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Integrated Pharmacognostical and Neuropharmacological Evaluation of *Pueraria montana* (Lour.) Merr. for Anxiolytic Potential.

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Abstract

Background*:** The long-term use of synthetic anxiolytic drugs is associated with adverse effects such as sedation, tolerance, and dependence. Therefore, plant-based alternatives with better safety profiles are being investigated. ***Objective*:** To evaluate the anxiolytic activity of the ethanolic extract of *Pueraria montana* (Lour.) Merr. (PMEE) using pharmacognostical, phytochemical, computational, and *in-vivo* studies. ***Methods: Pharmacognostical and phytochemical evaluation of PMEE was performed using microscopy, physicochemical analysis, TLC, HRMS, and GC-MS. Molecular docking was conducted against the GABA receptor (PDB ID: 6HUO). Acute oral toxicity was evaluated according to OECD guideline 425. Anxiolytic activity was assessed in Swiss albino mice using Elevated Plus Maze (EPM) and Light–Dark Box tests at doses of 100, 200, and 400 mg/kg. ***Results***: TLC analysis showed a characteristic puerarin spot at Rf 0.71. HRMS identified major phytoconstituents including puerarin, rutin, quercetin, kaempferol, glycitin, mirificin, and β-sitosterol. Molecular docking revealed strong receptor binding, with mirificin showing the highest docking score (−9.7308 kcal/mol). No mortality or toxic effects were observed up to 5000 mg/kg. In the EPM model, PMEE at 400 mg/kg increased open-arm time to 124.8 ± 5.84 sec compared to 102.4 ± 5.18 sec in normal controls animals. In the Light–Dark Box test, PMEE at 400 mg/kg increased light-compartment retention time to 142.6 ± 7.14 sec compared to 126.8 ± 5.14 in normal control. ***Conclusion***: PMEE showed significant dose-dependent anxiolytic activity with good safety, supporting its potential as a natural anti-anxiety agents.

Keywords: GABA, Puerarin, β-sitosterol, Mirificin, Anxiolytic.

Abbreviations

PMEE – *Pueraria montana* (Lour.) Merr. Ethanolic Extract, GABA – Gamma Aminobutyric Acid, EPM – Elevated Plus Maze, LDB – Light–Dark Box, WHO – World Health Organization, HCl – Hydrochloric Acid, mL – Millilitre, HRMS – High-Resolution Mass Spectrometry, GC-MS – Gas Chromatography–Mass Spectrometry, TLC – Thin Layer Chromatography, mg –

Milligram, kg – Kilogram, cm – Centimetre, mm – Millimetre, µm – Micrometre, % – Percentage, NaOH – Sodium Hydroxide, HNO_3 – Nitric Acid, NH_3 – Ammonia, $FeCl_3$ – Ferric Chloride, UV – Ultraviolet, nm – Nanometre, °C – Degree Celsius, hrs – Hours, CPCSEA – Committee for the Purpose of Control and Supervision of Experiments on Animals, IAEC – Institutional Animal Ethics Committee, OECD – Organisation for Economic Co-operation and Development, p.o. – Per Oral, RT – Retention Time, m/z – Mass-to-Charge Ratio, RMSD – Root Mean Square Deviation, ADMET – Absorption, Distribution, Metabolism, Excretion and Toxicity, TPSA – Topological Polar Surface Area, MOE – Molecular Operating Environment, PDB – Protein Data Bank, NIST – National Institute of Standards and Technology, EI – Electron Ionization, Q-TOF – Quadrupole Time-of-Flight, CYP – Cytochrome P450, w/w – Weight by Weight.

1. Introduction

Anxiety impacts the ability to remain calm and impairs sleep and social interaction as well as everyday functioning. Though synthetic anxiolytics help with anxiety, their long-term use often leads to sedation, dependence, tolerance, withdrawal, and impaired motor function. Due to this, in current scenario need to focusing on plant-derived anxiolytics that provide multi-target biological action with the greater safety margin [1]. Based on their use in traditional medicine, medicinal plants are considered to have pharmacological value. However, for their true value to the modern field of medicine to be established, they must undergo thorough research. The process of standardizing the medicine must begin with the correct identification of the plant and continue to include chemical and biological analyses [2]. This is especially important with *Pueraria montana* (Lour.) Merr., which contains a diverse collection of many classes of secondary metabolites that include isoflavones, flavonoids, glycosides, coumestans, pterocarpans, and phytosterols (Table 1 and Table 2) [3]. *Pueraria montana* (Lour.) Merr. extracts are important sources of isoflavones. In this research, the use of HRMS for the analysis of ethanolic *Pueraria montana* (Lour.) Merr. extracts provided partial identification of the major secondary metabolites in the extract as puerarin, genistin, biochanin A, ononin, glycitin, mirificin, coumestrol, medicarpin, tectoridin, tectorigenin, kaempferol, quercetin, rutin, catechin, β-sitosterol, and stigmasterol. GC-MS was the secondary method of choice for the identification of the non-polar, lipophilic, and volatile phytochemicals of the extract. Together, the methods applied in this study provided a basis for the preliminary and broad phytochemical and bioactive potential of the extract.

Computational tools were integrated into the preliminary screening of the identified constituents. Using molecular docking and ADME profiling, drug-likeness was assessed with respect to oral bioavailability, gastrointestinal absorption, permeability across the blood–brain barrier (BBB), P-glycoprotein and cytochrome P450 (CYP) inhibition, and predicted bioavailability. For this reason, the current research included an integrated pharmacognostical, phytochemical, computational, and *in-vivo* study to assess the ethanolic extract of *Pueraria montana* (Lour.) Merr. for its anxiolytic activity. The study entailed procedural standardization of pharmacognosy, high-resolution mass spectrometry (HRMS) and gas chromatography-mass spectrometry (GC-MS) profiling, molecular docking studies with predictions of ADME, an assessment of acute oral toxicity, and the evaluation of behavior in light–dark box and elevated plus maze models of anxiety after administering the extract.

2. Materials and Methods

2.1 Chemicals and reagents

The research used the following chemicals and reagents obtained from the Central Chemical Store, S.D. College of Pharmacy & Vocational Studies, Bhopa Road, Muzaffarnagar, Uttar Pradesh, India: ethanol (S-Merck), n-hexane (Qualigens), ethyl acetate (Loba Chemie), chloroform (Merck), petroleum ether (Qualigens), methanol (CGP Laboratory), acetone (Numex), toluene (Merck), benzene (Rankem), acetonitrile (Qualigens), distilled water, coconut water, Mayer's reagent (Merck), Dragendorff's reagent (Merck), Wagner's reagent (Merck), Hager's reagent, Fehling's solution (Loba Chemie), Benedict's reagent (S-Merck), Biuret reagent (S-Merck), Millon's reagent (S-Merck), ferric chloride (Blulex), Molisch's reagent (Loba Chemie), anthrone solution (Blulex), β-naphthol (Blulex), magnesium ribbon (Qualigens), sodium hydroxide (Loba Chemie), dilute ammonia (Numex), hydrochloric acid (S-Merck), concentrated sulphuric acid (Merck), nitric acid, picric acid, glacial acetic acid (Numex), acetic anhydride (Qualigens), copper sulphate (Merck), iodine (Merck), potassium iodide (Numex), sodium bicarbonate solution (SD Fine), safranin (SD Fine), lactophenol (Merck), lactochloral reagent (Qualigens), and Silica Gel G for TLC (Fisher Scientific).

2.2 Plant material collection and authentication

Leaves, stems, and branches of *Pueraria montana* (Lour.) Merr. from Rishikesh, Uttarakhand, India was collected. Dr. Subhash Kumar, Assistant Professor of Botany at Ch. Chhotu Ram (P.G.) College, Muzaffarnagar, Uttar Pradesh, India, validated the plant materials. The validation certificate was provided with a voucher/ reference number BOT/Auth/04 and dated 20 January 2023. The validated plant materials were used for pharmacognostical, phytochemical, analytical, computational, and pharmacological studies.

2.3 Preparation of plant material

The aerial parts were gathered and their collected parts were washed with clean water to eliminate dust and other impurities. The dried material was ground and then passed through a screen to obtain a uniform coarse powder and was kept in an airtight container [4].

2.4 Pharmacognostical evaluation

Macroscopic, microscopic, and physicochemical parameters were assessed for the aerial parts of *Pueraria montana* (Lour.) Merr. Parameters of characteristics include odor, taste, texture, surface features, and fracture patterns. For thin transverse sections of leaves and stems were made and were cleared and stained, then examined under a light microscope [5,6]. Standard pharmacognostical procedures were employed to determine foreign matter, moisture content, ash, extractive values, swelling index, and foaming index. Foreign matter was assessed visually and with a 10× magnifier. Moisture content was determined by drying the powdered sample at 105 °C.

2.5 Extraction procedure

2.5.1 Hot extraction method

The powdered drug was first defatted with n-hexane, followed by sequential extraction of the same marc using ethyl acetate, chloroform, ethanol, and finally distilled water in the order of increasing polarity. Each extraction was carried out in a Soxhlet apparatus for 6–8 hours, corresponding to approximately 10–15 siphon cycles, and continued until the solvent in the siphon tube became nearly colourless, indicating complete extraction. After completion of each extraction step, the solvent was concentrated under reduced pressure using a rotary vacuum

evaporator and further dried to obtain the respective extracts. The dried extracts were weighed individually, and the extractive yields were recorded separately [4] (Table 5).

2.5.2 Cold extraction method

For cold maceration, 2 g of loosely packed, coarsely powdered, air-dried plant material was used. Each of n-hexane, ethyl acetate, chloroform, ethanol, and distilled water was used separately for cold maceration. Each flask was shaken for 6 hours and then set to rest for 18 hours with occasional shaking. To prevent the loss of solvent, the extract was filtered and the filtrate was dried at 105 °C until a constant weight was achieved. The dried filtrate was then cooled in a desiccator and weighed. The percentage extractive value was calculated based on the dry weight (Table 5) [7].

The ethanolic extract of *Pueraria montana* (Lour.) Merr. was selected for further analytical and pharmacological evaluation and abbreviated as PMEE.

2.6 Preliminary phytochemical screening

Qualitative phytochemical screening for alkaloids, carbohydrates, proteins, steroids, flavonoids, and saponin glycosides and tannins was carried out on the prepared extracts. Alkaloids were tested using Dragendorff's, Mayer's, Hager's, and Wagner's reagents. Carbohydrates were analyzed using Fehling's and Benedict's tests. Biuret and Millon's tests were used to analyze proteins. Salkowski and Liebermann–Burchard reactions were used to test steroids. Flavonoids were analyzed using Shinoda and the sulphuric acid tests. The foam test was used for saponins whereas tannins were analyzed using ferric chloride solution (Table 6 & Table 7) [7].

2.7 Fluorescence analysis

The powdered drug was treated separately with sodium hydroxide, dilute ammonia, picric acid, concentrated hydrochloric acid, sulfuric acid, nitric acid, water, methanol, chloroform, petroleum ether and coconut water, and then fluorescence analysis was conducted [8]. The treated samples were then viewed under visible light and ultraviolet light at 254 nm and 365 nm. Changes in color were recorded and associated with fluorescence characteristics of the powdered drug (Table 8 & Table 9, Fig.3 & Fig.4).

2.8 Thin-layer chromatography

Silica Gel G plates were used to perform thin-layer chromatography. PMEE was applied as spots on TLC plates that were developed in Chloroform: Methanol (9:1) solvent systems [9]. The developed plates were dried and then visualized after exposure to iodine vapour (Fig.5).

2.9 HRMS analysis

PMEE was analyzed on an Agilent 6500 series Q-TOF/BIO-QTOF mass spectrometer. A 3.5 µL injection was made in Q-TOF mode. Retention time, m/z, calculated m/z, ion type, molecular formula, and spectral library comparison were considered in interpreting the detected compounds [10]. The most prominent phytoconstituents were identified with high accuracy mass criteria along with library support (Table 8, Fig. 6).

2.10 GC-MS analysis

GC-MS analysis of non-polar fraction was conducted using Shimadzu GC-MS-TQ8050 NX system with Elite-5MS column of 30 m × 0.25 mm. Helium was used as the carrier gas flowing at 1.0 mL/min. The injector temperature was set to 250 °C with a split ratio of 5:1. The column temperature was increased at 3 °C/min to 240 °C, then from 240 °C to 270 °C at a 5 °C/min rate. Mass spectral detection was done using electron ionization of 70 eV with the source of the ions and the transfer line maintained at 200 °C. Mass scans were done at 50-650 m/z [11]. Identification of compounds was done tentatively using the NIST17R mass spectral library based on retention time and fragmentation patterns (Table 11, Fig.7).

2.11 Molecular docking study

Interactions and binding affinities of major HRMS-analyzed phytoconstituents from *Pueraria montana* (Lour.) Merr. were assessed using MOE (Molecular Operating Environment) 2022 [12]. The analyzed compounds included: puerarin, genistin, biochanin A, glycitin, mirificin, coumestrol, medicarpin, tectoridin, tectorigenin, kaempferol, quercetin, rutin, catechin, and β-sitosterol. Molecular structures in the form of SDF files were downloaded from PubChem and processed using the MOE QuickPrep application [13-15]. Components of ligand preparation include 3D protonation, structure refinement, charge assignment, and energy minimization, using the Amber10: EHT. For the alignment of the PDB 6HUO structure of the Human GABA receptor 1-beta-3-gamma-2 (α1β3γ2) in MOE [16-19], the receptor was prepared by the removal of water, heteroatoms and terminal groups. Missing protons and structural protontation were performed along with structure minimization. Docking was performed using the Triangle Matcher placement and London dG scoring functions. Force field refinement was applied to the docked poses. Selection of top poses was based on dock score, interaction energy, and ligand pose interaction RSM distance. MOE's ligand interaction tool was used to analyze hydrogen, H-π, π-H, π-cation, and hydrophobic interactions (Table 12).

2.12 In silico ADME and drug-likeness profiling

SwissADME was used to predict the ADME and drug-likeness properties of several phytoconstituents. Each compound was individually submitted to SwissADME in their canonical SMILES forms [20-22]. The parameters measured were the molecular weight, number of heavy atoms, number of aromatic heavy atoms, fraction of Csp^3, number of rotatable bonds, number of hydrogen-bond acceptors, number of hydrogen-bond donors, molar refractivity, TPSA, consensus log P, absorption in the gastrointestinal tract, brain-blood barrier (BBB) permeability, P-glycoprotein substrate, CYP inhibition, violation of Lipinski rule, bioavailability score, and skin permeation coefficient log Kp (Table 13).

2.13 Experimental Animals and Ethical Approval

The study was conducted on Swiss albino male mice weighing 20–30 g to minimize sex-related behavioral variability and avoid confounding effects of estrogen-sensitive phytoconstituents. A total of 45 mice were used, 9 for the acute oral toxicity study and 36 for behavioral evaluation. Animals were housed under standard laboratory conditions (25 ± 2 °C, 50–60% relative humidity) with a 12-hour light/dark cycle and provided standard feed and water ad libitum. The Institutional Animal Ethics Committee of S. D. College of Pharmacy and Vocational Studies, Muzaffarnagar, Uttar Pradesh, India, approved the study (approval number: SDCOP&VS/AH/CPCSEA/03/05, dated December 29, 2022; registered institution number: 876/Po/Re/S/05/CPCSEA). All procedures adhered to CPCSEA guidelines.

2.14 Acute Oral Toxicity Study

Acute oral toxicity of PMEE was conducted in accordance with OECD Guideline 425. The acute toxicity study was performed using fixed-dose groups at 300, 2000, and 5000 mg/kg to obtain preliminary safety information Three groups of 3 mice each received PMEE suspended in 1% gum acacia at 300, 2000, and 5000 mg/kg via oral gavage. Animals were observed individually for the first 30 min, periodically over 4 hours, at 24 hours, and daily for 14 days for mortality and behavioral/physiological changes (stance, fur, eyes/mucous membranes, salivation, tearing, urination, defecation, gait, tremors, convulsions, sensory responses, and righting reflex). Body weights were recorded throughout, and gross necropsy was performed at study end (Table S5, Fig.26 – Fig.30).

2.15 Treatment Design for Anxiolytic Activity

PMEE (100, 200, and 400 mg/kg, p.o.) and diazepam (1 mg/kg, p.o.) were suspended in 1% gum acacia and administered 30 min prior to behavioral testing. Animals were randomly assigned to six groups (6 mice per group). No anxiogenic induction procedures were applied; naïve animals were used throughout (Table 3).

2.16 Experimental Procedure for Behavioral Evaluation

Each mouse was weighed before treatment and monitored during the study. Behavioral testing was conducted during the light phase of the light/dark cycle. Group size (n = 6) was selected based on previous literature; however, no a priori power calculation was performed.

2.16.1 Light–Dark Box Test (LDB)

The LDB consisted of a rectangular box (45 × 20 × 20 cm) divided into two compartments: a dark chamber (one-third of the box, black-painted, with a lid) and a light chamber (two-thirds, white). The compartments were connected by a 13.5 × 5 cm opening. Illumination was provided by two 40 W light bulbs placed 50 cm above the floor, producing ~1000 lux in the light chamber. The light chamber was further divided into center and periphery zones (6 cm from the wall). Each mouse was individually placed in the light compartment and observed for 5 min. Metrics recorded included time spent in the light compartment and number of transitions between compartments. Increased time in the light compartment and number of transitions were considered indicative of anxiolytic behavior. The apparatus was cleaned with 5% ethanol between trials [23].

2.16.2 Elevated Plus Maze Test (EPM)

The elevated plus-maze (Insight, Brazil) consists of two open arms and two closed arms elevated 40 cm above the floor. The raised plus-maze is a frequently used technique for assessing rodent anxiety. Mice naturally avoid heights and open spaces, making this apparatus a widely used model for evaluating anxiety-like behavior. Each mouse was placed at the center of the maze with its nose pointing toward one of the closed arms, 30 minutes after oral administration of vehicle, PMEE (100, 200, or 400 mg/kg), or diazepam (1.0 mg/kg). The total time spent in open and closed arms, as well as the number of entries into each arm, was recorded individually for 5 minutes. The maze was cleaned with a 5% ethanol solution after each trial to eliminate olfactory cues. Behavioral scoring was conducted for each mouse to generate individual data used for statistical analysis [24,25].

All data from individual mice (n = 6 per group) were used to calculate the mean, standard deviation (SD), standard error of the mean (SEM), and 95% confidence intervals (CI). SEM was calculated as [26,27]:

$$SEM = \frac{SD}{\sqrt{n}}$$

and the 95% confidence interval was calculated using the t-distribution [28]:

$$CI = Mean \pm t_{0.025,df} \times SEM$$

where $t_{0.025,df}$ is the critical t-value for 95% confidence with degrees of freedom $df = N - 1$ per group.

For statistical comparison across groups, **one-way ANOVA** was performed. The **sum of squares between (SSB)** was calculated as:

$$SSB = \sum_{i=1}^{k} n_i (\bar{x}_i - \bar{x})^2$$

and the sum of squares within (SSW) as [29,30]:

$$SSW = \sum_{i=1}^{k} \sum_{j=1}^{n_i} (x_{ij} - \bar{x}_i)^2$$

where k is the number of groups, n_i is the number of mice in group i, $\bar{x}_i$ is the group mean, and $\bar{x}$ is the overall mean. The **mean squares** were then computed as:

$$MS_{between} = \frac{SSB}{df_{between}}, \qquad MS_{within} = \frac{SSW}{df_{within}},$$

with degrees of freedom $df_{between} = k - 1$ and $df_{within} = N_{total} - k$. The F-value was calculated as:

$$F = \frac{MS_{between}}{MS_{within}}$$

Effect size (η^2) was calculated as [31]:

$$\eta^2 = \frac{SSB}{SSB + SSW}$$

and p-values were derived from the F-distribution with the corresponding degrees of freedom. All calculations were performed for **each individual mouse**, and the raw per-animal data, along with means, SEMs, 95% CIs, F-values, degrees of freedom, p-values, and effect sizes, are provided in the **Supplementary Material** (Table S1-S3).

3. Result and discussion

Macroscopic Evaluation

The fresh leaves of *Pueraria montana* (Lour.) Merr. have been assessed for their external and sensory characteristics. The leaves were found to have a green color, a smooth texture, a characteristic odor, and a bitter taste. Broad, simple, reticulate venation with an entire margin is how the leaves were found morphologically. The observations are shown in Table 4.

Microscopic Identification

A microscopic study of the fresh stem and leaf sections of *Pueraria montana* (Lour.) Merr. showed the stem and leaf sections possessed typical Oliver characters. Sections revealed showed epidermis, endodermis, pericycle, vascular bundles, xylem, phloem and their elements. Well-formed vascular tissues confirmed the dicot nature of the plant (Fig.1 & Fig.2).

TLC Analysis

The TLC profile of the ethanolic leaf extract of *Pueraria montana* (Lour.) Merr., developed in Chloroform: Methanol (9:1), showed a spot at Rf 0.71, indicating the presence of puerarin (Fig.5). Published literature on puerarin demonstrates a similar Rf value 0.71 [32].The presence of puerarin was evaluated with HRMS.

HR-MS evaluation of ethanolic extract of *Pueraria montana* (Lour.) Merr.

High-resolution mass spectrometry (HR-MS) was used to identify and characterize the phytoconstituents present in ethanolic extract of *Pueraria montana* (Lour.) Merr. (Table 10, Fig. 6).

GC-MS analysis

Gas chromatography mass spectrometry (GC-MS) was used to identify and characterize the phytoconstituents present in non-polar fraction of *Pueraria montana* (Lour.) Merr. (Table 11, Fig. 7).

In-silico anxiolytic activity (molecular docking)

Molecular docking was performed on the human heteromeric GABA(A) receptor alpha1beta3gamma2L (PDB ID: 6HUO) to explore the potential anxiolytic interactions of major HRMS-identified phytoconstituents from *Pueraria montana* (Lour.) Merr. Among the tested compounds, mirificin showed the highest docking score of −9.7308 kcal/mol, followed by rutin (−8.9862 kcal/mol), tectoridin (−8.7727 kcal/mol), glycitin (−8.4140 kcal/mol), genistin (−7.9279 kcal/mol), puerarin (−7.6546 kcal/mol), and β-sitosterol (−7.5615 kcal/mol). These values suggest favorable predicted binding affinities toward the GABA(A) receptor binding pocket. The docked phytoconstituents exhibited interactions with key amino acid residues including MET58, GLU138, ASP458, THR437, LYS105, GLU182, ASP48, TYR160, HIS102, and LYS156. Specifically, mirificin interacted with MET58, ASP458, and THR437; rutin showed multiple interactions with GLU138, GLU182, and LYS156; tectoridin formed hydrogen bonds and π-cation contacts with TYR160, ASP43, HIS102, and LYS156; and glycitin interacted with GLU138, GLU182, LYS105, THR437, and TYR282.

For comparative evaluation, standard anxiolytic and GABA(A)-modulating ligands were also docked against the same receptor model. Alprazolam exhibited a docking score of −5.7425 kcal/mol with a hydrogen-bond donor interaction involving ALA45. Diazepam showed a docking score of −6.1251 kcal/mol and interacted with GLU138 through hydrogen-bond donation. Bicuculline, a competitive GABA(A) receptor antagonist, demonstrated a docking

score of −7.1713 kcal/mol and formed hydrogen-bond acceptor interactions with ARG180 and LYS156. Flumazenil, a benzodiazepine-site antagonist, exhibited a docking score of −5.2879 kcal/mol and showed a π-H interaction with TYR282. Notably, several phytoconstituents, including rutin, glycitin, and tectoridin, shared interactions with residues such as GLU138, LYS156, and TYR282 that were also involved in the binding of reference ligands, suggesting potential occupancy of similar receptor regions. However, docking scores and interaction patterns alone cannot establish binding-site specificity or functional agonist, antagonist, or modulatory activity. The RMSD values ranged from 0.4717 to 1.8363 Å, indicating stable docking orientations and reliable ligand positioning within the receptor binding site (Table 12, Fig.8-26). Overall, these docking results suggest that certain phytoconstituents, particularly mirificin, rutin, puerarin, tectoridin, and glycitin, possess favorable predicted binding characteristics toward the GABA(A) receptor and warrant further investigation. However, molecular docking provides only a computational prediction of ligand–receptor association and should not be interpreted as confirmation of receptor-level activity or mechanism of action. Functional validation through receptor-specific studies, including antagonist-based experiments using bicuculline or flumazenil and electrophysiological assays, would be necessary to determine whether these compounds modulate GABA(A) receptor function and contribute to the observed anxiolytic effects.

In silico ADMET and drug-likeness profiling

The ADMET profiling and drug-like characteristics of some phytochemicals are provided in Table 8. Molecular weight is in the range 268.22 to 610.52 g/mol, while TPSA is in the range 20.23 to 269.43 Å^2. Most of the compounds evaluated had good physicochemical properties needed for oral drug-like characteristics. Among these compounds, Biochanin A, Glycitin, Coumestrol, Medicarpin, Tectorigenin, Kaempferol, Quercetin, and Catechin had no Lipinski violations. Most of these compounds had a bioavailability score close to 0.55. This indicates good oral bioavailability. From the analysis, Biochanin A, Coumestrol, Medicarpin, Tectorigenin, Kaempferol, Quercetin, and Catechin are likely to be absorbed from the gastrointestinal tract (Table 13), thus supporting the planned oral dosing of the ethanolic extract during the *in vivo* study.

Acute oral toxicity

Acute oral toxicity of PMEE was evaluated in Swiss albino mice administered oral doses of 100, 200, and 400 mg/kg. All mice were observed individually for the first 30 minutes, periodically over 4 hours, at 24 hours, and daily for 14 days for behavioral and physiological parameters including stance, fur, eyes/mucous membranes, salivation, tearing, urination, defecation, gait, tremors, convulsions, sensory responses. No mortality or adverse clinical signs were observed in any group as show in table 14. Body weights were recorded throughout the study. Histological examination of major organs, including brain, liver, kidney, stomach, and lungs, revealed normal tissue architecture without necrosis, inflammation, or degeneration in any group, confirming the safety of PMEE at the tested doses (Supplementary Table S5 and Fig. 26–30).

Elevated plus-maze test (EPM)

Behavioral assessment using the Elevated Plus Maze (EPM) showed a dose-dependent anxiolytic effect of PMEE. Mice treated with PMEE at 400 mg/kg spent the most time in the

open arms (124.8 ± 5.84 sec), exceeding the standard diazepam group (108.6 ± 5.24 sec) and representing approximately 115% of the diazepam effect. Lower doses of PMEE (100 and 200 mg/kg) produced intermediate increases in open-arm time (58.42 ± 3.62 sec and 71.65 ± 4.12 sec, corresponding to 54% and 66% of the diazepam effect, respectively) as show in table 15 and fig.31. Closed-arm entries and time spent in closed arms indicated that general locomotor activity was not impaired. One-way ANOVA confirmed a significant effect of treatment on open-arm exploration ($F(4,25) = 42.31$, $p < 0.001$, $\eta^2 = 0.87$) (Supplementary Table S1).

Light dark box test

In the Light–Dark Box (LDB) test, PMEE produced dose-dependent increases in time spent in the illuminated compartment. PMEE 400 mg/kg resulted in the longest light-compartment time (142.6 ± 7.14 sec), exceeding diazepam (131.4 ± 6.08 sec) and representing approximately 109% of the standard drug effect. Lower doses (100 and 200 mg/kg) showed moderate increases (88.52 ± 11.84 sec and 104.76 ± 10.92 sec, corresponding to 67% and 80% of the diazepam effect, respectively) shown in table 16 and fig.32. One-way ANOVA indicated a significant effect of treatment ($F(4,25) = 15.27$, $p < 0.001$, $\eta^2 = 0.71$) (Supplementary Table S2).

Conclusion

The present work investigated the various pharmacological and biological properties of *Pueraria montana* (Lour.) Merr. ethanolic extract. As a first step in this investigation, a series of studies aimed at performing a pharmacognostic characterization of PMEE were conducted, and included analyses of the morphology and anatomy of the plant, quantitative evaluations of its physicochemical properties, and stability assessments. In addition, preliminary phytochemical screening and elucidation of the chemical profile of PMEE through high-resolution mass spectrometry and gas chromatography mass spectrometry, revealed the presence of several phytoconstituents with potential biological activities. The major phytoconstituents of PMEE were subjected to molecular docking studies to evaluate their interactions with $GABA_A$ receptor as a potential mechanism of anxiety. The estimated binding affinities of the phytoconstituents were compared to the benzodiazepines and the results suggested that some of the examined phytoconstituents may act as anxiolytics-like effect, and would contribute to the overall observed anxious effect of PMEE. The results of the acute toxicity studies indicated that there were no observable adverse effects related to the treatment in any of the study subjects, and the extract is safe for use in traditional medicine. In addition, the results of the histopathological studies showed that sampled tissues were preserved and supported the conclusions related to the safety of PMEE. Behavioral assessment via the Elevated Plus Maze and the Light–Dark Box confirmed a significant and dose-dependent response of the anxiolytic effect. The highest response was seen at the 400 mg/kg/day level, resulting in a significant increase in both the open arm and the light compartment, to levels comparable and in some cases surpassing those seen in the control (diazepam) group in the experiment. When considered cumulatively, these results indicate that PMEE demonstrates significant anxiolytic activity, which is reasonable in light of its favorable profile of safety. However, based on these results, the mechanism of PMEE cannot be solely attributed to a GABAergic system. Aside from modulation at the level of binding to the GABA(A) receptor and other potential systems, it can include the serotonergic, dopaminergic, and noradrenergic systems, the glutamatergic system, and the antioxidant and neuroprotective systems. Future

research using an array of receptor-selective antagonists (e.g., flumazenil and bicuculline), neurotransmitter assessments, electrophysiological techniques, and bioactivity-guided fractionation studies, is warranted to determine what the effective constituent(s) are, and to describe the mechanisms of action and the extent of the potential anxiolytic effects of *Pueraria montana* (Lour.) Merr.

Credit Authorship Contribution Statement

Piyush Kumar Singhal: Writing original draft, Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Anju Pal:** Visualization, Supervision**, Raghav Dixit:** Formal analysis, Data curation**. Bhuwanendra Singh:** Data curation, Visualization, Supervision, Resource. **Mohammad Shami:** Investigation, Validation, Writing – review & editing. **Mahima**: Methodology, Conceptualization, Software, Writing – review & editing, Visualization. **Vaishali:** Investigation, Biological activity evaluation. **Arvind Kumar:** Data curation, Formal analysis. **Vibhu Sahani:** Writing – review & editing, Visualization.

Conflict of interest statement

The authors declare that there is no conflict of interest**.**

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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List of Tables

Table 1: Variation in major chemical composition of different *Pueraria* species on the basis of geography.

Pueraria species	Location	Phytoconstituents	% Content	Reference
Pueraria lobata (Willd.) Ohwi	China, Japan, Korea	Puerarin	1.5–3.5	[33]
		Daidzein	0.3–0.7	
		Daidzin	0.2–0.6	

		Genistein	0.1–0.4	
		Starch	10–15	
Pueraria mirifica Airy Shaw & Suvatab.	Thailand, Myanmar	Miroestrol	0.002–0.005	[34]
		Deoxymiroestrol	0.001–0.004	
		Isoflavones (Daidzein, Genistein)	0.1–0.5	
		Coumestrol	0.01–0.05	
Pueraria tuberosa (Willd.) DC	India, Nepal, Bangladesh	Total Isoflavones	0.8–2.0	[35]
		Starch	20–30	
		Saponins	1–3	
		Sterols	0.5–1.2	
Pueraria phaseoloides (Roxb.) Benth.	Southeast Asia, Pacific Islands, Africa	Puerarin	0.3–0.8	[36]
		Polyphenols	2–5	
		Proteins	10–15	
Pueraria thomsonii Benth.	China (Yunnan, Sichuan, Hunan)	Puerarin	1.0–3.2	[37]
		Daidzin	0.3–0.5	
		Isoorientin	0.1–0.3	
Pueraria peduncularis (Graham ex Benth.) Benth.	South China, Vietnam, Laos	Puerarin	0.5–1.8	[38]
		Formononetin	0.2–0.4	
		Biochanin A	0.05–0.2	
Pueraria montana (Lour.) Merr.	East Asia to Southeast Asia	Puerarin	1.0–2.5	[39]
		Daidzein	0.2–0.6	
		Genistin	0.05–0.2	
Pueraria edulis Pamp.	China (Guangxi, Guangdong)	Puerarin	1.5–2.8	[40]
		Daidzin	0.3–0.5	
		Starch	12–20	
		Puerarin	1.2–2.5	[41]

Pueraria candollei Grah. ex Benth.	Thailand, Laos, Cambodia	Miroestrol	0.002–0.005	
		Daidzein	0.1–0.4	
Pueraria hirsuta (Willd.) Merr.	India (Assam), Myanmar	Isoflavones	0.5–1.0	[42]
		Triterpenoids	0.2–0.6	
Pueraria wallichii DC	Himalayas (India, Nepal, Bhutan)	Puerarin	0.5–1.8	[43]

Table 2: Major phytoconstituents reported from *Pueraria* species and their neuropharmacological activities documented in the literature.

S. No.	Phytoconstituent	Activity (Literature Reported)	References
1	Puerarin	Anxiolytic-like, antidepressant-like, neuroprotective, cognition-enhancing, antioxidant, anti-neuroinflammatory	[44-46]
2	Daidzein	Neuroprotective, antioxidant, estrogen-mediated CNS modulation, anti-inflammatory	[47-49]
3	Daidzin	Neuroprotective, antioxidant, anti-alcohol dependence activity	[50-52]
4	Genistein	Anxiolytic-like, neuroprotective, antioxidant, anti-inflammatory, memory-enhancing	[53]
5	Genistin	Antioxidant, neuroprotective, potential anxiolytic activity	[54]
6	Glycitin	Antioxidant, neuroprotective, anti-inflammatory potential	[55]
7	Mirificin	Antioxidant and potential neuroprotective activity	[56]
8	Miroestrol	Phytoestrogenic, neuroprotective, cognition-supporting activity	[57]
9	Deoxymiroestrol	Estrogen receptor modulation, neuroprotective potential	[58,59]
10	Coumestrol	Phytoestrogenic, neuroprotective, antioxidant activity	[60]

11	Formononetin	Anxiolytic-like, antidepressant-like, neuroprotective, anti-inflammatory	[61,62]
12	Biochanin A	GABAergic modulation, anxiolytic-like, neuroprotective, antioxidant activity	[63]
13	Tectoridin	Neuroprotective, antioxidant, anti-inflammatory, potential anxiolytic activity	[64]
14	Tectorigenin	Neuroprotective, anti-neuroinflammatory, antioxidant, cognitive protective activity	[65]
15	Kaempferol	Anxiolytic-like, antidepressant-like, neuroprotective, antioxidant	[66]
16	Quercetin	Anxiolytic-like, neuroprotective, antioxidant, anti-inflammatory, cognition-enhancing	[67]
17	Rutin	Anxiolytic-like, neuroprotective, antioxidant, anti-inflammatory, memory-enhancing	[67]
18	Catechin	Neuroprotective, antioxidant, anti-stress, cognitive protective activity	[68]
19	Medicarpin	Neuroprotective, antioxidant, anti-inflammatory activity	[69]
20	β-Sitosterol	Anxiolytic-like, antidepressant-like, neuroprotective, anti-inflammatory	[70]
21	Isoorientin	Neuroprotective, antioxidant, anti-inflammatory activity	[71]
22	Saponins	Adaptogenic, neuroprotective, anti-stress activity	[72]
23	Polyphenols	Antioxidant, neuroprotective, free radical scavenging activity	[73]
24	Sterols	Antioxidant, anti-inflammatory, neuroprotective potential	[74]

Table 3: Groups of animals for Light & Dark model and their details

Group Details	**Inducing dose / Inducing Factor**	**Group details**	**Number of Animal Required**
Normal Control	NA	1% Gum acacia, p. o. (Volume 1ml/100g)	6

Disease Group	Dark-Light Box and EPM	Anxiety will develop using Light- Dark Test Model and EPM	6
Positive Control (Standard)	Dark-Light Box and EPM	Diazepam (1.0 mg/kg, p. o.) (Volume 1ml/100g)	6
Test Group I	Dark-Light Box and EPM	PMEE (100 mg/kg, p. o) (Volume 1ml/100g)	6
Test Group II	Dark-Light Box and EPM	PMEE (200 mg/kg, p. o.) (Volume 1ml/100g)	6
Test Group III	Dark-Light Box and EPM	PMEE (400 mg/kg, p. o.) (Volume 1ml/100g)	6

Table 4: Botanical and sensory characteristics of leaves of *Pueraria montana* (Lour.) Merr.

S.No.	Parameters	Observations
1.	Colour	Green
2.	Odour	Characteristic
3.	Taste	Bitter
4.	Touch/Texture	Smooth
5.	Shape	Broadly ovate to heart-shaped
6.	Size	Medium to large
7.	Surface	Smooth
8.	Venation	Reticulate
9.	Margin	Entire
10.	Apex	Acute to acuminate

Table 5: Physicochemical parameters of *Pueraria montana* (Lour.) Merr. Stem and Leaves

S.NO	Parameters	Value
1.	Moisture content (Leaves)	0.16±0.0464
2.	Moisture content (Stem)	0.2±0.00
3.	Total Ash (Leaves)	0.23±0.092
4.	Acid Insoluble Ash (Leaves)	0.16±0.094
5.	Water Soluble Ash (Leaves)	0.2±0.00
6.	Total Ash (Stem)	0.26±0.0943
7.	Acid Insoluble Ash (Stem)	0.26±0.0469

8.	Water Soluble Ash (Stem)	0.2±0.00
9.	Foreign Organic matter (Leaves)	No Adulterants
10.	Foreign organic matter (Stem)	No Adulteration
Hot extraction value (Successive)		
11.	n-Hexane (% w/w)	16.34
12.	Ethyl acetate (% w/w)	11.76
13.	Chloroform (% w/w)	10.54
14.	Ethanol extract (% w/w)	9.24
15.	Water (% w/w)	6.72
Cold extraction value (Maceration) ***(% w/w)***		
16.	n-Hexane (% w/w)	3.6
17.	Ethyl acetate (% w/w)	1.32
18.	Chloroform (% w/w)	1.72
19.	Ethanol extract (% w/w)	2.16
20.	Water (% w/w)	0.2
21.	Foaming Index (Leaves)	2.53±0.093
22.	Foaming Index (Stem)	3.53±0.209
23.	Swelling Index (Leaves)	1.2±0.94
24.	Swelling Index (Stem)	1.86±1.89

Table 6: Phytochemicals Present in the Leaves of *Pueraria montana* (Lour.) Merr.

Secondary Metabolites	Test	n-hexane	Ethyl acetate	Chloroform	Ethanol	Water
Alkaloids	Dragendorff's reagent	(+)	(+)	(+)	(+)	(+)
	Mayer's reagent	(-)	(+)	(+)	(-)	(-)
	Hager's reagent	(-)	(+)	(-)	(-)	(+)
	Wegner's reagent	(+)	(+)	(-)	(+)	(+)
Carbohydrates	Fehling's test	(-)	(+)	(+)	(-)	(-)
	Benedict's test	(+)	(-)	(+)	(-)	(+)
Proteins	Biuret test	(-)	(-)	(-)	(-)	(-)
	Millon's test	(-)	(+)	(+)	(+)	(-)
Steroids	Salkowski reaction	(-)	(+)	(+)	(-)	(+)
	Liebermann–Burchard reaction	(+)	(+)	(+)	(+)	(-)
Flavonoids	Shinoda test	(+)	(+)	(-)	(+)	(+)

	Sulphuric acid test	(-)	(-)	(+)	(+)	(+)
Saponin glycosides	**Foam test**	(-)	(-)	(-)	(-)	(-)
Tannins	**$FeCl_3$ test**	(+)	(-)	(+)	(+)	(-)

Note: **Positive Result: (+), Negative Result: (-).**

Table 7: Phytochemicals Present in the Stem of *Pueraria montana* (Lour.) Merr.

Secondary Metabolites	**Test**	**n-hexane**	**Ethyl acetate**	**Chloroform**	**Ethanol**	**Water**
Alkaloids	**Dragendorff's reagent**	(-)	(+)	(-)	(-)	(+)
	Mayer's reagent	(+)	(-)	(+)	(+)	(-)
	Hager's reagent	(+)	(+)	(+)	(-)	(-)
	Wegner's reagent	(-)	(-)	(-)	(-)	(-)
Carbohydrates	**Fehling's test**	(+)	(-)	(+)	(+)	(+)
	Benedict's test	(+)	(+)	(-)	(-)	(+)
Proteins	**Biuret test**	(+)	(+)	(-)	(+)	(-)
	Millon's test	(-)	(-)	(+)	(+)	(+)
Steroids	**Salkowski reaction**	(+)	(+)	(-)	(+)	(+)
	Liebermann–Burchard reaction	(-)	(-)	(+)	(+)	(-)
Flavonoids	**Shinoda test**	(+)	(+)	(-)	(+)	(+)
	Sulphuric acid test	(-)	(+)	(-)	(+)	(+)
Saponin glycosides	**Foam test**	(-)	(-)	(-)	(-)	(-)
Tannins	**$FeCl_3$ test**	(-)	(+)	(-)	(-)	(+)

Note: **Positive Result: (+), Negative Result: (-).**

Table 8: Results of Fluorescence tests of Leaves of *Pueraria montana* (Lour.) Merr.

S. No	**Drug Powder with Testing Reagent**	**Vis Radiation**	**U.V Radiation At 254 nm**	**U.V Radiation At 365 nm**
1	Drug Powder Tested with Sodium Hydroxide	Light Green	Dark Green	Brown

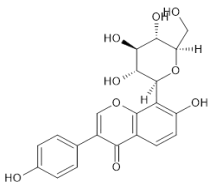
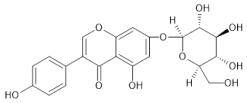
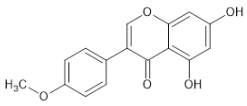
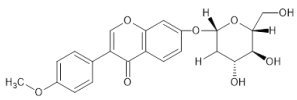
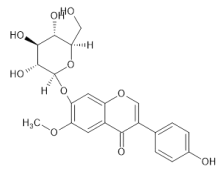
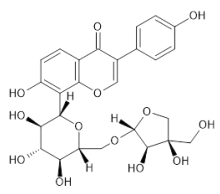
2	Drug Powder with NH_3	Light Green	Dark Green	Black Colour
3	Drug Powder with Picric Acid	Light Green	Yellowish Green	Dark Green
4	Drug Powder with Concentrated Hydrochloric Acid	Blackish In Colour	Black Colour	Brownish Black
5	Powder Drug with Sulphuric Acid	Black	Black In Colour	Dark Black
6	Powder Drug with Water	Light Green	Dark Green	Dark Green In Colour
7	Powder Drug with Methanol	Light Green	Dark Green	Dark Green
8	Powder Drug with Nitric Acid	Reddish Orange	Dark Green	Brown
9	Powder Drug with Chloroform	Dark Green	Blackish	Black
10	Powder Drug with Petroleum Ether	Green	Dark Blue	Blue
11	Powdered Drug with Coconut Water	Green	Dark Green	Bluish

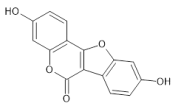
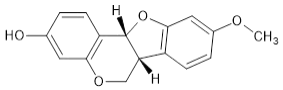
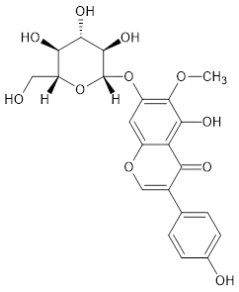
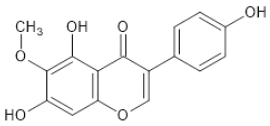
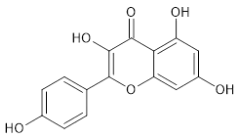
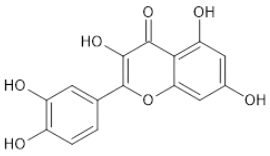
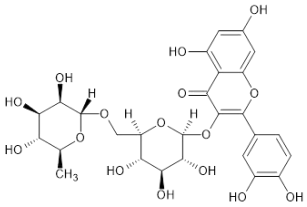
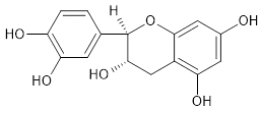
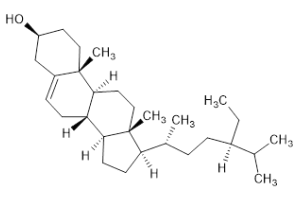
Table 9: Results of Fluorescence tests of Stem of *Pueraria montana* (Lour.) Merr.

S.No	Drug Powder with Testing Reagent	Vis Radiation	U.V Radiation At 254 nm	U.V Radiation At 365 nm
1.	Drug Powder Tested with Sodium Hydroxide	Brown	Light Green	Dark Brown
2.	Drug Powder with NH_3	Light Brown	Dark Greenish	Dark Brown
3.	Drug Powder with Picric Acid	Yellowish	Light Green	Light Brown
4.	Drug Powder with Concentrated Hydrochloric Acid	Brown Colour	Dark Black	Black Colour
5.	Powder Drug with Sulphuric Acid	Blackish Colour	Dark Blacked	Blueish

6.	Powder Drug with Water	Light Brown Colour	Light Greenish Colour	Dark Brown Colour
7.	Powder Drug with Methanol	Light Brown Colour	Green Colour	Brownish
8.	Powder Drug with Nitric Acid	Brownish	Green Colour	Light Brown Somewhat White
9.	Powder Drug with Chloroform	Brown Colour	Dark Green	Dark Brown Colour
10.	Powder Drug with Petroleum Ether	Brownish Colour	Light Green	Dark Brown Colour

Table 10: Major Phytochemical constituents identified using HR-MS

Compound Structure	Compound	Plant Source	Molecular Formula	RT (min)	Observed m/z	Calculated m/z	Ion Type	Compound Class	Identification Basis
	Puerarin	*Pueraria montana* (Lour.) Merr.	C21H20O9	0.428	417.118	417.1180	[M+H]+	Isoflavone C-glycoside	HRMS + NIST Library
	Genistin	*Pueraria montana* (Lour.) Merr.	C21H20O10	0.517	433.113	433.1129	[M+H]+	Isoflavone glycoside	HRMS + NIST Library
	Biochanin A	*Pueraria montana* (Lour.) Merr.	C16H12O5	0.589	285.076	285.0757	[M+H]+	Isoflavone	HRMS + NIST Library
	Ononin	*Pueraria montana* (Lour.) Merr.	C22H22O9	0.672	431.134	431.1337	[M+H]+	Isoflavone glycoside	HRMS + NIST Library
	Glycitin	*Pueraria montana* (Lour.) Merr.	C22H22O10	0.777	447.128	447.1286	[M+H]+	Isoflavone glycoside	HRMS + NIST Library
	Mirificin	*Pueraria montana* (Lour.) Merr.	C26H28O14	0.833	565.155	565.1552	[M+H]+	Isoflavone derivative	HRMS + NIST Library

	Coumestrol	*Pueraria montana* (Lour.) Merr.	C15H8O5	0.915	269.045	269.0445	[M+H]+	Coumestan	HRMS + NIST Library
	Medicarpin	*Pueraria montana* (Lour.) Merr.	C16H14O4	1.105	271.096	271.0965	[M+H]+	Pterocarpan	HRMS + NIST Library
	Tectoridin	*Pueraria montana* (Lour.) Merr.	C22H22O11	1.310	463.123	463.1235	[M+H]+	Isoflavone glycoside	HRMS + NIST Library
	Tectorigenin	*Pueraria montana* (Lour.) Merr.	C16H12O6	1.462	301.071	301.0706	[M+H]+	Isoflavone	HRMS + NIST Library
	Kaempferol	*Pueraria montana* (Lour.) Merr.	C15H10O6	1.588	287.055	287.0550	[M+H]+	Flavonol	HRMS + NIST Library
	Quercetin	*Pueraria montana* (Lour.) Merr.	C15H10O7	1.672	303.050	303.0499	[M+H]+	Flavonol	HRMS + NIST Library
	Rutin	*Pueraria montana* (Lour.) Merr.	C27H30O16	1.741	611.161	611.1607	[M+H]+	Flavonoid glycoside	HRMS + NIST Library
	Catechin	*Pueraria montana* (Lour.) Merr.	C15H14O6	1.836	291.086	291.0863	[M+H]+	Flavanol	HRMS + NIST Library
	β-Sitosterol	*Pueraria montana* (Lour.) Merr.	C29H50O	1.912	415.393	415.3934	[M+H]+	Phytosterol	HRMS + NIST Library

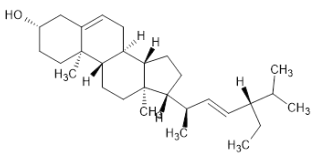
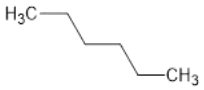
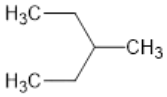
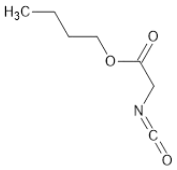
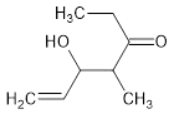
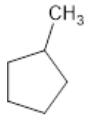
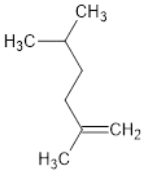
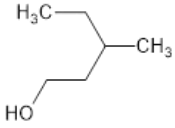
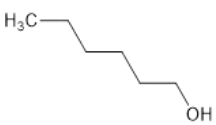
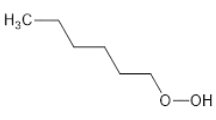
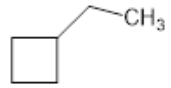
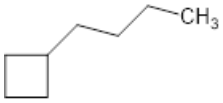
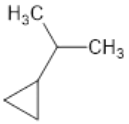
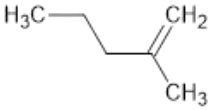
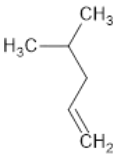
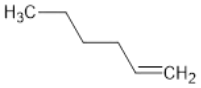
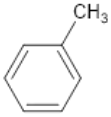
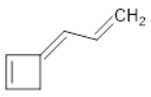
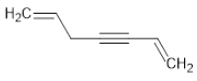
	Stigmasterol	*Pueraria montana* (Lour.) Merr.	C29H48O	1.987	413.377	413.3778	[M+H]+	Phytosterol	HRMS + NIST Library

Table 11: Phytochemical Constitunet Identified Using GC-MS

Compound Structure	Compound	Plant Source	Molecular Formula	RT (min)	% Content in Aerial Part	Class of Compound
	n-Hexane	*Pueraria montana* (Lour.) Merr.	C6H14	2.58	13.15	Aliphatic hydrocarbon (alkane)
	3-Methyl- Pentane	*Pueraria montana* (Lour.) Merr.	C6H14	2.58	13.15	Branched alkane
	Butyl isocyanatoacetate	*Pueraria montana* (Lour.) Merr.	C7H11NO3	2.58	13.15	Ester/Isocyanate derivative
	5-Hydroxy-4-methyl-6-hepten-3-one	*Pueraria montana* (Lour.) Merr.	C8H14O2	2.58	13.15	Oxygenated compound (unsaturated ketone/alcohol)
	Cyclopentane, methyl-	*Pueraria montana* (Lour.) Merr.	C6H12	2.66	13.28	Cycloalkane
	1-Hexene, 2,5-dimethyl-	*Pueraria montana* (Lour.) Merr.	C8H16	2.66	13.28	Alkene (unsaturated hydrocarbon)
	(S)-(+)-3-Methyl-1-pentanol	*Pueraria montana* (Lour.) Merr.	C6H14O	2.66	13.28	Alcohol (aliphatic alcohol)
	1-Hexanol	*Pueraria montana* (Lour.) Merr.	C6H14O	2.74	13.28	Alcohol (primary alcohol)
	Hydroperoxide, hexyl	*Pueraria montana* (Lour.) Merr.	C6H14O2	2.66	13.28	Organic peroxide

	Cyclobutane, ethyl-	*Pueraria montana* (Lour.) Merr.	C6H12	2.74	24.20	Cycloalkane
	Cyclobutane, butyl-	*Pueraria montana* (Lour.) Merr.	C8H16	2.74	24.20	Cycloalkane
	Propane, 2-cyclopropyl-	*Pueraria montana* (Lour.) Merr.	C6H12	2.74	24.20	Cycloalkane (bicyclic hydrocarbon)
	Cyclohexane	*Pueraria montana* (Lour.) Merr.	C6H12	2.90	42.51	Cycloalkane
	1-Pentene, 2-methyl-	*Pueraria montana* (Lour.) Merr.	C6H12	2.90	42.51	Alkene (unsaturated hydrocarbon)
	1-Pentene, 4-methyl-	*Pueraria montana* (Lour.) Merr.	C6H12	2.90	42.51	Alkene (unsaturated hydrocarbon)
	1-Hexene	*Pueraria montana* (Lour.) Merr.	C6H12	2.90	42.51	Alkene
	Spiro[2,4]hepta-4,6-diene	*Pueraria montana* (Lour.) Merr.	C7H8	3.72	3.72	Cyclic diene (unsaturated hydrocarbon)
	Toluene	*Pueraria montana* (Lour.) Merr.	C7H8	3.72	3.72	Aromatic hydrocarbon
	1,3,5-Cycloheptatriene	*Pueraria montana* (Lour.) Merr.	C7H8	3.72	3.72	Cyclic polyene (conjugated hydrocarbon)
	Cyclobutene, 2-propenylidene-	*Pueraria montana* (Lour.) Merr.	C7H8	3.72	3.72	Unsaturated cyclic hydrocarbon
	1,6-Heptadien-3-yne	*Pueraria montana* (Lour.) Merr.	C7H8	3.72	3.72	Unsaturated hydrocarbon (diene–yne)

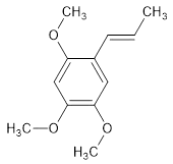
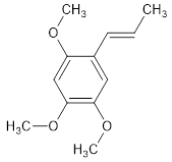
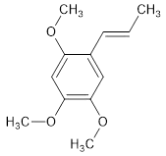
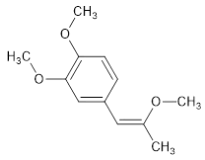
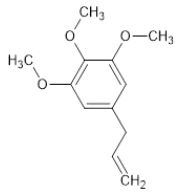
	Asarone	*Pueraria montana* (Lour.) Merr.	C12H16O3	10.33	2.99	Phenylpropanoid (aromatic ether)
	α-Asarone	*Pueraria montana* (Lour.) Merr.	C12H16O3	10.33	2.99	Phenylpropanoid (aromatic ether)
	Benzene, 1,2,4-trimethoxy-5-(1-propenyl)-	*Pueraria montana* (Lour.) Merr.	C12H16O3	10.33	2.99	Phenylpropanoid derivative
	1,2-Dimethoxy-4-(2-methoxy-1-propenyl)benzene	*Pueraria montana* (Lour.) Merr.	C12H16O3	10.33	2.99	Phenylpropanoid derivative
	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	*Pueraria montana* (Lour.) Merr.	C12H16O3	10.33	2.99	Phenylpropanoid derivative

Table 12: Molecular docking of HRMS-identified compounds from *Pueraria montana* (Lour.) Merr. with GABA receptor (PDB ID: 6HUO).

S. No.	Compound Name	PubChem ID	Ligand	Receptor	Interaction	Distance (Å)	E (kcal/mol)	Docking Score	RMSD
1	Puerarin	5281807	O2	SD MET58 (A)	H-donor	3.94	−0.5	−7.6546	1.0809
			C14	OE2 GLU138 (A)	H-donor	3.28	−0.7		
2	Genistin	5281377	O3	O THR434 (G)	H-donor	2.94	−1.1	−7.9279	1.8291
			O10	OD1 ASP48 (E)	H-donor	3.26	−0.5		
			O8	5-ring HIS142 (A)	H-pi	3.90	−3.8		
3	Biochanin A	5280373	O3	N GLU182 (E)	H-acceptor	3.48	−0.8	−6.3992	0.7805
4	Glycitin	187808	O4	OE2 GLU138 (A)	H-donor	2.78	−1.5	−8.4140	1.4928
			O5	O GLU182 (E)	H-donor	2.88	−0.8		
			O4	NZ LYS105 (A)	H-acceptor	3.15	−3.4		
			O9	OG1 THR437 (G)	H-acceptor	3.06	−0.9		
			6-ring	CE2 TYR282 (A)	pi-H	4.21	−0.5		
5	Mirificin	21676217	O4	SD MET58 (A)	H-donor	3.01	−1.3	−9.7308	1.4190
			O13	OD2 ASP458 (G)	H-donor	2.76	−1.1		
			O8	OG1 THR437 (G)	H-acceptor	2.92	−1.0		
6	Coumestrol	5281707	O5	OD2 ASP458 (G)	H-donor	2.74	−3.5	−6.5169	0.4717
7	Medicarpin	336327	O4	O ASP48 (E)	H-donor	3.08	−1.4	−6.4531	1.4890
8	Tectoridin	5281810	O3	O TYR160 (A)	H-donor	3.10	−0.6	−8.7727	1.7753
			O6	OD2 ASP43 (E)	H-donor	3.08	−0.8		
			O5	NE2 HIS102 (A)	H-acceptor	2.89	−1.2		
			6-ring	NZ LYS156 (A)	pi-cation	3.63	−0.5		
9	Tectorigenin	5281811	O3	O ALA45 (E)	H-donor	2.98	−1.4	−6.8628	0.9104
			O2	N GLU182 (E)	H-acceptor	3.04	−0.6		

10	Kaempferol	5280863	O4	NZ LYS105 (A)	H-acceptor	2.96	−10.4	−6.2382	0.6954
			6-ring	CA MET49 (E)	pi-H	4.23	−0.5		
11	Quercetin	5280343	O6	O ILE47 (E)	H-donor	2.95	−1.5	−6.4577	1.6209
			O7	SD MET58 (A)	H-donor	3.30	−0.8		
12	Rutin	5280805	O4	OE2 GLU138(A)	H-donor	2.88	−1.3	−8.9862	1.4329
			O8	O GLU82 (E)	H-donor	2.70	−1.3		
			O5	S156 (A)	H-acceptor	2.99	−2.2		
			O7	NZ LYS156 (A)	H-acceptor	3.00	−2.7		
			O8	N GLU182 (E)	H-acceptor	2.82	−0.7		
13	Catechin	9064	O5	O MET58 (A)	H-donor	3.10	−0.5	−6.5266	1.8363
			O6	O ASP48 (E)	H-donor	2.97	−2.4		
			O5	NZ LYS105 (A)	H-acceptor	2.87	−4.3		
			O6	NZ LYS105 (A)	H-acceptor	3.03	−0.8		
14	β-Sitosterol	222284	O1	OD2 ASP458 (G)	H-donor	2.68	−0.7	−7.5615	0.9908
15	Alprazolam	2118	C12	O ALA45 (E)	H-donor	3.21	-0.7	-5.7425	1.4705
16	Bicuculline	10237	O6	NH2 ARG180 (E)	H-acceptor	2.86	-3.1	-7.1713	1.8558
			N7	NZ LYS156 (A)	H-acceptor	2.93	-4.5		
17	Diazepam	3016	C11	OE2 GLU138 (A)	H-donor	3.37	-0.6	-6.1251	1.0095
18	Flumazenil	3373	6-ring	CE2 TYR282 (A)	π-H	4.26	-0.5	-5.2879	0.9654

Table 13: In silico ADMET and drug-likeness profiling of selected phytochemicals predicted using Swiss ADME.

Compound Name	Entry No.	MW (g/mol)	Heavy Atoms	Arom. Heavy Atoms	Frac Csp3	Rotatable Bonds	H-bond Acceptors	H-bond Donors	Molar Refract	TPSA (Å²)	Log Po/w	Lipinski Rule	Bioavail. Score	GI Absorp	BBB Perm	P-gp Substr	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	Log Kp (cm/s)
Puerarin	1	416.38	30	16	0.29	3	9	6	104.59	160.82	0.23	Yes, 1 violation	0.55	Low	No	No	No	No	No	No	No	−8.83
Genistin	2	432.38	31	16	0.29	4	10	6	106.11	170.05	0.42	Yes, 1 violation	0.55	Low	No	Yes	No	No	No	No	No	−8.33
Biochanin A	3	284.26	21	16	0.06	2	5	2	78.46	79.90	2.44	Yes, 0 violation	0.55	High	No	No	Yes	No	No	Yes	Yes	−5.91
Glycitin	4	446.40	32	16	0.32	5	10	5	110.58	159.05	0.56	Yes, 0 violation	0.55	Low	No	No	No	No	No	No	Yes	−8.57
Mirificin	5	548.49	39	16	0.42	6	13	8	131.04	219.74	−1.10	No, 3 violations	0.17	Low	No	No	No	No	No	No	No	−10.90
Coumestrol	6	268.22	20	17	0.00	0	5	2	73.81	83.81	2.46	Yes, 0 violation	0.55	High	No	No	Yes	No	No	Yes	No	−5.98
Medicarpin	7	270.28	20	12	0.25	1	4	1	73.17	47.92	2.53	Yes, 0 violation	0.55	High	Yes	Yes	Yes	Yes	No	Yes	Yes	−5.98
Tectoridin	8	462.40	33	16	0.32	5	11	6	112.60	179.28	0.35	No, 2 violations	0.17	Low	No	Yes	No	No	No	No	Yes	−8.53
Tectorigenin	9	300.26	22	16	0.06	2	6	3	80.48	100.13	2.06	Yes, 0 violation	0.55	High	No	No	Yes	No	No	Yes	Yes	−6.26
Kaempferol	10	286.24	21	16	0.00	1	6	4	76.01	111.13	1.58	Yes, 0 violation	0.55	High	No	No	Yes	No	No	Yes	Yes	−6.70
Quercetin	11	302.24	22	16	0.00	1	7	5	78.03	131.36	1.23	Yes, 0 violation	0.55	High	No	No	Yes	No	No	Yes	Yes	−7.05
Rutin	12	610.52	43	16	0.44	6	16	10	141.38	269.43	−1.51	No, 3 violations	0.17	Low	No	Yes	No	No	No	No	No	−10.26
Catechin	13	290.27	21	12	0.20	1	6	5	74.33	110.38	0.83	Yes, 0 violation	0.55	High	No	Yes	No	No	No	No	No	−7.82
β-Sitosterol	14	414.71	30	0	0.93	6	1	1	133.23	20.23	7.24	Yes, 1 violation	0.55	Low	No	No	No	No	No	No	No	−2.20

Table 14: Details of acute oral toxicity groups.

Group No.	Toxic Group Details	Toxic Dose (mg/kg)	Death of Mice	Number of Animals Taken
T1	Group-1	300	0	3
T2	Group-2	2000	0	3
T3	Group-3	5000	0	3

Table 15: Data of Elevated plus maze

Groups	Open Arm Entries (Counts/5 min)	Closed Arm Entries (Counts/5 min)	Time Spent in Open Arm (Sec/5 min)	Time Spent in Closed Arm (Sec/5 min)
Normal Control	9.42 ± 0.62	10.24 ± 0.84	102.4 ± 5.18	197.6 ± 5.22
Diazepam	11.24 ± 1.32***	10.82 ± 0.84***	108.6 ± 5.24***	189.4 ± 5.18***
PMEE 100 mg/kg	6.84 ± 0.52*	14.35 ± 1.28*	58.42 ± 3.62*	232.4 ± 3.69*
PMEE 200 mg/kg	8.15 ± 0.61**	13.12 ± 1.14**	71.65 ± 4.12**	218.7 ± 4.08**
PMEE 400 mg/kg	12.48 ± 0.86***	9.64 ± 0.72***	124.8 ± 5.84***	175.2 ± 5.42***

Where, Values are expressed as Mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test. Data normality was assessed using the Shapiro–Wilk test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with disease control group.

Table 16: Data of light dark box test

Groups	Time Spent in Light Box (Sec)
Normal Control	126.8 ± 5.14
Standard (Diazepam)	131.4 ± 6.08***
PMEE 100 mg/kg	88.52 ± 11.84*
PMEE 200 mg/kg	104.76 ± 10.92**
PMEE 400 mg/kg	142.6 ± 7.14***

Where, Values are expressed as Mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test. Data normality was assessed using the Shapiro–Wilk test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with disease control group.

List of Figures

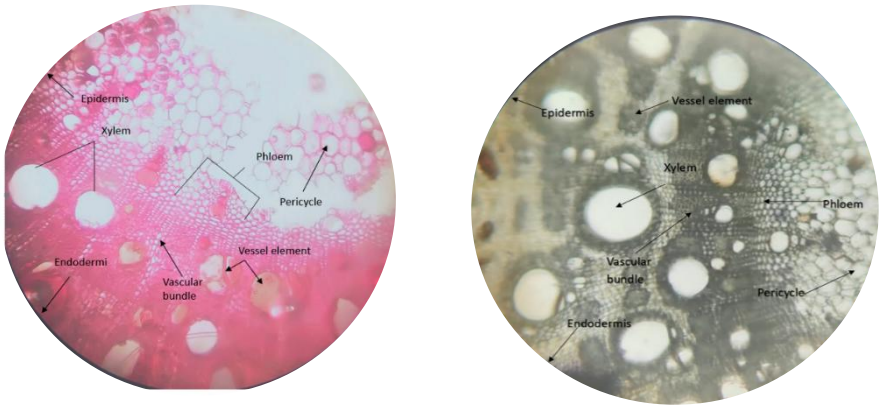


Fig. 1: T.S. of *Pueraria montana* (Lour.) Merr. Stem with different staining

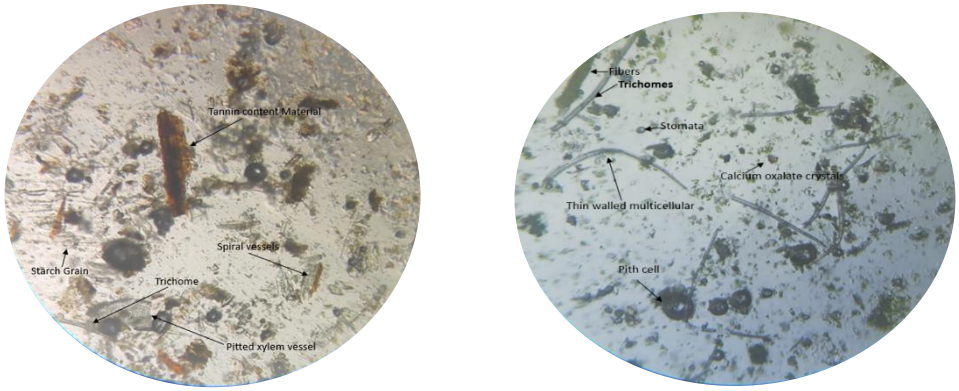


Fig. 2: Powder Microscopy of *Pueraria montana* (Lour.) Merr. with different staining

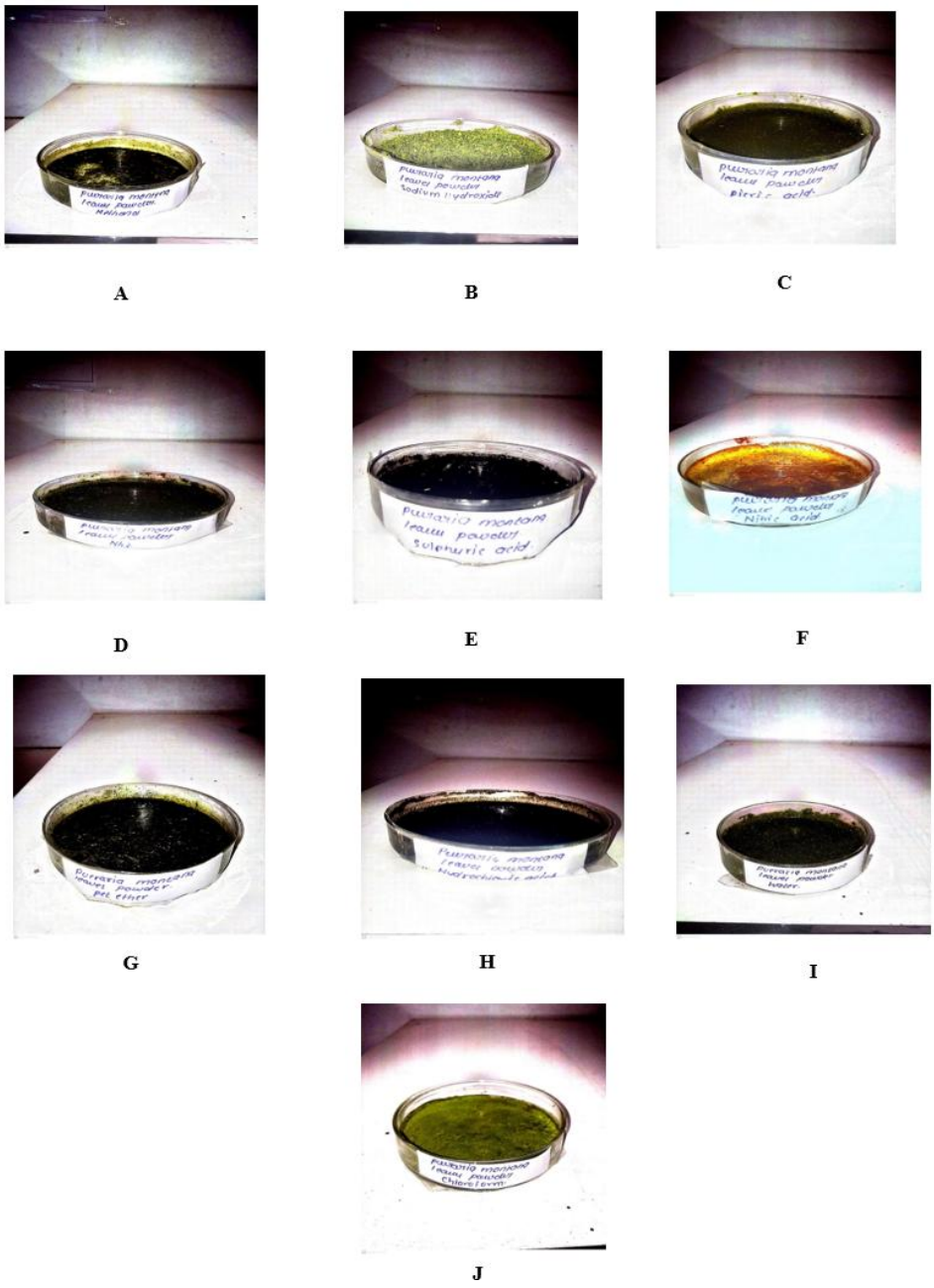


Fig. 3: Different picture of Powdered Drug with Various Extracts to show the Fluorescence Analysis (Leaves)

A

B

C

D

E

F

G

H

I

J

Fig. 4: Different picture of Powdered Drug with Various Extracts to show the Fluorescence Analysis (Stem)

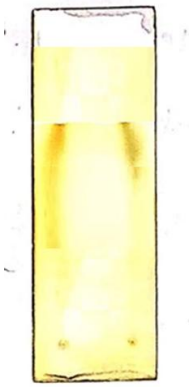


Fig. 5: TLC of *Pueraria montana* (Lour.) Merr.

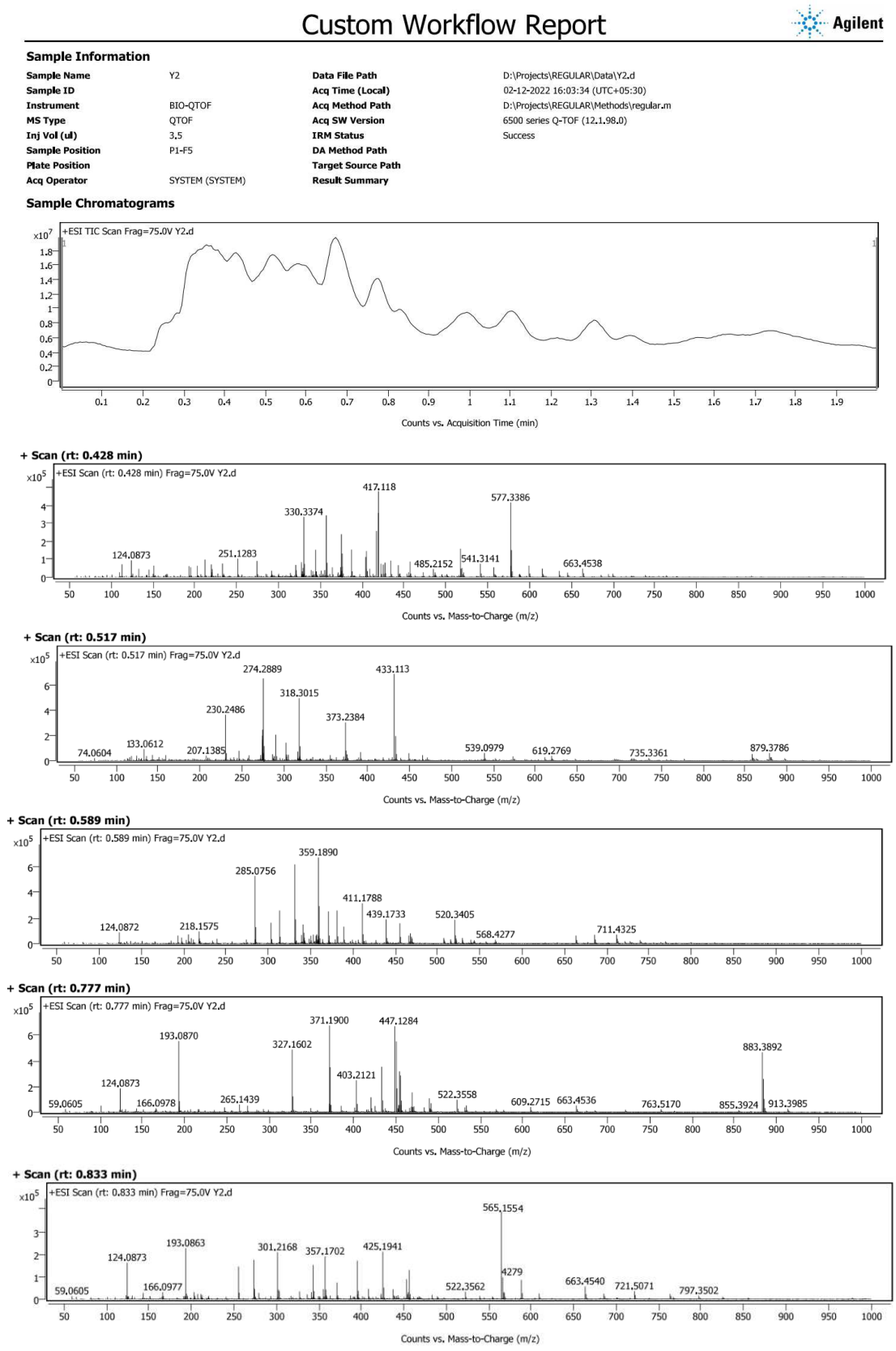


Fig. 6: HRMS chromatographic profile of the ethanolic extract showing major phytoconstituents with corresponding mass peaks.

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Sample Header

Data File:	ms_1
Original Data Path:	C:\Xcalibur\Data
Sample Type:	Unknown
Sample ID:	t
Sample Name:	
Acquisition Date:	06/08/23 06:50:14 PM
Run Time(min):	23.74
Injection Volume(µl):	1.00
Scans:	6981
Low Mass(m/z):	50
High Mass(m/z):	650
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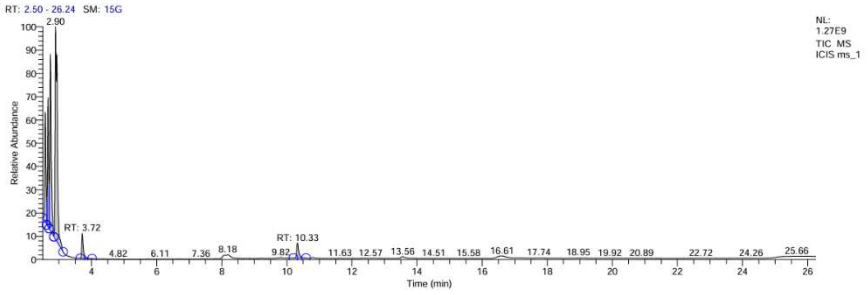

Qual Peak Table

RT	Peak Area	Area %	Peak Height
2.58	1646058151.83	13.15	602962354.31
2.66	1661940617.24	13.28	706576042.72
2.74	3028686336.83	24.20	966065602.26
2.90	5321440371.26	42.51	1163913959.56
3.72	485116727.88	3.88	135654039.71
10.33	374557657.27	2.99	82864701.14

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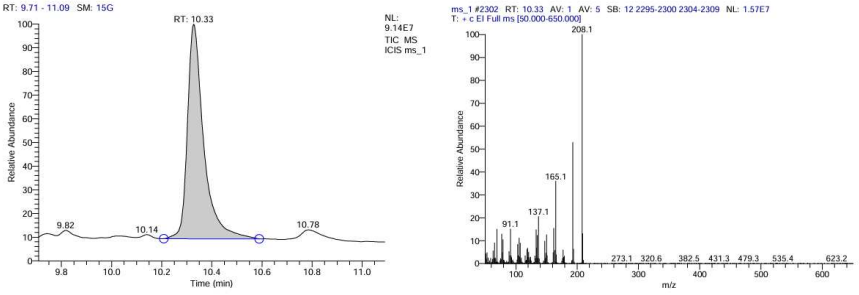

Library Search Results Table

Compound Name	RT	Molecular Formula	Cas #
Asarone	10.33	C12H16O3	2883-98-9
á-Asarone	10.33	C12H16O3	5273-86-9
Benzene, 1,2,4-trimethoxy-5-(1-propenyl)-	10.33	C12H16O3	494-40-6
1,2-Dimethoxy-4-(2-methoxy-1-propenyl)benzene	10.33	C12H16O3	NA
Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	10.33	C12H16O3	487-11-6

Fig. 7: GCMS chromatographic profile of the non-polar fraction showing major phytoconstituents with corresponding mass peaks.

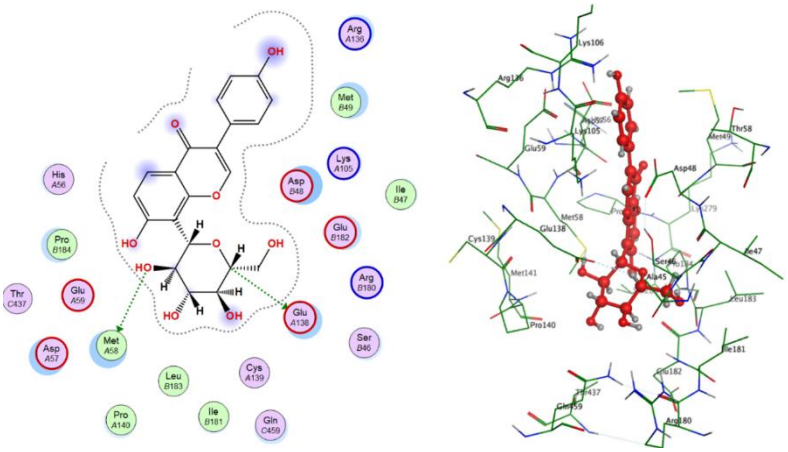

Fig. 8: Interaction diagram of Puerarin with 6HUO.

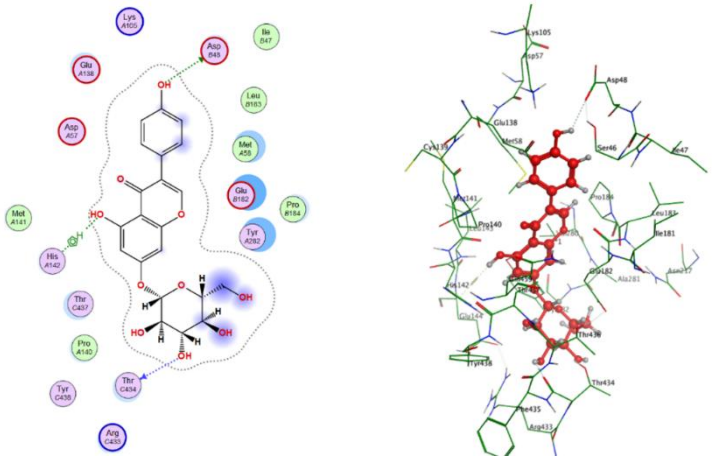

Fig. 9: Interaction diagram of Genistin with 6HUO.

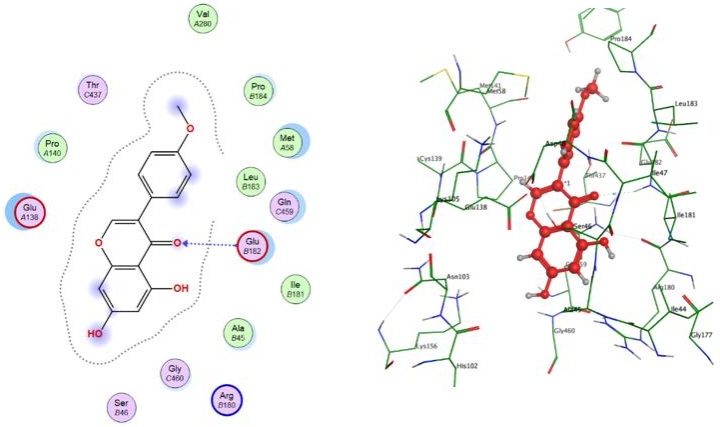

Fig. 10: Interaction diagram of Biochanin A with 6HUO.

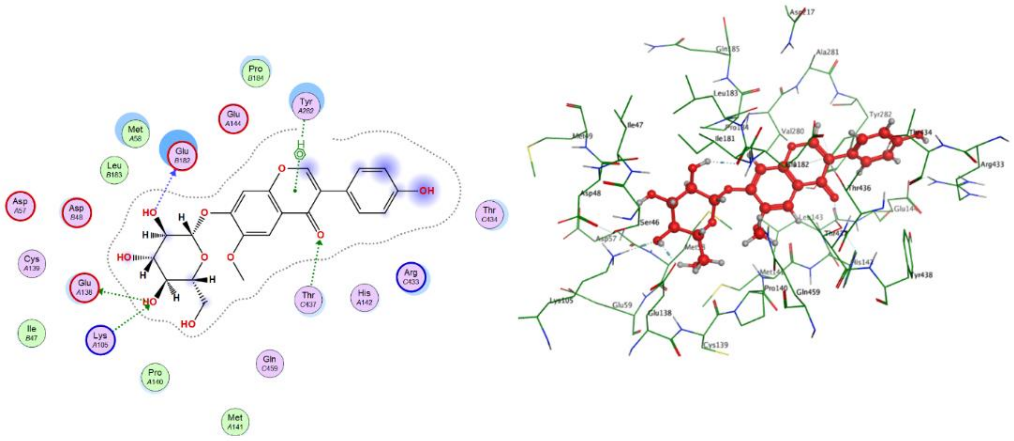

Fig. 11: Interaction diagram of Glycitin with 6HUO.

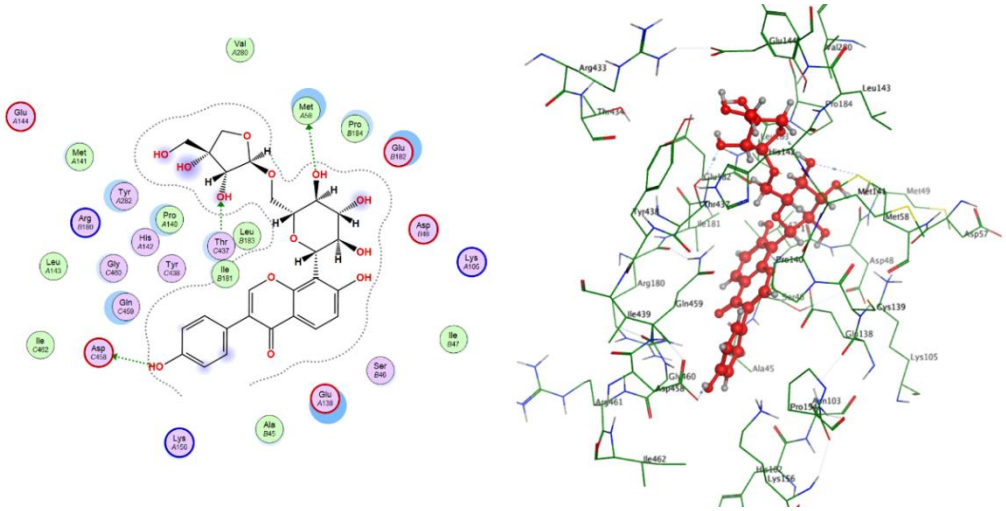

Fig. 12: Interaction diagram of Mirificin with 6HUO.

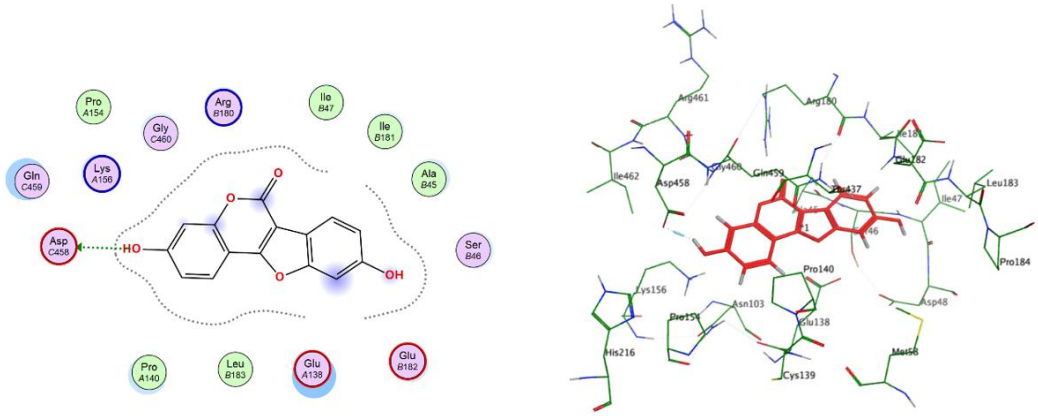

Fig. 13: Interaction diagram of Coumestrol with 6HUO.

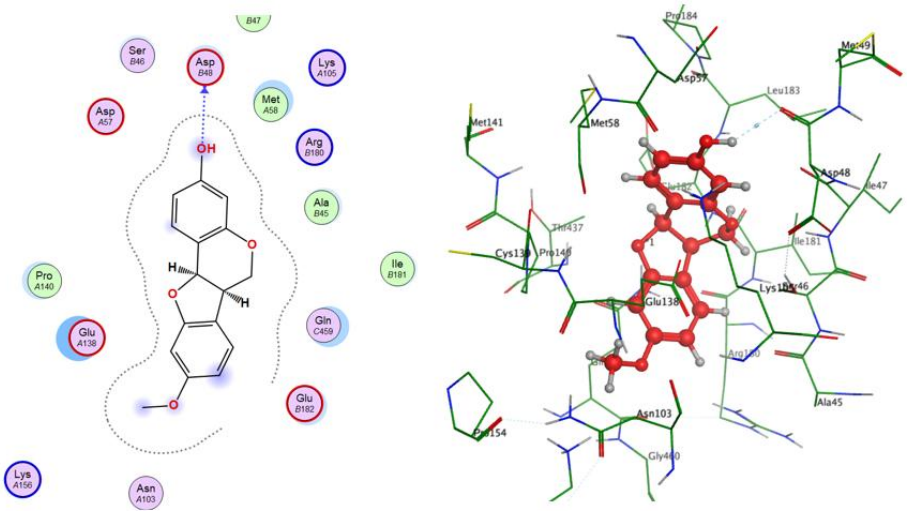

Fig. 14: Interaction diagram of Medicarpin with 6HUO.

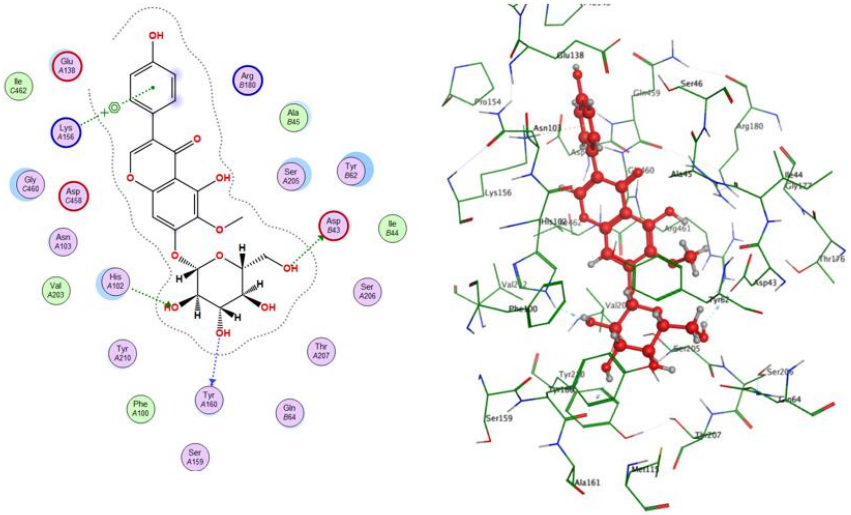

Fig. 15: Interaction diagram of Tectoridin with 6HUO.

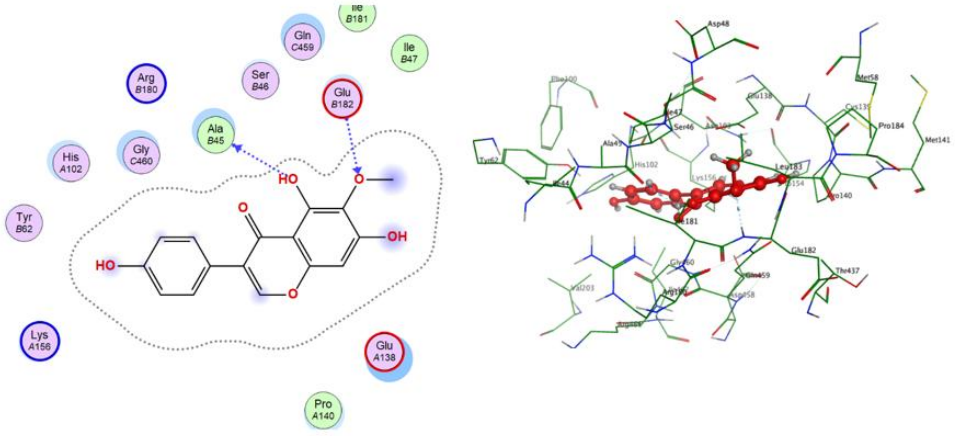

Fig. 16: Interaction diagram of Tectorigenin with 6HUO.

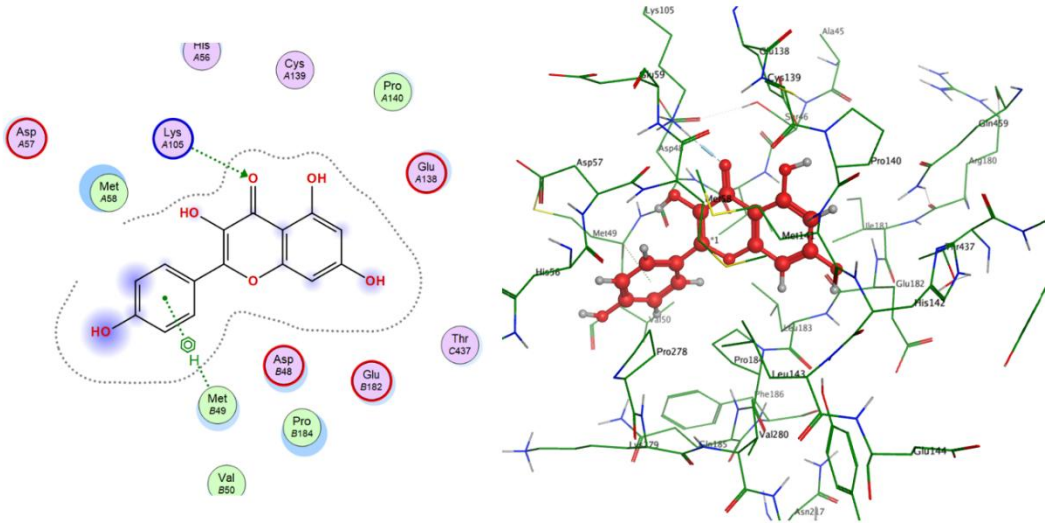

Fig. 17: Interaction diagram of Kaempferol with 6HUO.

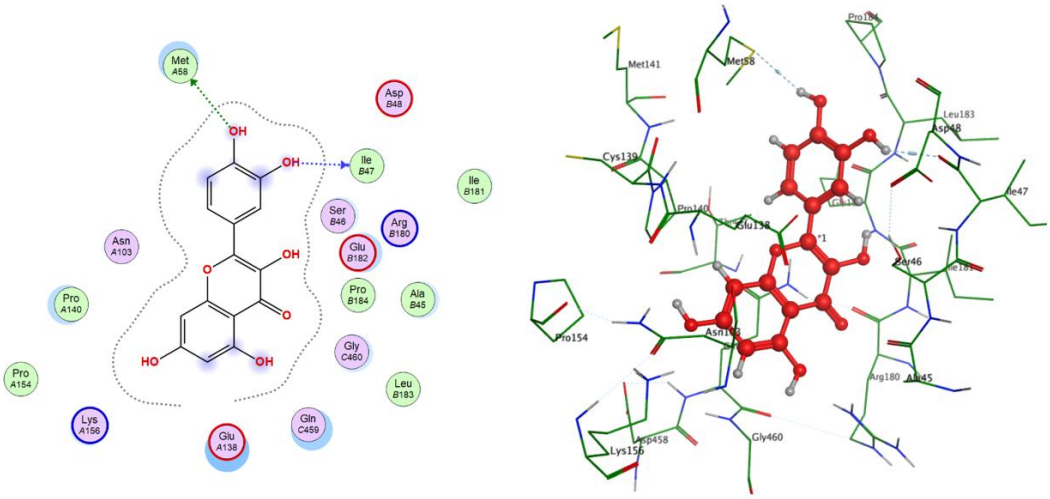

Fig. 18: Interaction diagram of Quercetin with 6HUO.

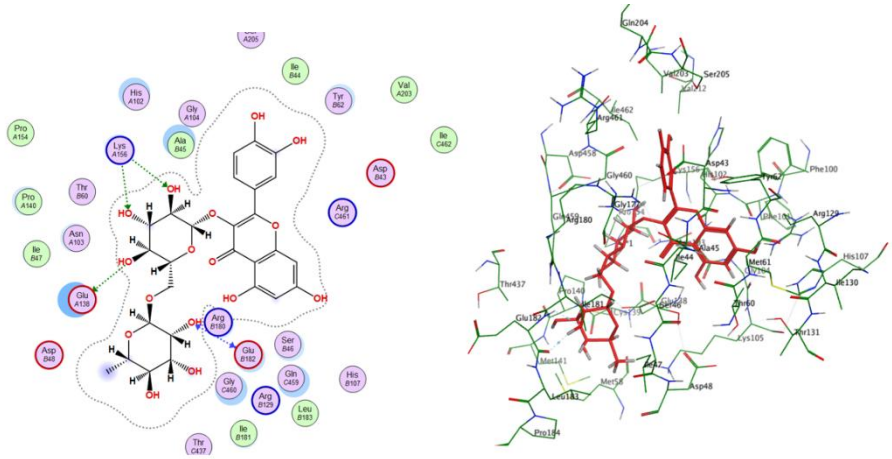

Fig. 19: Interaction diagram of Rutin with 6HUO.

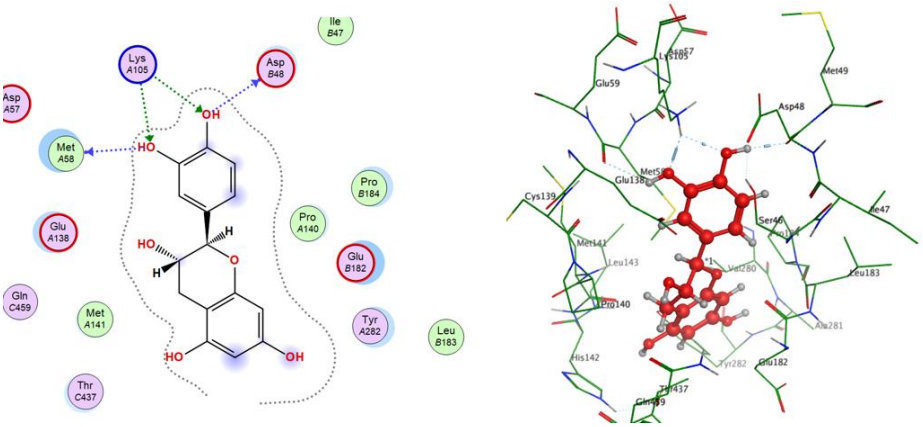

Fig. 20: Interaction diagram of Catechin with 6HUO.

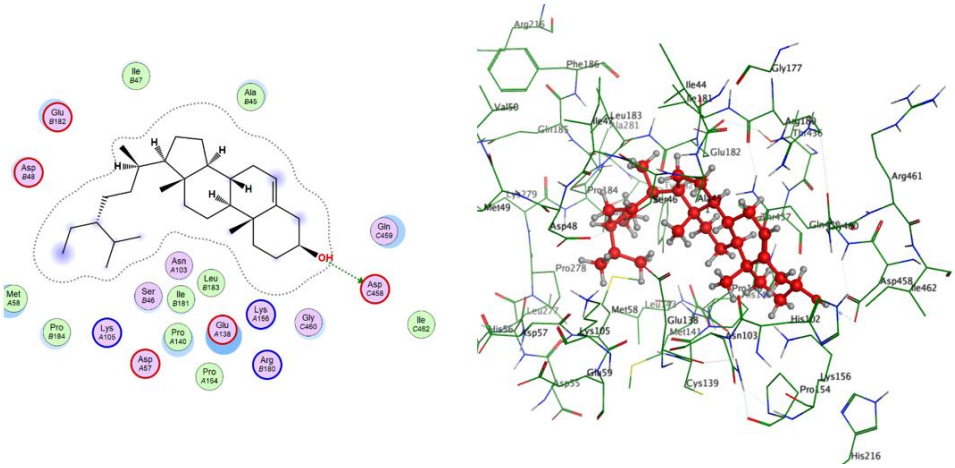

Fig. 21: Interaction diagram of β-Sitosterol with 6HUO.

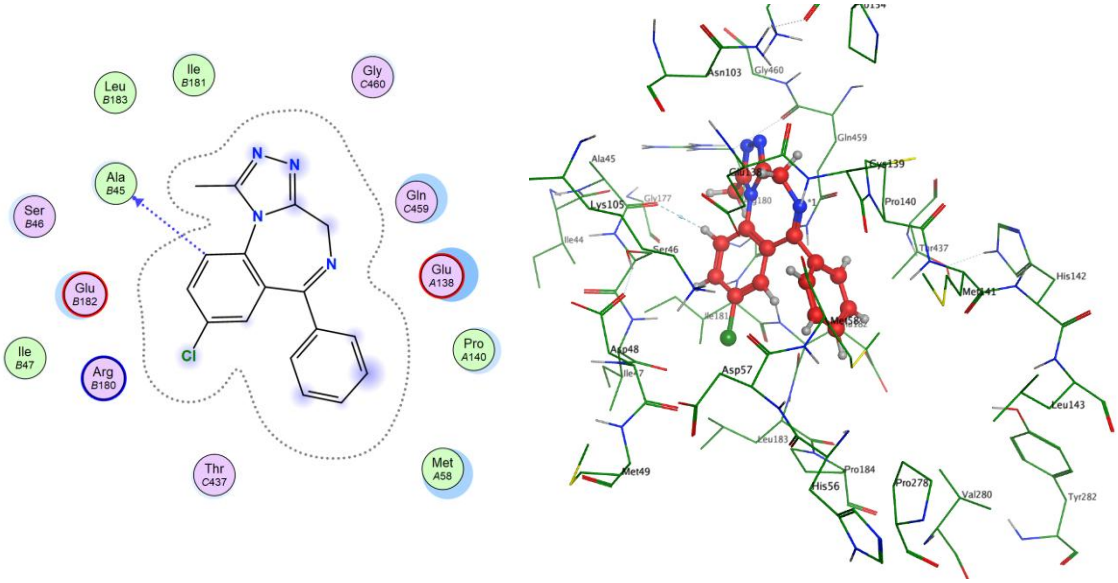

Fig. 22: Interaction diagram of Alprazolam with 6HUO.

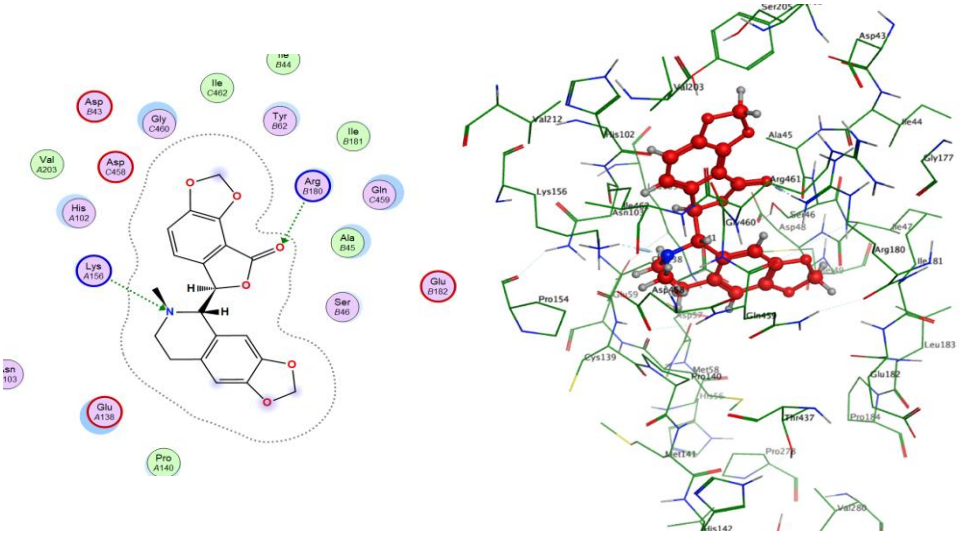

Fig. 23: Interaction diagram of Bicuculline with 6HUO.

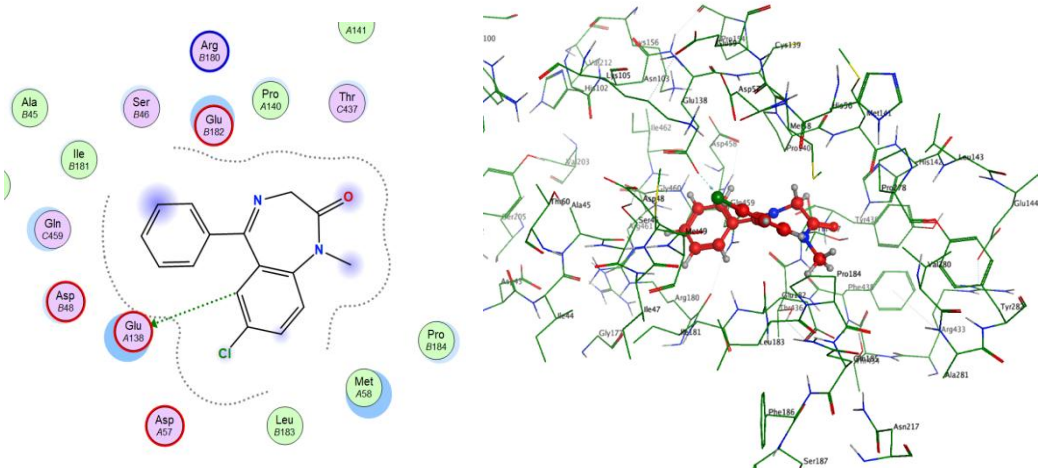

Fig. 24: Interaction diagram of Diazepam with 6HUO.

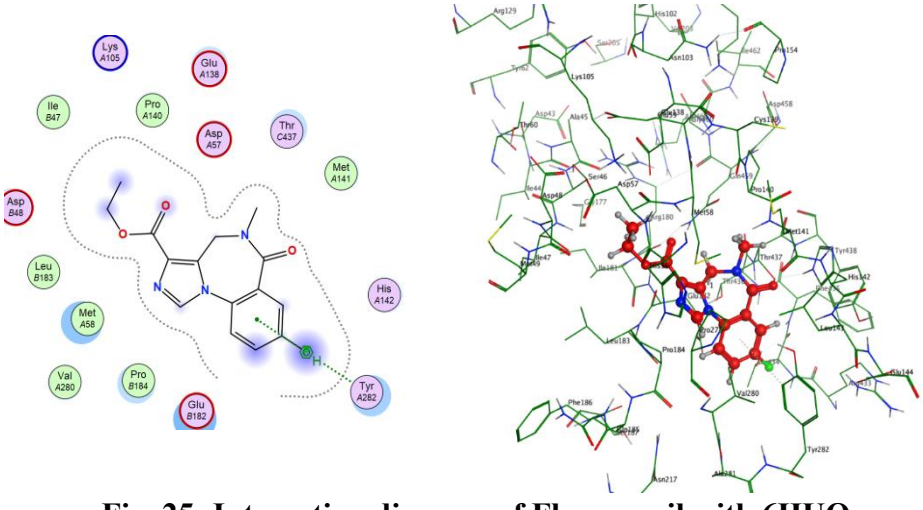

Fig. 25: Interaction diagram of Flumazenil with 6HUO.

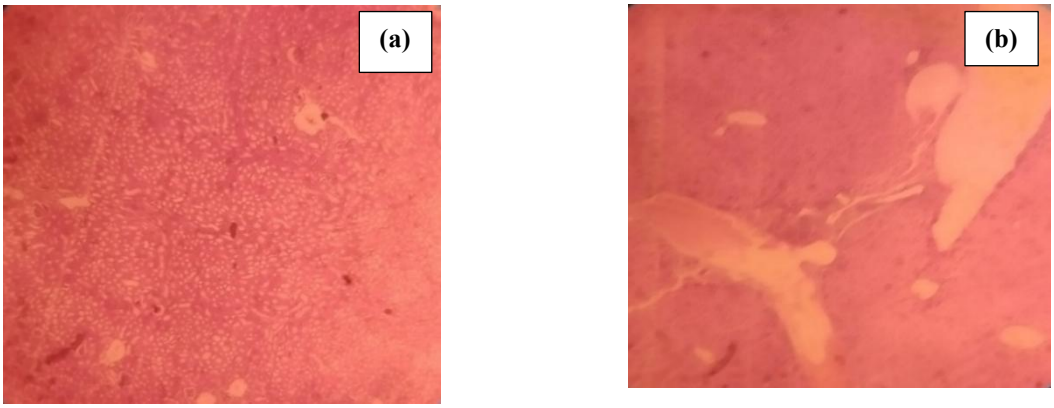

Fig. 26: Histology of Test (a) & Standard (b) of the Brain

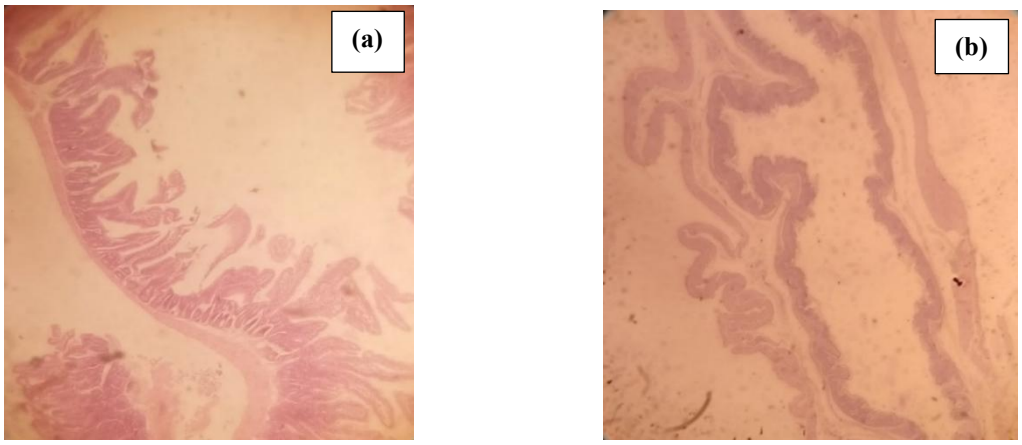

Fig. 27: Histology of Test (a) & Standard (b) of the Stomach

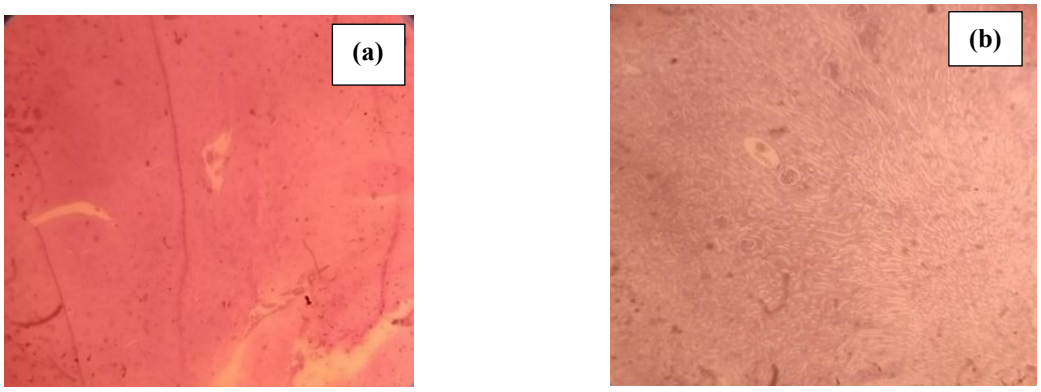

Fig. 28: Histology of Test (a) & Standard (b) of the Kidney

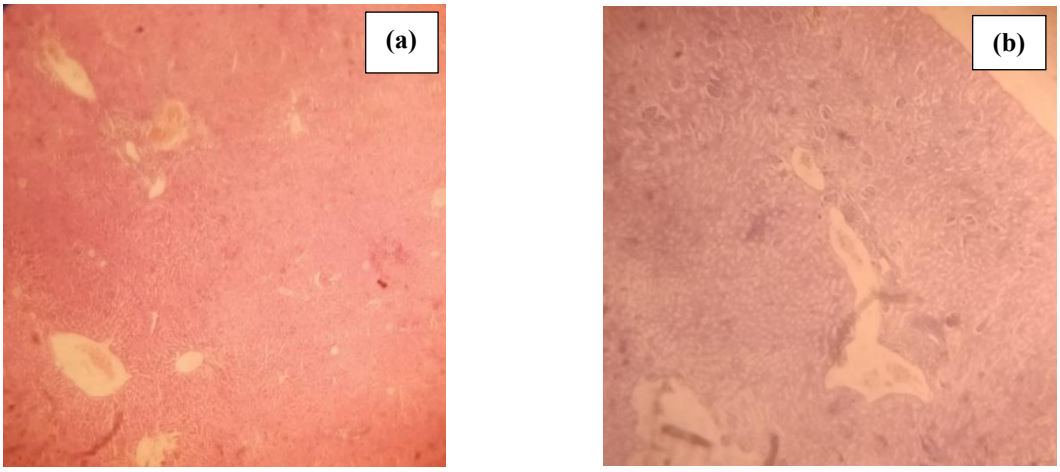

Fig. 29: Histology of Test (a) & Standard (b) of the Liver

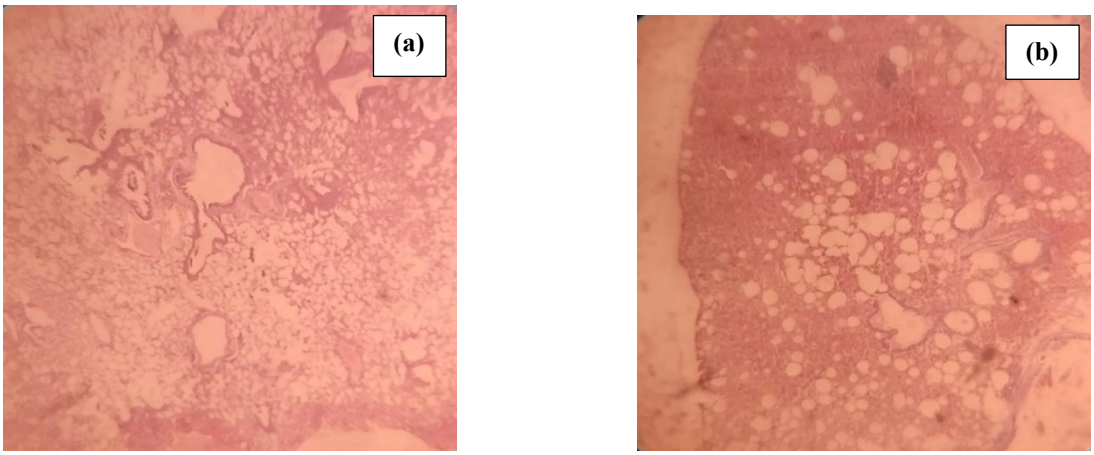

Fig. 30: Histology of Test (a) & Standard (b) of the Lungs

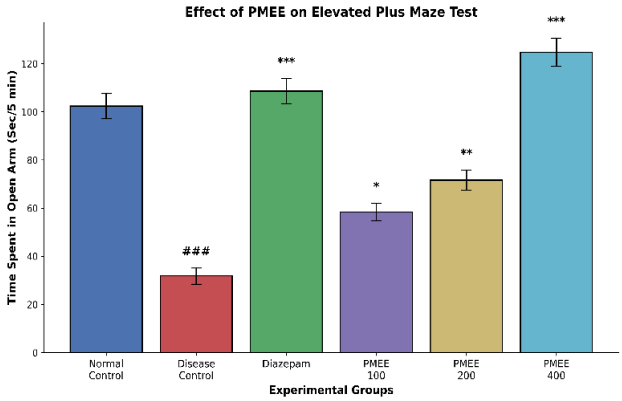

Fig. 31: Effect of PMEE on anxiety-related behaviour in experimental animals using the Elevated Plus Maze model.

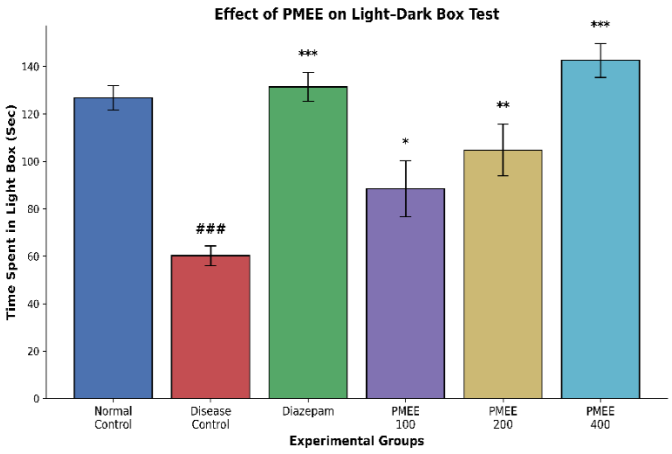

Fig. 32: Effect of PMEE on anxiety-associated parameters in experimental animals using the Light–Dark Box test.

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