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RESEARCH ARTICLE

Pharmacological and *In-Silico* Investigations of Anxiolytic-like Effects of *Phyllanthus Fraternus*: A Probable Involvement of GABA-A Receptor

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Abstract: ***Background*:** *Phyllanthus fraternus* Webster Linn (family, Euphorbiaceae) is used as a traditional medication for the treatment of various disorders and has therapeutic implications.

Objective- This study intends to investigate the anxiolytic potential of *Phyllanthus fraternus* standardized extract and prediction of the probable role of its marker phytoconstituents.

***Methods*:** We tested the standardized hydro-ethanolic extract of *Phyllanthus fraternus* (whole plant) for the Elevated plus-maze model (EPM) and Light & Dark Exploration test as classical models for anxiety. Phyto-chemical HPTLC fingerprint analysis was performed for the detection of two classes of compounds lignans and tannins. HPTLC analysis of the standardized extract was performed using phyllanthin hypophyllanthin and corilagin as marker compounds. Additionally, GABA receptor antagonism was studied in other sets of experiments to assess the involvement of this receptor in the anxiolytic-like effects produced by *Phyllanthus fraternus*.

***Results*:** The lower doses of the lignan and tannin-rich extract of the *Phyllanthus fraternus* possess significant anxiolytic-like activity compared to the standard diazepam. Additionally, the results of the present study suggested that high doses (400mg/kg) of *Phyllanthus fraternus* have exerted some sedative-like effects. Phytochemical screening and HPTLC fingerprint analysis indicate the presence of lignans and tannins, whereas HPLC analysis of the standardized extract revealed the presence of marker lignan (Hypophyllanthin) and Tannin (Corilagin). The anxiolytic-like effect of *Phyllanthus fraternus* observed in the mice models were blocked by Flumazenil indicating the involvement of $GABA_A$ receptors in the modulation of this effect. Our molecular docking studies also supported probable anxiolytic and sedative effects.

***Conclusion*:** To summarize, results support the use of *Phyllanthus fraternus* in the anxiety-like symptoms/disease condition and suggest its anxiolytic-like effect governed by the GABA-A receptors.

Keywords: *Phyllanthus fraternus*, anxiety, phyllanthin, hypophyllanthin, corilagin, molecular docking simulations.

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1. INTRODUCTION

Anxiety is considered to be an unpleasant state of mental health, which also affects physical health and ultimately affecting longevity. Anxiety significantly reduces the quality of life. Anxiety disorders are often accompanied by social phobia, selective mutism, panic attack, specific phobia and separation anxiety disorder, *etc*. [1].

The benzodiazepines (BZD) are classically used to treat anxiety disorders and are believed to act via GABA receptors[1]. However, BZD are reported to cause clinical adverse effects like amnesia, psychomotor impairment, sedation and ataxia that limit their use in anxiety therapies [1]. Therefore, a need to search for effective anxiolytics with lesser or no tolerability has initiated us to focus on herbal remedies.

Phyllanthus fraternus Webster Linn [PF] (family, Euphorbiaceae) is used as a traditional medication for the treatment of various hepatic disorders by tribals [2-5]. PF is one of the key plants of traditional medicines, which has therapeutic implications in pain management and some other diseases [2-6]. Phytochemical investigations have reported the presence of various classes of phyto compounds [7]. The PF extracts have reported a wide range of pharmacological effects Fig. (**1**). Aqueous extract of PF has reported a protective effect against bromobenzene induced mitochondrial dysfunction in the rat's liver [8] and earlier reports revealed that

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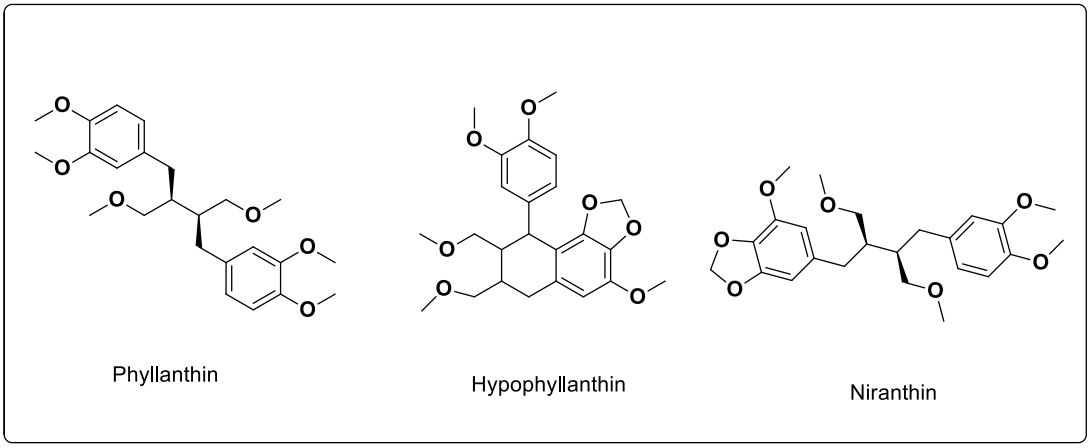

Fig. (1). General Potential phytoconstituents from *Phyllanthus* species [16].

alcohol, thioacetamide or carbon tetrachloride induced mitochondrial dysfunction could be prevented by prior administration of an aqueous extract of PF [9]. The therapeutic potential of *Phyllanthus* utilized to lessen or prevent the free radicals led to their deterioration, thereby helping in preventing subsequent pathological and biochemical changes observed during cerebral ischemia [10]. The PF extract has also reported protective effects against nephrotoxicity induced by bromobenzene [8]. PF has abilities to reduce drug-related toxicity, and therefore, co-administration of aqueous extract of PF can boost the therapeutic potential of anticancer drugs [11]. The Phyllanthus species have also reported the anti-inflammatory and analgesic potential [4, 5]. Several reports that have recognized molecular modeling techniques for a variety of bioactivities [13] and analgesic activity pronounced antinociception of PF extract [12-15].

In our recent study, we evaluated the anxiolytic potential of *Phyllanthus amarus* [16]. Our previous study suggests that the *P. fraternus* extract has no significant signs of toxicity and has CNS depressive property without affecting motor coordination [17]. In this study, *Phyllanthus fraternus* extract was evaluated for the potential anxiolytic effects and results were compared with the effects produced by diazepam in well-known pharmacological screening animal models utilized for accessing the effectiveness of anxiolytics [17]. Additionally, the involvement of the GABA-A receptor in the anxiolytic-like effect of *Phyllanthus fraternus* was explored by pretreatment with Flumazenil, a selective competitive antagonist of the GABA-A receptor [18]. The molecular docking studies were also performed to support/reconfirm the findings.

2. MATERIALS AND METHOD

2.1. Animals Study Design

For the present study, we utilized 8-10 weeks old Swiss albino mice weighing 20-25 g of both sex (male: female) in a 1:1 ratio in each group (n= 6 per group such as 3 males and 3 females) [16]. Mice were housed under standard laboratory conditions of temperature and 12/12 hours light and dark cycle in a group of six. All the experiments were conducted at the time from 9.00 to 15.00 hours. Animals had free access to water and food during acclimatization, deprived of food but not water 12 hours before the experiments. The animals acclimatized to laboratory conditions for not less than 10 days. The experimental protocols followed/utilized were in accordance with the ethical principles and guidelines, and they were approved by an institutional animal ethical committee constituted for the purpose of the control and supervision of experimental animals (CPCSEA) by the ministry of Environmental and Forests, Government of India, New Delhi (Reference- CPCSEA / IAEC/ GCOPK - MAR/01/2012) [16].

2.2. Phyllanthus fraternus Extract [PFE]

2.2.1. Collection and Authentication of Plant Materials

The whole plant of *Phyllanthus fraternus* Webster family- Euphorbiaceae was collected from different parts of Karad taluka [villages like Saidapur and Banawadi] western Maharashtra in months of September – October. The collected species were verified by the Botanical Survey of India [BSI], Pune. The plant species deposited as a herbarium was identified and authenticated by BSI, Pune [Reference No. BSI/WC/Tech./2012/644].

2.2.2. Preparation of Hydroethanolic Extract of Phyllanthus fraternus Whole Plant (PFE)

The shade dried plant components such as dried leaves, stems and roots were collected and further utilized for the preparation of standardized extract in the current study. Further, these components were subjected to mincing and extraction with 70% Ethanol:water (70:30 proportions). Then, the extracted marc was allowed to stir and further macerate at room temperature conditions (22-28^{o}C) for a period of 15 days. In order to get the desired semisolid level, we evaporated the ethanolic portion and further concentrated the extract (yield 5-7%) and stored in a refrigerator. We dissolved the standardized extract of P. fraternus in DMSO in order to achieve the desired concentrations just before all the experiments. The standardized extract of *Phyllanthus fraternus* whole plant was further phyto-chemically verified by HPTLC analysis [7, 15]. In this study, three doses (100, 200

and 400 mg/kg) of the *Phyllanthus fraternus* extract (PFE) were evaluated for the potential anxiolytic effect in animal models utilized for accessing the effectiveness of anxiolytics. The extract dissolved in dimethyl sulphoxide (DMSO, procured from Loba chemicals, Mumbai) to the desired concentration just before use.

2.3. Assessment of Anxiolytic Activity Using Elevated Plus Maze Test

Elevated plus maze (EPM) as described previously for mice [19,20], which consists of two open arms (37 X 5) and two enclosed arms (37 X 5 X 12) with 12 cm high wall , were placed in a way that the open arms were opposite to each other and so were the closed arms. All four arms were linked with a central square of 5 X 5 cm. The wooden apparatus was elevated to a height of 25 cm above the floor. The mice were placed individually at the centre of the EPM apparatus facing towards the open arm; the parameters, i.e., the number of entries and time spent in open arm and closed arms, were recorded for 5 minutes. The test was carried out anytime from 10:00 to 15:00 hours, *i.e*., at a fixed time of the day. The rationale of this study is that anxiolytics are expected to increase the number of entries into and time spent on open arm as animals tend to avoid open arms due to fear-provoking nature. After each trial, the EPM was cleaned with hydrogen peroxide. The Control group received DMSO.

2.4. Light and Dark Exploration Test (L & D)

As L & D test represents the natural habit of animals to prefer the dark place, these animals upon exposure to the brightly illuminated area, try to escape [16]. The L & D E apparatus consists of two compartments of a wooden box with dimensions of 45X27X27 cm: one dark compartment and the other brightly light. The box was dimly illuminated with 10 Watt white bulb and was open-topped. Upon treatment with anxiolytic agents, mice tend to spend more time in the light compartment as anxiolytics reduced the natural aversion to the light compartment and the anxiogenic agents, on the contrary, increased the proportion of time in the dark compartment. Naïve mice were placed individually at the centre of the light compartment. The parameters like the number of crossing between two compartments and time spent in the light and dark compartment were observed and recorded for the next 5 minutes. Diazepam 2mg/kg (intraperitoneally) was used as a reference standard.

2.5. Assessment of Motor Activity

The rota-rod apparatus from Ambala (Rota Rod apparatus model- K19616-2 Inco, Ambala) was used for motor activity assessments [16, 21]. It consists of three compartments and bar; the compartments are separated by disks. The constant rotation speed of the bar was 22 rpm. The mice were selected a day (24 h) before, eliminating those that did not remain on the bar for two consecutive sessions of 150–200 seconds. Selected animals were treated with PFE, Diazepam or the same volume of vehicle 30 minutes before the test. The test was performed on the rota-rod apparatus for a cut-off time of 150 seconds. The results were recorded as the time in seconds for which mice remained on the rota-rod.

2.6. Assessment of Probable Involvement of GABA Receptor

Flumazenil (FLU), a selective competitive antagonist of the GABA-A receptor, was used in the current study to evaluate the involvement of this receptor in the anxiolytic effects produced by *P. fraternus* extracts [22]. We gave the standard dose of 2.5 mg/kg of Flumazenil (Flu) along with test groups and Diazepam (DZP) so as to evaluate EPM and L&D models as per the aforementioned protocols [16].

2.7. Molecular Docking studies

2.7.1. Ligand Preparation

2D structures of ligands (Phyto-constituents) were prepared and converted into 3D by Chem Draw Ultra 8.0 software. Further, we optimized all 3D structures using MMFF, molecular mechanics protocols [16]. For that process, the parameter, *i.e*, the maximum number of cycles was set as 10,000 with a convergence criteria of 0.01 and constant of 1.0 (medium's dielectric constant which is 1 for in vacuo). MMFF was selected from Force Field drop-down list. The MMFF atomic charges were automatically selected for the MMFF Force Field. The values 20 and 10 were selected for electrostatic and vdW interaction, respectively. Conformers of the compounds were generated by a systematic search method. The docking results were ranked according to the decreasing docking energies of the different possible conformers for each of the ligands.

2.7.2. Preparation of Target Protein

We performed all our molecular docking simulations using the popular Vlife MDS 4.6.1 version. The crystal structure of a human gamma-aminobutyric acid receptor, the GABA (A) R-beta3 homopentamer receptor (PDB Code: 3COF) was taken from the protein database and further optimized as per our previously published protocols [16,23]. $GABA_AR$, the human β3 homopentamer, was co-crystallised with benzamidine, a novel agonist. The $GABA_AR$-β3cryst has a closed β9-β10 loop in an agonist-bound state, however the pore is shut, consistent with a de-sensitised conformation. The $GABA_AR$-β3cryst was in accordance with the electrophysiological recordings of benzamidine-induced desensitising currents measured in HEK cells at saturating concentrations of 10 mM, which approached those used in crystallization (33 mM). Moreover, in heteromeric $GABA_ARs$, exchanging the β-subunit intracellular border with the correspondent nAChR residues ablated desensitization. Therefore, by using Vlife MDS, we added hydrogens to our protein molecules [16].

2.7.3. Molecular Docking

All docking simulations were performed using Vlife QSAR software (4.6.1), and BioPredict tools for GRIP docking. Docking parameters were set as defaults [16]. The num-

ber of placements was set as 30 and ligand-wise results were given as 5 to obtain five top poses for each ligand.

2.8. High-Performance Thin Layer Chromatography (HPTLC) Studies

The HPTLC chromatogram plate development, fingerprint analysis and marker overlaying were done with the help of experts of Anchrom HPTLC laboratories, Mumbai. We utilized pre-coated silica gel 60 F-254 (0.2 mm thickness) HPTLC plates of 10 x 10 cm from Merck, Germany. The Camag Linomat V sample applicator was used for applying the samples on the plate of 7 mm bands, 15 mm apart from the edges. The plates were placed at a distance of 80mm at 25 ± 5° C in a Camag trough glass chamber. The saturation time was 30 min and after development, the plates were dried in a hot-air oven, viewed in a Camag UV chamber and the chromatograms were scanned with a Camag TLC Scanner. The Rf values and fingerprint data were recorded using WINCATS software. Initially, the solvent system used was toluene: ethyl acetate in varying ratios (2: 1, 85:15 v/v), but the plate was not well resolved. Of the various mobile phases tried, toluene: chloroform: ethanol (4:4:1, v/v) gave the best resolution for the development of a common chromatogram for the analysis of the components of the extracts under study. Well-defined spots were obtained when the chamber was saturated with the mobile phase for more than 20 minutes, at room temperature. HPTLC fingerprint study demonstrated a unique fingerprint pattern for a similar solvent system. HPTLC chromatogram of *P. fraternus* extracts was used for detection of standard markers in *P. fraternus* extract and fingerprint matching with help of three markers [phyllanthin (P), hypophyllanthin (H) and corilagin (C)] already available at Anchrom laboratories Mumbai.

2.9. Statistical Analysis

A Graph pad Prism software version 6.01©, 1992–2012 was used for statistical calculation. We represented all data in terms of mean ± SEM (standard error of the mean). Statistical significance (P-value < 0.05) was obtained with the control [16].

3. RESULTS AND DISCUSSION

The number of entries and time spent in an open arm of EPM model and light compartment of L & D exploration test and the score of motor activity after drug treatment in the control, (PFE 100, 200 and 400mg/kg) and Diazepam 2mg/kg treated animals are as per SI. Figs. (**1a**, **b**, **2a**, **b** and **3**). The combination of PFE doses decreased anxiety in mice as indicated by an increase in the number of entries and time spent in the open arm in the EPM paradigm as well as in the light compartment of L&D E test compared with saline-treated animals ($p< 0.001$). The number of entries and time spent in the open arm in the EPM paradigm as well as in the light compartment of light and dark test are comparable with EPM and L&D results of diazepam. The result of animals treated with PFE does not show a significant change in motor co-ordination compared with the results for the control group and the diazepam-treated mice SI. Fig. (**3**).

Three behavioral animal models were used in the present study, the EPM model, the L&D model and the LM activity, which assessed the anxiolytic potential of various doses of the *P. fraternus* in mice. These are classic tests and standard behavioral animal models utilized for obtaining data on anxiety and psychomotor performance. Additionally, these models have the ability to produce an anxiety condition in normal rodents in a reproducible pattern [24].

Both in the EPM test and L & D Exploration test, flumazenil reversed the effect of *P. fraternus* extract and diazepam on the number of entries and time spent in the open arm of EPM apparatus and light compartment of L&D Exploration apparatus, suggestive of a possible mechanism of action of *P. fraternus* via the GABAA receptor. The summarized results of the EPM test and L & D Exploration test are presented in SI Fig. (**1a, b** and **2a, b**), respectively.

In the EPM test, when an animal spends more time in open arms, it is assumed that it is in a good mood and free from anxiety. This validated, widely used behavioral test uses an elevated maze alley, which evokes an approach-avoidance conflict so the rodents spend more time in the closed arm when placed in the EPM [15]. Based on these claims, the EPM tests are a reliable means of identifying selective anxiolytic effects of drugs and can be utilized as a tool for assessing the efficacy of anxiety-modulating drugs or mouse genotypes [24]. In this study, three doses of *P. fraternus* extract (100, 200 and 400 mg/kg) produced significant effects in a dose-dependent manner compared to the diazepam group. Results of all three PFE treated dosage groups indicated that the observed anxiolytic activity did not impair the motor coordination activity as compared to diazepam-treated animals. The EPM test is one of the most popular tests for the search of new benzodiazepine-like anxiolytic agents [15]. With this reference, the activity of the extract of *P. fraternus* relieving anxiety in this model may suggest a possible positive modulation of the GABA-A/benzodiazepine receptor complex [21, 24-25]. The natural habit of animals of liking the dark place is the basis of the Light and Dark Test. During a 5-min period, animals permitted freely to explore a new atmosphere comprised of two different compartments: protected (dark) and unprotected (light). Anxiolytics tend to increase the number of entries and the time spent in the bright compartment to change the natural habit of animals to be in light . In the current scenario, the extracts of the *P. fraternus* in three different dosage forms (100, 200 and 400 mg/kg), separately, produced a significant effect in a dose-dependent manner as compared with the control diazepam [16, 26]. The phytochemical screening of *P. fraternus* extracts showed the presence of two major classes of compounds, lignans and tannins, along with phenolic compounds such as flavonoids, alkaloids, glycosides, steroids, saponins and proteins, [27] evident from HPTLC chromatogram plates *P. fraternus* extract. The HPTLC plate pictures are depicted under specific heading of wavelength (for details see SI. Fig. **4**). The result of this study proposes that the *P. fraternus* broadly possesses anxiolytic properties.

Table 1. Summary of Molecular Docking interactions of compounds reported in *P. fraternus* with Crystal structure of a human gamma-aminobutyric acid receptor, the GABA (A) R-beta3 homopentamer receptor (PDB Code: 3COF).

Molecule Name	Final Energy -(kcal/mol)	Final GRMS	Dock Score	HB Interactions	Hydrophobic Interactions	Van Der Waals Interaction
Diazepam (Std)	55.7428	0.9361	-63.1568	ASP17E	GLN90A	GLN90A
Lignans						
Phyllanthin	72.4109	0.9493	-59.1110	-	-	ASP17E
Hypophyllanthin	61.9501	0.6978	-44.7208	-	ILE25A	-
Niranthin	81.2560	0.9113	-62.1714	ASP17E	GLN90A	ASP24A
Nirtetralin	81.2297	0.7657	-48.4268	-	ILE25A	ASP89A
Tannins						
Ellagic acid	135.664	0.9323	-42.8061	-	TYR23A	ASP89A
Gallic acid	67.1261	0.7169	-58.5756	-	ILE25A	ARG26A
Gallocatechin	126.6973	0.5947	-58.97	-	-	ARG26A
Isocolliragin	201.8633	0.9147	-34.11	-	ILE25A	LEU20A
Catechin	128.5199	0.9805	-60.3729	-	ILE25A	LEU20A
Corilagin	293.3464	0.9094	-14.7823	-	-	TYR23A

*GRMS: Gradient Root Mean Square.

However, it is worthy to note that, with more analysis of single components of this extract in future; we will get more insights into its bioactivity. More studies that are investigational will be required in order to achieve an extensive Phyto-constituents analysis for anxiolytic potential. The phytochemical HPTLC fingerprint analysis of the *P. fraternus* extract signifies that the anxiolytic activity of *P. fraternus* extract could be due to the presence of lignans and tannins. The HPTLC fingerprint analysis of PFE extracts showed significant peaks, as shown in SI. Figs. (**5A**, **B** and **C**). The HPTLC chromatogram of *P. fraternus* extract utilized the standard marker compounds, phyllanthin and hypophyllanthin for the detection of lignans, and corilagin as the standard marker compounds for the detection of tannins, as shown in SI. Figs. (**5A**, **B** and **C**). The results of HPTLC chromatographic analysis revealed that PF extracts contained a considerable amount of lignans (hypophyllanthin) and tannins (corilagin). Phyllanthin was not detected in the HPTLC fingerprint analysis. Therefore, the presence of these phyto-constituents might be the cause of their bioactivity and thereby anxiolytic potential.

The docking analysis of the selected lignans and tannins previously reported with the GABA AR-B3 Crysthomopentamer receptor is summarized in Table **1**. The present study extends molecular docking analysis with an attempt to set a logical correlation for in-vivo outcomes with the in-silico study. The outcomes of molecular docking studies on GABA AR-B3CRYSTHOMOPENTAMER receptor are summarized Table **1** and depicted in SI. Fig. (**6**). Currently, our molecular docking simulations suggested the probable role of interaction of tannins and lignans with the GABA/benzodiazepine receptor complex in the brain. It can be said that lignans, niranthin, and hypopyllanthin are deeply embedded in the allosteric site surrounded by highly electro-negative residues (docking scores: -62.1714 and -44.7208 kcal/mol, respectively) [16]. The study also demonstrates that tannins may also possess prominent activity against GABA receptors as compared to the standard drug, *i.e*., diazepam, since catechin, gallocatechin and gallic acid have docking scores comparable with that of diazepam.

The interaction poses of main compounds such as phyllanthin, hypophyllanthin, niranthin and isocollaragin with GABA receptor are shown in SI. Fig. (**6A-E**). 2D interaction of diazepam and niranthin against GABA (A) R-beta3 homopentamer receptor is depicted in SI. Fig. (**7**). Exact mechanisms will also be elucidated in further studies. The lower doses of *P. fraternus* shoe significant anxiolytic activity without affecting locomotor activity, however, the effects of the extract at a higher dose appear to be potentially sedative. Hence, the present study signifies that the extract of the *P. fraternus* may have potential clinical application in the management of anxiety-like disorders.

CONCLUSION

To conclude, we have investigated the anxiolytic-like effect of *P. fraternus,* suggesting the probable involvement of GABA-A receptor.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

We took permissions to carry out our experimental protocols, adhering all the ethical guidelines as stated and cleared by the institutional animal ethical committee. The approved protocol number was CPCSEA / IAEC/ GCOPK - MAR/01/2012.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Data for this research will be made available upon request and its SI is also available on the Journals Online page.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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