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# Exploring the anxiolytic potential of *Breynia androgyna* (L.) a phytopharmacological, *in vivo* and *in silico* investigation

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Anxiety is a state of distress, affecting people worldwide. It is usually mitigated by conventional anxiolytic medications that produce dependency and adverse effects, thus highlighting the need for safer alternatives derived from natural sources. *Breynia androgyna*, a perennial shrub of the family Phyllanthaceae, has profound medicinal benefits, but its anxiolytic potential remains inadequately explored. The current study, hence, envisaged exploring the anxiolytic potential of the ethanolic leaf extract of *Breynia androgyna* (EEBA) and its acetone fraction (AEBA) through experimental and computational approaches. Phytochemical screening and quantification of total phenolic and flavonoid content were carried out using established procedures. EEBA and AEBA (200 and 400 mg/Kg) were administered per orally to Wistar albino rats for a period of 14 days, and the possible anxiolytic activity was assessed using the Elevated Plus Maze, Light and Dark Model, Mirror Chamber Test, and Opto-Varimex Open Field Test. One-way ANOVA was used to analyse the data, followed by Dunnett's test. Molecular docking was conducted *in silico* to examine interactions between the phytoconstituents and the $GABA_A$-$Cl^-$ ion channel receptor. Flavonoids, tannins, phenolics, and triterpenoids were detected in the extract and biofraction, with AEBA displaying higher phenolic (54.35 mg GAE/g) and flavonoid (67.81 mg QUE/g) content than EEBA. AEBA displayed superior *in vitro* antioxidant activity and produced significant anxiolytic effects at 400 mg/Kg ($P < 0.05$) across all behavioural models. Molecular docking studies revealed strong interactions between major phytochemicals and the $GABA_A$ receptor. In conclusion, the acetone fraction of *Breynia androgyna* demonstrated notable anxiolytic potential, probably attributed to its enriched flavonoid content, supporting its relevance as a promising natural anxiolytic therapeutic.

**Keywords:** Elevated Plus Maze, Light and Dark Model, Mirror Chamber, Opto-Varimex, $GABA_A$

## Introduction

The Global Burden of Diseases (GBD) states that anxiety disorders rank sixth in terms of disease burden worldwide[1]. Despite its prevalence and profound impact on psychological health, current treatments are undermined. Benzodiazepines offer a quick onset of action, notable efficacy and acceptable tolerability; hence, these medications have been widely used since the 1960s. However, its prolonged use can produce adverse effects, cognitive impairment and drug dependence[2]. Recent research has highlighted the significance of neurotransmitters like serotonin (5-hydroxytryptamine), norepinephrine, GABA, glutamate, and dopamine emphasising their contributions to the neurobiological mechanisms underlying mood and anxiety disorders[3]. In the central nervous system (CNS), GABA is the most predominant inhibitory neurotransmitter. The central amygdala consists of more than 90% of GABAergic neurons and plays a significant role in processing stress, anxiety and fear[4]. Many secondary metabolites of medicinal plants have demonstrated considerable efficacy in mitigating the symptoms of stress, anxiety and neurological disorders and flavonoids are noteworthy secondary metabolites that contribute to anxiolytic effects[5]. Flavonoids have been found to modulate the BZD-site of $GABA_A$ receptors and produce sedative, anxiolytic or anticonvulsant effects due to their binding affinity to $GABA_A$ receptors[6]. Physiologically, the human body possesses inherent antioxidant defence systems, comprising both enzymatic and non-enzymatic mechanisms that function synergistically to safeguard against stress and oxidative damage[7]. Antioxidants neutralise free radicals that damage DNA, proteins, and membrane lipids, while also modifying gene expression[8]. Plants rich in polyphenols and flavonoids exhibit strong antioxidant properties, effectively neutralising cellular free radicals and assisting in the prevention and treatment of oxidative stress-related disorders[9].

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Phytoconstituents from medicinal plants can thus act synergistically on one or more physiological targets and possibly combat diseases holistically, thereby proving advantageous over traditional allopathic medicines[10]. *Breynia androgyna* (L.) (synonym *Sauropus androgynus*), belonging to the family Phyllanthaceae, is a distinct medicinal shrub, widely distributed in Southeast Asia[11]. Several metabolites of this plant have been identified, confirming its therapeutic benefits and medicinal value. Ethnobotanical research confirms its traditional use in the treatment of a variety of diseases, *viz.,* diabetes, weight loss, diarrhoea, cough, ulcers, etc.[12]. Despite the potential benefits, there is a noticeable gap in scientific literature regarding the anxiolytic activity of this plant. Hence, the present study sought to unveil the therapeutic potential of *Breynia androgyna* (L.) in the management of anxiety disorder using phytopharmacological, *in vitro*, *in vivo,* and *in silico* approaches.

## Materials & Methods

### Drug solutions and reagents used

Diazepam was obtained from Ranbaxy Laboratories Ltd, N-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, acetone, and sodium nitroprusside were procured from Molychem Pvt. Ltd, and all the other chemicals used were of analytical grade.

### Collection and authentication of plant material

Fresh leaves of *Breynia androgyna* L. were collected from Mapusa, Goa, in December 2023. The leaves were identified, confirmed, and authenticated by Dr. Ashish V. Prabhugaonkar, Assistant Professor in Botany from Dhempe College of Arts and Science, Goa, dated 17/11/2023. The herbarium sample was submitted to the department of botany, DCT's Dhempe College of Arts & Science, Miramar, Panjim, bearing voucher specimen No. DD/BA/GCP/MAPUSA/23.

### Preparation of ethanolic and acetone biofraction

1.2 Kg of dried powdered leaves of *Breynia androgyna* (L.) were defatted by refluxing with petroleum ether. The dried marc was refluxed thrice using ethanol for one and a half hours each. After condensation, the solvent portion was decanted and filtered using Whatman no. 1 filter paper. The ethanolic leaf extract *Breynia androgyna* (L.) (EEBA) was obtained by completely evaporating the solvent from the filtrate. 80 g of EEBA was refluxed with acetone for one and a half hours. The process was repeated thrice, the solvent was filtered, the filtrate was evaporated and air-dried to yield the acetone enriched fraction of EEBA (i.e., AEBA). EEBA and AEBA samples were stored at 4°C until further use.

### Preliminary phytochemical analysis

#### *Qualitative phytochemical investigation*

The preliminary phytochemical screening of EEBA and AEBA was carried out to identify the different phytochemicals present in the extract and its biofraction[13].

#### *Quantitative phytochemical investigation*

The extract (EEBA) and its biofraction (AEBA) were subjected to the determination of total flavonoid content and total phenolic content.

#### *Total flavonoid content*

The total flavonoid content of EEBA and AEBA was estimated using aluminium chloride colorimetric method, and the absorbances of the samples were measured at 415 nm using a UV-VIS spectrophotometer[14].

The total phenolic content of EEBA and AEBA was evaluated using the Folin-Coicalteau's reagent and the intensity of blue colour formed due to the polyphenol was measured at 760 nm using a UV-VIS spectrophotometer[15].

### In vitro antioxidant activity

#### *DPPH radical scavenging assay*

The antioxidant activity of the EEBA, AEBA, and standard ascorbic acid was assessed using DPPH free radicals. The absorbances of the samples were recorded at 517 nm using a UV-Visible spectrophotometer after 30 min of reaction, and the scavenging activity was calculated as follows, and $IC_{50}$ values were determined[16-18].

% of DPPH scavenging activity =

$$[(A_{control} - A_{sample}) / A_{control}] \times 100$$

#### *Nitric oxide radical scavenging assay*

Nitric oxide scavenging activity of EEBA, AEBA and standard ascorbic acid was measured using the Griess reaction, in accordance with the procedure described by Kalhor *et al.*[19]. The Nitric oxide scavenging activity was calculated as follows.

% of Nitric oxide scavenging activity =
$[(A_{control} - A_{sample}) / A_{control}] \times 100$

***Hydrogen peroxide radical scavenging assay***

Hydrogen peroxide radical scavenging activity of EEBA, AEBA and standard was determined in accordance with the method described by Albayrak[20].

% of Hydrogen peroxide scavenging activity
$= [(A_{control} - A_{sample}) / A_{control}] \times 100$

**Procurement of animals and Ethics committee approval**

Wistar albino rats weighing about 200-250 g used in this study were procured from the National Institute of Biosciences, Pune (CPCSEA Reg No.: 1091/GO/Bt/07/CPCSEA), India. The animals were housed in polypropylene cages maintained under standard conditions (temperature 25°C ± 2°C and relative humidity 55 ± 10 %). The animals were provided with pellets and water *ad libitum*. A 12-hour light-dark cycle was maintained. All the animals were acclimatised to laboratory conditions for a week before the trial began (CCSEA Guidelines). The Institutional Animal Ethics Committee of Goa College of Pharmacy, Panaji, Goa, approved the animal study vide Protocol No. GCP/IAEC/2023/02. All experiments were carried out in compliance with the CCSEA guidelines, Government of India, ensuring sufficient care was taken to minimise the suffering of animals.

**Experimental design and screening tests for anxiolytic activity**

***Acute toxicity***

The report of the oral acute toxicity of the leaf extract of *Breynia androgyna* (L.) suggests it is safe, as no mortality was observed up to 2000 mg/Kg. Thus, 200 mg/Kg and 400 mg/Kg were selected as doses for the screening of *in vivo* anxiolytic activity[21]. To evaluate the anxiolytic potential of EEBA and AEBA, the rats were segregated into six groups, *viz.* Group I (control, distilled water), Group II (standard Diazepam, 2 mg/Kg body weight), Group III and IV (EEBA at doses of 200 mg/Kg and 400 mg/Kg respectively, p.o.), Group V and VI ( AEBA at doses of 200 mg/Kg and 400 mg/Kg respectively, p.o.), and administered the samples for a period of 14 days. After one hour of dose administration, the rats were exposed to EPM, LDT, MCA and OFT on 1$^{st}$, 7$^{th}$ and 14$^{th}$ day.

***Elevated plus maze (EPM)***

The EPM apparatus comprised of two open and two enclosed arms that were positioned opposite each other at a height of 50 cm above the ground. The test was conducted in accordance with the technique explained by Rodríguez-Landa for assessing anxiety behaviour[22]. Each rat was individually placed at the centre of the apparatus. The entries made by the rat in the open and closed arms were monitored over a 5 min trial period, using the Smart Version 3.0.02 Panlab Harvard Apparatus software. The percent number of open arm entries (%OAE) and percent time spent in the open arm (%TSOA) were calculated as indices of anxiolytic activity.

***Light and Dark model (LDM)***

To execute the Light and Dark model, the method used was similar to that described by Guillen-Ruiz *et al.*[23]. It is a well-known model for assessing anxiolytic and anxiogenic drugs. The light and dark model consists of a wooden box measuring 80 x 40 cm base x 40 cm high; it has two chambers (40 cm x 40 cm x 40 cm), one darkened and the other brightly lit with a 40-watt bulb. The partition separating the two chambers has a doorway (10 cm x 10 cm) for the passage of rats. Each rat was placed at the passageway facing the lit compartment, and the number of entries (NEL) and time spent (TSL) in the light chamber were monitored over a 5 min test period.

***Mirror chamber apparatus (MCA)***

The anxiolytic activity of EEBA and AEBA was evaluated using a mirror chamber apparatus as per the procedures described by Liu *et al.*[24] and Fernandes e Mendonca *et al.*[25]. The MCA consists of a wooden box with an open chamber with four-sided mirrored walls and one mirrored floor (76 cm × 57 cm × 35 cm), which is connected to a non-mirrored alleyway (57 cm × 12 cm × 35 cm). The mirror chamber consists of four pieces of mirror glass (three side panels and a floor panel) and one mirror glass placed just in front of the opening of the mirrored area. Each rat was placed individually in the corner of the mirror chamber and observed for a 5 min period. The number of entries into the mirror chamber (NEM) and the time spent in the mirror chamber (TSM) were considered indices to monitor potential anxiolytic activity.

***Optovarimex open field test***

The apparatus consisted of a central chamber (44 cm × 33 cm × 20 cm) with photocells equipped at the two opposite walls. After one hour of

administration, each rat was placed individually in the Optovarimex apparatus, and the locomotor activity in terms of distance travelled (DT) and resting time (RT) of each rat were monitored for a period of 3 min[26,27]. The activity was analysed using the Auto-Track system ATM-3 software.

**Identification of phytoconstituents in EEBA and AEBA by LC-MS analysis**

The Liquid chromatography-mass spectrometry analysis of EEBA and AEBA was carried out using ACQUITY UPLC H CLASS (Waters, UK, model DBA064). 5 µL of EEBA or AEBA was injected into the C18 Waters, Acquity BEH column (2.1×100 mm and particle size of 1.7 µm). The solvent system of the mobile phase consisted of solvent A: 0.1% Formic Acid and LC-MS grade water, along with solvent B: 0.1% formic acid and acetonitrile. The standardised gradient method was programmed as time (min)/%B - 0/5; 5/5; 30/90; 35/90; 36/5 and 45/5 with a stop time at 45 min to ensure all the compounds were eluted out at a flow rate of 0.2 mL/min. The mass spectrometer used was SYNAPT-XS HDMS (Waters, UK) in ESI positive ion mode.

***In silico* molecular docking**

***Preparation of the protein***

The 3D structure of the Human $GABA_A$ receptor, alpha1-beta2-gamma2 subtype (PDB ID 6D6T, 3.86 Å, electron microscopy) was acquired from the Protein Bank database and downloaded in the *.pdb file.

***Preparation of the ligand***

The 3D structures of identified phytoconstituents of *Breynia androgyna* (L.) leaves were obtained from the PubChem Open Chemistry Database. The Pymol software program was used to convert the *.sdf files of the 3D structures of the identified compounds into *.pdb files.

***Docking and visualisation of protein-ligand interactions***

Docking simulations between the flavonoid compounds and the target protein was performed using AutoDockTools 1.5.7, and the interactions were visualised using BIOVIA Discovery Studio 2024 Client.

**Statistical analysis**

Results of all the above-mentioned models were statistically analysed using one-way ANOVA by Dunnett's test, in which the results obtained from experimental samples were compared with the control. Each result was represented as mean ± SEM, with statistical significance of *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001, for anxiolytic (n=6) and antioxidant (n=3) activities.

## Results

The yield of EEBA was reported to be 132.0 g (11 % w/w). Moreover, that of AEBA was reported to be 44.40 g (55.5 % w/w).

**Preliminary Phytochemical Analysis**

Qualitative phytochemical screening results of EECC & AECC revealed the presence of the following phytochemicals (Table 1).

***Total Flavonoid Content (TFC) assay***

The total content of flavonoids in AEBA was found to be 67.81 mg QUE/g, which was greater than that of EEBA (57.97 mg QE/g).

***Total Phenolic Content (TPC) assay***

The study showed that the total phenolic content of EEBA and AEBA was 33.39 and 54.35 mg GAE/g, respectively.

***In vitro* antioxidant activity**

The results of the DPPH, nitric oxide and hydrogen peroxide radical scavenging activity of EEBA and AEBA are depicted in Table 2.

Table 1 — Preliminary Phytochemical analysis.

| Chemical tests | EEBA | AEBA |
|---|---|---|
| Carbohydrates | + | + |
| Protein | + | + |
| Tannins | + | + |
| Saponins | - | - |
| Steroids and terpenoids | + | + |
| Alkaloids | + | - |
| Flavonoids | + | + |

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

Table 2 — DPPH, Nitric oxide and Hydrogen peroxide Free radical scavenging activity of EEBA and AEBA.

| Samples | Free radical scavenging assays ($IC_{50}$ = µg/mL) | | |
|---|---|---|---|
| | DPPH | Nitric oxide | Hydrogen peroxide |
| EEBA | 67.73±2.45**** | 91.10±6.89**** | 52.50±1.06**** |
| AEBA | 56.28±0.09**** | 78.46±0.40**** | 36.74±3.56*** |
| Standard Ascorbic Acid | 6.27±0.29 | 4.07±0.60 | 6.11±0.50 |

Each value is expressed as mean ± SEM (n=3). One way ANOVA, ***$P$<0.001, ****$P$<0.0001.

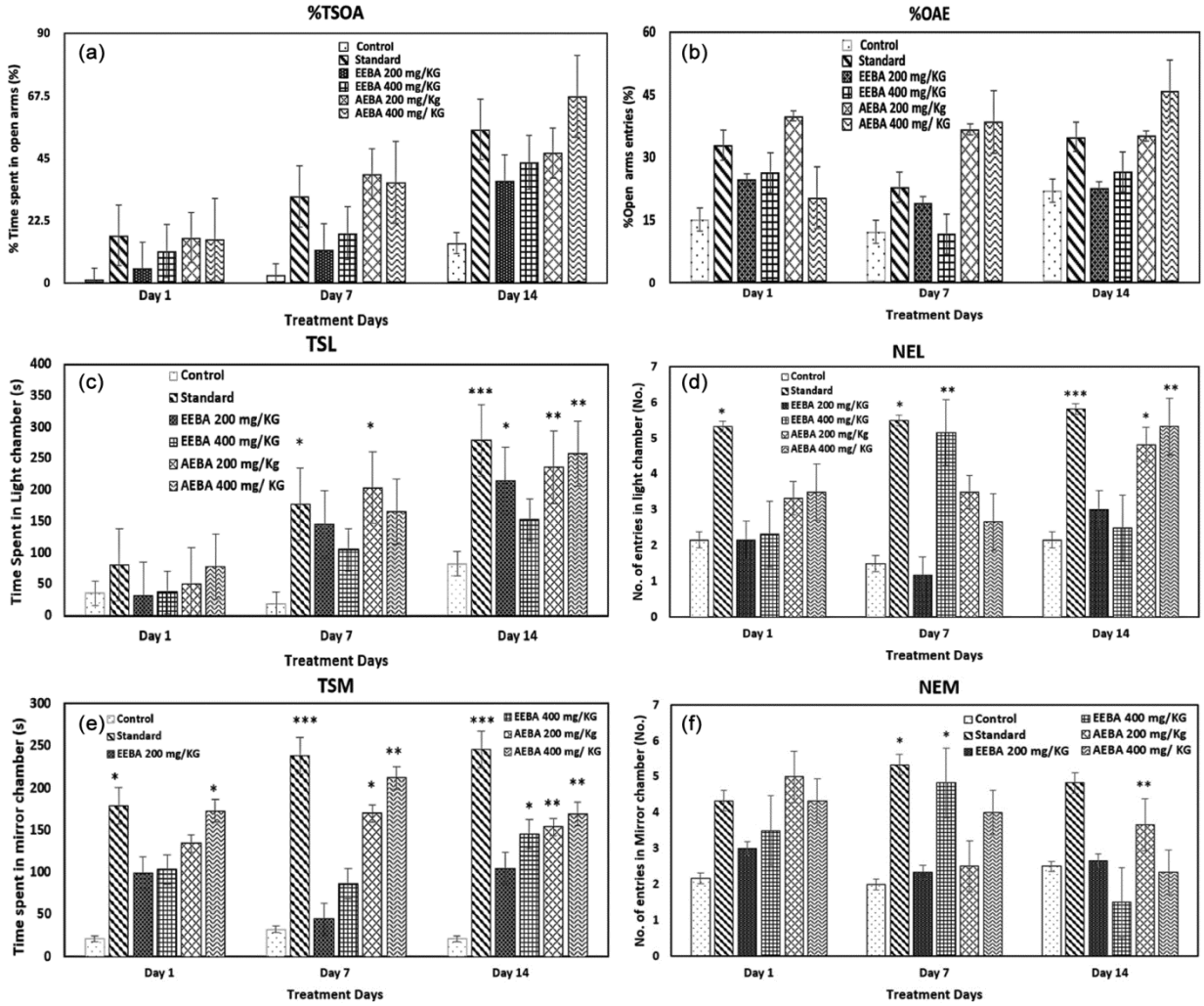


Fig. 1 — Effects of EEBA and AEBA in the Elevated plus maze test, Light and Dark model and Mirror Chamber apparatus. Where, (a) indicates percent time spent in open arms, (b) indicates percent open arm entries, (c) indicates time spent in light chamber, (d) indicates number of entries in light chamber, (e) indicates time spent in mirror chamber and (f) indicates number of entries in mirror chamber. Values are presented as mean ± SEM, n = 6 rats in each group. *P* values of **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant compared to the control (One way ANOVA followed by Dunnett's t-test).

***In vivo* anxiolytic study**

The findings of the anxiolytic activity study of EEBA and AEBA using various animal screening models like the Elevated plus maze test, Light and Dark model, and Mirror Chamber apparatus, and Optovarimex (open field test) have been illustrated in Figs. 1 and 2. Each value is presented as the mean ± SEM; n = 6 rats per group. **P*<0.05, considered significant compared to the control in the (Dunnett's t-test).

**LC-MC analysis**

A total of four compounds were identified by LC-MS analysis. i.e., Afzelin (MW: 432.4g/mol), Astragalin (MW: 448.4g/mol), Diosgenin (MW: 414.6g/mol) and Neophytadiene (MW: 278.5g/mol).

***In silico* molecular docking**

*The* molecular docking analyses of the identified compounds, *viz*. afzelin, astragalin, diosgenin, and neophytadiene from *Breynia androgyna* (L.) and Diazepam (standard) with $GABA_A$-BZD receptor (PDB ID: 6X3X) *in silico* have been depicted in Table 3 and Fig. 3.

## Discussion

Anxiety is a prevalent psychological disorder that impacts millions of people globally. It is characterised by an uncomfortable emotional or fearful state of mind, in which the individual perceives a threat that may be either specific or undefined[25,27]. A critical aspect of prospective new anxiolytic and

antidepressant agents is the emphasis on developing compounds that offer a rapid onset of action, exhibit fewer side effects, and maintain a wide safety margin[28]. Diazepam, a benzodiazepine (BZD), is widely used as a standard drug for its anxiolytic and central nervous system depressant effects. It functions by amplifying both presynaptic and postsynaptic inhibition through its interaction with a specific BZD receptor, which forms an essential part of the $GABA_A$ receptor $Cl^-$ channel complex[29]. The therapeutic effects of BZDs are achieved through positive allosteric modulation of $GABA_A$-BZD-$Cl^-$ channel

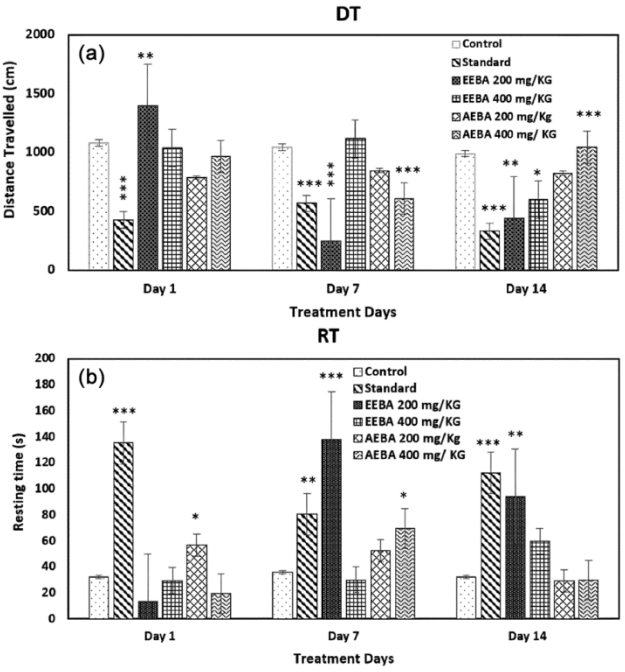


Fig. 2 — Effects of EEBA and AEBA in Optovarimex-autotrack system. Where, (a) indicates Distance travelled (DT) in cm and (b) indicates Resting time (RT) in seconds. Values are represented as mean ± SEM (n = 6). *P* values of **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant compared to the control (One way ANOVA followed by Dunnett's t-test).

receptors, which enhance the inhibitory effects on neurons of the CNS by hyperpolarising them[30]. The present study was aimed at investigating the neurobehavioral effects of the ethanolic extract of *Breynia androgyna* (L.) leaves (EEBA) and its bio-enriched acetone fraction (AEBA) at two doses each, i.e. 200 mg/Kg and 400 mg/Kg of BW of rats, using well-validated animal models. The qualitative phytochemical screening of EEBA and AEBA confirmed the presence of carbohydrates, proteins, steroids, terpenoids, flavonoids, alkaloids, glycosides, and tannins, as noted in Table 1. Several experimental studies on various plant extracts have demonstrated that flavonoids, alkaloids, and terpenoids are responsible for their anxiolytic and sedative properties[28]. The results of the total flavonoid and phenolic content revealed that the extract and its biofraction are a rich reservoir of flavonoids and phenolic compounds. Flavonoids are secondary metabolites from plants that can reduce anxiety and neuroinflammation by modulating the $GABA_A$-$Cl^-$ channel complex, altering monoaminergic neurotransmitters and boosting levels of dopamine, serotonin, and other monoamines in the CNS[31,32]. *In vitro* antioxidant activity study revealed that EEBA and AEBA were able to efficiently scavenge the DPPH, nitric oxide and hydrogen peroxide free radicals as compared to the standard ascorbic acid. The results illustrated that the bio-fraction exhibited substantial *in vitro* radical scavenging in all three assays performed (see Table 2).

The evaluation of the putative anxiolytic and locomotor activities of EEBA and AEBA was performed using the most commonly used validated rat models, including the EPM, Light and Dark model, Mirror Chamber apparatus, and Optovarimex open field system. The activities in each animal model were recorded on the 1st, 7th and 14th day after one hour of treatment. As depicted in Fig. 1(a), in the

Table 3 — Ligand interaction and binding affinity of Diazepam, Afzelin, Astragalin, Diosgenin, Neophytadiene.

| Compounds | PubChem CID | Ligand Binding Sites | Binding Affinity (Kcal/mol) |
|---|---|---|---|
| Standard (Diazepam) | 3016 | ALA161, TYR210, SER159, TYR160, PHE77, PHE100, HIS102, ASN60, VAL203, GLN204, SER206, TYR58, SER205 | -7.0 |
| Afzelin | 5316673 | ILE202, TYR58, GLN204, ASP192, TRP196, ASN60, SER61, SER195, GLU138, ASN103, LYS156, HIS102, VAL203, VAL212 | -7.4 |
| Astragalin | 5282102 | ASP192, SER195, ASN60, SER61, GLU138, ASN103, VAL203, LYS156, PRO140, PRO154 | -7.2 |
| Diosgenin | 99474 | LYS156, SER195, GLU138, SER61, ASP192, ASN60, HIS102, VAL203, ILE202 | -7.6 |
| Neophytadiene | 10446 | PRO140, GLU138, ASN103, SER195, ASN60, ASP192, VAL203, ILE202, LYS156, HIS102, VAL212, THR214 | -5.0 |

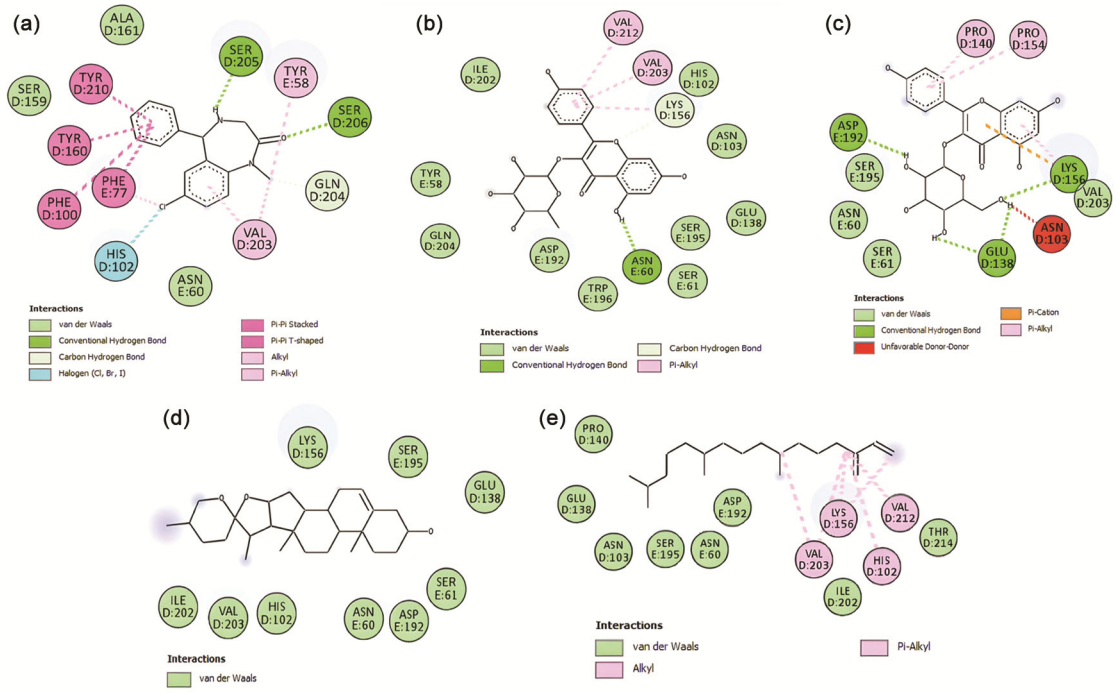


Fig. 3 — Two-dimensional interaction of (a) Diazepam (b) Afzelin (c) Astragalin (d) Diosgenin (e) Neophytadiene with various amino acids of $GABA_A$-BZD receptor.

elevated plus maze, AEBA 200 mg/Kg exhibited a significant increase in percent time spent in open arms ($P$<0.05) on day 7. Similarly, a significant increase in %OAE was observed for AEBA 400mg/Kg ($P$<0.05) on day 7, as observed in Fig. 1(b). However, on day 14, AEBA 400mg/Kg exhibited a substantial increase in %TSOA and %OAE. From these findings, we can infer that both the acetone enriched dose groups have shown a potential anxiolytic effect, i.e., effects that are significantly similar to those of Diazepam compared with the control. Thus, the treatment groups effectively exhibited anxiolytic behaviour by conflicting the anxiogenic environment of the elevated plus maze apparatus[33].

In the Light and Dark model, AEBA 200 mg/Kg depicted a significant increase in time spent in the light chamber (TSL) on the 7$^{th}$ ($P$<0.05) and 14$^{th}$ day ($P$<0.01) as seen in Fig. 1(c). Similarly, on day 14 a significant increase was observed for AEBA 400mg/Kg in TSL with ($P$<0.01) comparable to standard Diazepam. On day 14, both AEBA 200 mg/Kg and AEBA 400mg/Kg depicted a significant increase in number of light chamber entries with $P$<0.05 and $P$<0.01, respectively as seen in Fig. 1 (d). From the results obtained, we can state that AEBA 200mg/Kg and AEBA 400mg/Kg have demonstrated anxiolytic effects in rats in the light and dark model. The results also revealed that AEBA 400 mg/Kg performed better than AEBA 200mg/Kg, suggesting that a higher dose may produce better calming effects. In a similar type of study, Shah corroborated his findings of increased time spent in the light chamber with increased anxiolytic effects[34], thus validating anxiolytic activity of the EEBA and AEBA.

For the Mirror chamber test, it was observed that AEBA 400 mg/Kg exhibited a significant increase in TSM on 1$^{st}$ ($P$<0.05), 7$^{th}$ ($P$<0.01) and 14$^{th}$ ($P$ <0.01) day of trial, as displayed in Fig. 1(e). Similarly, AEBA 200mg/Kg showed significant activity ($P$<0.01) for TSM and NEM on day 14 as depicted in Figs. 1(e) and 1(f). Thus, EEBA 400mg/Kg, AEBA 200mg and 400mg/Kg have exhibited anxiolytic activity using the mirror chamber apparatus, as evident by a significant increase in the number of entries and time spent in the mirror chamber, which was corroborated with the anxiolytic activity of *Hybanthus enneaspermus* Linn. extract and biofractions reported by Fernandes e Mendonça[25]. The locomotor activity of rats treated with EEBA, AEBA and Diazepam was assessed using the Optovarimex open field system and parameters such as Distance Travelled (DT) and Resting Time (RT) were recorded. As observed in Fig. 2(a), EEBA 200mg/Kg has shown a significant decrease in DT on day 7 ($P$<0.0001) and 14

($P$<0.01), when compared to the control. The resting time as seen in Fig. 2(b), was also significantly increased on days 7 ($P$<0.001) and 14 ($P$<0.01). AEBA 400 mg/Kg showed a significant decrease in DT ($P$<0.001) and a significant increase in RT ($P$<0.05) on day 7. The results obtained for spontaneous motor activity indicate that, test groups EEBA 200mg/Kg and AEBA 400mg/Kg have shown a significant decline ($P$<0.001) in DT along with an increase ($P$< 0.001) in RT when compared with the control. The movement of an experimental animal in an unfamiliar environment reflects its motor activity, and a decline in locomotion indicates a depressive impact on the CNS[35]. The results of the open field test probably indicate that it may also have CNS depressant activity. Therefore, the open field test, associates anxiety-related behaviour with a reduction in locomotor activity, indicating that a potential sedative effect could be mediated by the $GABA_A$-$Cl^-$ channel complex, as reported by Cesário[36].

Recent advances in pharmacotherapy indicate that many phytochemicals can synergistically enhance the efficacy of natural products and conventional drugs, highlighting their therapeutic roles in supporting brain health and treating anxiety, mood disorders, cognitive decline and neurodegenerative diseases[37]. The LC-MS analysis of the leaf extract of *Breynia androgyna* and its biofraction revealed the presence of Afzelin, Astragalin, Diosgenin, and Neophytadine, amongst others. Kciuk reported the significant translational potential of Afzelin in mitigating neurodegenerative diseases associated with oxidative stress[38]. Yu documented that the anxiolytic effects of Astragalin were attributed to its modulation of the anterior cingulate cortex (ACC) and the lateral hypothalamus, thereby inhibiting neuronal excitability and contributing to central nervous system depressant effects[39]. In another investigation, Adnan identified astragalin as having the highest binding affinity with human serotonin receptors in an *in silico* antidepressant analysis[40]. Ben-Azu highlighted the neuroprotective and anti-apoptotic properties of the natural steroidal saponin Diosgenin against different neurological disorders[41]. Furthermore, Malik reported that Diosgenin exhibited anxiolytic and antidepressant-like properties, as well as enhances cognitive function[42]. Gonzalez-Rivera explored the involvement of the GABAergic system in the anxiolytic-like and anticonvulsant effects of neophytadiene[43]. To deduce the probable mode of action of EEBA and AEBA, the phytoconstituents identified by the LC-MS study were docked *in silico* with the $GABA_A$-$Cl^-$receptor. Docking with the receptor revealed that Diosgenin, Afzelin, Astragalin, and Neophytadiene displayed promising binding affinities to the $GABA_A$-BZD-$Cl^-$receptor with similar amino acid interactions to those of the standard Diazepam (refer to Fig 3). The plant steroid, Diosgenin, demonstrated a remarkable binding affinity of -7.6 Kcal/mol with the receptor, while Afzelin, Astragalin and Neophytadiene exhibited promising binding affinities of -7.4 Kcal/mol, -7.2 Kcal/mol, and -5.0 Kcal/mol respectively as compared to standard Diazepam (Table 3). The remarkable binding affinities of the prominent phytoconstituents with the $GABA_A$-BZD-$Cl^-$ receptor could account for mediating the anxiolytic effect[44]. Ben-Azu revealed that Diosgenin alleviated anxiety by plausibly modulating cholinergic and GABAergic pathways[45]. The significant *in silico* binding affinity of Diosgenin with the $GABA_A$-BZD-$Cl^-$ ion channel receptor could contribute to the molecular mechanisms underlying its anxiolytic potential. Based on the results of our study, it is evident that the leaf extract of *Breynia androgyna* and its biofraction have anxiolytic activity, with AEBA at a dose of 400 mg/Kg demonstrating remarkable anxiolytic potential. It can thus be corroborated that the enriched phytoconstituents present could synergistically mitigate anxiety by binding and modulating the $GABA_A$-$Cl^-$ ion channel receptors.

## Conclusion

The study successfully evaluated and demonstrated the anxiolytic potential of *Breynia androgyna* (L.). The acetone enriched fraction (AEBA) exhibited significant antioxidant and anxiolytic activities, as evidenced by phytochemical analyses, free radical scavenging properties, *in vivo* behavioural outcomes, and *in silico* receptor interaction studies. Flavonoids within AEBA displayed strong affinity for the $GABA_A$ chloride ion-channel complex, offering a plausible mechanism for the observed anxiolytic effects. Together, these findings fill the existing gap in scientific literature and highlight the remarkable potential of the plant as promising therapeutics in anxiety management.

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## Ethical Statement

For animal studies: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution. The study was approved by the Institutional Animal Ethics Committee with Approval No. GCP/IAEC/2023/02. Refer Annexure - II.

For *in vitro* or *in silico* studies: This study did not involve human participants or animals and therefore did not require ethical approval.

## Conflict of Interest

The authors have no known competing interests.

## Consent to Publish declaration

Not applicable. This manuscript does not contain any person's data in any form (including images, videos, or case details).

## Consent to Participate declaration

Not applicable.

## Clinical trial registration

Not applicable.

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