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RESEARCH ARTICLE

An Insight Into the Anxiolytic Effects of Lignans (Phyllanthin and Hypophyllanthin) and Tannin (Corilagin) Rich Extracts of *Phyllanthus amarus* : An *In-Silico* and In-vivo approaches

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Abstract: The extracts and the compounds isolated from *Phyllanthus amarus* Schumm and Thonn (Family: Euphorbiaceae) have shown a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diurectic properties

***Background*:** This investigation was aimed at exploring the anxiolytic potential of *Phyllanthus amarus* standardized extracts and predict probable role of marker phyto constitutents.

***Objective and Methods*:** Three standardized extracts of *Phyllanthus amarus* plant viz. standardized aqueous extract of *Phyllanthus amarus* whole plant (PAAE), standardized methanolic extract of *P. amarus* leaf (PAME) and the standardized hydro-methanolic extract of *P. amarus* leaf (PAHME) were tested in the classical animal models of anxiety: Elevated plus-maze model and Light & Dark Exploration test.

***Results*:** The lower doses of the tannin rich extract (PAHME) of the *P. amarus* possess significant anxiolytic activity compared to lignin rich (PAME) and aqueous extracts (PAAE), while at a higher dose (400mg/kg) the results of all three extracts appears to be potentially sedative. While the molecular docking studies support these probable anxiolytic, the sedative effects of the *Phyllanthus amarus* extracts could be due to the interaction of tannins and lignans with the GABA-benzodiazepine receptor complex.

***Conclusion*:** The results of the present study indicate that the tannin-rich extract of the *P. amarus* may have potential clinical applications in the management of anxiety. It can be further studied for optimum dosage to be used as a future of anti-anxiety drug development or as a standardized Phytomedicine.

Keywords: *Phyllanthus amarus*, anxiety, phyllanthin, hypophyllanthin, corilagin.

1. INTRODUCTION

Anxiety, an unpleasant state of inner turmoil, not only affects the physical health but also the longevity. The excess of anxiety can significantly reduce the quality of life. The American Psychiatric Association (APA), says that "there are sharing of features of fear and anxiety in the Anxiety Disorders"[1]. These disorders are often accompanied by Social Phobia, Selective Mutism, Panic Attack, Specific Phobia and Separation Anxiety Disorder, *etc*. [2]. It has been known that only two-thirds of the anxiety patients respond to the available drug treatment. The benzodiazepines (BZD) group, constitutes majority of portion of anxiolytic substances, which are proposed to act via BZD receptors presented on the GABAA pentameric complex [3]. However, the use of classical BZD can significantly cause other clinical adverse effects such as myorelaxation, amnesia, psychomotor impairment, sedation, and ataxia, etc., which ultimately limits their use in anxiety treatments. Henceforth, there is a need to search for more effective anxiolytics with lesser or no tolerability [4].

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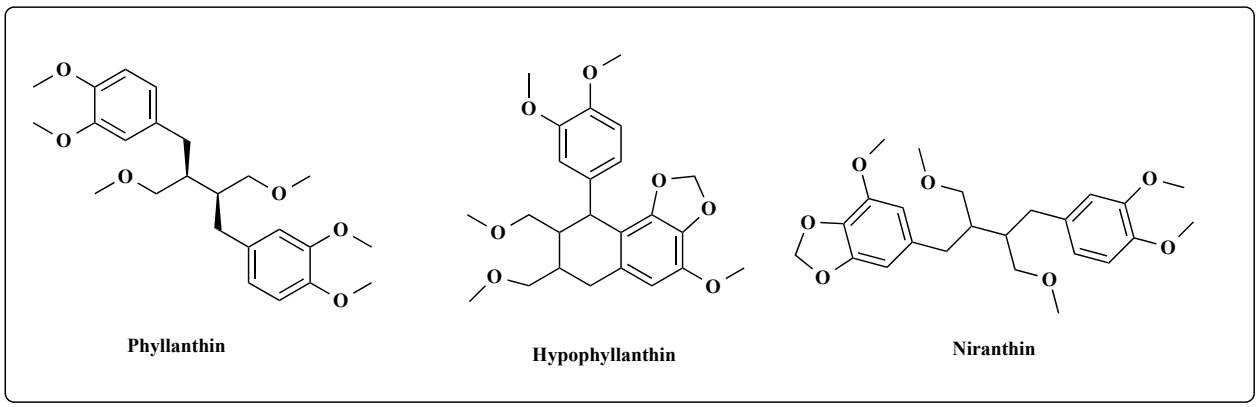

Fig. (1) Potential phytoconstituents from *Phyllanthus amarus* species.

Phyllanthus amarus Schumm and Thonn (Family: Euphorbiaceae) commonly known as bhumi amla, are traditionally used to treat different ailments including, but not limited to, flu, dropsy, diabetes, and jaundice [5]. *Phyllanthus amarus* (Euphorbiaceae) plant can typically be accessed from tropical regions of the world; which include the southern parts of China and India [6]. This genus has remarkable significance in treatments made with medicines from the traditional medicine system. *P.amarus (Phyllanthus amarus)* has been reported in the literature for a number of biological properties including anti-hepatic, anti-bacterial, antiviral, anti-diarrheal, and anti-plasmodial properties Fig. (**1**) [6-35]. The various extracts of *P.amarus* have also been reported to possess hepatoprotective (evaluated against Oxidative stresses induced by alloxan in rats), anti-inflammatory, anti-carcinogenic as well as anti-tumorogenic potentials [11,12]. One of the studies in literature demonstrated hypoglycemic effect on the alloxan induced diabetes mellitus by methanolic extracts of *P. amarus* [13]. The aqueous extract of *P. amarus* was investigated to have potential for the treatment of nervous debility and epilepsy. In our previous studies, we have investigated and demonstrated the pain modulatory potential and antifibromyalgic activity of different extracts of *P. amarus* [14,15]. Enticed by our literature survey, we have investigated the anxiolytic potentials of *P. amarus* extracts.

2. MATERIALS AND METHODS

2.1. Animals

In our present study, we have utilized Swiss albino mice, aged between 8-10 weeks, weighing between 20-25g, of either sex. We have maintained the standard lab conditions including the temperature and 12/12 hour light and dark cycle for mice, which were grouped in 6. We have taken necessary precautions to provide free food and water facility to them. We have carried out all the experiments at the time (9.00 to 15.00 hours). Before, 12 hours of experiment; we specifically made arrangement to deprive the animals from food but not water. All the animals were allowed to acclimatize to specific lab conditions for a minimum of 10 days. We have taken permissions for carrying out our experimental protocols, which were adhering to the ethical guidelines as stated and cleared by institutional animal ethical committee. The approved protocol number was RCP/18-19/P-19.

2.2. Chemicals

We have received a gift sample of standardized *P. amarus* aqueous extract of whole plant (PAAE) from Chemiloids Ltd., Vijayawada, India (Reference No. SR/KN/CL/1/2012-L12030241). Natural Remedies Pvt. Ltd., Bangalore also gifted us the standardized methanolic extract of P. amarus leaf (PAME) (>2.5% phyllanthin and Hypophy llanthin) and hydromethanolic extract of P. amarus leaf (PAHME) (>5% Corilagin) with referenced (No. FP1112042 – PA/11LOT05) and (No. FP1102034–PA/11LOT/02), respectively. We have procured the necessary DMSO (dimethylsulphoxide) and NaCl (sodium chloride) from commercial available sources. The standard Flumazenil (FLU) was obtained from the Neon Laboratories Ltd, Mumbai.

2.3. Evaluation of Anxiolytic Potential using Elevated Plus Maze Test

In the present study, the elevated plus maze (EPM) apparatus as described by Pellow et al.[16] and for mice as specified by Lister RG was utilized to assess anxiolytic potential of P. amarus extracts. We have used the popular EPM test for the assessments of behavioral aspects for anxiety. When we placed rodents on the EPM, due to fear to height they were subjected to anxiety. The manifestations to anxiety can easily be accessed by looking at rodents to stay at safer places and a decremental motor activity. Typical, EPM apparatus accompanies the two open arms (37X5) and two enclosed arms (37X5X12) with 12 cm high wall arranged. This arrangement makes sure that same types of arms will be opposite to each other. A central square (5X5 cm) was connected to arms. We have kept the wooden apparatus to a 25 cm height above the floor. All mice were allowed to place separately in EPM center in a such a way that they faced the open arm. For a period of 5 minutes each, we recorded the time spent in open and enclosed arm. We have ultilized each animal only single time and furthermore, we conducted test protocol during specific time of day as mentioned in section **2.1**. A simple rational behind this is that the open arms are more fear-provoking and that the ratio of the time spent on open, closed arms or entries into open-closed arms reflect the relative "safety" of closed arms compared with the relative "fearfulness" of open arms. It has been believed that anxiolytics will cause increment into and time spent on open arms of EPM apparatus. For cleansing of the EPM apparatus, we utilized hydrogen peroxide [17-18].

2.4. Light and Dark Exploration Test

Light and Dark Exploration (L & D E) test method represents the natural habit of animals like the dark place, i.e., they tend to avoid entry into and reduce spontaneous exploratory behavior in the brightly illuminated area; a natural tendency when a rat/mice is exposed to an unfamiliar environment. During a 5-min period, animals were permitted to freely investigate a new atmosphere comprised of two different compartments: protected (dark) and unprotected (light). Anxiolytic compounds change the natural habit of animals to light and increase the time spent in the light compartment. The dark and other bright are the 2 compartments boxes of the L&D apparatus. As there will be reduction of aversion to light compartments by anxiolytics, rodents will spent more time in that compartment. As opposite, to above fact, the agents causing the anxiety, will force rodents to spent more time in the dark compartment. This apparatus is made of wood and having dimensions of 45X27X27 cm. This box was allowed to open and illuminated with incandescent lamps, 65 lx. We have recorded for the duration of 5 minutes, the number of crossings and time spent in L & D compartments, after individually placing naïve mice in the center of the L compartment [19].

2.5. Assessment of Motor Activity

It has been well reported that barbiturates, benzodiazepines like compounds may result into the impairment of Rota-rod apparatus. For evaluation of effects on motor coordination, we utilized the Rota-rod apparatus. This Rota Rod apparatus, model - K19616-2 Inco, Ambala has a bar and is subdivided into 3 compartments by discs. We allowed the rotation of the bar at the speed of 22 rpm. Those mice, which did not remain on the bar for two consecutive periods of 150–200 seconds, were excluded, eliminated before 24 hours of experiment. After proper selection we have intraperitoneally administered the drugs, 30 minutes before conducting the test. Results were representing the time for which animals were supposed to stay on the Rota-rod (Cut off time=150 S).

2.6. Docking Methodology and *In-silico* Boiled Egg Model Analysis

2.6.1. Ligand Preparation

We allowed to convert 2D structures into 3D structures by utilizing the popular sketching Chem Draw Ultra 8.0. These 3D structures were then constructed to energy minimization process by using batch optimization for a set of molecules. The MMFF is used for molecular mechanics. The parameter was set as 10,000 as maximum number of cycles, 0.01 as Convergence Criteria, 1.0 as Constant (medium's dielectric constant was 1 for in vacuo). MMFF was selected from Force Field drop down list, for which the MMFF atomic charges were automatically selected. The value was set for 20 and 10 respectively for electrostatic and vdW interaction. The systematic search method was utilized for generation of the conformers [36-37]. We have ranked the docking results according to the decrements in docking energies of the different possible conformers for each of the ligands [38-39].

2.6.2. Preparation of Target Protein

Molecular docking studies were performed using Vlife MDS 4.6.1 version. In recent studies from coauthor of present paper varied targets were screened for biological activities, which formed the basis for present molecular screening methodology [36-42]. 3D X-ray crystallographic structure of the GABA-(A) homopentamer receptor (PDB Code: 3COF) was retrieved from the Protein Data Bank (www.rcsb.com) [20]. The receptor was extracted by X-ray diffraction method at a resolution of 2.97 A°. Benzamidine, a novel agonist, is co-crystallised with GABAA R. GABAA R-β3cryst has a closed β9-β10 loop, being in an agonist-bound state, but the pore was shut, consistent with a desensitized conformation. GABAAR-β3cryst was in agreement with our electrophysiological recordings of benzamidine-induced desensitising currents measured in HEK cells at saturating concentrations (10 mM), which were used in crystallisation (33 mM). Furthermore, in heteromeric GABAARs, swapping the β-subunit intracellular border with the equivalent nAChR residues ablates desensitization. Thus, by using Vlife MDS the protein molecules was reconstructed by addition of hydrogen in protein. The protein molecules were saved into the mole2 format and used forfurther processing.

2.6.3. In-silico Boiled Egg Model Analysis

We have evaluated the in-silico boiled-egg model analysis of the main phytoconstituents such as Lignans (Niranthin, Phyllanthin and Hypophyllanthin) for predictions of passive intestinal absorption and brain penetration, as a function of lipophilicity and apparent polarity (described by WLOGP and TPSA, respectively) using popular web based tool called "Swissadme"(http://www.swissadme.ch/faq.php) [21]. It was found that ligans (Phyllanthin and Hypophyllanthin) falls in yellow zone of boiled-egg model, indicating their ability to cross BBB (Blood-Brain-Barrier) (See **SI**).

2.7. Assessment of Involvement of GABA Receptor

In order to evaluate the participations of this abovementioned receptor for anxiolytic-like effects produced by *P. Amarus* extracts (PAAE, PAHME, PAME); we used popular BZD antagonist, Flumazenil (FLU). Flumazenil (FLU) at a dose of 2.5mg/kg was given along with test groups and Diazepam (DZP) to evaluate the effects utilizing EPM model and L&D Exploration apparatus per procedures given above (Sections 2.3 and 2.4).

2.8. Statistical Analysis

For performing the statistical calculation of the present study Graph pad Prism software version 6.01©, 1992–2012 was utilized. We have expressed all data mentioned in this study in terms of mean ± SEM (standard error of the mean). We have conducted one way ANOVA (one-way analysis of variance) for the analysis of statistical data along with the treatment with Dunnett's multiple comparison test. We have set statistical significance at P value < 0.05 by following comparison made to the control.

3. RESULTS

3.1. Effects of *P. amarus* aqueous extract (PAAE) on the EPM Model and L & D Exploration Test

The number of entries in an open and closed arms of the EPM apparatus after drug treatment in Control, PAAE 100mg/kg, PAAE 200mg/kg, PAAE 400mg/kg, and Diazepam 2mg/kg treated animals were presented in the **SI Fig. 1a**. The PAAE doses demonstrated anxiolytic potential in mice as indicated by the increase in number of entries in open arm of EPM paradigm compared with control group ($p< 0.001$). Also, the number of entries reported in open arm of EPM paradigm for PAAE 400mg/kg dose were comparable with the number of entries observed for diazepam. The time spent in an open and closed arms of the EPM apparatus after drug treatment in Control, PAAE 100mg/kg, PAAE 200mg/kg, PAAE 400mg/kg, and Diazepam 2mg/kg treated animals were presented in the **SI, Fig. 1b**. The PAAE dose demonstrated anxiolytic potential in mice as indicated by the increase in time spent in open arm of the EPM paradigm compared with control group ($p< 0.001$). Also, the time spent in open arm of EPM paradigm for PAAE 400mg/kg dose was comparable with the results for diazepam. The number of entries in the light compartment of L & D exploration test after drug treatment in Control, PAAE 100mg/kg, PAAE 200mg/kg, PAAE 400mg/kg, and Diazepam 2mg/kg treated animals were presented in the **SI Fig. 2a**. The PAAE doses demonstrated anxiolytic potential in mice as indicated by the increase in number of entries in the light compartment of L&D exploration test compared with control group ($p< 0.001$). For PAAE 400mg/kg treated mice, the number of entries in light compartment of L&D E test apparatus were comparable with the results for diazepam treated mice. The time spent in the dark and light compartments of L & D exploration test after drug treatment in Control, PAAE 100mg/kg, PAAE 200mg/kg, PAAE 400mg/kg, and Diazepam 2mg/kg treated animals were as per (**SI Fig. 2b).** The PAAE doses decreased anxiety in mice as indicated by the increase in the time spent in light compartment of L&D Exploration test compared with normal saline treated animals ($p< 0.001$). Also, the time spent in light compartment was comparable with the results for diazepam.

The score of motor activity after drug treatment in Control, PAAE 100mg/kg, PAAE 200mg/kg, PAAE 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI Fig. 3**. The results for PAAE 100mg/kg and PAAE 200mg/kg treated animals did not show significant change in locomotor activity compared with the results for control group. Also, the results for PAAE 400mg/kg treated animals were comparable with the results for diazepam treated mice.

3.2. Effect of *P. amarus* Hydroxyl Methanolic Extract (PAHME) on EPM Model and L & D Exploration Test

The number of entries in an open and closed arms of the EPM apparatus after drug treatment in Control, PAHME 100mg/kg, PAHME 200mg/kg, PAHME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI Fig. 4a**. Three doses of PAHME decreased anxiety in mice as indicated by the increase in the number of entries in open arm of the EPM paradigm with DMSO treated animals ($p< 0.001$). Also, the number of entries reported in open arm of EPM paradigm for PAHME 400mg/kg were comparable with the results for diazepam treated mice. The time spent in an open and closed arms of the EPM apparatus after drug treatment in Control, PAHME 100mg/kg, PAHME 200mg/kg, PAHME 400mg/kg, and Diazepam 2mg/kg treated animals were as per (**SI Fig. 4b**). Three doses of PAHME decreased anxiety in mice as indicated by the increase in time spent in open arm in EPM paradigm as compared with DMSO treated animals ($p< 0.001$). Also, the time spent in open arm of EPM paradigm for PAHME 400mg/kg was comparable with the time spent for diazepam treated mice. The number of entries in the light compartment of L & D exploration test after drug treatment in Control, PAHME 100mg/kg, PAHME 200mg/kg, PAHME 400mg/kg, and Diazepam 2mg/kg treated animals were as per (**SI Fig. 5a**). Three doses of PAHME decreased anxiety in mice as indicated by the increase in number of entries in light compartment of L&D E test compared with DMSO treated animals ($p< 0.001$). Also, the number of entries in light compartment of L & D Exploration test apparatus for PAHME 400mg/kg treated mice were comparable with the results of diazepam treated mice. The time spent in the dark and light compartment of L & D exploration test after drug treatment in Control, PAHME 100mg/kg, PAHME 200mg/kg, PAHME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI Fig. 5b**.

Three doses of PAHME decreased anxiety in mice as indicated by the increase in time spent in light compartment of L&D E test compared with DMSO treated animals ($p< 0.001$). Also, the time spent in light compartment of L &D Exploration test apparatus for PAHME 400mg/kg were comparable with the results of diazepam treated mice.

The score of motor activity after drug treatment in Control, PAHME 100mg/kg, PAHME 200mg/kg, PAHME 400mg/kg, and Diazepam 2mg/kg treated animals were as per, **SI Fig. 6**. The results of PAHME 100mg/kg and PAHME 200mg/kg treated animals do not show significant change in locomotor activity when compared with the results for control group. Also, the results for PAHME 400mg/kg treated animals were comparable with the results for diazepam treated mice.

3.3. Effect of *P. amarus* Methanolic Extract (PAME) on EPM Model and L & D Exploration Test

The number of entries in closed and open arms of EPM model after drug treatment in Control, PAME 100mg/kg, PAME 200mg/kg, PAME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI, Fig. 7a**. The PAME doses decreased anxiety in mice as indicated by the increase in the number of entries in open arm in EPM paradigm compared with DMSO treated animals ($p< 0.001$). For the PAME 400mg/kg treated animals, the number of entries in an open arm in EPM paradigm were comparable with the results of diazepam treated mice. The time spent in an open and closed arm of EPM model after drug treatment in Control, PAME 100mg/kg, PAME 200mg/kg, PAME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI, Fig. 7b**.

The PAME doses decreased anxiety in mice as indicated by the increase in time spent in open arm in EPM paradigm

compared with DMSO treated animals ($p< 0.001$). Also, for PAME 400mg/kg treated mice, the time spent in an open arm in EPM paradigm were comparable with the results of diazepam treated animals.

The number of entries in the light compartment of L & D exploration test after drug treatment in Control, PAME 100mg/kg, PAME 200mg/kg, PAME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI, Fig. 8a**. The PAME doses decreased anxiety in mice as indicated by the increase in number of entries in light compartment of L&D E test compared with DMSO treated animals ($p< 0.001$). For PAME 400mg/kg treated mice, the number of entries in light compartment of L&D E test apparatus were comparable with the results of diazepam treated animals. The time spent in dark and light compartment of L & D exploration test after drug treatment in Control, PAME 100mg/kg, PAME 200mg/kg, PAME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI ,Fig. 8b,9, 10a-10b**.

The PAME doses decreased anxiety in mice as indicated by the increase in time spent in light compartment of L&D E test compared with DMSO treated animals ($p< 0.001$). For PAME 400mg/kg treated mice, the time spent in light compartment of L&D E test was comparable with the results of diazepam treated mice.

The score of motor activity after drug treatment in Control, PAME 100mg/kg, PAME 200mg/kg, PAME 400mg/kg, and Diazepam 2mg/kg treated animals were as per **SI, Fig. 9**. The results of animals treated with PAME 100mg/kg and PAME 200mg/kg do not show significant change in locomotor activity when compared with the results for control group. Moreover, the results for PAME 400mg/kg treated animals were comparable with the results for diazepam treated mice.

3.4. Docking Study Results

In the current study, the molecular docking study was performed by using Vlife QSAR software. The software uses BioPredict tools to perform the GRIP docking. For performing these studies set the docking parameter as Exhaustive and input Rotation Angle step size of 30° by which the ligand were rotated for different poses. Input Number of Placements was 30 and Ligand wise results were obtained of top 5 poses for each ligand. In-silico docking studies were performed. Molecular docking gave us an opportunity to virtually screen a variety of phytochemical constituents of *Phyllanthus amarus* based on their binding orientation and binding abilities with a target molecule [22-25, 38-42]. Docking data directed us to select compounds with strong affinity for the target site. Generally, all the compounds have shown good docking results. Scattered trends of binding energies have been observed amongst the phytochemicals docked [36-42]. Molecular docking analysis against receptor contains Crystal structure of a human gamma-aminobutyric acid receptor, the GABA (A) R-beta3 homopentamer injuries to the extent of 47–70% whereas, the co-administration of benzamidine prevented it significantly. It was observed that Niranthin (docking score: -62.1714 Kcal/mol) and Catechin (docking score: -60.3729 Kcal/mol) have shown best docking score compared to the standard drug Diazepam (docking score: -63.1568 Kcal/mol).

The results of the interactions of ligands (Phyllanthus compounds) with the GABA receptor are summarized in **SI, Table 1 and 2.** The 2D Interactions of Niranthin and Diazepam molecules against the active site of GABA (A) R-beta3 homopentamer receptor is shown in **SI, fig. 11**. **SI, Fig. 12**, depicts the ligand interaction diagrams for poses of Niranthin and Diazepam on protein (pdb id: 3COF). Additionally, the 3D Interaction Poses of marker phyllanthus (Phyllanthin, Hypophyllanthin and Collarigin) GABA receptor is depicted in **SI, Fig. 13**.

3.5. Blockade of the Anxiolytic Effect of *P. amarus* by Flumazenil

Both in the EPM test and L & D Exploration test, Flumazenil reversed the effect of diazepam and all three extracts of *P. amarus* (at a dose of 400 mg/kg) on the number of number of entries and time spent in the open arm of EPM apparatus and light compartment of L&D Exploration apparatus, suggestive of possible mechanism of action of *P. amarus* via GABAA receptor. The summarized results of EPM test and L & D Exploration test are shown in **SI, fig. 14a** and **14b** respectively.

4. DISCUSSION

In order to evaluate the anxiolytic potential of *P. amarus* extracts, we have studied 3 behavioral animal models of anxiety ; EPM apparatus, L&D E test, and LM activity. As they minimize confounding factors of other conditioned assays and produce reproducible paradigm for creation of anxiety in normal rodents, henceforth we utilized the aforementioned models. In this way, we would be able to screen central nervous system actions giving more details about anxiety and psychomotor performance. As EPM is based on the behavioral aspects of animals; when exposed to an elevated maze alley, which henceforth provide an approach-avoidance conflict. Animals were observed to spend more time in the closed arms as compared to placing of open arms. When an animal spends more time in open arms it is assumed that it is in good mood and free from anxiety. In this study, three extracts of the *P. amarus* in three different dosage forms (100, 200, and 400 mg/kg), separately, produced significant effect in a dose dependent manner compared to Diazepam group. Also, results of 100 and 200 mg/kg groups showed that the anxiolytic activity was without any impairments in motor activity.

For better search of new benzodiazepine-like anxiolytic agents, we utilized the well-reported EPM test protocol. From our current study, a possible positive modulation of the GABA-A/benzodiazepine receptor complex in relieving anxiety has been noted as we tested and recorded the effects of the extracts of the *P. amarus* (in three different dosage forms 100, 200, and 400 mg/kg)[26-27].

Anxiolytics tend to increase the number of entries, time spent, and rears in the bright arena of L & D E test apparatus. However, in this model, compared to anxiety control, three extracts of the *P. amarus* in three different doses (100, 200, and 400 mg/kg), separately, produced significant effect in a

dose dependent manner compared to Diazepam group, i.e. increase the time spent on the animals in the light box.

It was noted that the reduction of spontaneous motor activity could be related to the calmness/sedative effect. In the present study, the motor activity results demonstrated dose-dependent sedative activity of all three extracts of *P. amarus*. [28].

Extracts of *P. amarus* consisting of various phytochemicals including alkaloids, glycosides, steroids, proteins, phenolic compounds, saponins, tannins and flavonoids. The results obtained in our study would suggest the probable anxiolytic potential of *P. amarus*. This potential may be due to synergistic action of these phytochemicals [29,30]. From our current analysis (HPTLC studies; **SI Figs. 15a-15c,16**) of phytoconstituents, it was clear that the PAAE extract was rich in tannins and lignans, Lignan rich in the PAME extract and tannin rich PAHME extract. As ligans are major phytoconstituents of phyllanthus species, they are reported for a number of biological activities such as immunomodulatory activity, anti-inflammatory, antioxidant, anti-arthritic and analgesic activities [31-35]**. Kassuya et al. [32]** reported the purified lignans isolated from the extracts of phyllanthus such as phyltetralin, nirtetralin and niranthin for their anti-inflammatory effects. Furthermore, there are several reports for mainly niranthin, which was found to have interference with PAF induced inflammatory response. Corilagin present in the extracts of phyllanthus also reported the anti-hyperalgesic activity**.**

From our previous study, the analysis made for phytochemical finger print suggests the role of *Phyllanthus* extracts in the modulation of hyperalgesia and which have thought to be due to the presence of lignans and tannins. SI, Figures 15a-15c, depict the HPTLC finger print analysis, showing significant peaks corresponding to each extract. SI, Fig. 16, depicts the HPTLC chromatograms with respect to standard markers phyllanthin and hypophyllanthin. SI, **Fig. 17**, shows the HPTLC chromatograms, for assessment of tannoid content with respect to standard marker corilagin. Our HPTLC fingerprint analysis and screening clearly states majority of lignans (phyllanthin and hypophyllanthin) and tannin (corilagin) in extracts of *Phyllanthus amarus*. The molecular docking studies have revealed that these effects of the *Phyllanthus amarus* extracts could be due to the interaction of tannins and lignans with the GABA/benzodiazepine receptor complex in the brain. It is noteworthy to mention that Niranthin was observed as deeply embedded in the allosteric site surrounded by highly electronegative residues and forms hydrogen bonding; having docking score of -62.1714. It demonstrates that Niranthin possesses most prominent activity against GABA receptors as compared to standard drug, i.e., Diazepam. Also, Catechin, Gallocatechin, Gallic acid, and Phyllathin have docking scores comparable with that of Diazepam. In-silico boiled egg models clearly demonstrated the passing of Nirathin, phyllanthin and hypophyllanthin via BBB barrier, **SI, Fig. 18**. Hence, further investigations are deemed necessary for elucidating the exact mechanism and detailed investigation of efficacy of these bioactive compounds.

The lower doses of the tannin-rich extract (PAHME) of the *P. amarus* possess significant anxiolytic activity compared to lignin-rich and aqueous extracts, while at a higher dose (400mg/kg) the results of all three extracts appears to be potentially sedative. Hence, our current in-silico analysis and in-vivo study suggests that the extract of the *P. amarus* rich in tannins might have anxiolytic activity and could be successfully used for the treatment of anxiety after careful optimization of dosage in the future.

Currently, our research group is working on the design, synthesis and biological activity studies [36-53]. We have recently also published a viewpoint on COVID-19 as a part of same activity [54].

CONCLUSION

The results of the present study indicated that the tannin-rich extract of the *P. amarus* may have potential clinical applications in the management of anxiety. It can be further studied for optimum dosage to be used as a future of anti-anxiety drug development or as a standardized Phytomedicine.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

We have taken permissions for carrying out our experimental protocols, which were adhering to the ethical guidelines as stated and cleared by the institutional animal ethical committee. The approved protocol number was **RCP/18-19/P-19**.

HUMAN AND ANIMAL RIGHTS

The approved protocol number was RCP/18-19/P-19.

RESEARCH INVOLVING PLANTS

None.

CONSENT FOR PUBLICATION

None

CONFLICT OF INTEREST

None

AVAILABILITY OF DATA AND MATERIALS

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

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SUPPORTIVE/SUPPLEMENTARY MATERIAL

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