3287686**	**10.38**	**596168**	**9.72**	**Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7- methyl-4-methylene-1-(1-methylethyl)-, (1 alpha,4a alpha,8a alpha)-**

Table 3. Chemical constituents of Black Pepper in GC-MS analysis (Continued).

Peak	Rt (min)	Peak area	% Peak area	Peak Intensity	% Peak intensity	Chemical
37	34.283	530162	1.67	71196	1.16	Veridiflorol
38	34.426	92949	0.29	17424	0.28	Beta-guaiene
39	34.722	75224	0.24	5213	0.09	1-(3,4-Dimethoxyphenyl)-2-[(4,6-Ddimethyl-2-Pyrimidinyl)Amino]-1,5-Ddihydro-4H-Imidazole- 4-one
40	34.935	172726	0.55	31688	0.52	Ledene
41	35.215	159085	0.5	29885	0.49	Beta-copies-4 alpha-ol
42	35.47	124127	0.39	20738	0.34	Beta-copies-4 alpha-ol
43	36.767	101273	0.32	14562	0.24	Pentalene, octahydro-1,4-diiodo-
44	37.191	136241	0.43	20772	0.34	Androstan-17-one, 3-ethyl-3-hydroxy-, (5 alpha)-
45	37.983	113287	0.36	18635	0.3	(E,1'RS,2'RS,4'SR,7'SR)-1-(2',5',5'-trimethyl-3'-oxatricyclo[5.1.0.0(2,4)]oct-4'-yl)-3-methyl-1,3- butadiene
46	38.795	234331	0.74	39785	0.65	1H-Cycloprop[E]Azulen-7-ol, Decahydro-1,1,7- Trimethyl-4-Methylene-
47	41.758	78865	0.25	16648	0.27	Pogostol
48	45.475	73993	0.23	12721	0.21	2,3-Bornanediol
49	49.413	104705	0.33	17333	0.28	9,12-Octadecadienoic acid (Z, Z)-, methyl ester
50	49.604	106873	0.34	18774	0.31	Cyclopropaneoctanoic acid, 2-octyl-, methyl ester

Table 4. Potential active compounds for anxiolytic activity

No	Metabolite	Lipinski rule	Toxicity level	Receptor	Smile
1	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aβ,8aα)-	yes	5	Acetylcholinesterase	CC1=C[C@@H]([C@H](C(C)C)CC2)[C@H](C2=C)CC1
2	Trans-caryophyllene	yes	5	Cannabinoid receptor 2	CC1=CCCC(=C)C2CC(C2CC1)(C)C
3	Torreyol	yes	5	Butyrylcholinesterase	CC1=CC2C(CCC(C2CC1)(C)O)C(C)C
4	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methyl ethyl)-, (1 alpha,4a alpha,8a alpha)-	yes	5	Cannabinoid receptor 2	CC1=C[C@@H]([C@H](C(C)C)CC2)[C@H](C2=C)CC1
5	beta-Caryophyllene Oxide	yes	5	Serotonin transporter	CC1(CC2C1CCC3(C(O3)CCC2=C)C)C
6	(+)-delta-Cadinene	yes	5	Cannabinoid receptor 2	CC1=CC2C(CCC(=C2CC1)C)C(C)C
7	(-)-Alloaromadendrene	Yes	5	Cannabinoid receptor 2	CC1CCC2C1C3C(C3(C)C)CCC2=C
8	Beta-selinene	yes	5	Cannabinoid receptor 2	CC(=C)C1CCC2(CCCC(=C)C2C1)C
9	Germacrene D	yes	5	Cannabinoid receptor 2	CC1=CCCC(=C)C=CC(CC1)C(C)C
10	Alpha-humulene	yes	5	Cannabinoid receptor 2	CC1=CCC(C=CCC(=CCC1)C)(C)C
11	**piperin**	**yes**	**4**	**Monoamine oxidase B**	**C1CCN(CC1)C(=O)C=CC=CC2=CC3=C(C=C2)OCO3**

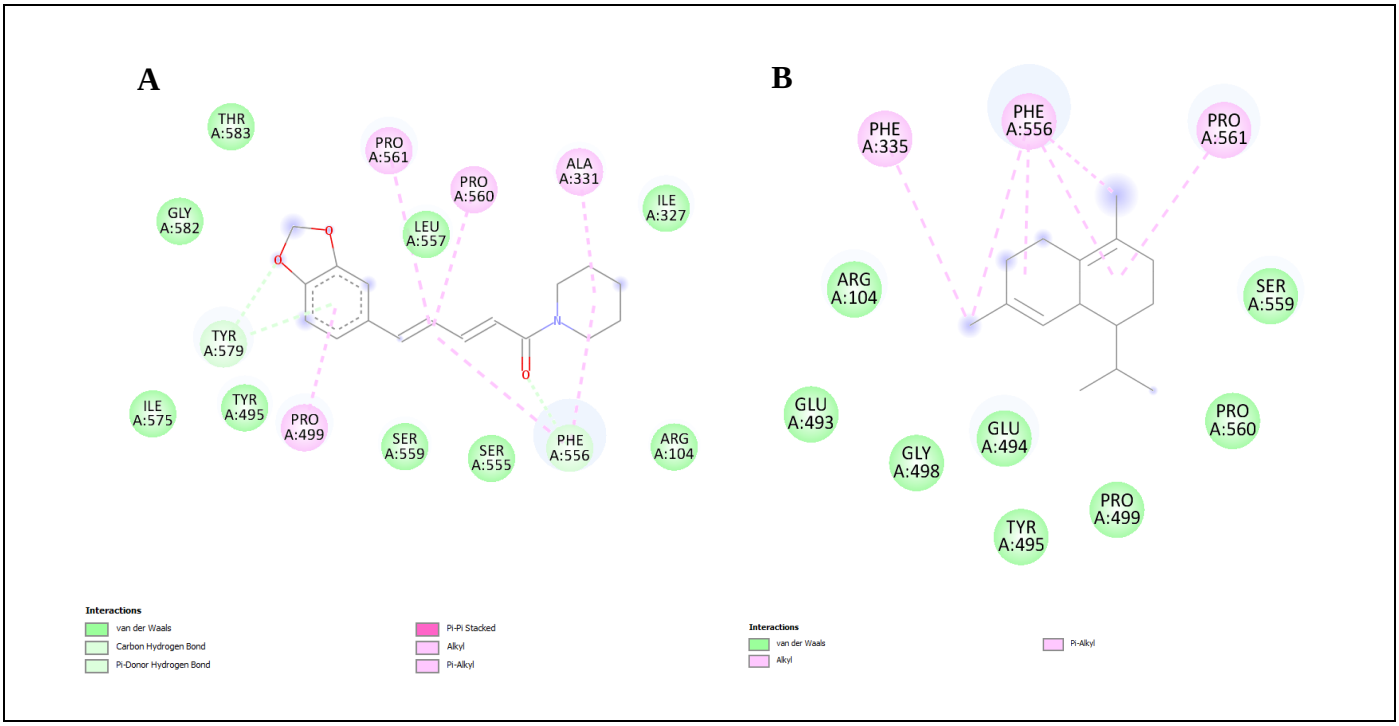

Figure 4. Molecular docking visualisation 5i6x with piperin (A) and (+)-delta-Cadinene (B)

the ligand's nonpolar groups-suggesting a potentially similar binding mode. For the serotonin transporter, (+)-δ-cadinene demonstrated the highest affinity (–7.3 kcal/mol), forming interactions with essential residues shared with the reference ligand, namely Pro 561, Tyr 495, Pro 499, Ser 559, and Phe 556 (Figure 4), with Phe A:556 serving as an aromatic anchor through consistent π–π stacking across all ligands. Piperine, as a native ligand, is attributed to its ability to complement core hydrophobic contacts with a distinct hydrogen-bonding network that stabilises its binding pose. In contrast, (+)-delta-Cadinene showed weaker performance due to its reliance on hydrophobic interactions alone. Additionally, Glu A:493 was identified as a discrete electrostatic hotspot, indicating a potential site for enhancing ligand selectivity via charged or polar substitutions.

Docking with butyrylcholinesterase revealed that 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)], achieved the strongest affinity (–8.2 kcal/mol) and engaged critical amino acids similar to those of the reference ligand, including Trp 82, His 438, Gly 439, Glu 197, Gly 116, and Asp 70 (Figure 5). It showed that Piperine and 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)] conserved binding pocket defined by Asp A:70, His A:438, and Trp A:82 but rely on distinct interaction. Piperine, as a native ligand, exhibited a more favourable binding mode, supported by a directional hydrogen bond with Glu A:197 and a π–π stacking interaction with Trp A:82. In the other hand, 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)] was predominantly stabilised by hydrophobic π-π-alkyl and π-π-sigma contacts and lacked polar interactions. These findings highlight the potential of these metabolites as promising anxiolytic candidates based on their favorable binding characteristics.

4. DISCUSSIONS

The anxiolytic activity of BPE was evaluated using the Rotarod and FST tests. The Rotarod measures motor coordination and balance following administration of CNS-active compounds, as animals with intact motor function can maintain their position on a rotating rod [16]. Rotarod is not a direct method for measuring anxiety, but it can be used as a stressor; it works by influencing emotional areas of the brain (e.g., the amygdala) to increase anxiety [17], and the resulting stress mediators can ultimately alter motor performance [18]. Fall latency is used to infer anxiolytic activity, where a shorter fall time indicates a more substantial anxiolytic effect. Corresponding values are expressed as the percentage of maximum possible effect (%MPE) [13].

The FST method was used to determine the immobility time, a condition in which mice do not move except for the movements necessary to keep their heads above water [19]. Observations focused on the last four minutes, when immobility becomes stable [15]. Increased immobility is associated with anxiolytic action, as reduced anxiety diminishes escape-driven activity [20].

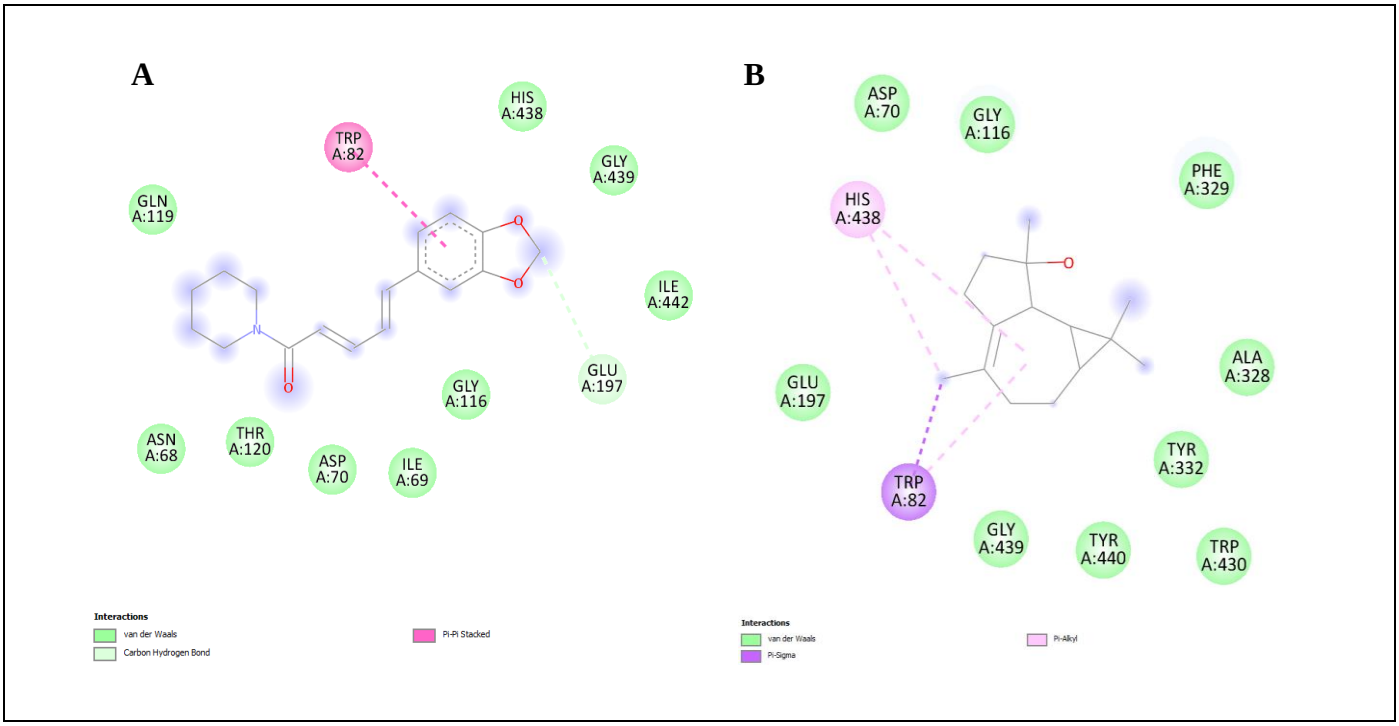

Figure 5. Molecular docking visualisation 1P0I with piperin (A) and 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]- (B)

Alprazolam served as the positive control due to its established anxiolytic efficacy. Consistent with previous findings, alprazolam reduced fall latency on the Rotarod [16], and increased immobility in the FST [21]. Its rapid onset (15 - 30 minutes) corresponds with the early decline in fall time, while the maximal effect observed at 90 minutes aligns with its peak plasma concentration (1 - 2 hours) [22].

In the BPE-treated groups (100 and 200 mg/kg BW), the lowest fall times (%MPE peak) occurred at 120 minutes, with FST outcomes supporting the Rotarod findings. This effect is thought to involve piperine, the primary bioactive compound in black pepper extract [10], which reaches its peak plasma concentration approximately two hours after oral administration [23].

Anxiolytic activity of BPE is supported by previous research showing that high piperine content in *Piper nigrum* L. methanolic extract can produce anxiolytic effects by increasing the time spent in the open arm of the elevated plus-maze (EPM) method [12]. Previous studies have also shown that piperine can increase the number of entries and the time spent in the open arms in the EPM method. Piperine may exhibit anxiolytic activity in non-stressed rats through GABAergic enhancement and nitricergic inhibition; however, in stressed rats, its effects are likely primarily mediated through nitricergic inhibition [11]. However, many other studies have shown that the anxiolytic activity of *Piper nigrum* is derived from their essential oil compound. For example, β-Caryophyllene, one of the major volatile components in black pepper, acting as a CB2 receptor agonist, produces several behavioral effects in mice that are linked to anxiety and depression [24]. Limonene exhibits anxiolytic effects by increasing dopamine and enhancing GABA release; however, these actions are blocked when the adenosine A2A receptor was inhibited [25].

Taken together, these findings suggest that the anxiolytic effects of black pepper are not solely mediated by piperine, but also by a broader spectrum of volatile constituents capable of acting on multiple neurochemical pathways. This multi-component pharmacological pattern provides an important biological context for interpreting the results of the current study. The presence of GABAergic, nitricergic, dopaminergic, and cannabinoid-related behavioral effects observed *in vivo* suggests that several molecular targets may be involved, an interpretation further clarified through in-silico analyses.

However, besides piperine, other components in black pepper may also contribute to its anxiolytic activity, particularly those derived from the essential oil group. Of the 50 compounds identified by GC-MS, the profiling reveals that trans-caryophyllene is the most abundant compound, contributing 21.25% of the total peak area and 22.79% of the peak intensity. Additional significant components include beta-selinene (3.2%), alloaromadendrene (3.8%), delta-cadinene (3.31%), and germacrene-D (2.95%). These sesquiterpenes are reported in the literature for their therapeutic effects, including modulation of neural pathways, which could

contribute to the anxiolytic activity. The presence of compounds such as Torrey (10.5%), (-)-caryophyllene oxide (7.07%), and delta-element (0.93%) also suggests a synergistic role in the pharmacological profile, especially lipophilic compounds. However, on the other hand, only 10 met the criteria and exhibited anxiolytic activity. The in-silico findings of this study further support the anxiolytic potential of black pepper and provide preliminary insight into the molecular pathways that may underlie its activity. Consistent with the Results, SwissTargetPrediction identified several central nervous system-related targets, including the cannabinoid CB2 receptor, serotonin transporter (SERT), monoamine oxidase B (MAO-B), and butyrylcholinesterase (BuChE).

Protein 5zty, known as the Cannabinoid 2 receptor (CB2R), has garnered attention in anxiety modulation due to its distinct pharmacological profile compared to the Cannabinoid 1 receptor (CB1R). Unlike CB1R, which is predominantly expressed in the central nervous system and associated with psychoactive effects, CB2R is primarily found in peripheral tissues and immune cells, suggesting a role in modulating anxiety without the psychotropic effects typically related to cannabinoids [26]. Recent studies indicate that activation of CB2R can exert anxiolytic effects, particularly in models of anxiety disorders. For instance, chronic blockade of CB2R has been shown to induce anxiolytic-like actions, which are associated with alterations in $GABA_A$ receptor functionality, highlighting the interplay between cannabinoid signaling and GABAergic neurotransmission in anxiety regulation [27]. The involvement of the endocannabinoid system in anxiety is further supported by evidence showing that cannabinoids can modulate neurotransmitter release in brain regions implicated in anxiety, such as the amygdala and prefrontal cortex [28]. Activation of CB2R has been linked to reduced anxiety-like behaviors in various animal models, suggesting that selective CB2R agonists could serve as potential therapeutic agents for anxiety disorders [29].

Protein 5i6x, known as serotonin transporter (SERT), plays a crucial role in the regulation of serotonin levels in the brain, which is directly linked to mood and anxiety disorders. Recent studies have explored the potential anxiolytic effects of various natural compounds, particularly those derived from plants such as black pepper. The essential oils and bioactive compounds from black pepper have been shown to influence serotonergic transmission, suggesting a pathway through which these compounds may exert their anxiolytic effects. Ghosh et al. demonstrated that the essential oil extracted from the fruits of *Piper nigrum* exhibits significant anxiolytic and antidepressant-like effects in mice, primarily through serotonergic mechanisms rather than the GABAergic system [30]. This finding aligns with the broader literature, indicating that many essential oils exert their anxiolytic effects by modulating serotonin receptors [31]. The involvement of the serotonergic system in mediating these effects is further supported by studies indicating that various plant extracts can enhance serotonin receptor activity, leading to reduced anxiety behaviours in animal models. The primary bioactive compound in black pepper, piperine, has been extensively studied for its pharmacological properties. It has been reported to enhance the bioavailability of various drugs and may also influence neurotransmitter systems, including serotonin [32]. The interaction of piperine with the serotonin transporter could enhance serotonin signaling, thereby contributing to its anxiolytic effects.

Additionally, black pepper has been recognized for its antioxidant properties. It may also play a role in its therapeutic effects by reducing oxidative stress, a known contributor to anxiety and mood disorders [33]. Moreover, genomic studies of *Piper nigrum* have provided insights into the biosynthesis of piperine and other secondary metabolites that may have therapeutic potential [34]. Understanding the metabolic pathways involved in producing these compounds could lead to the development of more effective anxiolytic therapies derived from natural sources. The comprehensive review by Salehi et al. highlights the diverse biological activities of *Piper* species, including their neuropharmacological effects, which further supports the potential of *Piper nigrum* as an anxiolytic agent [6].

1P0I is identified as Butyrylcholinesterase, which plays a crucial serotonin transporter (SERT) plays a vital role in the regulation of serotonin levels in the brain, which is directly linked to anxiety and mood disorders. Inhibition of SERT has been associated with anxiolytic effects, as it increases serotonin availability in the synaptic cleft, thereby enhancing serotonergic neurotransmission. This mechanism is a target for various pharmacological agents used in the treatment of anxiety disorders. Recent studies have highlighted the potential of natural compounds, including those derived from plants, to act as SERT inhibitors. For instance, certain polyphenolic compounds found in various plant extracts have demonstrated significant inhibitory effects on cholinesterase, which are enzymes that can influence neurotransmitter levels, including serotonin [35]. The presence of these compounds in plants like *Bergenia pacumbis* suggests a broader therapeutic potential for managing cognitive and mood disorders through natural means [36]. One specific compound of interest is 1H-Cyclopropeazulen-7-ol, decahedron-1,1,7-trimethyl-4-methylene, which has been identified in the essential oils of plants such as *Piper nigrum* [37]. This compound exhibits various biological activities, including anti-inflammatory and antioxidant properties, which may contribute to its potential anxiolytic effects [38].

The presence of this compound in black pepper indicates that it could play a role in modulating the serotonergic system, possibly through its interaction with SERT or related pathways [39].

Moreover, butyrylcholinesterase (Bu-ChE) inhibitors have gained attention for their role in enhancing cognitive function and potentially alleviating anxiety symptoms. The inhibition of Bu-ChE can lead to increased levels of acetylcholine, which may indirectly influence serotonin pathways and contribute to anxiolytic effects [40]. Compounds such as β-caryophyllene oxide, derived from various plants, have shown promising Bu-ChE inhibitory activity, suggesting they might benefit anxiety management.

Overall, these docking outcomes suggest that multiple *P. nigrum* constituents may exert anxiolytic effects through complementary pathways, including cannabinoid, serotonergic, and cholinergic systems. The identification of germacrene D, (+)-δ-cadinene, and a cycloprop[e]azulene derivative as strong ligands for these targets offers a mechanistic rationale that aligns well with the behavioral outcomes observed in animal models, supporting the concept that the anxiolytic effects of black pepper arise from synergistic, multi-target interactions rather than from a single active compound.

However, the extract utilized in this study was not standardized, and no mechanistic or receptor-based *in vitro* assays were conducted. These limitations prevent definitive confirmation of the molecular pathways involved. Therefore, while the in-silico findings provide meaningful hypotheses and directions for future investigation, experimental validation remains essential.

5. CONCLUSIONS

Black pepper extract (BPE) demonstrated measurable anxiolytic activity *in vivo*, and the in-silico findings of this study suggest that several secondary metabolites, including Germacrene D, (+)-δ-Cadinene, and 1H-Cycloprop[e]azulen-7-ol derivatives, may contribute to this effect in addition to piperine. Their strong predicted interactions with CB2, SERT, MAO-B, and Bu-ChE highlight the potential involvement of multiple neuropharmacological pathways.

These results strengthen the rationale for exploring black pepper as a source of anxiolytic candidates. Future work should focus on isolating these active compounds, confirming their receptor interactions through *in vitro* assays, and conducting targeted *in vivo* studies to clarify their mechanisms and therapeutic relevance.

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Author contribution

Fransiska Maria Christianty: Conceptualization (lead); original drafting (lead); analysis (lead). Mohammad Hilmi Afthoni: *In silico* analysis, data interpretation, writing, review, and editing, design (equal). Fitrotun Khasanah: Methodology, *in vivo* analysis (equal). Diana Holidah: *In vivo* analysis, data interpretation, writing (supporting). Ika Puspita Dewi: *In vivo* analysis, writing (supporting). Fifteen Aprila Fajrin: Conceptualization (supporting); original drafting (supporting), review and editing (equal).

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Conflict of interest

None to declare.

Ethics approval

The research protocol was approved by the Ethical Committee of Medical Research, Dental Faculty, University of Jember (No.1978/UN25.8/KEPK/DL/2023).

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