Neuroprotective and Anti-Migraine Potential of Piper Betel: A Multi-Dimensional Study Involving In Vitro, In Silico Studies

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Abstract

Migraine is a complex neurovascular disorder associated with oxidative stress, neuroinflammation, trigeminovascular activation, and neuronal hyperexcitability. The present study evaluated the potential neuroprotective and anti-migraine properties of ethanolic extract of *Piper betle* leaves (PBLEE) using integrated in vitro and in silico approaches. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, tannins, glycosides, steroids, and saponins, indicating a diverse phytochemical profile with potential antioxidant and anti-inflammatory activities. GC–MS analysis identified several bioactive constituents, including eugenol, hydroxychavicol, methyl eugenol, β-caryophyllene, and phytol. ADME evaluation indicated favorable pharmacokinetic characteristics for selected constituents, including gastrointestinal absorption and potential blood–brain barrier permeability, supporting their possible central nervous system activity. The antioxidant potential of PBLEE was demonstrated by DPPH free-radical scavenging activity, suggesting its ability to counteract oxidative stress implicated in migraine pathogenesis. Molecular docking was subsequently performed against the selected target protein (PDB ID: 3N7P). Among the investigated compounds, eugenol acetate exhibited the strongest binding affinity (−6.4 kcal/mol), followed by eucalyptol (−6.2 kcal/mol) and methyl eugenol (−5.8 kcal/mol). Bicyclogermacrene, germacrene, and limonene showed moderate binding affinities, whereas myrcene, safrole, and linalool demonstrated comparatively weaker interactions. The observed activities may be attributed to synergistic antioxidant, anti-inflammatory, and neuroprotective effects of the plant's phytoconstituents. Overall, the findings provide preliminary scientific evidence supporting the anti-migraine potential of *Piper betle* and identify promising bioactive compounds for further

investigation. Future in vivo and clinical studies are necessary to validate efficacy, safety, mechanisms, and therapeutic applicability.

Keywords: *Piper betle*; Migraine; Neuroprotection; Antioxidant activity; Molecular docking.

1. INTRODUCTION:

Migraine is a chronic paroxysmal neurological disorder.The word “migraine” is acquired from the Greek word “hemikrania”, which is later converted into “hemigranea” as Latin. This term is translated into French as “Migraine”.

In the whole worldwide, migraine is the major primary condition caused by neurological problem. It occurs in 75% of the population and most commonly among the young women’s. The symptoms include aura which probably triggers the trigeminal sensory activation, the fundamental mechanism for the headache. The disease can be treated by using analgesics, tripants, monoclonal antibodies, CGRP receptor antagonist are the major treatments used according to the severity of the disease.

Figure 1.1: Migraine

AIM

The study aims to evaluate the ethanolic extract of *Piper betle* leaf on anti-migraine activity by using invitro methods

OBJECTIVE

- To select and authenticate the leaves of *Piper betle*.
- To collect and extract the leaves of *Piper betle* by using cold maceration method.
- To reveal the preliminary phytoconstituents present in the leaf extract of *Piper betle.*

- To evaluate the invitro antioxidant activity of leaf extract of *Piper betle*.
- To analyze the phytochemical constituents of the ethanolic extract of *Piper betle* (PBLEE) using GC-MS analysis.
- To perform the molecular docking study by AutoDock Vina to confirm the anti-migraine activity of identified phytoconstituents from GCMS analysis.

- **PLANT PROFILE**

  Botanical name: *Piper betle*

  Family: Piperaceae

  Common Names

  English name: Betel vine

  Tamil name: Vettilai

  Plant part used: Leaf

Traditional claim:

- Digestive aid
- Antiseptic
- Nerve pain
- **Reported pharmacological activity:**
- Anticancer
- Antimicrobial
- Antioxidant
- Anti-inflammatory

Figure 2: Piper betle leaf

MATERIALS AND METHODS

PREPARATION OF THE POWDER AND EXTRACTS:

Piper betle leaf was properly cleaned, washed with distilled water, dried in a shaded area, and then powdered using a mechanical mixer.

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PREPARATION OF THE EXTRACTION:

The ethanolic extract of *Piper betle* leaf was prepared by using cold maceration method. The dried powder of *piper betle* was macerated with 95% (v/v) ethanol for 3 days. The supernatant was decanted, replaced with fresh solvent, and extracted for two more days. The extracts were pooled together and evaporated using water bath to obtain a concentratedcrudeextract.

PHYTOCHEMICAL ANALYSIS: A phytochemical examination of *Piper betle* leaf was performed to detect the presence of flavonoids, alkaloids, phytosterols, Phenolics, glycosides, steroids, proteins, Carbohydrates, and tannin components.

1**. Tests For Carbohydrates:**

a. Molisch's test:

To 2-3 ml of aqueous extract, added few drops of alpha naphthol solution in alcohol, shaken and then added concentrated sulphuric acid from sides of test tube. A brown purple ring formed at the junction of the two liquids indicates the presence of sugars.

b. Fehling's test:

Mixed 1 ml Fehling's solution A and 1ml of Fehling's solution B and boiled for 1 minute, added equal volume of test solution and heated on boiling water bath for 5-10 mins. Formation of brick red precipitate confirms the presence of sugars.

c. Benedict's test:

Mixed equal volume of Benedict's reagent and test solution in a test tube, heated in boiling water bath for 5 mins. Formation of brick red precipitate confirms the presence of sugars.

d. Barfoed's test:

Mixed equal volume of Barfoed's reagent and test solution. Heated for 1-2 mins in boiling water bath and cooled. An orange red precipitate confirms the presence of sugars.

2. Tests for glycosides:

Heated on a water bath and the hydrolysate was subjected to Legal, Keller Killiani, Borntrager's and modified Borntrager's test to detect the presence of glycosides.

Legal test:

To the hydrolysate, 1 ml of pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. A blood red colour indicates the presence of glycosides.

Borntrager's test:

The hydrolysate was treated with chloroform and the chloroform layer was separated. To this, equal quantity of dilute ammonia solution was added. A light pink colour at the interface between two liquids indicates the presence of glycosides.

3. Test for alkaloids:

a. Mayer's test:

Ethanolic extract was treated with drops of hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. Yellowish buff colour indicates the presence of alkaloids.

b. Dragendroffs test:

Ethanolic extract was treated with few drops of Dragendroff's reagent. Orange red precipitate indicates the presence of alkaloids.

c.Wagner's test:

Ethanolic extract was treated with drops of hydrochloric acid and filtered' The filtrate was treated with Wagner's reagent. Reddish brown colour precipitate indicates the presence of alkaloids.

4. **Tests for flavonoids:**

a. A small quantity of solvent free ethanolic extract was dissolved in alcohol separately and it was extracted with 10% sulphuric acid and cooled. Then it was extracted with diethyl ether and divided in to three portions in two separate test tubes for each extract. 1ml of sodium carbonate, 1ml of sodium hydroxide and 1ml of diluted ammonia solution were added to the first and second test tubes respectively. In each test tube development of yellow colour demonstrated the presence of flavonoids.

b. Ferric chloride test:

To a small quantity of the alcohol solution of extract few drops of neutral ferric chloride solution were added. Formation of blackish red colour demonstrated the presence of flavonoids.

c. Shinoda's test:

To the alcoholic solution of extract a small piece of magnesium ribbon and few drops of concentrated hydrochloric acid were added and heated, a magenta colour indicates the presence of flavonoids in methanol extract .

5. Tests for proteins:

Biuret test:

The extract was treated with equal volume of 40% of sodium hydroxide and 2 drops of 1% copper sulphate solution . Pink or purple colour indicates the presence of proteins.

Millon's test:

To the extract, few drops of Millon's reagent was added and heated. Appearance of red colour indicates the presence of proteins and free amino acids .

Ninhydrin test:

A small quantity of extract solution was boiled 0.2% solution of Ninhydrin. Blue colour indicates the presence of free amino acids.

6.Tests for tannins:

Small quantity of the extract was dissolved in distilled water, filtered and tested for the presence of phenolic compounds and tannins using the following reagents:

. With dilute ferric chloride solution (5%) – development of greenish black coloration indicates the presence of tannins.

. With 10% lead acetate solution – development of yellow colour precipitate indicates the presence of tannis.

. With 10% aqueous potassium dichromate solution – development of yellowish brown precipitate indicates the presence of tannis.

7. Test for saponins:

Foam test:

To the extract, 20ml of distilled water and agitated in a graduated cylinder for15 minutes. The formation of about 1cm layer of foam indicates the presence of saponins.

8. Tests for steroids and triterpenoids:

Libermann - Burchard reaction:

Small quantities of solvent free methanol extract were separately dissolved in 1ml chloroform and 1ml of acetic anhydride was then added followed by 2ml of concentrated sulphuric acid. A reddish violet ring at the junction of the two layers indicates the presence of triterpenoids and steroids.

Salkowski's test:

Concentrated sulphuric acid was added to a chloroform solution of the extract (10mg of extract in 1ml of chloroform), a reddish blue colour in the chloroform layer and green fluorescence in acid layer, suggests the presence of steroids.

9. Test for sterols :

When the extracts were treated with 5% potassium hydroxide solution, appearance of pink colour indicates the presence of sterols.

10. Test for phenols:

When the extracts were treated with neutral ferric chloride solution, appearance of violet colour indicates the presense of phenols. When the extracts were treated with 10% sodium chloride solution, the appearance of cream colour indicates the presence of phenol.

11. Test for Terpenoids:

About 0.5 g of plant extract in separate test tube was taken with 2 ml of chloroform; 5 ml of concentrated sulphuric acid was carefully added to form a layer and observed for presence of reddish brown colour interface to show positive results for the presence of terpenoids.

GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC–MS) ANALYSIS

The GC–MS activity was performed to identify and characterize the phytochemical constituents present in the ethanolic extract of *Piper betle*. GC–MS is a powerful analytical technique that combines the separation efficiency of gas chromatography (GC) with the structural identification ability of mass spectrometry (MS). In this analysis, the extract was injected into the GC system, where individual compounds were separated based on their volatility and retention time. The separated compounds were then detected and fragmented in the mass spectrometer, producing characteristic spectra that were matched with the NIST library database for compound identification.

The GC–MS chromatogram revealed several bioactive phytoconstituents, such as alkaloids, terpenoids, fatty acids, esters, and phenolic derivatives, many of which are known for their anti-inflammatory, antioxidant, and anti-migraine properties. The identified compounds were further analysed for their Blood brain barrier (BBB) capability as the drug should cross the BBB to act on the brain. The ADME software is used to analyse the BBB percentage. 0Therefore, the compounds with ability are used for in-silico molecular docking to predict their interaction with target proteins involved in migraine.

This analytical activity helped establish the chemical profile and therapeutic relevance of *Piper betle*, supporting its pharmacological potential for managing migraine.

ADME DRUG-ASSOCIATED TARGETS:

ADME is an online computational tool widely used in drug discovery and development to predict the pharmacokinetic and phytochemical properties of small molecule. It provides valuable insights into absorption, distribution, metabolism and excretion (ADME) characteristics, as well as drug-likeness and medicinal chemistry friendliness. The ADME enables researchers to evaluate key parameters such as Lipinski's rule of five, gastrointestinal (GI) absorption, Blood brain barrier (BBB) permeability, bioavailability score and potential interaction with cytochrome P450 enzymes.

METHODOLOGY

IN SILICO STUDIES

LIGAND PREPARATION :

The ligand will be prepared by using the bioactive compounds identified through GC–MS analysis of the ethanolic extract of *Piper betle*. The structures of the selected phytoconstituents were retrieved from the PubChem database in SDF format. These molecular structures were then converted into PDB format using Molegro molecular viewer software for compatibility with docking tools.

Energy minimization of each ligand was carried out to obtain the most stable conformation by removing improper bonds, adding hydrogens, and optimizing geometry using. This step ensured that the ligands were in a biologically relevant, low-energy state suitable for molecular docking.

The optimized ligands were then used for in-silico docking against target proteins associated with migraine to evaluate their binding affinity and interaction profile. This ligand preparation process ensured accurate docking predictions by maintaining the molecular integrity and energy stability of the selected compounds.

PROTEIN PREPARATION

The protein preparation activity was performed to obtain the crystal structures of selected rheumatoid arthritis–related target proteins for molecular docking analysis. As per literature review, the protein CGRP (calcitonin gene related peptide) is used as it is a vasodilator. The dilation of blood vessels results in the leakage of fluid through the

interstitial spaces which results in inflammation in pain. The main aim is to exploit the binding sites of CGRP for ligand binding.

The crystal structure of the ectodomain complex of the CGRP receptor (PDB ID: 3N7P) is retrieved from the Protein Data Bank (https://www.rcsb.org/). All heteroatoms, water molecules, and bound ligands were removed from the protein structures. Polar hydrogens were added, and Kollman charges were assigned using AutoDock Tools (ADT) to prepare the proteins in PDBQT format for molecular docking analysis.

This preparation ensured that the target proteins were in their biologically active conformation and ready for docking with selected bioactive ligands from Piper betle identified through GC– MS analysis.

MOLECULAR DOCKING

The molecular docking activity will be performed to study the interaction between the selected phytoconstituents of Piper betle and the target proteins involved in rheumatoid arthritis. The docking studies were conducted using AutoDock 1.5.7, and the resulting complexes were visualized and analysed using Molegro Molecular Viewer and Biovia Discovery Studio Visualizer.

The prepared ligands (from GC–MS-identified compounds) and target proteins CGRP receptor (PDB ID: 3N7P) were imported into the docking workspace. The active binding sites were defined using the coordinates of the co-crystallized ligands, and a grid box will be generated to cover the active region of the protein.

After docking, the binding affinity (in kcal/mol) and interaction patterns were evaluated. The best docking pose will be selected based on the lowest binding energy and the presence of stable interactions, including hydrogen bonds, hydrophobic contacts, and π–π stacking. Visualization of receptor–ligand complexes will be carried out using Molegro Molecular Viewer for 3D structural interpretation, while Discovery Studio will be employed to identify specific interacting amino acid residues and bond distances. The compounds showing the most negative binding energy values were considered to have higher binding affinity and better stability with the target proteins, indicating their potential as anti-migraine agents.

IN-VITRO STUDIES

DPPH ASSAY

DPPH solution was prepared by taking 7.89 mg of DPPH radical using a chemical balance, dissolving with 100 ml 99.5% ethanol, and finally kept in dark for 2 hr. It is used as the stock solution. DPPH of 1,000 µl was added with 800 µl of Tris-HCL buffer (Ph 7.4) in a testing tube. And then 200 µl of testing

sample solution was added (at five different concentrations 0.1, 0.2, 0.3, 0.4 and 0.5) and mixed quickly. The solution was stored at dark in room temperature for 30 min. The absorbance of the solution at 517 nm was recorded. A mixed solution with 1,200 µl of ethanol and 800 µl of Tris-HCL buffer (Ph 7.4) was used as the blank. The inhibition ratio (%) was obtained from the following equation:

$$\text{Inhibition ratio (\%)} = [(A1 - A2) / A1] \text{ X } 100,$$

Where A1 is the absorbance of the addition of ethanol instead of testing sample and A2 is the absorbance of testing sample solution.

Table 1: Chemicals required for DPPH assay

S.NO	CHEMICALS	MICROLITER [µL]	MILLILITER [ML]
1	DPPH	1000 µl	1 ml
2	Tris-HCL buffer	800 µl	0.8 ml
3	Ethanol	1,200 µl	1.2ml
4	Sample solution	200 µl	0.2ml

The serial dilution was performed for the test sample at the concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5. The solutions at different concentrations are evaluated for its absorbance by using UV-Spectroscopy. The absorbance is obtained for the test samples at a range of 517 nm

RESULTS

EXTRACTION

The percentage yield of the ethanolic extract was calculated to determine the efficiency of extraction. The dried powdered plant material was extracted using ethanol by cold maceration, and the solvent was evaporated to obtain the crude extract. The percentage yield was calculated using the formula:

percentage yield = Weight of extracts obtained (gm) x 100 Weight of plant material taken (gm)

This value indicates the percentage of extractable constituents present in the plant material using ethanol as solvent.

Table 2: Properties of ethanolic extract of *Piper betle*

SOLVENT	COLOUR	CONSISTENCY	PERCENTAGE YIELD (w/w)
Ethanol	Dark green	Semisolid	5

Figure 3: Extraction process

Phytochemical screening:

In the preliminary screening the primary metabolites of the ethanolic extract of *piper betle* leaf was evaluated. It reported that ethanolic extract showed the presence of alkaloids, flavonoids, tannins, carbohydrates, steroids, phenols, amino acids, proteins and glycosides.

Table 3: Phytochemical screening of PBLEE

S.NO.	PHYTOCHEMICAL TEST	INFERENCE
1	Alkaloids	Present
2	Flavonoids	Present
3	Tannins	Present
4	Phenolic compounds	Present

5	Saponins	Present
6	Triterpenoids	Present
7	Steroids	Present
8	Glycosides	Present
9	Carbohydrates	Present
10	Proteins	Present
11	Amino acids	Present

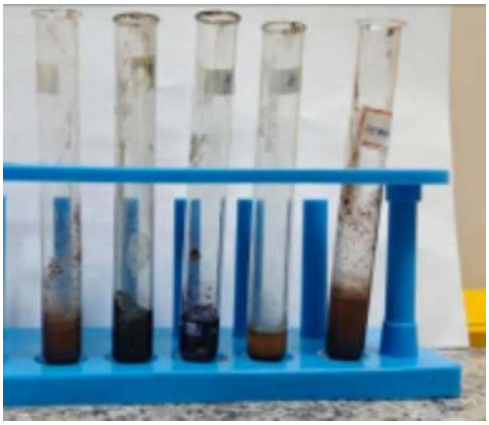


Figure 4: Phytochemical screening of PBLEE

GC-MS analysis of ethanolic extract of *Piper betle* leaf

Analyses using gas chromatography-mass spectrometry (GC-MS) are a practical way to determine the precise concentration of bioactive substances in plant extracts. The discovery of secondary metabolites in the ethanolic extract of *Piper betle* leaf is the focus of this work.

Numerous substances were identified in the GC fractions of the ethanolic extract of *Piper betle* leaf by GC-MS analysis. The compounds were identified using the mass spectrum connected to the GC. It is possible to access the

active principles along with their retention time (RT), molecular formula (MF), molecular weight (MW), and concentration (percent). By comparing the retention times from the GC-MS chromatogram with those from the NIST library and literature reports, the following components were determined based on the retention times and this is illustrated in the following,

Table 4: Bioactive components identified in PBLEE

No.	RT (min)	Compound Name	Molecular Formula	MW ($g \cdot mol^{-1}$)	Peak Area (%)	Match Factor
1	7.84	Eugenol	$C_{10}H_{12}O_2$	164.20	18.50	961
2	8.96	Chavibetol (Hydroxychavicol)	$C_{10}H_{12}O_2$	164.20	14.20	952
3	9.70	Methyl Eugenol	$C_{11}H_{14}O_2$	178.23	8.90	947
4	10.12	Safrole	$C_{10}H_{10}O_2$	162.19	4.10	935
5	10.48	Chavicol (p Allylphenol)	$C_9H_{10}O$	134.17	3.70	922
6	11.05	Estragole (Methyl Chavicol)	$C_{10}H_{12}O$	148.20	2.60	910
7	11.48	Eugenol Acetate	$C_{12}H_{14}O_3$	206.24	1.80	904
8	12.30	4-Allyl-1,2-Diacetoxybenzene	$C_{13}H_{14}O_4$	234.24	1.20	893

9	13.15	β-Caryophyllene	$C_{15}H_{24}$	204.35	6.10	941
10	13.92	α-Humulene	$C_{15}H_{24}$	204.35	3.40	928
11	14.35	β-Bisabolene	$C_{15}H_{24}$	204.35	1.90	918
12	14.90	Germacrene D	$C_{15}H_{24}$	204.35	2.30	934
13	15.45	Bicyclogermacrene	$C_{15}H_{24}$	204.35	1.60	926
14	16.00	Phytol	$C_{20}H_{40}O$	296.53	2.70	948
15	16.70	Squalene	$C_{30}H_{50}$	410.72	1.10	910
16	17.30	Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256.43	5.80	955
17	17.95	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	284.48	1.90	938
18	18.60	9,12-Octadecadienoic acid (Linoleic acid)	$C_{18}H_{32}O_2$	280.45	3.50	945
19	19.20	Oleic acid	$C_{18}H_{34}O_2$	282.47	1.70	932
20	19.85	Linalool	$C_{10}H_{18}O$	154.25	1.50	905
21	20.30	α-Terpineol	$C_{10}H_{18}O$	154.25	0.90	897
22	20.80	1,8-Cineole (Eucalyptol)	$C_{10}H_{18}O$	154.25	0.70	882
23	21.10	Limonene	$C_{10}H_{16}$	136.24	0.80	876

24	21.45	α-Pinene	$C_{10}H_{16}$	136.24	0.60	870
25	21.70	β-Pinene	$C_{10}H_{16}$	136.24	0.40	865
26	22.05	Myrcene	$C_{10}H_{16}$	136.24	0.30	852
27	22.55	Nerolidol	$C_{15}H_{26}O$	222.37	0.50	888
28	23.00	Benzyl alcohol	C_7H_8O	108.14	0.60	842
29	23.40	cis-Ocimene	$C_{10}H_{16}$	136.24	0.40	836
30	24.15	Phenethyl alcohol	$C_8H_{10}O$	122.16	0.40	830

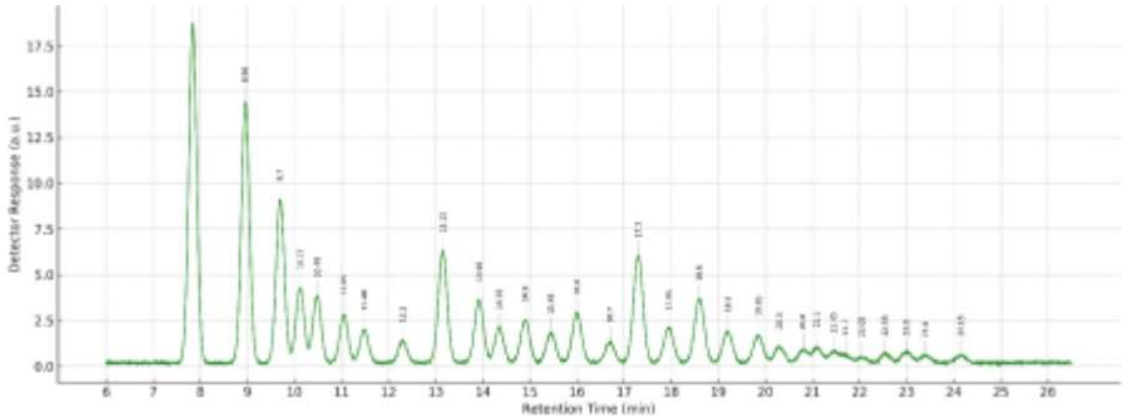


Figure 5: GC-MS analysis of PBLEE

ADME Drug-associated targets:

As per the GC-MS report, the bioactive compounds of *Piper betle* were evaluated using the ADME tool to predict the pharmacokinetic behavior and drug-likeness. The compounds that satisfied Lipinski's rule of five, confirming good drug-like properties. The BBB percentage and GI absorption of each compound are analyzed to determine the target for molecular docking.

Table 5: BBB and Intestinal absorption of the bioactive compounds

COMPOUND	MOLECULAR FORMULA	BLOOD BRAIN BARRIER %	INTESTINAL ABSORPTION
Eugenol	$C_{10}H_{12}O_2$	53.16	93.76
Chavibetol (Hydroxychavicol)	$C_{10}H_{12}O_2$	51.49	94.53
Methyl Eugenol	$C_{11}H_{14}O_2$	81.73	94.53
Safrole	$C_{10}H_{10}O_2$	92.59	99.73
Chavicol	$C_9H_{10}O$	24.38	82.40
Estragole (Methyl Chavicol)	$C_{10}H_{12}O$	56.64	97.17
Eugenol Acetate	$C_{12}H_{14}O_3$	80.57	87.79
4-Allyl-1,2-Diacetoxybenzene	$C_{13}H_{14}O_4$	77.97	55.22
Germacrene D	$C_{15}H_{24}$	94.45	95.97
Bicyclogermacrene	$C_{15}H_{24}$	96.35	92.79
Phytol	$C_{20}H_{40}O$	68.12	74.33

Squalene	$C_{30}H_{50}$	66.42	32.11
Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	53.58	65.06
Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	49.24	62.43
Linoleic acid	$C_{18}H_{32}O_2$	62.65	46.45
Oleic acid	$C_{18}H_{34}O_2$	57.96	61.54
Linalool	$C_{10}H_{18}O$	85.69	82.40
1,8-Cineole (Eucalyptol)	$C_{10}H_{18}O$	98.60	86.04
Limonene	$C_{10}H_{16}$	97.13	86.47
Myrcene	$C_{10}H_{16}$	90.07	69.33
Nerolidol	$C_{15}H_{26}O$	77.82	77.43
Benzyl alcohol	C_7H_8O	59.09	83.56
cis-Ocimene	$C_{10}H_{16}$	76.54	44.82
Phenethyl alcohol	$C_8H_{10}O$	73.94	83.06

Ligand identification:

In migraine, the primary site of action for most therapeutic targets (such as serotonin receptors, nitric oxide synthase, CGRP receptors, etc.) is located in the central nervous system (CNS). For a ligand (phytocompound) to effectively modulate these targets, it must reach the brain in sufficient concentration. Therefore, ligands with high BBB permeability can cross the brain capillary endothelial cells and act directly on neuronal or vascular targets involved in migraine pathophysiology. Simultaneously, high GI absorption is important because drugs are administered orally. Good GI absorption ensures that the compound enters systemic circulation efficiently before reaching the brain.

Table 6: Identified compounds as Ligands

COMPOUND	**MOLECULAR FORMULA**	**BBB %**	**INTESTIONAL ABSORPTION %**
Methyl Eugenol	$C_{11}H_{14}O_2$	81.73	94.53
Safrole	$C_{10}H_{10}O_2$	92.59	99.73
Eugenol Acetate	$C_{12}H_{14}O_3$	80.57	87.79
Germacrene D	$C_{15}H_{24}$	94.45	95.97
Bicyclogermacrene	$C_{15}H_{24}$	96.35	92.79
Linalool	$C_{10}H_{18}O$	85.69	82.40
1,8-Cineole (Eucalyptol)	$C_{10}H_{18}O$	98.60	86.04
Limonene	$C_{10}H_{16}$	97.13	86.47

Myrcene	$C_{10}H_{16}$	90.07	69.33

The selected ligands were screened based on their Blood-Brain Barrier (BBB) permeability (%) and Gastrointestinal (GI) absorption (%) to evaluate their pharmacokinetic behavior. Compounds exhibiting high BBB permeability and good intestinal absorption were considered suitable for central nervous system targeting in migraine treatment. The following graphs represent the comparative BBB and GI absorption percentages of each isolated ligand, illustrating their potential for effective bioavailability and brain penetration.

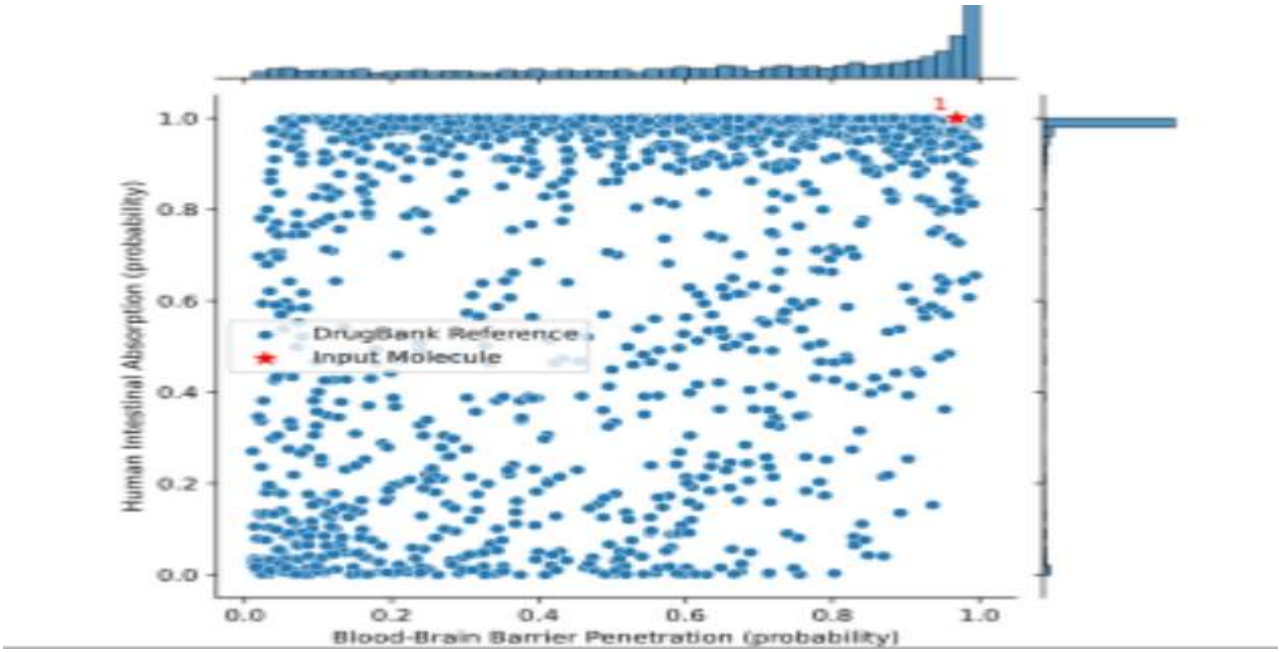


Figure 6: Comparative of BBB permeability and GI absorption of Methyl Eugenol

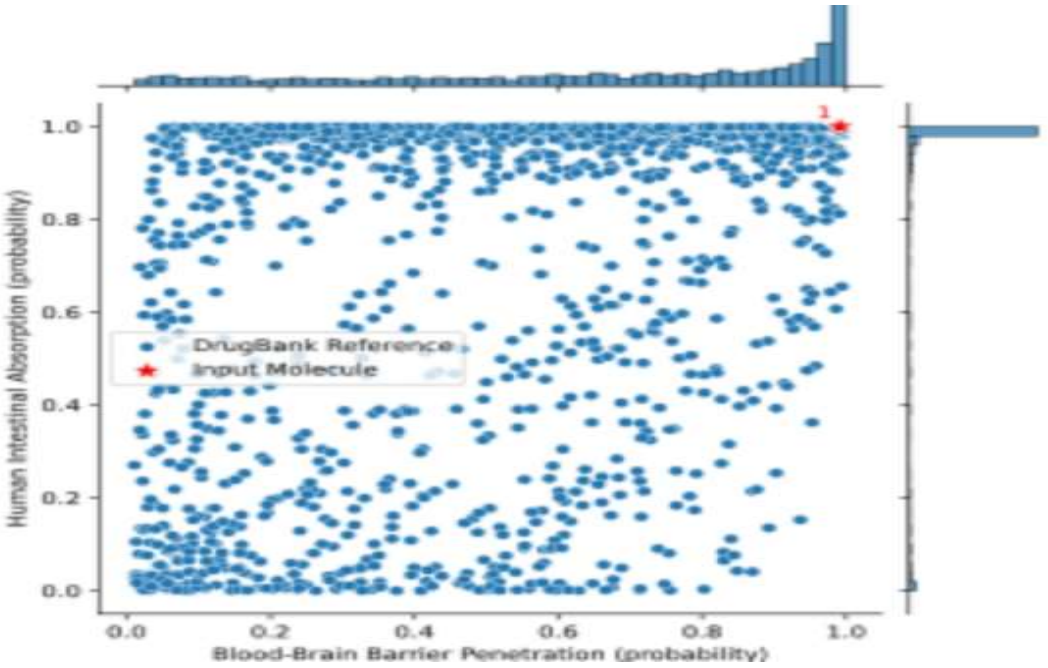


Figure 7: Comparative of BBB permeability and GI absorption of safrole

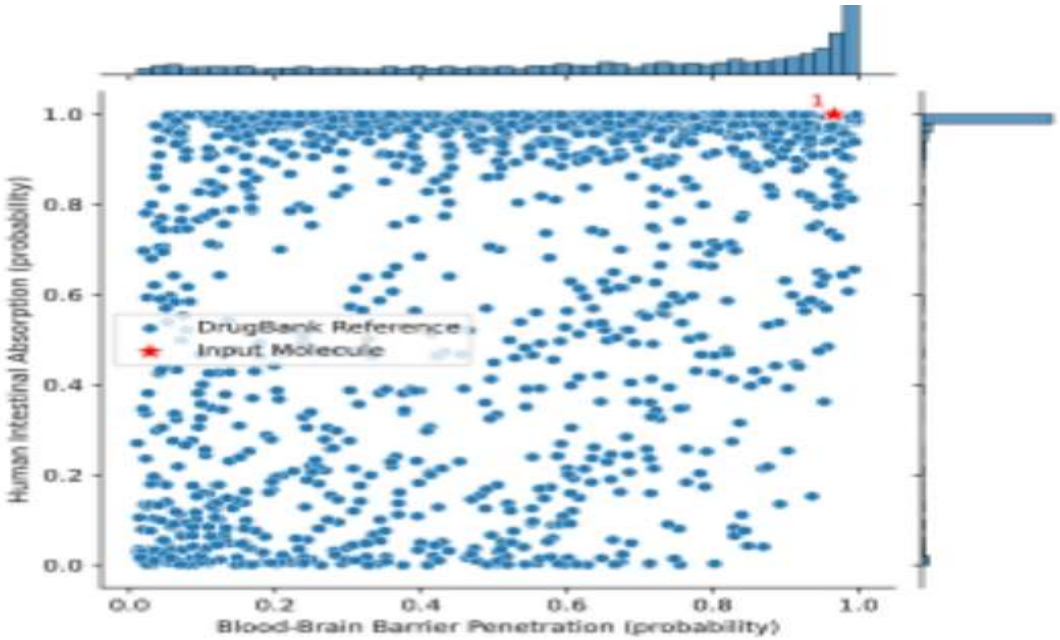


Figure 8: Comparative of BBB permeability and GI absorption of Eugenol Acetate

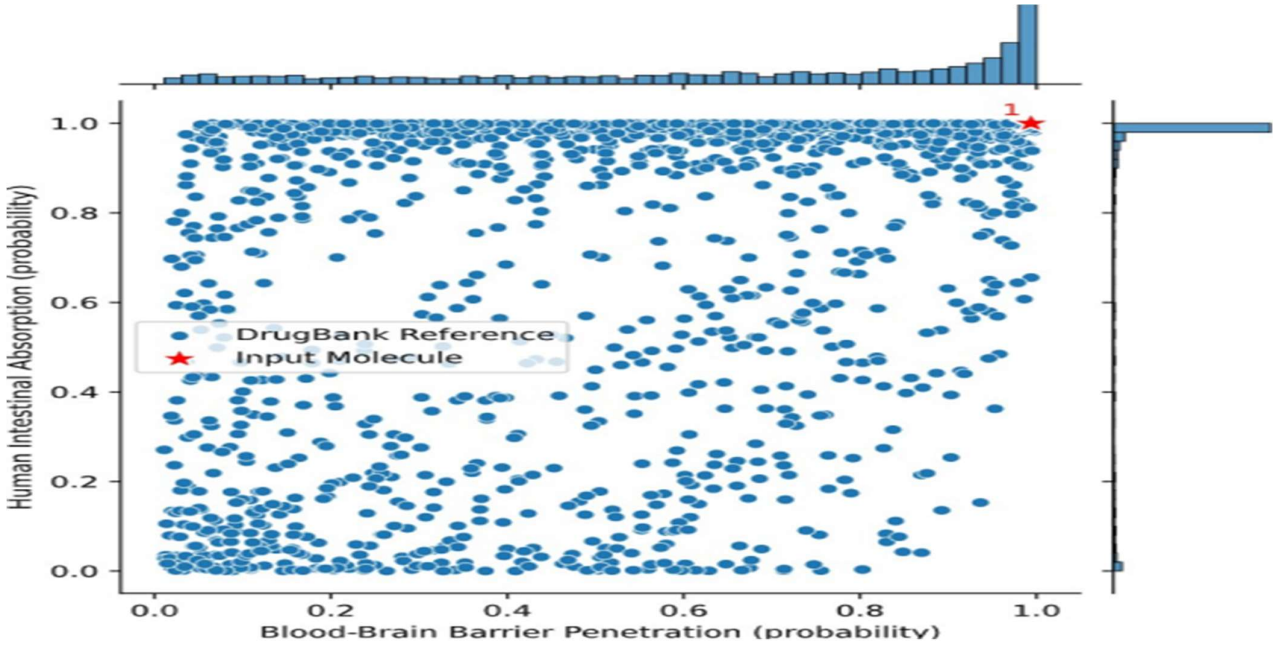


Figure 9: Comparative of BBB permeability and GI absorption of Germacrene D

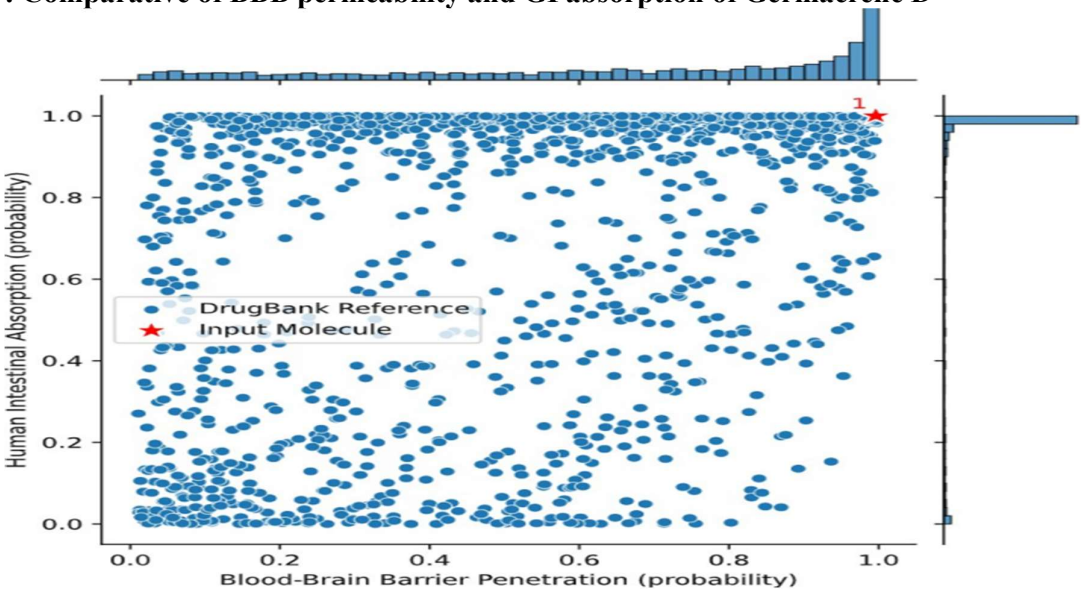


Figure 10: Comparative of BBB permeability and GI absorption of Bicyclogermacrene

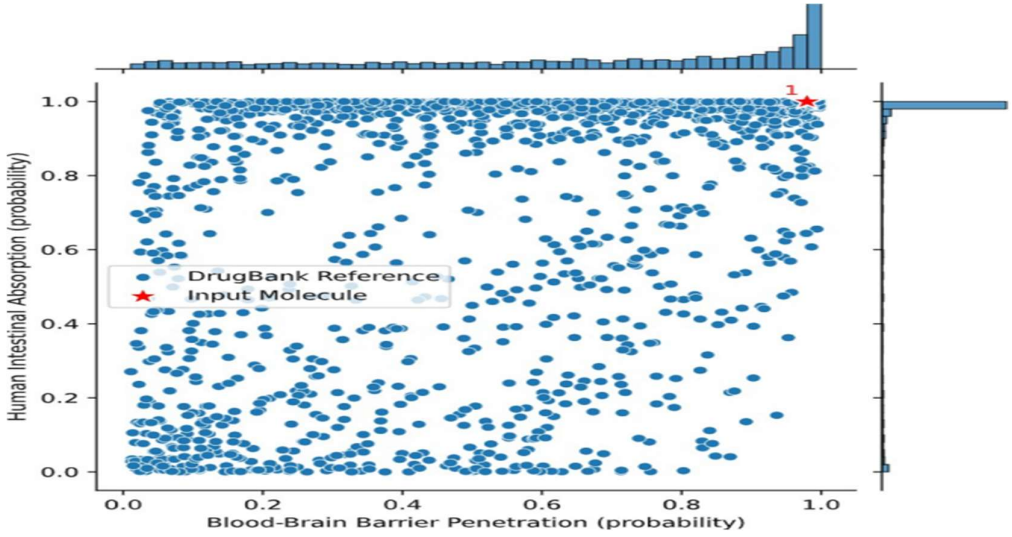


Figure 11: Comparative of BBB permeability and GI absorption of Linalool

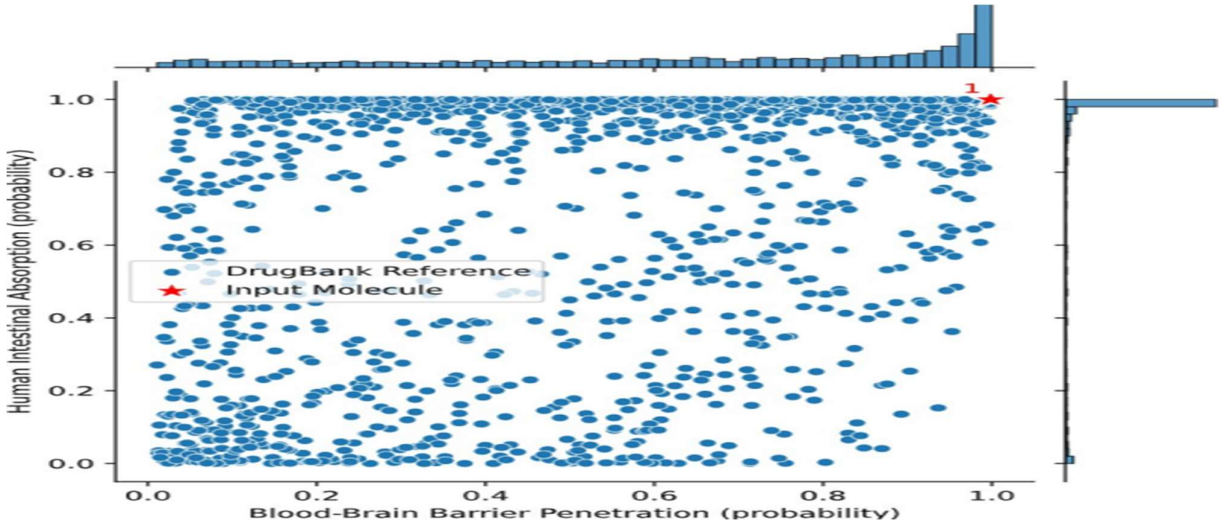


Figure 12: Comparative of BBB permeability and GI absorption of Eucalyptol

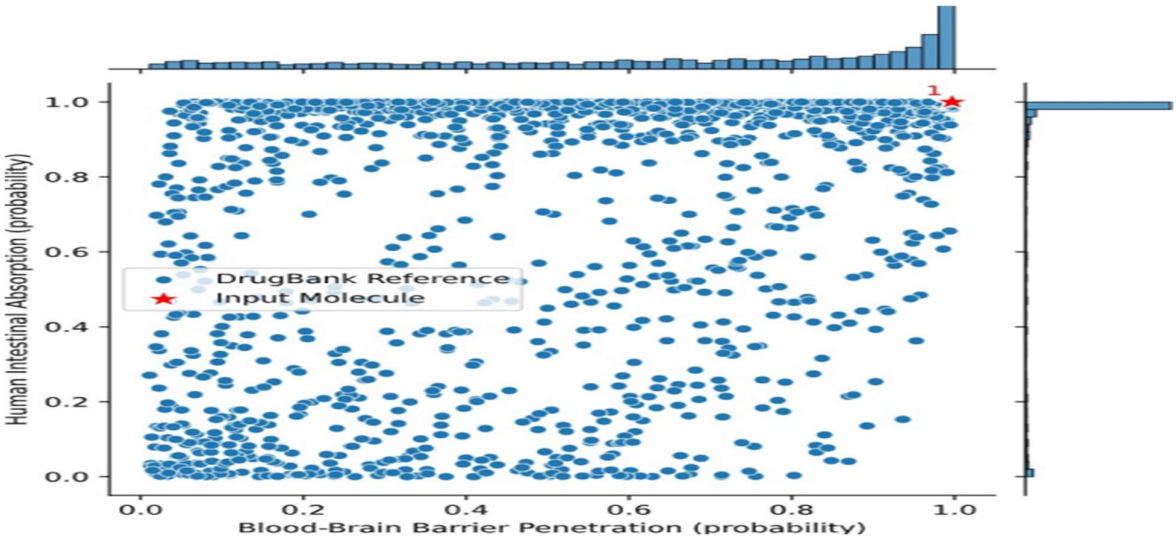


Figure 13: Comparative of BBB permeability and GI absorption of Limonene

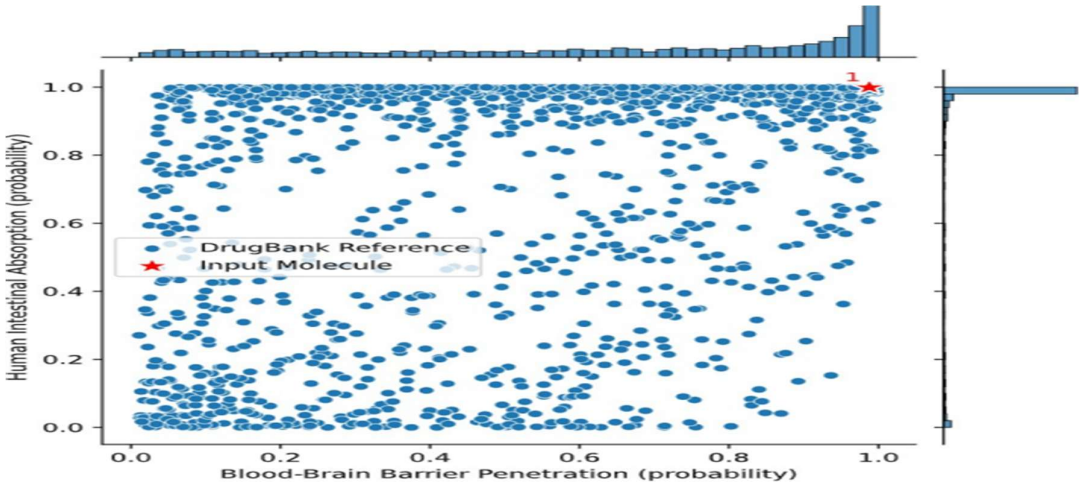


Figure 14: Comparative of BBB permeability and GI absorption of Myrcene

IN VITRO STUDIES:

Table 7: DPPH radical scavenging of PBLEE

Conc (µg/mL)	Abs (Control)	Abs (Extract)	Abs (Std) (Ascorbic Acid)	% Inhibition (Extract)	% Inhibition (Std)
20	0.800	0.396	0.333	43.43 %	58.36%
40	0.800	0.354	0.208	49.43 %	74.06%
60	0.800	0.283	0.173	59.64 %	78.40%
80	0.800	0.239	0.148	65.80 %	81.56%
100	0.800	0.100	0.088	85.73 %	89.07%
IC_{50}	—	—	—	44.82 µg/mL	31.70 µg/mL

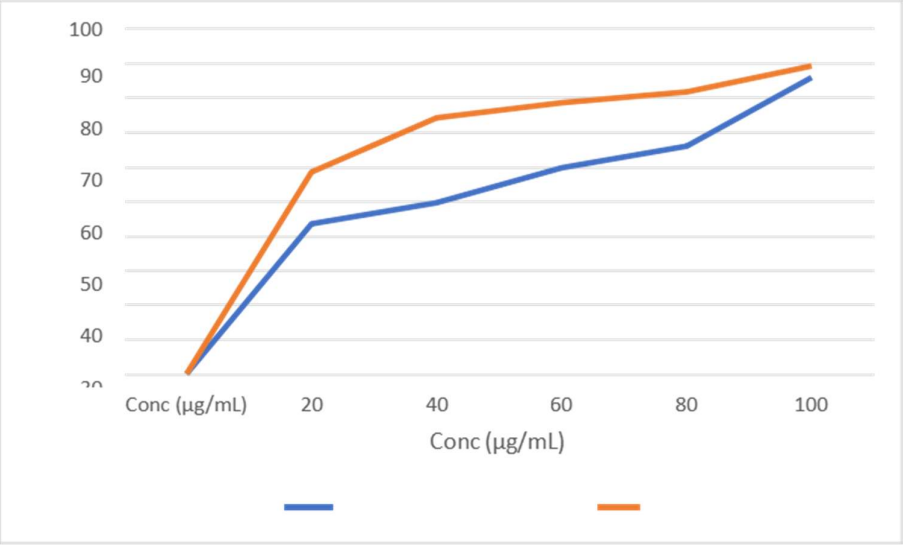


Figure 15: DPPH free radical scavenging

The ethanolic extract of *Piper betle* showed concentration-dependent DPPH radical scavenging activity. Maximum % inhibition was observed at 100 µg/mL (85.73 %), compared to standard ascorbic acid (89.07% at 100 µg/mL). The IC_{50} value of the extract (44.82 µg/mL) indicates moderate antioxidant potency when compared to ascorbic acid (31.70 µg/mL).

The DPPH assay demonstrated that the ethanolic extract of *Piper betle* possesses significant free-radical scavenging activity, which increased with rising concentration. The extract showed notable antioxidant potential, although slightly lower than the standard ascorbic acid.

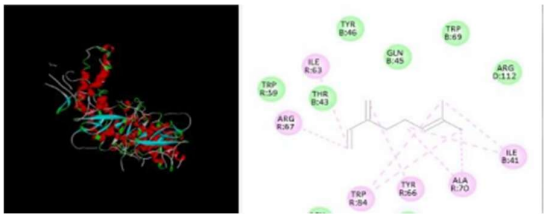
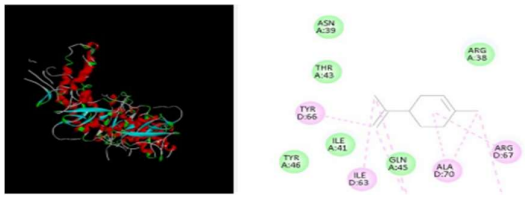
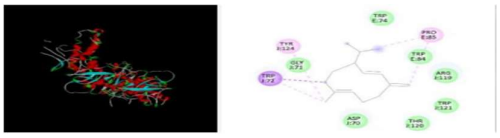
The strong reduction in DPPH absorbance suggests the presence of phenolic compounds, flavonoids, alkaloids, and other phytoconstituents capable of donating hydrogen atoms or electrons to neutralize free radicals. Since oxidative stress plays a major role in inflammation and migraine progression, these antioxidant properties support the plant's therapeutic relevance in anti-migraine activity.

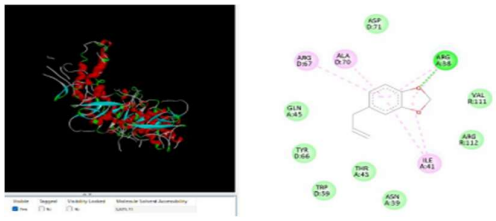
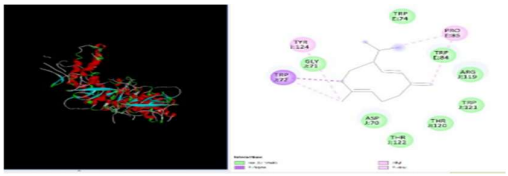
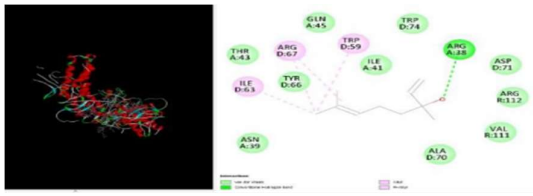
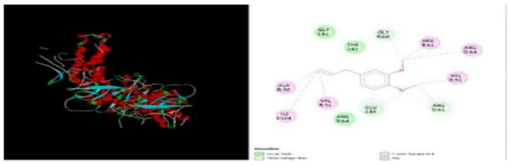
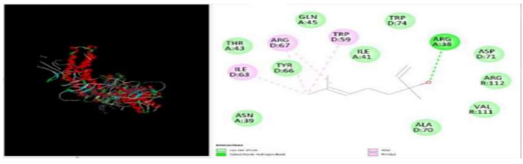
These findings are consistent with reports that phytochemicals in medicinal plants help mitigate oxidative damage and protect biological systems by scavenging reactive oxygen species.

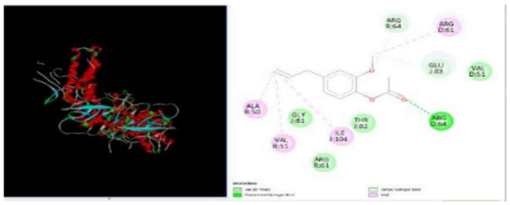
IN SILICO STUDIES

The molecular docking study was performed to evaluate the binding affinity of selected phytoconstituents with the target protein (PDB ID: 3N7P). The compounds were docked into the active site of the protein using standard docking protocols, and their interactions were assessed based on binding energy values. The results obtained from the docking analysis are summarized in the table below, where binding energy (kcal/mol) indicates the strength of ligand–protein interaction.

Table 8: Docking results of *Piper Betle*

S NO	COMPOUND NAME	DOCKING IMAGE PROTEIN – 3N7P	BINDING ENERGY
1	MYRCENE		-4.55
2	LIMONENE		-5.18
3	GERMACRENE		-5.68

4	SAFROLE		-4.56
5	BICYCLOGERMACRENE		-5.77
6	LINALOOL		-4.59
7	METHYL EUGENOL		-5.8
8	EUCALYPTOL		-6.2

9	EUGENOL ACETATE		-6.4

DISCUSSION

The present study was designed to evaluate the neuroprotective and anti-migraine potential of the ethanolic extract of *Piper betle* leaves (PBLEE) using in-silico, in-vitro experimental models.

The phytochemical analysis of PBLEE revealed the presence of several bioactive constituents such as alkaloids, flavonoids, phenols, tannins, glycosides, steroids, and saponins, which are known to possess antioxidant and anti-inflammatory activities. GC–MS profiling further identified major compounds including eugenol, hydroxychavicol, methyl eugenol, β-caryophyllene, and phytol, all of which have been previously reported for their neuroprotective and Vaso modulatory properties. The ADME analysis demonstrated that these compounds possess favourable blood–brain barrier permeability and high gastrointestinal absorption, confirming their potential for central nervous system activity.

The antioxidant potential assessed by DPPH assay confirmed that PBLEE has strong free radical scavenging activity, which is essential to counteract oxidative stress implicated in migraine attacks. The presence of phenolic and flavonoid compounds may contribute to this activity, reducing neuronal hyperexcitability and protecting against oxidative damage to brain tissues.

The above in vitro study suggested that *Piper betle* possesses significant pharmacological potential that may contribute to anti-migraine activity through mechanisms such as antioxidant, anti-inflammatory, and neuroprotective effects. Based on these promising preliminary findings, the present research has been designed to further evaluate and validate the in vivo anti-migraine therapeutic efficacy of *Piper betle*. The study aims to investigate its effectiveness using suitable

animal models of migraine, focusing on behavioural, biochemical, and molecular parameters. This approach will help to establish a scientific basis for its traditional use in migraine management and to elucidate the possible mechanisms of action involved in its neuroprotective and anti-migraine effects.

In the present study, molecular docking analysis was carried out to investigate the interaction of selected phytoconstituents with the target protein (PDB ID: 3N7P). Binding energy values were used as the primary parameter to evaluate ligand–protein affinity, where more negative values indicate stronger and more stable interactions.

Among all the tested compounds, **eugenol acetate (-6.4 kcal/mol)** exhibited the highest binding affinity, followed by **eucalyptol (-6.2 kcal/mol)** and **methyl eugenol (-5.8 kcal/mol)**. These compounds demonstrated significantly stronger interactions with the active site of the protein, suggesting their potential as effective bioactive molecules. The higher binding affinity may be attributed to favorable hydrophobic interactions and possible stabilization within the binding pocket of the protein.

Additionally, **bicyclogermacrene (-5.77 kcal/mol)** and **germacrene (-5.68 kcal/mol)** also showed good binding affinity, indicating moderate stability of the ligand–protein complexes. **Limonene (-5.18 kcal/mol)** displayed intermediate interaction strength, suggesting a comparatively lower but still notable contribution to biological activity.

In contrast, compounds such as **myrcene (-4.55 kcal/mol)**, **safrole (-4.56 kcal/mol)**, and **linalool (-4.59 kcal/mol)** exhibited weaker binding energies, indicating less stable interactions with the protein. This reduced affinity may be due to their simpler molecular structures and limited ability to form strong interactions within the active site.

The observed differences in binding affinity among the compounds can be explained by variations in their chemical structures, functional groups, and molecular conformations. Compounds containing aromatic rings, oxygenated functional groups, or greater structural complexity tend to exhibit enhanced interaction potential through hydrogen bonding, van der Waals forces, and hydrophobic interactions.

Overall, the results indicate that **eugenol acetate, eucalyptol, and methyl eugenol** are the most promising candidates among the selected phytoconstituents based on their superior binding energies. These findings suggest that these compounds may play a significant role in the pharmacological activity of the plant source from which they are derived.

However, it is important to note that molecular docking studies provide only a theoretical prediction of ligand–protein interactions. Therefore, further validation through in vitro and in vivo studies is essential to confirm their biological efficacy, safety, and mechanism of action

CONCLUSION

The present study comprehensively demonstrates that migraine is a complex neurovascular and neuroinflammatory disorder involving multiple interconnected mechanisms such as cortical spreading depression, trigeminovascular activation, neurotransmitter imbalance, and central sensitization. Conventional therapies, although effective, are often associated with adverse effects and limitations in long-term use, highlighting the need for safer and more sustainable alternatives.In this study, the investigation of the ethanolic extract of Piper betle leaf revealed significant pharmacological potential due to the presence of diverse phytoconstituents such as flavonoids, alkaloids, phenols, and tannins, which contribute to its antioxidant and anti-inflammatory properties .

Furthermore, the integration of in vitro,, and in silico approaches strengthened the scientific validity of the findings, particularly through GC-MS analysis and molecular docking studies that supported the interaction of bioactive compounds with migraine-related targets. Overall, the study suggests that Piper betle possesses promising anti-migraine activity and can serve as a potential natural therapeutic agent with fewer side effects. However, further clinical investigations are required to validate its efficacy and safety in humans and to establish standardized dosing regimens for its therapeutic application.

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