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Research article

Exploring antioxidative, cytotoxic and neuropharmacological insights into *Bixa orellana* leaves: Experimental and *in silico* approaches

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ABSTRACT

Background Study: The aim of this research was to examine possible antioxidant, cytotoxic and neurological activity of methanol and n-hexane extracts of *Bixa orellana* leaves. Additionally, we aimed to identify potential lead compounds through *in-silico* analysis.

Methods: In-vitro antioxidative properties were investigated through different assays, including: total phenolic content assay (TPC), total flavonoid content assay (TFC), DPPH free radical scavenging assay and reducing power assay. Also, the cytotoxic effect of the samples was assessed using the brine shrimp lethality test. In addition, anxiolytic, locomotor, and CNS depressant activities were assessed utilizing various established methods. Moreover, reported compounds were used in the *in silico* study to explore the best-fit phytoconstituents against gamma-aminobutyric acid ($GABA_A$) receptor.

Results: MBOL displayed substantial antioxidative activities in various established assays compared to NBOL. In brine shrimp lethality bioassay, both MBOL and NBOL revealed cytotoxic activity in a concentration-dependent approach. Again, in Elevated Plus Maze test, 200 and 400 mg/kg of NBOL and MBOL demonstrated significant anxiolytic activities evident from time spent in open arms. In addition, maximum number of head dipping was demonstrated by MBOL at 400 mg/kg (53.90 ± 1.16) in Hole Board test. NBOL and MBOL at both doses significantly diminished the magnitude of movements from the 2nd to 5th observation periods in Open Field test. Furthermore, in Hole Cross test, MBOL remarkably dwindled the locomotor activity at 120 min and 180 min (3.60 ± 0.40 and 2.40 ± 0.51) at 400 mg/kg. Finally, *in silico* analysis revealed 13 compounds as promising leads with strong binding affinity to $GABA_A$ receptor along with good pharmacokinetics and toxicity profiles.

Conclusion: Therefore, the present study's findings advocate the traditional usage of this plant and recommend both MBOL and NBOL as as a potential source of therapeutic candidate for the management of neurological disorders.

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Abbreviations

MBOL:	Methanol extract of *Bixa orellana* leaves
NBOL:	N- hexane extract of *Bixa orellana* leaves
CNS	Central nervous system
TPC	Total phenolic content
TFC	Total flavonoid content
LD50	Half lethal dose
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPM	Elevated Plus Maze
HBT	Hole Board Test
OFT	Open Field Test
HCT	Hole Cross Test
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
IC 50	Inhibitory concentration
$GABA_A$	Gamma-Amino Butyric Acid-A.

1. Introduction

Being a surging alarm for the health sector of humans, neuropsychiatric disorders result in the deterioration of the meaning and worth of life [1]. Depressive disorders are largely responsible for non-lethal health complications across the world [2], and anxiety ailments are in sixth place as per the World Health Organization (WHO) [3]. Depression associated with functional demobilization significantly influences the public as lasting and complicated disarray and uplifts morbidity and mortality rate [4]; conversely, anxiety is regarded as a recurrent psychiatric disorder throughout the whole world [5]. Actually, there is a supplementary diagnosis for anxiety disorder once examined with depression in terms of nearly fifty percent of the sufferers [6]. Unreliable prognosis, greater risk of suicidal propensity, the complexity of symptoms, and exacerbated feedback for therapy are the results of concomitant anxiety and depression [5]. There are some prevailing factors, namely psychological, genetic, environmental, and biological, that have been recognized to be responsible for the development of neuropsychiatric disorders like anxiety and depression, although their underlying cause is a paradox [7]. In addition, interruption of the antioxidant defense system makes oxidative stress or redox inequity, which results in the overproduction of reactive oxygen species (ROS) in the brain associated with cellular disparity [8] and consequently leads to cognitive worsening and injury of neurobiological mechanisms [9]. The consumption of antioxidants may contribute to the improvement of continuing tissue injury through the alleviation of aberrant generation of ROS in the central nervous system, although single antioxidant therapy may not be able to produce the desired effect [10]. Hence, continuous concurrent administration of antipsychotic medications and dietary supplements with antioxidants as a part of pharmacotherapy and polypharmacy are suggested for managing these life-threatening health problems which lead to aversion among the patients in terms of receiving medicines [7]. Biochanin A (BCA) is an O-methylated isoflavones obtained from different legumes including red clover (Trifolium pratense), soy, alfalfa sprouts, peanuts, and chick peas (Cicer arietinum) and is responsible for a variety of biological activities such as anti-proliferative, anti-inflammatory, antioxidant, neuro-protective, nephro-protective, and skin protective [11]. However, it is quite difficult to control anxiety, depression, chronic inflammation, and ROS through the currently prescribed antidepressant drugs including benzodiazepines, selective serotonin, and/or serotonin-norepinephrine reuptake inhibitors instantaneously because of their undesirable side effects such as sedation, sexual dysfunction, memory disturbances, abuse liability, amnesia, and daytime drowsiness [12]. In these circumstances, worldwide research attention lies in searching for prospective plant-derived bioactive compounds with multiple pharmacological targets [7]. Different phytochemicals obtained from medicinal herbs, fruits, or vegetables may be found to be effective in mitigating neurodegeneration and also in ameliorating memory and cognitive performance of the brain [13].

Bixa orellana (L) belongs to the family Bixaceae, locally recognized as the Annatto plant, is a tiny evergreen plant and is endemic to Central and Southern America. It is commonly known as Latkan in Bangladesh and is grown to a minor amount in Dhaka and Chattogram [14]. Two bioactive natural pigments bixin and norbixin are potent carotenoids isolated from the seeds of *B. orellana* and are utilized in the food, cosmetics, and pharmaceutical sectors [15]. Several polyphenol compounds namely catechin, chlorogenic acid, chrysin, butein, hypoaletin, and xanthoangelol are found in this plant and thought to be responsible for the inhibition of free radical production and neurodegeneration [16]. Other prominent compounds known as isobixin, bixol, beta-carotene, tryptophan, lutein, zeaxanthin, orellin, bornyl acetate, bixein, tomentosic acid, cryptoxanthin, isoledene, ishwarane, ellagic acid, salicylic acid, threonine, crocetin, copaene, and phenylalanine have been reported from this plant [17]. Various portions of this plant are utilized by traditional healers to address a range of ailments. It has been reported that the seed extract has astringent, febrifuge, and chemo-protective possession [18]. The root and bark are also used traditionally to treat fever, digestive aid, and gonorrhea [15]. For many years, leaf extracts in particular have been used to treat headaches, diarrhea, fever, different microbiological infections, heartburn, and indigestion [18,19]. Moreover, this plant's leaves have allegedly been used to cure a variety of neurological diseases [20]. However, the majority of these ethnopharmacological claims have yet to be confirmed scientifically. There haven't been many investigations that back up the neuropharmacological effects of *B. orellana* leaves. Consequently, the goal of the current investigation was to provide rational support and explore any prospective neuropharmacological features. To rationalize the pharmacological evidence of the

traditional usages of the plant, we evaluated the antioxidant, neuropharmacological and cytotoxic potentials of methanol and *n*-hexane extract of *B. orellana* leaves. Additionally, bioactive compounds reported from this plant was utilized in the *in silico* study against $GABA_A$ receptor to find out promising lead compounds responsible for neuropharmacological activities.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals were used: n-hexane, methanol, dimethyl sulfoxide (DMSO), and vincristine sulfate were obtained from Merck, Germany, whereas tween-80 was obtained from Loba Chemie Pvt. Ltd., India. Diazepam was collected from Square Pharmaceuticals Ltd., Bangladesh. These tests used exclusively analytical-grade substances.

2.2. Collection and identification of the plant material

In September 2017, *B. orellana* leaves were obtained from Sylhet, Bangladesh (located at latitude 24°53′11.1696°N and longitude 91°52′50.5992°E). Naimur Rahman, senior scientific officer, Bangladesh National Herbarium, Dhaka, then identified the samples that were collected. Moreover, a voucher specimen (DACB: 45170) was placed there for future use.

2.3. Preparation and extraction of the plant material

The leaves were collected, washed three to four times under running water from the faucet and then with distilled water before being dried for seven days at room temperature in the shade. Grinding the dried leaves in a laboratory mill resulted in pulverization of the leaves (Model 2000 LAB Eriez®) followed by 40-mesh sieving to achieve a fine powder. A hot extraction process using the soxhlet apparatus was used for the extraction purpose. A mixture of 250 g of plant powder and 1.5 L of n-hexane and methanol was used for extracting where 25 g was dissolved in 150 mL in each flask. Then, cotton plug was used to collect the filtrate along with Whatman no. 1 filter paper (pore size: 11 μm). Finally, *n*-hexane and methanol filtrates were subjected to vacuum-distillation at 68 and 76 °C, correspondingly in a rotary evaporator (Buchi Rotavapor Model R-124) and then dried by desiccator where the yield values were 3.6 and 4.8% w/w, respectively. Finally, the sticky (semi-solid) extract was sealed in an airtight vial and stored at 4 °C in the refrigerator to avoid fungal attack which was used in this study.

2.4. Experimental animals

Swiss albino mice obtained from the International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B), Mohakhali, Dhaka were both sexes and their average weight and age were 25–30 g and 6–7 weeks, respectively. The animals were kept in cleaned polypropylene cages in a typical setting with room temperature (25 ± 1 °C), humidity (50 ± 5%), and 12:12 h light-dark cycles. Next, the mice were fed rodent foods as well as sufficient distilled water. Before the experiment, a 7 days' acclimation period was given to all of the animals. The Ethical Review Committee of the Faculty of Biological Science and Technology at Jashore University of Science and Technology gave its approval to each and every experimental procedure and every *in vivo* trial was performed following their guidelines [ERC/FBS/JUST/2018–16].

2.5. Qualitative phytochemical screening

For freshly produced NBOL and MBOL, numerous qualitative tests were conducted through distinct color changes to determine the presence of various phytochemical groups [21].

2.6. Acute toxicity study

As part of the acute toxicity investigation, the median lethal dosage (LD_{50}) of experimental samples was calculated in accordance with OECD recommendations. The mice were allocated into three groups such as control, NBOL, and MBOL where number of mice in each group was five. Individual mice in each group were given test samples orally at different dosed (100–4000 mg/kg, b.w.). After that, the mice were monitored for 14 days to notice any signs of toxicity including coma, death, salivation, diarrhea, noisy breathing, refusal of food or water, aggressiveness, discharge from the eyes and ears, injury, weakness, and variations in locomotor activity [22].

2.7. In vitro antioxidant study

2.7.1. Total phenolic content (TPC) assay

Folin-Ciocalteu (FC) reagent was employed to assess TPC of plant extracts reported by Sarkar et al., 2022 [23] and Medha et al., 2022 [24], where gallic acid standard curve obtained from different concentrations in the range of 100–500 μg/mL, were used to estimate TPC and the findings were interpreted as milligram gallic acid equivalent per gram of dry extract (mg GAE/g).

2.7.2. Total flavonoid content (TFC) assay

TFC of NBOL and MBOL was measured using a standard quercetin calibration curve which was created through several concentrations of quercetin including 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL [23]. The absorbance at 510 nm was measured in comparison to a blank, and the TFC of plant extracts was expressed as milligrams of quercetin equivalent per gram of dry extract (mg QE/g).

2.7.3. DPPH radical scavenging assay

NBOL and MBOL's capacity to scavenge DPPH free radicals was evaluated using a slightly modified version of the technique reported by Ali et al., 2021 [9]. Standard and extract concentrations between 1024 and 2 μg/mL were created using the serial dilution method, and 6 mL of alcoholic DPPH solution (0.004% w/v) was combined with 2 mL of sample solutions from each concentration. After incubation for half an hour at room temperature, the absorbance was measured at a wavelength of 517 nm. Ultimately, the following formula was used to calculate % inhibition.

$$\%\ \text{Inhibition of DPPH} = (1 - A_1\ / A_0) \times 100$$

Where A_1 is the absorbance of sample or standard and A_0 is the absorbance of control.

2.7.4. Reducing power assay

The procedure described by Sarkar et al., 2022 [23] was used to carry out the reducing power assay. In this case, the absorbance of various standard and extract concentrations (ranging from 100 to 500 μg/mL) was recorded at 700 nm in comparison to a blank solution.

2.8. In vivo neuropharmacological study

2.8.1. Dosing groups

Randomly selected 120 mice were split into six groups (n = 5) for the *in vivo* study. The control group was given distilled water having 1% tween-80 and the positive control group was administered diazepam as the reference drug (1 mg/kg; b.w., p. o.), in contrast NBOL and MBOL (200 and 400 mg/kg, respectively) were provided to the remaining four groups.

2.8.2. Elevated plus maze (EPM) test

This test was conducted using the technique of Adnan et al., 2020 [25] for assessing exploration, anxiety, and motor behavior. The wooden EPM instrument featured two opposed closed arms that measured 50 × 10 × 30 cm in length, breadth, and height as well as two opposed open arms that measured 50 × 10 cm in length and width and formed the shape of a plus sign. It was kept at a height of 70 cm above the ground. Each mouse received treatment before being put in the maze's center, confronting one of the closed arms. The point at which the animal put all four paws on the arm was defined as an entry. During the observation period of 3 min, the time spent in both arms was recorded and every counting was continued at 0, 30, 60, 120, and 180 min following the respective treatment. The treatment was carried out in a soundproof environment. During the interval of each trial, the plus maze was completely cleaned with 70% ethanol.

2.8.3. Hole board test (HBT)

To execute HBT, the approaches described by Ali et al., 2021 [9] was used with a minor alteration. It is a well-known and reliable pharmacological approach for determining anxiolytic and/or anxiogenic action. The hole board is a 40 × 40 cm^2 hardwood board featuring 16 holes that are distributed uniformly apart (3 cm in diameter and 2.2 cm in depth). Grouping and treatment of mice were as like an elevated plus-maze test. After 1 h of oral treatment, a single mouse was kept in the center of the hole board and given 5 min to freely explore the apparatus. Finally, it was recorded how many times mice put their heads inside the holes.

2.8.4. Open field test (OFT)

This test was performed for the simultaneous measurement of locomotion, exploration, and anxiety where slightly modified method of Uddin et al., 2018 [26] was used. The open field apparatus with a dimension of 45 × 45 cm was constructed of squares that alternated between white and black color and the floor was divided into 8 × 8 equal squares. Following oral administration, each mouse was immediately put in the centre of the device. For a period of 3 min, the total number of squares visited by each mouse was noted at time intervals of 0, 30, 60, 120, and 180 min. The open field apparatus was first wiped with dry tissue paper and then with a 70% alcohol solution and left to dry in the air after each trial.

2.8.5. Hole cross test (HCT)

HCT was completed for the evaluation of locomotor and exploratory behavior as described by Sarkar et al., 2021 [21]. Here, the hole cross apparatus was a wooden cage having dimensions of 30 × 20 × 15 cm and, in the center of the cage, a wooden partition was fastened. In addition, a 3 cm diameter hole was built in the hub of the cage. Following oral administration of the test samples, the spontaneous movement (number of crossings) of a mouse across the opening from one compartment to another was recorded for 3 min at 0, 30, 60, 120, and 180 min time points, respectively.

2.9. Cytotoxic activity study

2.9.1. Brine shrimp lethality bioassay

The cytotoxic effect of NBOL and MBOL were evaluated by the brine shrimp lethality bioassay according to the methodology proposed by Sarkar et al., 2021 [27] with slight modification. In a tiny tank with sterile simulated seawater (Diluted 1 L of purified water with 38 g of iodine-free sea salt, then use 1 N NaOH to bring the pH to 8.5) and continuous aeration for 48 h, brine shrimp (Artemia salina) were hatched. After hatching, mature nauplii were taken from the hatching chamber and employed in the experiment. Different concentrations of the extracts (1000–31.25 μg/mL) were prepared by serial dilution technique with seawater whereas, for reference standard, vincristine sulfate was used at very low concentrations (5–0.312 μg/mL). Following that, 5 mL of saltwater with 10 nauplii were added to the plant extract solutions. After 24 h, with the help of a magnifying lens, the number of surviving nauplii present in each tube was counted on a dark background. Using Abbott's method, the lethality of brine shrimp nauplii was calculated in percentage [28].

2.10. Statistical analysis

The data were all displayed as mean ± SE (standard error). The mean values of the different groups were compared using a one-way ANOVA with Dunnett's test (P 0.05 vs. control) and was deemed to be statistically significant. Statistical Package for the Social Sciences (SPSS) software was used to analyze all of the data (version 20; IBM Corporation, New York, USA).

2.11. In silico study

2.11.1. Instruments and tools

In this study, we used Windows 10 Pro operating system and Intel Core i5-7200UCPU 2.7 GHz and DDR4 RAM. In addition, ligands and protein preparation were done by using BIOVIA Draw 2018, PyMOL DLP3D, Swiss PDB Viewer 4.10 and PyMOL. The molecular docking was performed by using Autodock 4.2.6. and ligand protein interactions were visualized by Discovery studio 4.5.

2.11.2. Ligand preparation

To collect all of the reported compounds of *B. orellana*, our primary concern was to search at different search engines such as Google Scholar, web of science, PubMed, and Scopus. This operation was done by searching the keywords, "*Bixa orellana*" and "reported compounds". From different articles, we found 172 compounds [supplementary file **S1**] proclaimed from different parts of the *B. orellana* [29–36]. To identify potential neurological effects against the $GABA_A$ receptor, those compounds were chosen for *in silico* screening. Then, all constituents were looked for using the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/), and any compounds that weren't found were drawn using BIOVIA Draw 2018 and saved in SDF format. Following that, utilizing the B3LYP correlation function theory of the DFT approach and the GaussView 5.0.8 software program, the energy of the molecules was minimized [37,38].

2.11.3. Protein preparation

Protein Data Bank (www.rcsb.org) was utilized for downloading the 3D X-ray crystallographic structure of $GABA_A$ (PDB ID: 6X3X) complexed with diazepam [39] as PDB format containing two α subunit (B and D), two β (A and C) and one γ subunit (E) where the complex of α subunit (D) and γ subunit (E) with benzodiazepine binding (BZD) site promotes the binding of GABA with this receptor. Except for α subunit (D) and γ subunit (E), unnecessary subunits and water molecules were removed by PyMOL DLP 3D [40]. Then free energy of those units was minimized through in vacuo with GROMOS 96 43B1 parameters set without reaction field implemented at Swiss-PDB Viewer 4.10 [41] and thus −37971.168 kJ/mol energy was minimized.

2.11.4. Molecular docking analysis and visualization

172 compounds reported from various parts of *B. orellana* were collected from different articles and virtually screened at the BZD site to identify potential candidates by using Autodock 4.2.6 [42]. During this process, Universal Force Field (UFF) along with the steepest descent optimization algorithm implemented in this software were used for further energy minimization of the bioactive compounds. Then all of the bioactive compounds were turned into pdbqt format with AutoDock tools by Python Molecular Viewer, required by AutoDock Vina [43]. After that, virtual screening was accomplished at the BZD site in terms of each bioactive molecule and this computation grid box size was kept at 36.2317, 44.0139, and 33.4758 Å dimensions through Vina search space facilities along with XYZ direction. The binding affinity of individual compound was represented by a negative score in the Autodock program; a larger negative energy denotes a stronger binding affinity. Finally, complexes of bioactive compounds with the BZD site were made by PyMOL [40] and opened it on Discovery studio 4.5 to understand the corresponding interacting amino acids and their subsequent bond types with this site.

2.11.5. ADMET analysis

SwissADME online server was utilized to analyze the physiochemical attributes (molecular weight (g/mol), number of hydrogen bond acceptors, the number of hydrogen bond donors and number of rotatable bonds), gastrointestinal absorption, blood brain barrier, number of Lipinski's violations, and drug-likeness [44,45]. Besides, lipophilicity and various toxicity parameters (LD_{50}, hepatotoxicity, and AMES toxicity) were anticipated by using pkCSM online server [46]. Compounds used as input were obtained from the PubChem

database using their canonical SMILES [47].

3. Result

3.1. Qualitative phytochemical screening

Many phytochemical categories were identified in NBOL and MBOL, according to the initial phytochemical screening. It was found that carbohydrates, flavonoids, phenolic compounds, tannins, alkaloids, glycosides, and gums were present in both extracts. Moreover, Anthraquinones and steroids were detected in NBOL but absent in MBOL. Additionally, MBOL demonstrated the presence of saponins and acidic compounds (Table 1).

3.2. Acute toxicity study

Over the course of the 14-day monitoring period, the maximum dose of 4000 mg/kg b.w. did not result any mortality, toxicity, or aberrant behavior. Additionally, the experience was the same for the control group suggesting that the lethal doses of NBOL and MBOL needed to generate acute toxicity are higher than 4000 mg/kg.

3.3. Antioxidant activity study

3.3.1. TPC assay

According to the Gallic acid calibration curve ($Y = 5.809x + 0.0499$; $R^2 = 0.9952$), the quantity of total phenolic compound found in MBOL and NBOL was 145.13 ± 0.34 and 109.55 ± 0.22 mg GAE/g dry extract, respectively.

3.3.2. TFC assay

Using the Quercetin calibration curve ($Y = 1.0705x + 0.1104$; $R^2 = 0.9922$), it was found that MBOL contained a greater quantity of TFC (122.46 ± 1.40 mg QE/g dry extract) than that of NBOL (70.15 ± 0.47 mg QE/g dry extract).

3.3.3. DPPH radical scavenging assay

In this experiment, NBOL and MBOL exhibited concentration-dependent scavenging activity in comparison to that of standard (ascorbic acid). Furthermore, IC_{50} values of NBOL and MBOL were about 62 and 30 μg/mL, respectively in contrast, the standard revealed an IC_{50} value of 5.6 μg/mL (Fig. 1).

3.3.4. Reducing power assay

The maximal absorbance was determined to be 1.573 and 0.863 exposed by the maximum concentration (500 μg/mL) of BHT and MBOL, respectively. The reducing ability of both extracts was disclosed in a concentration-dependent fashion (Fig. 2).

3.4. In vivo neuropharmacological study

3.4.1. Elevated plus maze test

Generally, the anxiolytic activity of a drug is defined by the enhancement of exploration in the open arms of the EPM tool. Compared to the control, the duration spent in open arms was significantly increased after NBOL and MBOL were administered at doses of 200 and 400 mg/kg, respectively suggesting anxiolytic activity (Fig. 3A). Besides, mice treated with both extracts spent less time in closed arms (Fig. 3B).

Table 1
Qualitative phytochemical screening of NBOL and MBOL.

Phytochemical group	NBOL	MBOL
Carbohydrate	+	+
Phenolic compound	+	+
Flavonoids	+	+
Tannins	+	+
Alkaloid	+	+
Glycosides	+	+
Saponins	–	+
Anthraquinone	+	–
Gums	+	+
Steroids	+	–
Acidic compounds	–	+

Here "+" denotes present and "-" denotes absent.

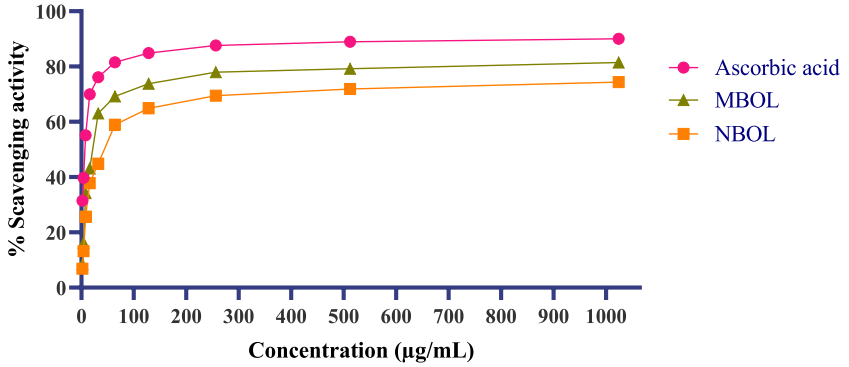


Fig. 1. Comparison of DPPH radical scavenging activity among ascorbic acid (standard), NBOL, and MBOL.

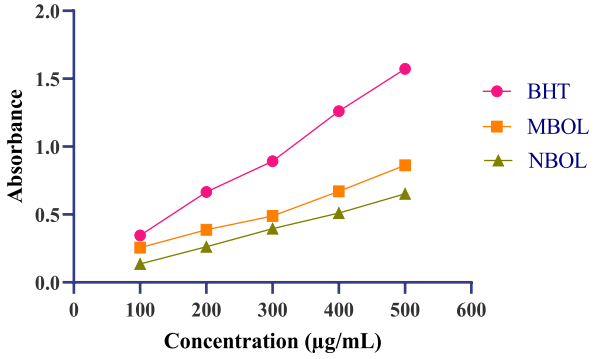


Fig. 2. Comparison of reducing power among BHT (standard), NBOL, and MBOL.

3.4.2. Hole board test

HBT was also employed to assess anxiolytic potential. In this test, diazepam as well as both extracts namely NBOL and MBOL increased the number of head dipping following a dose-dependent fashion and was comparable to that of control. Moreover, the maximum number of head dipping was exhibited by MBOL at the dose of 400 mg/kg (53.90 ± 1.16) (Fig. 4).

3.4.3. Open field test

In this test, NBOL and MBOL demonstrated a dose-dependent diminution in locomotor effect at the tested doses during all observation periods and the results were significant ($P < 0.05$) in comparison to control. Besides, both the lower and higher doses of NBOL and MBOL significantly dwindled the number of movements from the 2nd to 5th observation periods compared to that of diazepam (Fig. 5).

3.4.4. Hole cross test

Both the lower and higher doses of NBOL and MBOL gradually declined the number of movements of mice across the hole from the 1st to 5th observation periods compared to that of the control. In addition, significant reduction in the locomotion action in terms of the number of movements was demonstrated by MBOL 400 mg/kg at 120 min and 180 min (3.60 ± 0.40 and 2.40 ± 0.51) comparable to that of control indicating CNS depressant activity (Fig. 6).

3.5. Cytotoxic activity study

3.5.1. Brine shrimp lethality bioassay

NBOL, MBOL, together with vincristine sulfate revealed cytotoxic effect in a concentration-dependent approach [Fig. 7 (A, B)]. The MBOL displayed moderate toxicity having LC_{50} value of 176.16 µg/mL and NBOL disclosed minor toxicity (LC_{50} value of 603.15 µg/mL) whereas the LC_{50} value of vincristine sulfate was 0.364 µg/mL.

3.6. In silico study

3.6.1. Molecular docking of best ligands with $GABA_A$ receptor

Stigmasterol, a steroid derivative with hydroxyl group in the place of C-3 of the steroid skeleton displayed the maximum binding

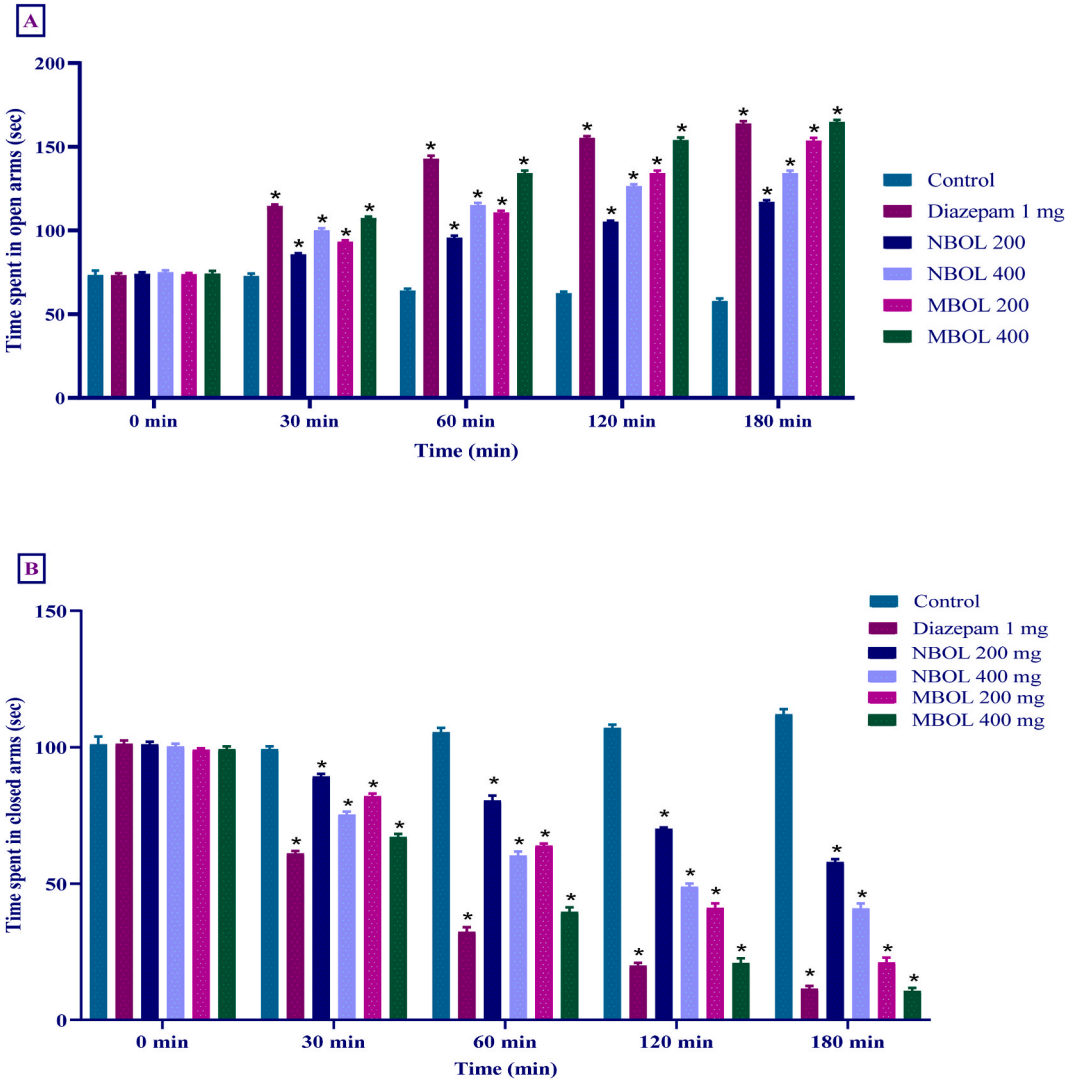


Fig. 3. Effects of NBOL and MBOL in the elevated plus maze test.
Values are presented as mean ± standard error of mean. n = 5 mice in each group. *P < 0.05, considered significant compared to the control in the (Dunnett's *t*-test). Here, Figure A indicates time spent in open arms and B indicates time spent in close arms.

affinity (−10.5 kcal mol^{-1}) with the BZD site of $GABA_A$ receptor among all of the bioactive compounds of *B. orellana*. Stigmasterol formed three different Pi-alkyl bonds with TYR58 of the γ subunit at the distance of 4.12, 5.47, and 4.23 Å, respectively. Besides, PHE77 of γ subunit interacted with stigmasterol by two Pi-alkyl and one Pi-Sigma bond. Furthermore, PHE100, HIS102, LYS156, TYR160, VAL203, and TYR210 of α subunit made numerous hydrophobic bonds with stigmasterol [Fig. 8 (A) and Table 2].

Cholest-5-en-3-ol, 24-propylidene-, (3. Beta.)- showed a binding affinity of −10.4 $kcalmol^{-1}$ with the BZD site of the $GABA_A$ receptor. Here, SER205 of α subunit made a hydrogen bond with the hydroxyl group at 2.37 Å. Besides, TYR58 of γ subunit interacted through two different Pi-alkyl bonds whereas PHE77 of γ subunit formed two Pi-alkyl bonds and one Pi-sigma bond with this ligand. On the other hand, VAL203, LYS156, PRO140, PRO154 and HIS102 of α subunit made numerous alkyl and Pi-alkyl bonds with various parts of cholest-5-en-3-ol, 24-propylidene-, (3. Beta.) [Fig. 8 (B) and Table 2)].

A binding affinity of −9.4 $kcalmol^{-1}$ was determined for αcadinol at the BZD site, indicating that it is stable there. At a distance of 2.52, the hydroxyl group of α-cadinol interacted with SER205 of the α subunit through a typical hydrogen bond. Additionally, TYR58 of γ subunit formed two Pi-alkyl bonds at 4.68 and 5.33 Å, respectively. Moreover, α-cadinol interacted with PHE77 of γ subunit by making two Pi-alkyl and one Pi-sigma bonds. Furthermore, HIS102 and VAL203 of α subunit made six different hydrophobic bonds with α-cadinol [Fig. 8 (C)and Table 2].

δ-Tocotrienol formed a strong Pi-donor hydrogen bond at 2.84 Å with SER205 of α subunit. In addition to, TYR-58 and PHE-77 of γ subunit formed two Pi-alkyl bonds at 4.69 and 3.93 Å, respectively and an additional Pi-Pi stacked interaction was also found with PHE-77 of γ subunit. On the other hand, different moieties of δ-tocotrienol formed various types of hydrophobic bonds with TYR210,

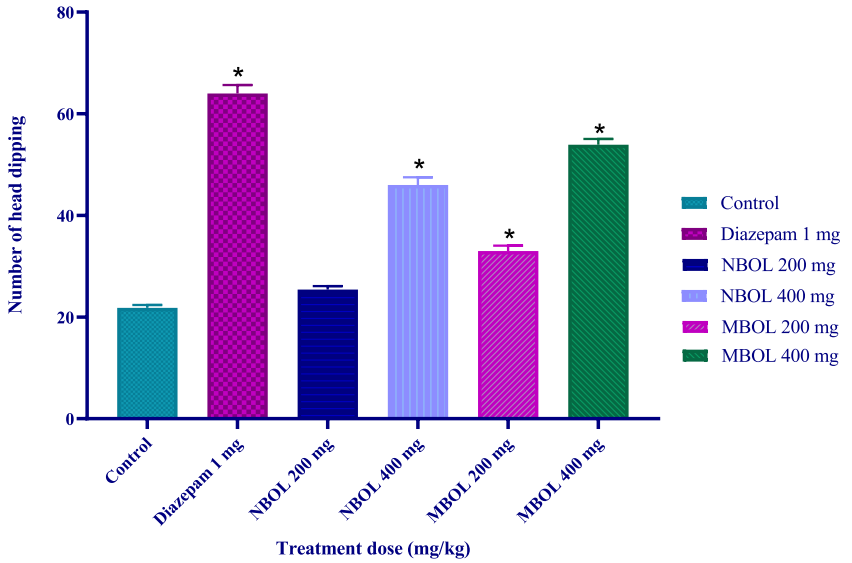


Fig. 4. Effects of NBOL and MBOL in the hole board test.
Values are presented as mean ±standard error of mean. n =5 mice in each group. *P <0.05, considered significant compared to the control in the (Dunnett's *t*-test).

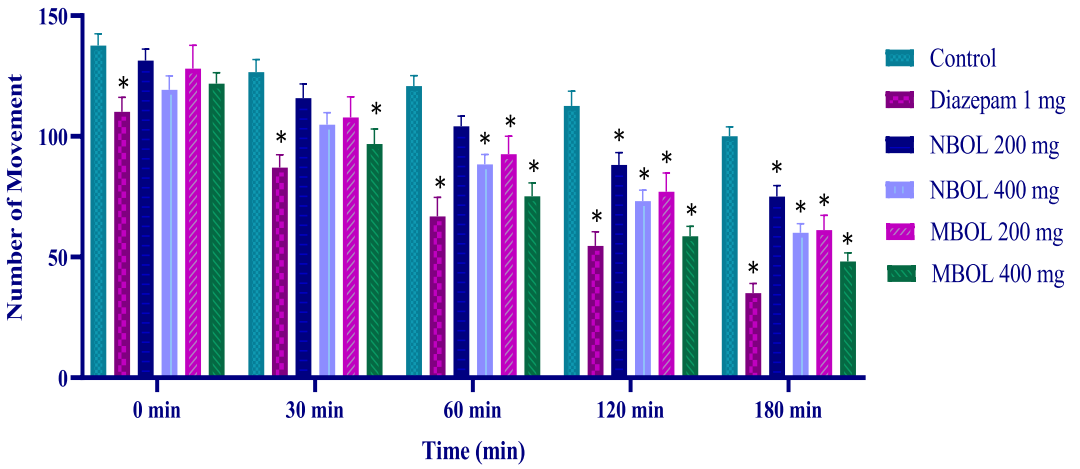


Fig. 5. Effects of diazepam (standard), NBOL and MBOL in the open field test.
Values are presented as mean ± standard error of mean. n = 5 mice in each group. *P < 0.05, considered significant compared to the control in the (Dunnett's *t*-test).

TYR160, VAL203, LYS156, VAL212, PHE100 and HIS102 amino acid residue of α subunit. It was found that δ-tocotrienol revealed a binding affinity of −9.9 kcalmol^{-1} with the BZD site [Fig. 8 (D) and Table 2].

It was also found that 4, 22-stigmastadiene-3-one, epi-bicyclosesquiphellandrene, γ-muurolene, γ-cadinene, δ-cadinene, β-cadinene, viridiflorene, *cis*-calamenene and δ-selinene formed different types of molecular interaction with the BZD sites of $GABA_A$ receptor. Besides, *cis*-calamenene made a hydrogen bond with SER205 of α subunit at 2.84 Å [Fig. 8 (E–M) and Table 2].

3.6.2. ADMET (absorption, distribution, metabolism, excretion, and toxicity) and drug-likeliness of ligands

From the projected data, it was explored that, α-cadinol had the highest GI absorption and was capable to cross the blood-brain barrier among 13 different compounds with no violations of Lipinski's rule of five. Moreover, stigmasterol and δ-tocotrienol were more lipophilic than others. Any of the compounds did not reveal AMES toxicity except *cis*-calamenene (Table 3).

4. Discussion

Plant-derived medications have a very promising future since they are more effective, inexpensive, and safe [48]. However, a

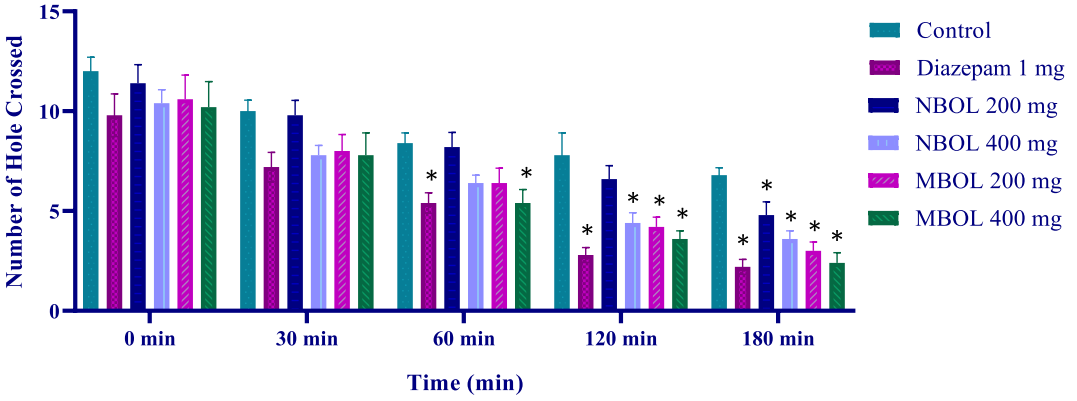


Fig. 6. Effects of diazepam (standard), NBOL and MBOL in the hole cross test.
Values are presented as mean ± standard error of mean. n = 5 mice in each group. *P < 0.05, considered significant compared to the control in the (Dunnett's *t*-test).

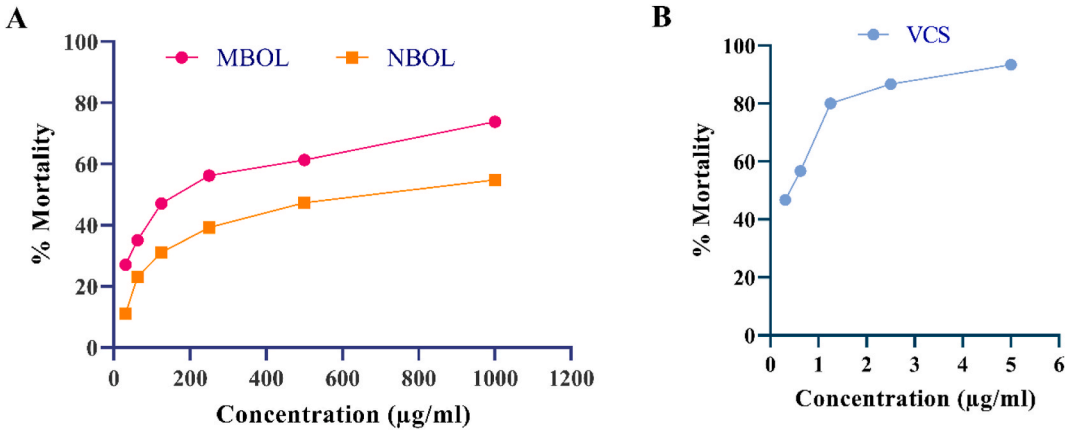


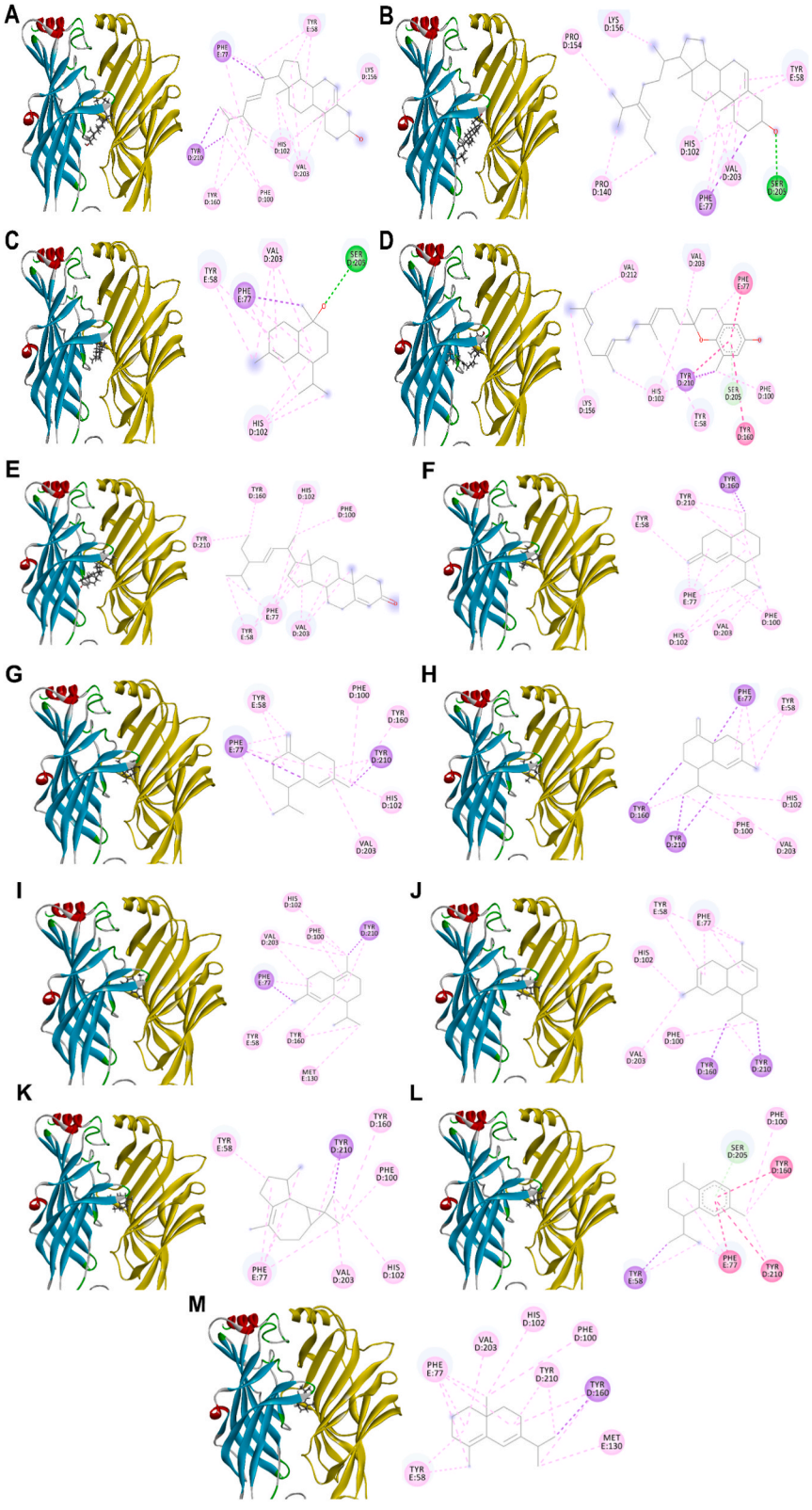
Fig. 7. Percentage of mortality of brine shrimp at different concentrations of MBOL, NBOL, and vincristine sulfate (VCS).

variety of experimental models and well-documented tests are required to develop a prospective lead molecule having widespread pharmacological properties [25]. Preliminary phytochemical investigation of this plant (leaves) found a variety of phytochemicals including carbohydrates, tannins, alkaloids, flavonoids, glycosides and gums in both extracts (Table 1). Moreover, acute toxicity study confirmed the safety and acceptability of the two selected doses used in *in vivo* experments.

The brain is extremely susceptible to imbalances caused by excessive cellular ROS generation and an inadequate antioxidant defense scheme, which may result in oxidative stress (OS) [49]. Persistent neuroinflammation is linked to OS from both physiological and pathological perspectives [50]. However, it is not startling that OS and neuropsychiatric disorders are related; several meticulous research has demonstrated that brain's cellular level of OS may revert normal brain functions, neuronal processes, and neurotransmissions [9]. There was a strong established relationship stuck between anxiety and oxidative stress, in which animals with an imbalanced redox system had chronic inflammation, neuro-inflammation, and recurring infections [51]. Antioxidant treatment may improve neuronal functioning by reducing ROS generation and altering redox-related signaling pathways [7].

Polyphenols are subordinate metabolites which have impending health benefits and are administered for boosting brain energy, scavenging ROS, and inhibiting the inflammatory demonstrating mechanism resulting in improved depression and anxiety [51,52]. Flavonoids are substances that can suppress neuroinflammation, activate the $GABA_A$-Cl channel complex, modify monoaminergic neurotransmitters, and enhance dopamine, serotonin, and monoamine levels in the CNS [9,23]. In our study, TPC and TFC assays were used to measure the quantity of polyphenols. Based on the result, the utmost amount of phenolics and flavonoids were found to be present in MBOL and those findings indicate that MBOL seems to be a promising therapeutic agent for antioxidant deficiency and neuropsychiatric disorders.

Evidence shows that oxidative stress influences anxiety-related behavior by interfering with brain activity and neurotransmission [51]. Using various approaches such as hydrogen transfer, chelating peroxidase metals, single oxygen transfer, and inactivating lipoxygenase, antioxidants can stop or deter the oxidation of a substrate [53]. In DPPH free radical scavenging assay, both extacts namely MBOL and NBOL demonstrated significant activity against DPPH free radicals comparable to that of standard (Fig. 1). Moreover, a substance with a reducing potential has the capacity to transfer electrons during redox processes, converting free radicals into more



(caption on next page)

Fig. 8. Paste and olive color represents α (D) and γ (E) subunit of $GABA_A$ respectively. 3D and 2D interaction of (A) stigmasterol, (B) cholest-5-en-3-ol, 24-propylidene-, (3. beta.)-, (C) α-cadinol, (D) δ-tocotrienol, (E) 4,22-stigmastadiene-3-one, (F) epi-bicyclosesquiphellandrene, (G) γ-muurolene, (H) γ-cadinene (I) δ-cadinene, (J) β-cadinene, (K) viridiflorene, (L) *cis*-calamenene and (M) δ-selinene, respectively with BZD site. Light violet, purple, and light pink colors of the 2D image indicate Pi-sigma bond, Pi-alkyl, and alkyl of that compound with BZD site. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Molecular interaction of the best ligands with BZD site of $GABA_A$.

Compounds	Molecular formula	Binding affinity ($kcalmol^{-1}$)	Interaction with amino Acids	
			Hydrogen bond interaction	Hydrophobic bond interaction
Stigmasterol (5280794)	$C_{29}H_{48}O$	−10.5		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:LYS156, D:TYR160, D:VAL203, D:TYR210
Cholest-5-en-3-ol, 24-propylidene-, (3. Beta.)-(6443745	$C_{30}H_{50}O$	−10.4	D:SER205(2.37 Å)	E:TYR58, E:PHE77, D:VAL203, D:LYS156, D:PRO140, D:PRO154, D:HIS102
4,22-Stigmastadiene-3-one (5364563)	$C_{29}H_{46}O$	−9.4		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
Epi-bicyclosesquiphellandrene (91747125)	$C_{15}H_{24}$	−9.6		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
γ-Muurolene (12313020	$C_{15}H_{24}$	−9.5		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
γ-Cadinene (92313)	$C_{15}H_{24}$	−9.8		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
δ-Cadinene (441005)	$C_{15}H_{24}$	−9.8		E:TYR58, E:PHE77, E: MET130, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
β-Cadinene (10657	$C_{15}H_{24}$	−9.5		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
α-Cadinol (10398656)	$C_{15}H_{26}O$	−9.4	D:SER205(2.52 Å)	E:TYR58, E:PHE77, D:HIS102, D:VAL203
δ-Tocotrienol (5282350)	$C_{27}H_{40}O_2$	−9.9	D:SER205(2.82 Å)	E:TYR58, E:PHE77, D:TYR210, D:TYR160, D:VAL203,D:LYS156,D:VAL212,D:PHE100, D:HIS102
Viridiflorene (10910653)	$C_{15}H_{24}$	−9.7		E:TYR58, E:PHE77, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210
Cis-calamenene (6429077	$C_{15}H_{22}$	−10.2	D:SER205 (2.84 Å)	E:TYR58, E:PHE77, D:TYR160, D:TYR210, D:PHE100
δ-Selinene (520383	$C_{15}H_{24}$	−9.8		E:TYR58, E:PHE77, E: MET130, D:PHE100, D:HIS102, D:TYR160, D:VAL203, D:TYR210

Table 3
ADMET analysis of the ligands with better binding characteristics.

Compound name	MW	NHA	NHD	LogP	NRB	GIA	LD50	BBB	HT	AT	NLV	DL
Stigmasterol	412.702	1	1	7.8008	5	Low	2.54	No	No	No	1	Yes
Cholest-5-en-3-ol, 24-propylidene-, (3. Beta.)-	426.729	1	1	8.335	6	Low	2.609	No	No	No	1	Yes
4,22-Stigmastadiene-3-one	410.686	1	0	8.009	5	Low	2.527	No	No	No	1	Yes
Epi-bicyclosesquiphellandrene	204.357	0	0	4.5811	1	Low	1.56	No	No	No	1	Yes
γ-Muurolene	204.357	0	0	4.5811	1	Low	1.54	No	No	No	1	Yes
γ-Cadinene	204.357	0	0	4.5811	1	Low	1.54	No	No	No	1	Yes
δ-Cadinene	204.357	0	0	4.7252	1	Low	1.552	No	No	No	1	Yes
β-Cadinene	204.357	0	0	4.5811	1	Low	1.586	No	No	No	1	Yes
α-Cadinol	222.372	1	1	3.7759	1	High	1.918	Yes	No	No	0	Yes
δ-Tocotrienol	396.615	2	1	7.98372	9	Low	2.024	No	No	No	1	Yes
Viridiflorene	204.357	0	0	4.415	0	Low	1.518	No	No	No	1	Yes
Cis-calamenene	202.341	0	0	4.63192	1	Low	2.069	No	No	Yes	1	Yes
δ-Selinene	204.357	0	0	4.8693	1	Low	1.647	No	No	No	1	Yes

MW-molecular weight (g/mol); **NHA**-no. of hydrogen bond acceptor; **NHD**-no. of hydrogen bond donor; **LogP**-predicted octanol/water partition coefficient; **NRB**- number of rotatable Bond; **GIA**-gastro intestinal absorption; **LD_{50}**-median lethal dose; **BBB**- blood brain barrier; **HT**-hepatotoxicity; **AT**- AMES toxicity; **NLV**- number of Lipinski's violation; **DL**-drug likeness.

inert or less reactive molecules. Nonetheless, by altering the ratio of protonated to deprotonated antioxidants, buffer systems can stabilize the radical cation and boost antioxidant activity. Many research investigations have been conducted to explore how the structure of polyphenols and their capacity for ferric reduction are interconnected. In a reducing power assay, two extracts demonstrated concentration-dependent antioxidant activity (Fig. 2). These findings show that antioxidant capacity of MBOL can be considered as a possible treatment strategy for anxiety and depression. Since polyphenols provide natural defense against various illnesses, regular antioxidant supplementation may give the desired effect.

Anxiolytic drugs exert their pharmacological effect in the brain through increasing GABA-ergic neurotransmission. EPM and HBT, well-recognized methods were used to measure the anxiolytic activity in this study [54]. The more time spent in the EPM device's open arms is thought to have an anxiolytic effect in this test [9]. As illustrated in Fig. 3A, the amount of time spent in the open arms was significantly lengthened ($P < 0.05$) by MBOL at both dosages (200 and 400 mg/kg) (153.6 ± 1.69 and 164.8 ± 1.28 s, respectively) at the 5th observation period. Consequently, mice treated with extract spent a shorter time in closed arms (Fig. 3B). Similarly, HBT was shaped to assess a mouse's exploratory behaviors and several dimensions of unconditioned behavior in an unknown setting [55]. More head dipping (hole poking) indicates higher levels of anxiolytic activity, while hesitation to poke the hole is a promising indicator of anxiety [56]. NBOL and MBOL at both doses augmented the incidence of head dipping where MBOL at the higher dose produced a substantial upsurge (53.9 ± 1.16) compared to that of diazepam (64 ± 1.67) (Fig. 4). The anxiolytic-like properties may be linked to activation of GABA-activated chloride channels which enhances GABA receptor sensitivity.

Evaluation of locomotor activity is regarded as a noteworthy tool for evaluating the impact of crude extract on the central nervous system. The movement of an experimental animal in an unfamiliar environment reflects its motor activity and a decline in locomotion indicates a CNS depressive impact. Furthermore, presynaptic inhibition of sensory afferent neurons by local GABAergic interneurons controls neuronal signal transduction [57]. It has been asserted that, burst amplitude of motor neurons is regulated by $GABA_A$ergic neurons in the course of vigorous locomotion as per pharmaceutical therapies [58]. Numerous studies have evaluated locomotor activity, among them OFT and HCT are the most accepted methods [59]. In OFT, both lower and higher doses of NBOL and MBOL revealed a significant decrease in the movement in a dose dependent manner in comparison to control ($P < 0.05$) during the whole observation period (Fig. 5). The decline in locomotor function caused by MBOL and NBOL in the experimental mice corresponded to the impact of CNS depressants. Equally, HCT is comparable to the OFT and used to measure locomotor activity. According to our findings, administration of NBOL and MBOL at various doses significantly reduced the impulsive locomotion of experimental rodents in the hole-cross test. Besides, MBOL (400 mg/kg) significantly diminished the locomotor activity in terms of the number of movements at 120 and 180 min (3.60 ± 0.40 and 2.40 ± 0.51) comparable to that of control ($P < 0.05$) indicating CNS depressant activity (Fig. 6). The previous study also reported that, methanol extract of *B. orellana* leaves displayed similar results in both open field as well as hole cross tests through time-dependent decline in locomotor activity [14]. It is conceivable that both the NBOL and MBOL extracts strengthen GABAergic inhibition in the CNS via membrane hyperpolarization as GABA and glutamate are indeed the predominant inhibitory neurotransmitters in the CNS and they reduce the firing rate of important neurons in the brain. It may also be due to the prolongation of the GABA-gated channel opening period or an increase in GABA affinity [26].

An easy, quick, and economical method for assessing the cytotoxic and antitumor effects of plant extracts is the brine shrimp lethality test [60]. A study of toxicity obtained from plant extracts is necessary to determine their safety as a medicinal agent and to identify both the intrinsic toxicity of the plant and the result of an acute overdose. This experiment has aided in determining the biological response of the natural plant extract. Additionally, it has supported in establishing the safety level of dose that may be administered to animal models. A crude plant extract is categorized as extremely toxic if its LC_{50} value is less than 100 μg/mL, moderately toxic if it is between 100 and 500 μg/mL, weakly toxic if it is between 500 and 1000 μg/mL, and safe or nontoxic if it is over 1000 μg/mL [61]. The findings of this experiment indicated that percent mortality augmented with the enhancement of the concentration of the experimental samples (Fig. 7). The cytotoxic bioactivity of MBOL and NBOL might be attributed to the presence of anti-tumor substances as well as flavonoid components in the plant extracts [62].

A crucial strategy that has been widely applied in the process of developing new drugs is molecular docking, a form of structure-based approach. It assesses the affinities of small molecules for binding to proteins and other macromolecules. Also, it is utilized to investigate the molecular mechanisms of action of multiple pharmacological actions. Hence, molecular docking analysis has recently been used in several studies to estimate the biological activities of chemical compounds with the respective receptors [63]. In our study, 172 bioactive compounds of *B. orellana* plant found in numerous articles were virtually screened against the $GABA_A$ receptor (PDB: 6X3X) to confirm the experimental results of the *in vivo* study. However, the docking analysis revealed that 13 compounds had the remarkable binding affinity with the $GABA_A$ receptor amongst 172 compounds (Table 2).

Moreover, based on the binding affinity against the $GABA_A$ receptor, 13 bioactive compounds were selected for further evaluation. In our experiment, all of the selected bioactive compounds revealed not more than one violation demonstrating good drug-likeness properties and thus being considered promising lead compounds for the activation of the $GABA_A$ receptor. Moreover, current study's findings revealed that none of the compounds showed hepatotoxicity and also a considerable range of LD_{50} values were observed indicating the safety of the compounds in the biological system. In addition, no compound showed AMES toxicity except *cis*-calamenene (Table 3).

To summarize, all 13 bioactive compounds could be considered potential therapeutic candidates characterized by anxiolytic as well as CNS depressant activities with good pharmacokinetic and toxicological properties though advanced studies are still being looked for.

5. Conclusion

To conclude, *B. orellana* leaves trained with multiple traditional usages could be regarded as a possible source of different bioactive compounds. In the present study, different pharmacological properties including antioxidative, cytotoxic, anxiolytic and CNS depressive have been revealed by both nonpolar and polar extracts through *in vitro* and *in vivo* assays. Moreover, the aforementioned usage of this plant has been rationalized through molecular docking and ADMET analysis where some compounds were found to be potential leads with prominent binding affinity against $GABA_A$ receptor along with remarkable pharmacokinetic and toxicological profiles. However, exploration of underlying mechanism of action is desired to be confirmed through the isolation and identification of

pure compounds.

Data availability statement

Data will be made available upon request.

CRediT authorship contribution statement

Kishore Kumar Sarkar: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Trina Mitra:** Methodology, Investigation, Data curation. **Md Aktaruzzaman:** Writing – original draft, Formal analysis. **Md Ahsan Abid:** Visualization, Software. **Md Asibur Rahman:** Formal analysis, Data curation. **Pradip Debnath:** Visualization, Validation, Data curation. **Samir Kumar Sadhu:** Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27001.

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