

Research Paper

Molecular docking, dynamic simulations, and network pharmacology approach to explore the therapeutic potential of *Zanthoxylum armatum* phytochemicals against Malaria

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Abstract: Malaria continues to be a global health challenge mainly due to drug resistance that necessitates the innovative approaches in pharmaceutical development. Therefore, in the present study, we investigated the therapeutic potential of natural plant-based phytochemicals from *Zanthoxylum armatum* against malaria. Based on pharmacokinetic and ADMET properties analysis, a total of 40 compounds were identified, and these were analyzed and screened in accordance with Lipinski rule of five. The Molecular docking, dynamic simulations, and network pharmacology approach was used to validate the role of selected phytochemicals against malarial target proteins such as PfAMA-1, Pf GST, and PfDHFR. Molecular docking experiments revealed the strong interactions of the three phytochemicals: Cuminal, Sabinene, and Phellandral with the key malarial targets. Molecular dynamic (MD) simulations further confirmed the stability and flexibility of these docked complexes. Furthermore, the network pharmacology analysis identified 23 common targets of these three active phytochemicals and malaria, some of these protein targets include ALB, CXCL8, NOS2, HMOX1, MPO, TNF, IL6, and PTPRC. These proteins are mainly involved in inflammatory response and oxidative stress, the key mechanisms involved in malarial infection, so regulating these targets may prove beneficial in treating *Plasmodium falciparum*-induced malaria. These findings provide strong basis for further *in-vitro* and *in-vivo* studies to validate the role of Cuminal, Sabinene, and Phellandral as substantial drug candidates against malaria.

Keywords: Malaria, *Zanthoxylum armatum*, *Plasmodium falciparum*, Molecular docking, MD simulations, Network pharmacology.

Resumen: La malaria sigue siendo un desafío de salud global principalmente debido al aumento de la resistencia a los medicamentos existentes, lo que resalta la necesidad de enfoques innovadores en el desarrollo de fármacos. Por lo tanto, el presente estudio investiga el potencial terapéutico de los fitoquímicos de origen vegetal de *Zanthoxylum armatum* como agentes antipalúdicos. Basado en el análisis de propiedades farmacocinéticas y ADMET, se identificaron y evaluaron un total de 40 compuestos de acuerdo con la regla de cinco de Lipinski. Se utilizó el acoplamiento molecular, las simulaciones dinámicas y el enfoque de farmacología de red para validar el papel de los fitoquímicos seleccionados contra proteínas diana del paludismo como PfAMA-1, Pf GST y PfDHFR. Los experimentos de acoplamiento molecular revelaron las fuertes interacciones de los tres fitoquímicos; Cuminal, sabineno y phelantral con los objetivos clave de la malaria. Las simulaciones de dinámica molecular (MD) confirmaron además la estabilidad y flexibilidad de estos complejos acoplados. Además, el análisis de farmacología de redes identificó 23 objetivos comunes de estos tres fitocompuestos activos y la malaria, algunos de estos objetivos proteicos incluyen ALB, CXCL8, NOS2, HMOX1, MPO, TNF, IL6 y PTPRC. Estas proteínas están principalmente involucradas en la respuesta inflamatoria y el estrés oxidativo, los mecanismos clave involucrados en la infección por malaria, por lo que regular estos objetivos puede resultar beneficioso en el tratamiento de la malaria inducida por *Plasmodium falciparum*. Estos hallazgos proporcionan una base sólida para estudios adicionales *in vitro* e *in vivo* que validen el papel del Cuminal, sabineno y phelantral como candidatos a fármacos sustanciales contra la malaria.

Palabras clave: Malaria, *Zanthoxylum armatum*, *Plasmodium falciparum*, Acoplamiento molecular, Simulación de dinámica molecular, Farmacología de red.



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INTRODUCTION

Malaria affects 219 million people worldwide, leading to 435,000 deaths annually (Citaristi, 2022). The highest number of malarial cases has been reported in Africa and certain Asian countries and the prevalence of the disease is increasing since 2015 (PAH, 2017a; PAH, 2017b; Basu and Sahi, 2017). *Plasmodium*, the causative agent of malaria, affects humans and accumulates malarial pigment in the form of an insoluble haemoglobin metabolite (Miller *et al.*, 1994; Phillips y Pasvol, 1992). These parasites can be found in various vertebrates, including red blood cells and human tissues. The deadliest and highly pathogenic form of *Plasmodium* is known as *Plasmodium falciparum* which causes malaria in humans (Prugnotte *et al.*, 2011; Daxon, 2019; Ogony, 2021). The main symptoms include chills, fever, and headaches and these symptoms arise primarily due to the destruction of erythrocytes (Tahita *et al.*, 2013; Greenwood, 2019; Fikadu and Ashenafi, 2023).

The mortality rate due to malaria has decreased due to the increased antimalarial drugs that is now safe, efficient, and cost-effective (Warrel *et al.*, 2017). Drugs mainly include quinine, chloroquine, pyrimethamine, sulfonamides, sulfones, and artemisinin derivatives. Two of the commonly used drugs artesunate and sulfadoxine mainly perform their antimalarial action via inhibiting protozoal glutathione S-transferase (GST) and dihydrofolate reductase (DHFR). However, the malaria parasite rapidly modifies itself, thus causing antimicrobial resistance and difficulties in the development of efficient vaccines and drugs (Narwal *et al.*, 2024). Therefore, scientists are striving hard to develop alternative, safer and more cost-effective treatment options against malaria.

Plant-based compounds such as flavonoids, terpenoids, alkaloids, tannins, steroids, resins, phenolic compounds, and other metabolites are frequently used in research related to drug development due to their antimicrobial, antioxidant, anti-inflammatory, antiviral, and anticancer properties (Guadie *et al.*, 2020; Malami *et al.*, 2022). *Zanthoxylum armatum* DC., also known as Indian prickly ash or Nepal pepper, belongs to the Rutaceae family (Sharma *et al.*, 2021). The species typically inhabits valleys and thickets mountains, wastelands, and forests. In traditional systems of medical treatment, *Z. armatum* tree bark, fruits, and seeds were widely used as carminatives, stomach, and anthelmintics, and the dried fruit served as a spice (Barkatullah *et al.*, 2014). Fruit and seeds were also used to treat dyspepsia and fever with an aromatic tonic useful for dental problems, and their ointment is used to treat infestations (Iqbal and Hamayun, 2004). The extract of *Z. armatum* has effective anti-skin sensitivity, loculicidal, anti-inflammatory, antibacterial, antifungal, and cytotoxic activities (Peana *et al.*, 2002; Khan *et al.*, 2008).

Therefore, the present study has been conducted to assess the inhibitory capabilities of 40 phytochemicals that include terpenoids, flavonoids, alkaloids, carbonyls, and coumarins, all sourced from *Z. armatum*. Three proteins / antigens were selected as a target against compounds based on *Z. armatum* and these include *P. falciparum* glutathione S-transferase (Pf GST), *P. falciparum* dihydrofolate reductase (PfDHFR), and *P. falciparum* apical membrane antigen 1 (PfAMA-1). Pf GST

acts as a peroxidase, and it is mainly overexpressed in chloroquine resistance strains. Therefore, it can act as a potential target for drugs that can be used in combination with chloroquine to effectively treat malaria. PfDHFR is mainly responsible for DNA and folate synthesis and various other metabolic pathways in the malarial parasite therefore, its inhibition may lead to suppression of the growth of plasmodium (Shamshad et al., 2022). Furthermore, PfAMA-1 is a type 1 integral membrane protein that consists of 3 domains and is involved in the entry of the parasite into the hepatocytes of the host to evoke the immune response (Obonyo, 2022). Therefore, its binding to cell surface receptors can be inhibited to prevent the entry of this pathogenic antigen inside cells.

In the present study, a pharmacokinetic analysis was performed to assess the drug-like properties and toxic potential of these phytochemicals. Molecular docking was carried out to identify the interactions between these compounds and target proteins from *P. falciparum* involved in the pathogenesis of malaria. The stability of the docked complexes was confirmed through molecular dynamic simulations and the network pharmacology approach was used to further anticipate the toxicity, classification, and bioactivity of the selected phytochemicals.

MATERIALS AND METHODS

Selection of Ligands and Receptors (accessed in April 2024)

The molecular structures of the 40 *Z. armatum*-based compounds (Ahmad, Misra and Gupta, 1993; Tiwary et al., 2007; Dhami et al., 2018) and 2 commonly used antimalarial drugs sulfadoxine (Francis et al., 2020) and artesunate (Khanal, 2021) were obtained from PubChem in SDF format and converted to PDB files using Biovia Discovery Studio v2021 software (ter Kuile et al., 2007; Phuyal et al., 2019). The necessary charges like water molecules that were removed and hydrogen atoms were added were incorporated to prepare the ligands and then saved in PDBQT format for additional analysis and computational assessments. The names, molecular weight, formulas, Pubchem IDs, and structures of the selected ligands are provided in the Supplementary Table 1. Three of the proteins were selected as targets against the 40 *Z. armatum*-based compounds, including Pf GST (PDB ID: 1OKT), Pf DHFR (PDB ID: 7F3Y), and Pf AMA-1 (PDB ID: 1Z40) and their 3D structures were obtained from RCSB PDB.

Subsequently, using Discovery Studio software for protein 3D structure refinements, the ligands and other small molecules or atoms were eliminated from the receptors. Further refinements were made by removing water molecules, adding polar hydrogen atoms, and assigning charges. The final refined receptor structure was saved in PDBQT format, for docking experiments (Figure 1). The canonical SMILES (Simplified Molecular Input Line Entry System) notations for each compound were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>).

Prediction of Activity Spectra for Substances (PASS) Analysis

The Prediction of Activity Spectra for Substances (PASS) analysis program (accessed in April 2025) was used to predict the biological properties, pharmacological characteristics, drug-likeness, potential side effects, and possible mode of action of the selected constituents. These predictions are made based on the structural and active relationships observed with established chemical entities (Khan et al., 2017). PASS Online (<http://www.pharmaexpert.ru/passonline/>), which offers probability of activity (Pa) and inactivity (Pi) for a broad range of pharmacological effects, was used to forecast the possible biological activities of a few chosen phytochemicals. Activities were only deemed noteworthy if their Pa was more than 0.7. Lipinski's Rule of Five, Veber's Rule, and pharmacokinetic properties such as gastrointestinal absorption, blood-brain barrier permeability, P-glycoprotein substrate/inhibitor status, and CYP inhibition profile were assessed using SwissADME (<http://www.swissadme.ch/>) (accessed in June 2024). ADMET-related metrics and toxicity evaluations were further analyzed through ProTox-II (https://tox-new.charite.de/protox_II) (accessed in July 2024), respectively. Each of these tools was chosen for its ability to accurately predict certain parameters that PASS alone does not offer.

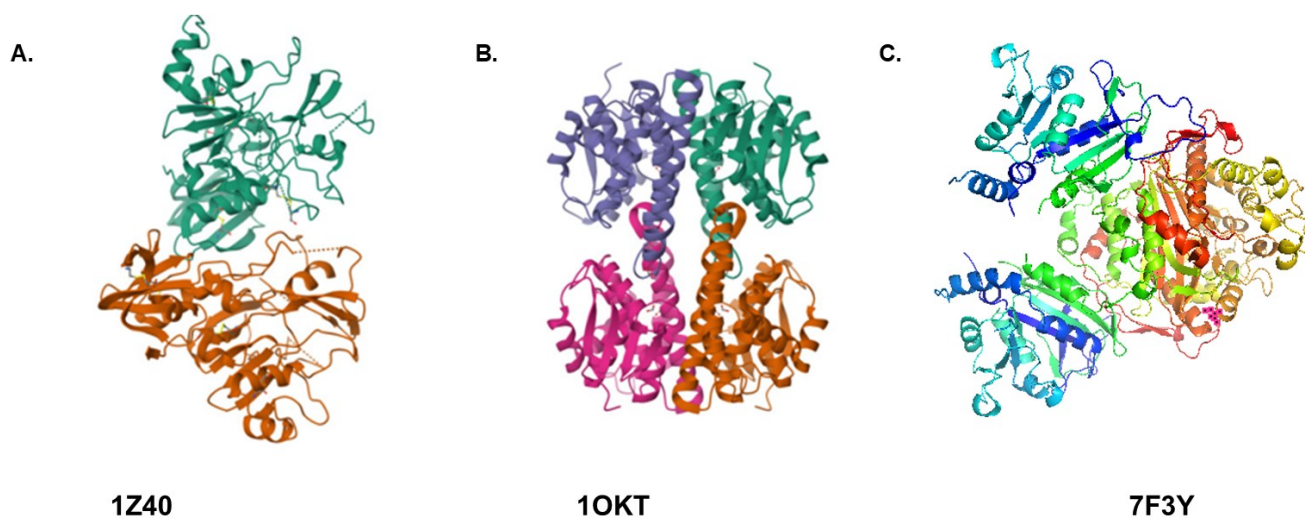


Figure 1. Representation of the 3-Dimensional structure of Malarial receptor proteins at the cell surface. **A.** *Pf* Anti-Malarial Antigen1 (*Pf* AMA-1), **B.** *Pf* Glutathione S-Transferase (*Pf* GST), **C.** *Pf* dihydrofolate reductase-thymidylate synthase (*Pf* DHFR-TS).

Lipinski's Rule of Five (Drug Likeness Analysis)

The pharmacokinetic properties of the selected compounds such as absorption, distribution, excretion, and metabolism were evaluated using Lipinski's rule of five using Molinspirationv2021, (<https://www.molinspiration.com/cgi-bin/properties>) (accessed on May 2024). The determination of Lipinski's rule of five was based on certain predefined criteria, including the logarithm of the partition coefficient between *n*-octanol and water ($\log P \leq 5$), molecular weight ($MW \leq 500$), the number of hydrogen bond donors ($\text{NOH} \leq 5$), hydrogen bond acceptor sites ($\text{NON} \leq 10$), topological polar surface area ($\text{TPSA} \leq 140 \text{ \AA}^2$), and the number of rotatable

bonds (≤ 10). It is important to note that, to maintain bioavailability, an orally active drug should not exceed a single Lipinski violation (Pollastri, 2010). Drug likeness was also evaluated based on the rules of Ghoshe, Veber, Egen, and Muegge (Arunkumar et al., 2018). The drug-like potential of a compound is the assessment of oral bioavailability and ADMET properties (Kumari et al., 2019). According to Muegge's rule, certain ranges of H-bond donors and acceptors can be used to suggest drug-like effects, the Egen rule is a method for predicting oral bioavailability using molecular polar surface area (PSA $\times 131 \times 2$) and Log P $\times 5.88$, the Ghosh's rule-based approach that emphasizes on molecular weight (MW < 480), Log P (1-3), and atom count (20-70) and in Veber's Rule, 10 rotatable bonds and 140 polar surface areas (PSA) are recommended to maximize oral bioavailability (Ndombera et al., 2019). Additional filters were employed to improve prediction accuracy. Veber's Rule highlights the significance of molecular flexibility and polarity, suggesting a maximum of 10 rotatable bonds and a polar surface area (PSA) of no more than 140 Å². Ghose's Rule highlights ideal physicochemical characteristics, indicating a molecular weight range of 160–480 Da, LogP from -0.4 to 5.6, molar refractivity between 40 and 130, and a total atom count between 20 and 70. Egan's filter employs PSA ≤ 131 Å² and LogP ≤ 5.88 to anticipate membrane permeability. Muegge's Rule combines several parameters such as MW (200–600), LogP (-2 to 5), H-bond donors (≤ 5), H-bond acceptors (≤ 10), and rotatable bonds (≤ 15) to recognize drug-like entities. Through the incorporation of these guidelines, compounds were evaluated to confirm advantageous pharmacokinetic properties and a high likelihood of success as orally active therapeutics.

Bioactivity Score Prediction

The bioactivity score indicates the likelihood that the studied complex could act as a potential drug (accessed on June 2024). The bioactivity score of phytochemicals against malarial target proteins was determined by a computational tool called Molinspiration version 2021.13. The structures were prepared through different options, and their physicochemical features and pharmacokinetic descriptors were calculated. The software indicates these 3 parameters; active, moderately active and inactive that if the score is higher than 0.0, the compound is active; if it is between -0.5 and 0.0, it is moderately active; and if it is less than -0.5, it is inactive (Jindal and Rani, 2023).

Toxicity Risk Assessment

The study provides an assessment of the anticipated adverse effects of a pharmaceutical compound on human health (Barile, 1996). The Protox-II server was used to evaluate the toxicological properties of phytochemicals found in *Z. armatum*, including aspects such as hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, and tumorigenicity. Toxicity categorizations were established in alignment with the criteria of the Global Harmonized System (GHS) criteria for the classification and labelling of chemical compounds. The LD50 classes of the active

compounds were also evaluated. The LD50 value is the dose of a drug or bioactive compound that kills 50% of the test substances (Banerjee et al., 2018).

Pharmacokinetic Activity Prediction

Using the online tool Swiss-ADME, predictive modelling was employed to assess the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of all phytochemical compounds and antimalarial drugs under investigation. The ADMET analysis focusses on blood-brain barrier (BBB) permeability, tissue distribution, gastrointestinal absorption, P-glycoprotein (P-gp) substrate, metabolic effects as an inhibitor of CYP3A4, and fluidity pertinent to plasma membrane absorption (Muhammed et al., 2021). PKCSM was used to evaluate the properties of pharmacokinetics (https://biosig.lab.uq.edu.au/pkcsm/prediction_single/).

Molecular Docking (accessed in August 2024)

The ligands were docked with receptors using AutoDock version 1.5.6. (Huey et al., 2012). AutoDock vina was used to automatically calculate the grid values. In this research, the Grid box size was 40×40×40 (x, y and the z) and spacing was 0.375 for 3 receptors. Docking was performed using the Lamarckian Genetic Algorithm with an exhaustiveness value of 8 and a maximum of 9 binding modes. The energy range was set to 4 kcal/mol, and the number of evaluations was adjusted to the default settings of AutoDock Vina. For PfAMA-1 (PDB ID:1Z40), the center of the grid box was set at the position 11.657, 12.407, and 63.734 for x, y, and z, respectively. For PfGST (PDB ID: 1OKT) the center was set at the position 50.718, 42.107, and -8.602 for the x, y and z axes, respectively. For PfDHFR (PDB ID: 7F3Y) the grid center was set at 1.265, -1.860, and -23.712 for x, y, and z, respectively. The configuration file containing Grid box values for each ligand was obtained and saved in txt. format. The output was obtained by generating a command or code and saved in PDBQT format. The results showed the binding affinity of ligand to the receptor that was Gibbs free energy in kcal mol⁻¹. The highest negative value was considered as the highest binding affinity for receptor proteins. For the above 50 values, the best fit was selected. All profiles were displayed in Discovery Studio and a 2D output image showing the interaction of the ligand with the receptor's amino acids was obtained (Vong et al., 2022).

Molecular Dynamic Simulations (accessed in June 2025)

Molecular dynamic simulations were performed to study the interactions and movements of docked complexes using the accelerated central processing unit (CPU) platform via GROMACS software (van der Spoel et al., 2005). The system was prepared by charm 36-jul 2022. ff. The docked complexes were separated using Chimera into protein and ligand. For proteins, the additional dock prep with library was done by Dunbrack 2010 rotamers, and for PfDHFR-TS and PfGST protein complexes, the Richardson rotamer library was used. For the analysis of the PfAMA-1 protein and the prepared ligand, GROMACS files were used from the Swiss Institute of Bioinformatics web server were used (Zoete et al., 2011). The

resulting input files were downloaded as GROMACS files. The initial position of the particle was assigned using GROMACS '.gro' files, while the topologies were added in interactions between particles. Long range electrostatic interactions were implemented using particle mesh Ewald method, with cutoff distance of 1.2 nm for van der Waals interactions. The equilibrium was continued in an isothermal-isobaric ensemble maintaining a temperature of 300K for a 100 ns duration. The simulations were executed in the MD integrator with a 2fs time step. For trajectory analysis, MD analysis packages were used to compute the root mean square fluctuation (RMSF), radius of gyration (Rg), and root mean square deviation (RMSD) of both the protein and protein-ligand complexes. These parameters showed deviations and stability of the protein and ligand complexes in the simulation. These dynamics were conducted in GROMACS.

Network Pharmacology Approach (accessed in September 2024)

Screening of the Potential Targets of Selected Compounds

The data of potential targets of active compounds were retrieved from Swiss Target Prediction (<http://www.swisstargetprediction.ch/>) by entering the canonical SMILES and specifying the species as homo sapiens. However, malarial targets were downloaded from GeneCard (<http://www.genecards.org/>) and DesGenet (<http://www.disgenet.org/>). The targets of these databases were merged, and repetitions were removed. The common names of the targets were also searched from UniProtKB (<https://www.uniprot.org/>). The mutual targets of the selected compounds and the malaria were achieved, and Venn diagram was constructed using the bioinformatics tool (<https://bioinformatics.psb.ugent.be/webtools/Venn>) (Tabassum et al., 2022).

Construction of Compound Target Network

Cytoscape V3.10.1 was used (<https://cytoscape.org/>) to build the compound-targets network to study the interactions among active compounds within the intricate biological system. In this network, the nodes represent the chemical constituents and targets, while the edges represent their interactions. The network analyzer function was employed to assess the basic characteristics of the network. Subsequently, the network was filtered based on the "degree," which indicates the number of connected nodes linked to a specific network node as a node attribute (Ram et al., 2023).

Prediction of Protein–Protein Interaction Network and Hub Genes

The STRING database (<https://stringdb.org/>) was used to evaluate the protein-protein interaction of 340 common genes, with the organism specified as "*Homo sapiens*". Cytoscape V3.10.1 was utilized to visualize the protein-protein interaction network. Identification of hub genes and nodes with elevated degrees within the network was carried out using the CytoHubba plugin (Tao et al., 2013).

Construction of target–compound–pathway network

The DAVID database (<https://david.ncifcrf.gov/tools.jsp>) provided the data for the analysis of the top hub genes, and a network was created to examine the mechanism within these pathways (Tabassum et al., 2022).

Gene Ontology and KEGG Pathway Analysis

The DAVID functional gene annotation resource database (<http://david.ncifcrf.gov/>) was used to conduct Gene Ontology and KEGG pathway analyses for the "*Homo sapiens*". It categorized gene functions into biological processes (BP), cellular components (CC), and molecular functions (MF) using Gene Ontology analysis, as well as performing enrichment pathway analysis for KEGG pathways. To visualize the results, top 20 GO annotations (BP, CC, and the MF) and KEGG pathways were selected using a cutoff method with a probability score below 5×10^{-2} . The bars and lollipop plots were created using Shiny GO (<http://bioinformatics.sdstate.edu/go/>) for the selected annotations and pathways (Pomazny et al., 2018).

RESULTS

Lipinski's Rule of Five (Drug Likeness Analysis)

This analysis provided the phytochemical properties of 40 phytoconstituents of *Z. armatum* (Shah et al., 2024). Tambuletin exhibited more than 3 violations while Lupeol, α -Amyrins, Nerol, *allo*-Aromadenderene, (*E*)-Nerolidol and β -caryophyllene showed 1 violation. The remaining compounds did not show any violations of Lipinski's rule (Supplementary Table 2). An ideal compound should not contain more than one violation. The results of the Ghoshe, Veber, Egen, and Muegge rules are provided in the (Supplementary Table 3).

Bioactivity Score Prediction

Prediction of the bioactivity score showed that the score is higher than 0.0, the compound is active; if it is between -0.5 and 0.0, it is moderately active; and if it is less than -0.5, it is inactive. Cuminal (-0.44), Sabinene (-0.33), and Phellandral (-0.04) are important moderately active ion channel modulators, while Phellandral also revealed the potential to act as inactive nuclear receptor ligand (-1.50) and a moderately active enzyme inhibitor (-0.27). Lupeol is an active GCPR ligand

(0.27), nuclear receptor inhibitor (0.85), protease inhibitor (0.15), and enzyme inhibitor (0.52) but is moderately active as a kinase inhibitor (-0.42). α -Amyrins and Tambuletin are active GPCR ligands (0.22, 0.10), nuclear (0.79,0.16), protease (0.19, 0.24), and enzyme inhibitors (0.60, 0.36), but moderately active ion channel modulators (-0.02, -0.04) and kinase inhibitors (-0.41, -0.18). The α -Terpinene is an active ion channel modulator (0.24), nuclear receptor inhibitor (0.24), protease inhibitor (0.52), enzyme inhibitor (0.11) and kinase inhibitor (0.29) but an inactive GPCR ligand (-0.96). Pyrimethamine and sulfadoxine are inactive nuclear-receptor ligands (-0.62). Details of all other phytochemicals are presented in (Table 1).

Table 1. Bioactivity Score prediction of phytochemicals of *Z. armatum*.

Phytoconstituents	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme Inhibitor
α -Fenchol	-0.56	-0.46	-1.71	-0.80	-0.67	-0.59
α -Terpinene	-0.96	0.24	0.29	0.24	0.52	0.11
α -Thujene	-0.86	-0.37	-1.73	-0.86	-0.82	-0.48
α -Thujone	-0.75	-0.50	-1.63	-1.08	-0.64	-0.53
α -pinene	-0.48	-0.43	-1.50	-0.62	-0.85	-0.34
α -Terpineol	-0.51	0.15	-1.45	-0.02	-0.78	0.14
β -Terpineol	-0.63	0.23	-1.60	-0.21	-0.92	-0.07
Camphor	-0.79	-0.56	-2.12	-1.21	-0.95	-0.52
Citral	-0.86	-0.25	-0.29	-0.42	-0.57	0.02
Citronellol	-0.81	-0.24	-1.16	-0.61	-0.83	-0.12
Citronellal	-0.83	-0.08	-1.30	-0.61	-0.50	-0.03
1'8-cineole	-0.93	0.01	-1.60	-1.07	-0.90	-0.15
Geraniol	-0.60	0.07	-1.32	-0.20	-1.03	0.28
γ -terpinene	-0.90	-0.24	-1.37	-0.33	-1.55	-0.07
Limonene	-0.91	-0.27	-2.01	-0.34	-1.38	-0.21
Linalool	-0.73	0.07	-1.26	-0.06	-0.94	0.07
Nerol	-0.60	0.07	-1.32	-0.20	-1.03	0.28
P-cymene	-1.18	-0.61	-1.40	-1.21	-1.42	-0.78
Piperitone	-0.99	-0.60	-2.12	-0.57	-0.96	-0.08
Sabinene	-1.15	-0.33	-1.79	-0.69	-0.78	-0.60
Tagetonol	-0.76	-0.13	-1.75	-0.28	-0.47	0.01
Terpinen-4-ol	-0.56	-0.04	-1.68	-0.20	-0.92	0.06
(Z)-linalool oxide	-0.67	-0.31	-0.95	-0.34	-0.93	0.12
allo-Aromadendrene	-0.67	-0.47	-0.98	-0.21	-0.67	-0.30
(E)-nerolidol	-0.17	0.21	-0.64	0.42	-0.43	0.39
α -Amyrins	0.22	-0.02	-0.41	0.79	0.19	0.60
lupeol	0.27	0.11	-0.42	0.85	0.15	0.52
Cis-ocimene	-0.98	-0.08	-1.26	-0.49	-1.24	0.06
Cuminal	-1.15	-0.44	-1.22	-0.86	-1.48	-0.64
Cinamaldehyde	-1.15	-0.44	-1.22	-0.86	-1.48	-0.64
Phellandral	-0.77	-0.07	-1.50	-0.36	-0.97	-0.27
2-tridecanone	-0.54	-0.18	-1.02	-0.41	-0.56	-0.07
Umbelliferon	-1.22	-0.72	-1.30	-0.92	-1.30	-0.35
Bergapten	-0.16	-0.20	-0.45	-0.03	-0.28	-0.35
3,5-diacetylTambulin	-0.21	-0.15	-0.16	-0.09	-0.32	0.14
Kaempferol	-0.10	-0.21	0.21	0.32	-0.27	0.26

Tambulin	-0.18	-0.16	0.11	0.10	-0.27	0.17
Tambuletin	0.10	-0.04	-0.18	0.16	0.24	0.36
Artesunate	0.08	0.04	-0.20	0.35	0.15	0.31
Sulfadoxine	0.26	-0.21	0.16	-0.62	-0.18	0.10

Pharmacokinetic Properties

The pharmacokinetic assessment of potential compounds includes essential factors that evaluate their performance in the human body. Gastrointestinal (GI) absorption signifies the likelihood of oral bioavailability, and strong GI absorption enhances the effectiveness of oral delivery. Blood-brain barrier (BBB) permeability indicates a compound's capacity to reach the central nervous system, which is vital for medications aimed at neurological disorders. The classification of a compound as a P-glycoprotein (P-gp) substrate dictates whether it can be expelled from cells, which may influence its absorption and distribution. Furthermore, the inhibition of cytochrome enzymes specifically CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 can affect the metabolism of other medications, raising potential concerns regarding drug-drug interactions and toxicity. The skin permeability coefficient (Log Kp) also sheds light on the likelihood of transdermal absorption, with more negative values signifying reduced permeability. Collectively, these factors offer a thorough insight into a compound's ADME (Absorption, Distribution, Metabolism, and Excretion) profile, critical for evaluating its drug-likeness and therapeutic potential.

The results revealed that β -caryophyllene 0.733, Lupeol 0.72, Tambulin, Tambuletin 0.7, Kaempferol 0.7, and 3,5-Diacetyltumbulin 0.7 were unable to cross the blood-brain barrier (BBB), while the remaining phytochemicals showed permeability to the BBB. Tambuletin showed positive responses against P-gp substrate and the remaining compounds exhibited negative results. Most of the substances were expected to inhibit the various classes of cytochromes (CYP450, CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4) for continuous plasma concentrations and increased bioavailability (Table 2). Among these results Tambuletin show positive response to p-gp substrate and show only favorable BBB permeability but lack p-gp substrate activity, making them strong candidate for further *in vivo* evaluation.

Toxicity Assessment Analysis

Using the Protox-II server the drug-likeness and toxicity potential of different ligands were estimated. The criteria for parameters are; High risk values is any probability > 0.8 in mutagenicity, hepatotoxicity, immunotoxicity, cytotoxicity, or carcinogenicity and $LD_{50} < 300$ mg/kg (Class I–III). Moderately risk probability includes 0.6-0.8 and LD_{50} between 300-200mg/kg (Class IV). Low risk includes < 0.6 and $LD > 2000$ mg/kg (Class V-VI). 10 compounds; α -Fenchol (0.96), α -Terpineol (0.96), (*Z*)-Linalool oxide (0.96), *allo*-Aromadendrene (0.96), β -Caryophyllene (0.96), α -Amyrins (0.98), 3,5-Diacetyltambuletin (0.90), Tambulin (0.98) and Tambuletin (0.87) were proven highly immunotoxic and Lupeol (0.57) was slightly immunotoxic. (*Z*)-linalool oxide (0.69), *allo*-Aromadendrene (0.69), β -Caryophyllene (0.69) were slightly hepatotoxic and Cinnamic aldehyde (0.72) and Bergapten (0.75) were mutagenic. Tambuletin was slightly cytotoxic (0.50) while Bergapten (0.84) showed carcinogenic properties and Umbelliferon (0.64) and P-Cymene (0.64) were also slightly carcinogenic. Sulfadoxine was also slightly carcinogenic (0.52) (Table 3).

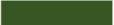



Table 2. Pharmacokinetics properties of phytochemicals of *Z. armatum*

Phytoconstituents	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log Kp
α-Fenchol	High	Yes	No	No	No	No	No	No	-4.99 cm/s
α-Terpinene	Low	Yes	No	No	No	No	No	No	-4.11 cm/s
α-Thujene	Low	Yes	No	No	No	No	No	No	-5.11 cm/s
α-Thujone	High	Yes	No	No	No	No	No	No	-5.62 cm/s
α-pinene	Low	Yes	No	No	No	Yes	No	No	-3.95 cm/s
α-Terpineol	High	Yes	No	No	No	No	No	No	-4.83 cm/s
β-Terpineol	High	Yes	No	No	No	No	No	No	-5.45 cm/s
Camphor	High	Yes	No	No	No	No	No	No	-5.67 cm/s
Citral	High	Yes	No	No	No	No	No	No	-5.08 cm/s
Citronellol	High	Yes	No	No	No	No	No	No	-4.52 cm/s
Citronellal	High	Yes	No	No	No	No	No	No	-4.88 cm/s
1'8-cineole	High	Yes	No	No	No	No	No	No	-5.30 cm/s
Geraniol	High	Yes	No	No	No	No	No	No	-4.11cm/s
γ-terpinene	High	Yes	No	No	No	No	No	No	-4.70 cm/s
Limonene	High	Yes	No	No	No	No	No	No	-3.94 cm/s
Linalool	Low	Yes	No	No	No	Yes	No	No	-3.89 cm/s
Nerol	High	Yes	No	No	No	No	No	No	-5.13cm/s
P-cymene	High	Yes	No	No	No	No	No	No	-4.71cm/s
Piperitone	Low	Yes	No	No	No	No	Yes	No	-4.12 cm/s
Sabinene	High	Yes	No	No	No	No	No	No	-5.71cm/s
Tagetonol	Low	Yes	No	No	No	No	No	No	-4.94 cm/s
Terpinen-4-ol	Low	Yes	No	No	No	No	No	No	-3.94 cm/s

Table 3. LD50 values and toxicity potential of phytochemicals of *Z. armatum*.

Ligands	LD50 potential	Toxicity class	Mutagenicity	Hepatotoxicity	Immunotoxicity	Cytotoxicity	Carcinogenicity
α -Fenchol	1190 mg/kg	Class 4	0.97	0.69	0.96	0.93	0.62
α -Terpinene	1680 mg/kg	Class 4	0.85	0.78	0.95	0.85	0.75
α -Thujene	5000 mg/kg	Class 5	0.78	0.86	0.98	0.73	0.55
α -Thujone	500 mg/kg	Class 4	0.86	0.76	0.99	0.67	0.62
α -Pinene	3700 mg/kg	Class 5	0.93	0.86	0.99	0.75	0.60
α -Terpineol	1190 mg/kg	Class 4	0.97	0.69	0.96	0.93	0.62
β -Terpineol	2000 mg/kg	Class 4	0.9	0.75	0.98	0.89	0.75
Citral	500 mg/kg	Class 4	0.98	0.69	0.99	0.82	0.88
Camphor	775 mg/kg	Class 4	0.94	0.72	0.96	0.61	0.68
Citronellal	2420 mg/kg	Class 5	0.99	0.70	0.99	0.82	0.83
Citronellol	3450 mg/kg	Class 5	0.96	0.84	0.99	0.86	0.65
1,8-cineole	2480 mg/kg	Class 5	0.96	0.86	0.99	0.75	0.68
Cis-ocimene	113 mg/kg	Class 3	0.90	0.83	0.99	0.75	0.51
Geraniol	2100 mg/kg	Class 5	0.97	0.79	0.99	0.85	0.76
γ -terpinene	2500 mg/kg	Class 5	0.92	0.83	0.98	0.82	0.60
Limonene	4400mg/kg	Class 5	0.97	0.76	0.95	0.82	0.65
Linalool	2200mg/kg	Class 5	0.95	0.76	0.99	0.82	0.64
Nerol	2100mg/kg	Class 5	0.97	0.79	0.99	0.85	0.76
P-cymene	3 mg/kg	Class 1	0.98	0.87	0.99	0.89	0.67
Piperitone	2450 mg/kg	Class 5	0.93	0.64	0.92	0.87	0.79
Sabinene	5000 mg/kg	Class 5	0.82	0.81	0.51	0.71	0.59
Tagetonol	2500 mg/kg	Class 5	0.87	0.68	0.99	0.83	0.54
Terpinen-4-ol	1016 mg/kg	Class 4	0.83	0.80	0.99	0.88	0.72S

(Z)-linalool oxide	1190 mg/kg	Class 4	0.97	0.69	0.96	0.93	0.62
allo-Aromadendrene	1190 mg/kg	Class 4	0.97	0.69	0.96	0.93	0.62
β-caryophyllene	1190 mg/kg	Class 4	0.97	0.69	0.96	0.93	0.62
(E)-nerolidol	5000 mg/kg	Class 5	0.91	0.81	0.99	0.81	0.65
α-Amyrins	70000 mg/kg	Class 6	0.92	0.75	0.98	0.95	0.63
lupeol	1190 mg/kg	Class 4	0.95	0.91	0.57	0.97	0.63
Cuminal	1320 mg/kg	Class 4	0.97	0.71	0.96	0.89	0.52
Cinamaldehyde	1850 mg/kg	Class 4	0.72	0.70	0.98	0.92	0.71
Phellandral	2300 mg/kg	Class 5	0.93	0.66	0.97	0.82	0.77
2-Tridecanone	5000 mg/kg	Class 5	0.97	0.69	0.99	0.73	0.63
Umbelliferon	10000 mg/kg	Class 6	0.83	0.68	0.97	0.68	0.64
Bergapten	8100 mg/kg	Class 6	0.75	0.79	0.83	0.85	0.84
3,5-diacetylTambulin	5000 mg/kg	Class 5	0.68	0.73	0.90	0.76	0.54
Kaempferol	3919 mg/kg	Class 5	0.52	0.68	0.96	0.98	0.72
Tambuletin	5000 mg/kg	Class 5	0.77	0.83	0.98	0.50	0.93
Tambulin	3919 mg/kg	Class 5	0.77	0.70	0.87	0.66	0.63
Artesunate	1000 mg/kg	Class 4	0.63	0.76	0.87	0.87	0.65
Sulfadoxine	5200 mg/kg	Class 6	0.72	0.50	0.99	0.93	0.52

	Strongly non-toxic
	Less non-toxic
	Less toxic
	Strongly toxic

Analysis of LD50 values revealed that α -Amyrins shows 70000mg/kg and class VI, Bergapten and Umbelliferon have high LD50 values (8100 and 10000 mg/kg) therefore, belong to class 6 that is nontoxic, Sabinene have 5000mg/kg and Phellandral have 2300mg/kg are categorized in class 5. Cuminal is placed in class 4 that could be harmful if swallowed have value of 1850mg/kg and P-Cymene belongs to class 1 have value 3mg/kg that is highly toxic. The details of all the compounds are presented in Table 3.

Based on pharmacokinetics analysis, various compounds that showed more than one Lipinski's violations, less bioactivity score and toxic potential were filtered out and the remaining compounds were selected for the molecular docking studies. The schematic representation of the study is shown in graphical abstract.

Molecular Docking

After the initial analysis, 21 phytochemicals were selected for further analysis and docked with 3 target proteins involved in the pathogenesis of malaria with the help of AutoDock vina v1.5.6. PfAMA-1 (1Z40) showed the highest binding affinities with Cuminal (-5.9Kcal/mol), Pf GST (1OKT) exhibited the highest binding interaction with Sabinene (-7.1 Kcal/mol), while PfDHFR (7F3Y) showed the highest binding affinities with Phellandral (-5.5 Kcal/mol) (Tables 4, 5 and 6; Figures 2 and 3).

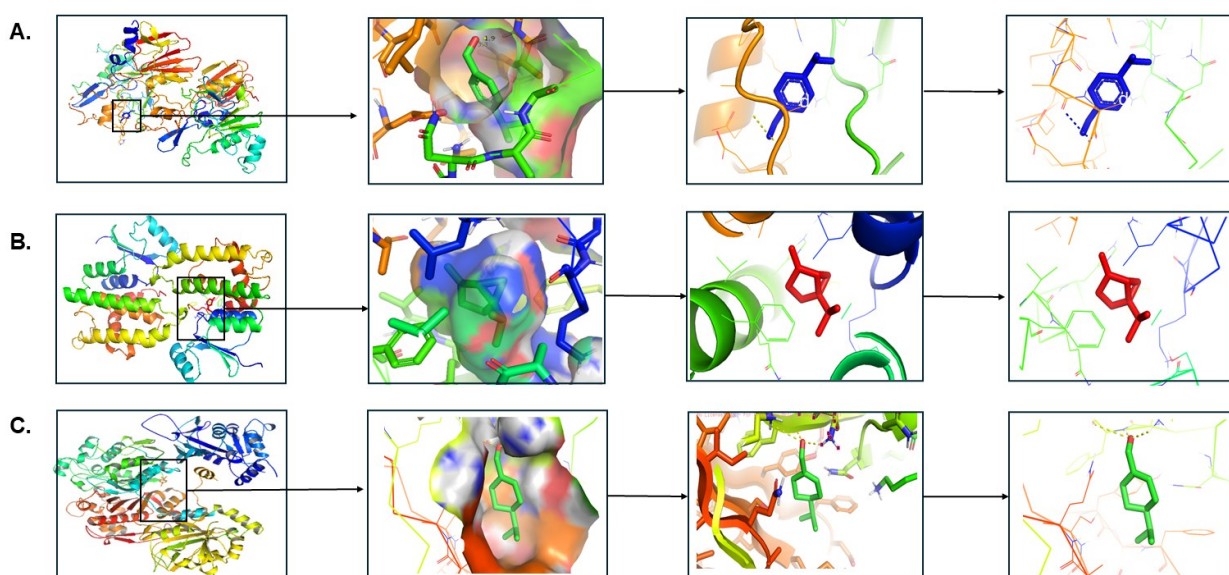


Figure 2. Different interactive docked poses of the selected phytochemical from *Z. armatum*. **A.** Pf Anti-Malarial Antigen1(Pf AMA1) with Cuminal, **B.** Pf Glutathione S-Transferase (Pf GST) with Sabinene **C.** Pf dihydrofolate reductase-thymidylate synthase (Pf DHFR-TS) with Phellandral.

Cuminal shows 3 amino acid interaction GIU350 (Conventional hydrogen bonding), PRO350 (Pi-Alkyl) And GLU365 (Pi-Anion). Sabinene shows an interaction with ALA164, LEU18, VAL103, HIS107, PHE100, LYS15 (Conventional

hydrogen bonding). Phellandral shows interactions with PHE499, TYR497(both with Pi-Alkyl) and PHE354 (Conventional hydrogen bonding). Sulfadoxine and Artesunate was used as standard drugs, sulfadoxine show no interaction with PfAMA-1 (1Z40) and PfGST (1OKT) but with Pf-Dihydrofolate reductase (7F3Y) as LYS353, GLN542 (both are conventional hydrogen bonding), LYS502, TYR497, SER504, ASN340, LYS359 (Pi-Anion). Artesunate show binding affinity with PfAMA-1 (1Z40) is -9.0 kcal/mol as PRO330, ARG128, SER392, LYS351, SER347, LYS395, TYR397, with Pf GST (1OKT) is -7.8 kcal/mol and interactions are ALA12, TYR211, VAL210, PHE10, LEU10, ALA41, PHE42, *ASP11* and with Pf-Dihydrofolate reductase (7F3Y) is -7.0 kcal/mol with interactions are PHE354, PHE499, LYS502, SER504, LYS302. It shows conventional hydrogen bonding, Pi-Anion and Pi-Alkyl, carbon hydrogen bonding and alkyl bonding. It only shows only unfavorable donor donor interaction with PfAMA-1 (1Z40). The docking poses of all the other phytochemicals are shown in tables 4, 5 and 6.

Table 4. Binding affinities of PfAMA-1 (1Z40) with selected phytochemicals from *Z. armatum*

Ligands	Binding Affinities Kcal/mol	Receptor protein	Interacting Amino acids residues
α -Terpinene	-5.4	PfAMA-1	PRO350, GLU365
α -pinene	-5.5	PfAMA-1	LYS364, LYS391, ARG389, ARG143, TYR390, LEU380
β -Terpineol	-5.6	PfAMA-1	PRO381, LEU380, ASP134, LYS391, ARG143, TYR390
Citral	-5.0	PfAMA-1	ILE332, TYR397, LYS351, LEU340, PHE329, PRO330
Citronellol	-4.6	PfAMA-1	SER345, LYS351, LEU340, PHE329, PRO330
Citronellal	-4.6	PfAMA-1	LYS364, ARG143, LEU380, ILE363, TYR360, ARG389, TYR390, LYS391
1'8-cineole	-5.6	PfAMA-1	ARG143, LYS364, LEU380, ARG389, TYR390
Cis-ocimene	-4.8	PfAMA-1	LEU380, ARG143, LYS364, ARG389, TYR390, LYS391
Geraniol	-4.8	PfAMA-1	LEU380, ARG143, LYS364, LYS391, TYR390
γ - terpinene	-5.6	PfAMA-1	GLU365, LEU357
Limonene	-5.1	PfAMA-1	LEU380, ARG143, LYS391, TYR390, ARG389
Linalool	-4.8	PfAMA-1	PRO330, LYS351, PHE329, LEU340, TYR397, LEU344
Nerol	-4.7	PfAMA-1	PHE329, PRO330, LYS351, LEU340, SER347
Piperitone	-5.4	PfAMA-1	ARG143, LYS391, LYS364, TYR390, LEU380
Sabinene	-5.6	PfAMA-1	LYS364, ARG143, LYS391, TYR390, ARG389
Tagetonol	-4.8	PfAMA-1	PRO330, LEU340, LEU344, TYR397, GLU336, ILE332
(<i>E</i>)-nerolidol	-5.7	PfAMA-1	LEU340, PHE329, PRO330, PRO350, LYS391
Cuminal	-5.9	PfAMA-1	PRO350, GLU354, GLU365
Phellandral	-5.5	PfAMA-1	LUE357, PRO330
2-tridecanone	-4.5	PfAMA-1	PRO330, TYR397, LEU340, LYS351
Sulfadoxine	-3.8	PfAMA-1	No interaction
Artesunate	-9.0	PfAMA-1	PHE354, PHE499, LYS502, SER504, LYS302

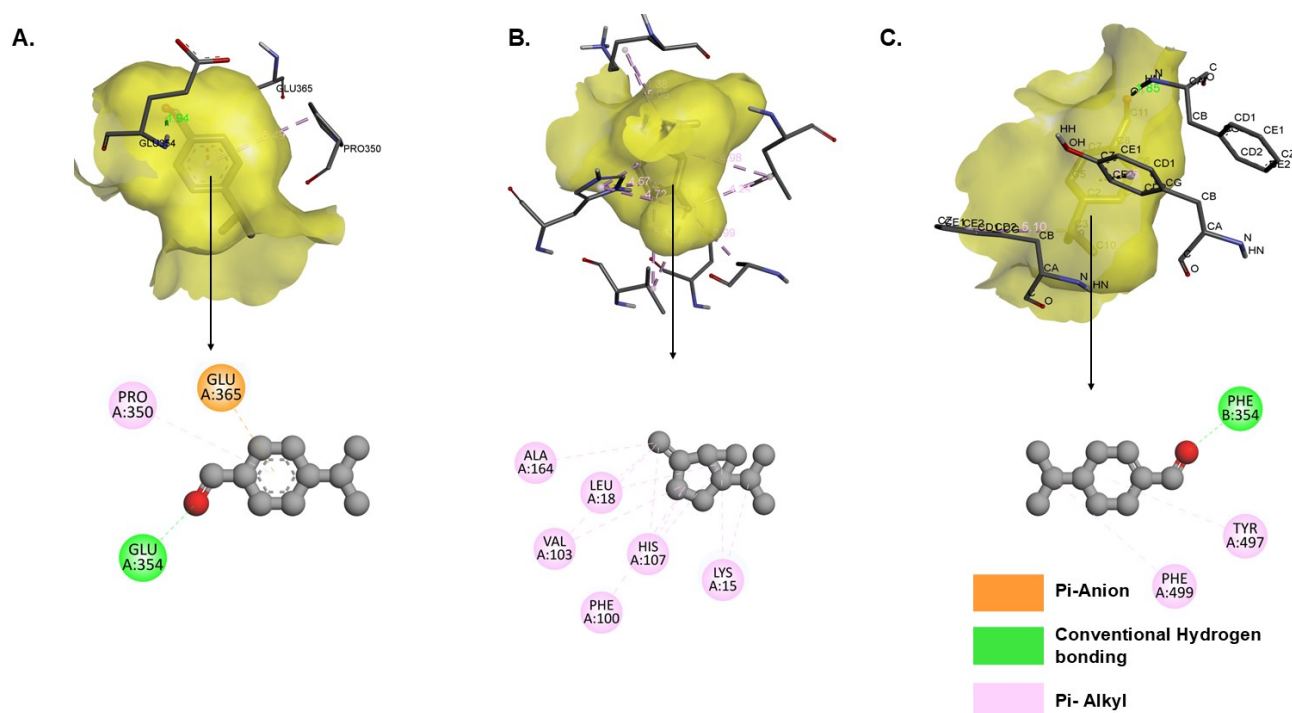


Figure 3. Molecular docking representations showing the interaction of selected phytochemicals from *Z. armatum* with key *Plasmodium falciparum* receptor proteins involved in malaria pathogenesis. **A.** *Pf* Anti-Malarial Antigen1 (*Pf*AMA-1) with Cuminal, **B.** *Pf* Glutathione S-Transferase (*Pf* GST) with Sabinene **C.** *Pf* dihydrofolate reductase-thymidylate synthase (*Pf* DHFR-TS) with Phellendral. These docked complexes highlight the potential of *Z. armatum* phytoconstituents as anti-malarial agents through multi-target inhibition.

Table 5. Docking analysis of *Pf* GST with selected phytochemicals of *Z. armatum*.

Ligand	Protein (10KT)	Binding affinity Kcal/mol	Amino acids residues interaction
1,8-cineole	<i>Pf</i> GST	-6.3	TYR211, ALA12, ALA41, PHE42, PHE10, LEU115
2-tridecanone	<i>Pf</i> GST	-4.8	PHE10, TYR211, LEU215, ALA12, PHE42, ALA41
α -Terpinene	<i>Pf</i> GST	-6.3	HIS107, PHE116, LYS15, GLN104, LEU118
α -Pinene	<i>Pf</i> GST	-6.8	VAL76, VAL103, LEU18, PHE100, HIS107
β -Terpineol	<i>Pf</i> GST	-6.0	HIS107, LYS15, PHE116, PHE100, LEU18
Cis-ocimene	<i>Pf</i> GST	-6.1	VAL103, VAL76, HIS107, TYR108, PHE116, PHE100, LEU18, LYS15
Citral	<i>Pf</i> GST	-5.3	PHE10, PHE42, LEU115, LYS175, TYR211, ALA12, TYR176
Citronellal	<i>Pf</i> GST	-5.6	LYS15, PHE176, HIS107
Citronellol	<i>Pf</i> GST	-5.0	PH10, PHE42, ALA12, ALA41, TYR176, TYR211, LEU115
Cuminal	<i>Pf</i> GST	-6.3	PHE45, GLN18
(<i>E</i>)-nerolidol	<i>Pf</i> GST	-6.9	TYE108, LYS15, PHE116, PHE100, HIS160, ALA164, LEU18, VAL103, HIS107
γ -terpinene	<i>Pf</i> GST	-6.4	PHE116, PHE100, GLN104, LEU18, LYS15
Geraniol	<i>Pf</i> GST	-5.8	TYR108, PHE116, HIS 107, LYS15, PHE 100
Limonene	<i>Pf</i> GST	-6.3	HIS107, LEU18, PHE116, PHE100, VAL76, LYS 15

Linalool	<i>Pf</i> GST	-5.4	TYR211, LEU215, TYR211, ASN 111, LYS175, PHE110
Nerol	<i>Pf</i> GST	-6.1	PHE100, PHE116, TYR108, HIS107, LYS15, GLY14(UNFAVORABLE ACCEPTOR-ACCEPTOR)
Phellandral	<i>Pf</i> GST	-6.3	PHE100, LYS15, LEU18, GLN104
Piperitone	<i>Pf</i> GST	-6.5	LYS15, LUE18, PHE116
Sabinene	<i>Pf</i> GST	-7.1	ASN 111, PHE110, LYS175
Tagetonol	<i>Pf</i> GST	-5.1	ASN111, TYR21, PHE110
Artesunate	<i>Pf</i> GST	-7.8	ALA12, TYR211, VAL210, PHE10, LEU10, ALA41, PHE42, ASP11(UNFAVORABLE ACCEPTOR-ACCEPTOR)
Sulfadoxine	<i>Pf</i> GST	-4.7	No interaction

Table 6. Docking analysis of *Pf*-DHFR with selected phytochemicals of *Z. armatum*.

Ligand	Binding affinities kcal/mol	Receptor (7F3Y)	Interactions
1,8-cineole	-5.3	<i>Pf</i> -DHFR	ALA541, LYS353, ASN340, TYR497, PHE499
2-Tridecanone	-3.6	<i>Pf</i> -DHFR	ALA541, LYS502, SER504
α-Terpinene	-5.2	<i>Pf</i> -DHFR	LYS353, TYR497, LYS502, ALA541
α-Pinene	-5.2	<i>Pf</i> -DHFR	LYS353, TYR497, LYS502, ALA541
β-Terpineol	-4.5	<i>Pf</i> -DHFR	PHE499, PHE353, LYS 353, TYR497, LYS502, ALA541
Cis-ocimene	-5.4	<i>Pf</i> -DHFR	ALA541, LYS502, TYR497
Citral	-4.5	<i>Pf</i> -DHFR	ALA541, LYS502
Citronellal	-4.4	<i>Pf</i> -DHFR	ALA541, LYS502, PHE354, LYS353, GLN542, TYR497, PHE499
Citronellol	-4.2	<i>Pf</i> -DHFR	LYS353, TYR497, ALA541, LYS502
Cuminal	-5.3	<i>Pf</i> -DHFR	ASN340, PHE499, GLN542, LYS353, PHE354
(E)-nerolidol	-5.0	<i>Pf</i> -DHFR	PHE499, LYS502, LYS353, GLN542, SER504, TYR497, ALA541
γ-terpinene	-5.2	<i>Pf</i> -DHFR	LYS353, PHE499, ASN340
Geraniol	-4.5	<i>Pf</i> -DHFR	LYS353, TYR497, PHE499, LYS502
Limonene	-5.2	<i>Pf</i> -DHFR	ALA541, TYR497, LYS502, PHE499, LYS353
Linalool	-4.3	<i>Pf</i> -DHFR	ALA541, TYR497, LYS502, PHE499, LYS353, PHE354
Nerol	-4.4	<i>Pf</i> -DHFR	TYR497, PHE499, LYS502
Phellandral	-5.5	<i>Pf</i> -DHFR	PHE354, PHE499, TYR457
Piperitone	-5.4	<i>Pf</i> -DHFR	LYS502, ALA541, TYR497, SER504, LYS353
Sabinene	-5.0	<i>Pf</i> -DHFR	LYS353, TYR497, PHE499
Tagetonol	-5.0	<i>Pf</i> -DHFR	TYR497, LYS353, ALA514
Sulfadoxine	-6.4	<i>Pf</i> -DHFR	LYS353, GLN542, LYS502, TYR497, SER504, ASN340, LYS359, LYS353
Artesunate	-7.0	<i>Pf</i> -DHFR	PHE354, SER504, LYS302, LYS 502, PHE499

Molecular Dynamic Simulations

The structural flexibility of the best docked complexes (highest binding affinities) was evaluated by GROMACS. On the basis of the results, the variation of Rg is caused by protein-ligand interactions of their binding sites. Rg is Radius of Gyration that describes protein compactness and stability in MD simulation. The Rg plots evaluate the protein structure's compactness over time. In the Sabinene–PfGST complex, the Rg remains consistently between approximately 2.555 and 2.565 nm, indicating that the protein maintains its structural integrity and stable tertiary conformation (Figure 5B). Likewise, the Cuminal complex exhibits minor variations, showing no significant signs of unfolding or collapse in the protein (Figure 4B). On the other hand, the Phellandral complex shows a gradual decline in Rg values from initial measurements to a stable range of 3.28–3.32 nm, suggesting that the protein becomes more compact following ligand binding (Figure 6B). This compaction signifies improved stability upon the formation of the complex.

The RMSD graphs for the three ligands Cuminal, Sabinene, and Phellandral demonstrate overall structural stability throughout the 50 ns MD simulation. The Sabinene–Pf GST complex displays the most stable pattern, with RMSD values slightly varying between 0.25 and 0.35 nm, indicating consistent ligand binding without significant conformational alterations (Figure 5A). Pf AMA-1 Cuminal's RMSD shows a comparable trend, achieving stability around 0.3–0.5 nm after some initial minor fluctuations (Figure 4A). The Phellandral–Pf DHFR complex experiences an initial increase in RMSD during the first 5 ns but subsequently stabilizes between 0.6 and 0.7 nm (Figure 6A), suggesting the system has reached equilibrium and that binding is thermodynamically favorable across all complexes.

SASA measurements offer insight into how much protein surface is accessible to the solvent. In the Sabinene complex, the SASA values hover around 230–235 nm², indicating that there has been no notable unfolding or exposure of hydrophobic areas (Figure 5C). A similar pattern is seen in the Cuminal–Pf AMA-1 complex, where stable SASA values demonstrate structural integrity and minimal changes in conformation (Figure 4C). Conversely, the Phellandral–Pf DHFR complex exhibits a downward trend in SASA over time, suggesting a decrease in solvent-accessible surface area due to enhanced compactness and stabilization of the protein structure (Figure 6C).

The RMSF analysis assesses the variability of individual amino acid residues. For all three complexes, minimal fluctuations are noted across most residues (<0.3 nm), especially in the regions of the active site, suggesting robust stabilization induced by the ligands. Somewhat increased fluctuations are confined to the terminal and loop regions, which is commonly observed in such simulations. The stability observed in the core and binding-site residues of the Cuminal, Sabinene, and Phellandral complexes supports their effective interaction and potential as promising bioactive antimalarial agents targeting Pf GST. The highest peak was observed for Cuminal at 350-400 residue index at 0.34nm (Figure 4D). For sabinene it was from

0 -50 at 37nm (Figure 5D). For Phellandral it was observed at 600 residue index at 0.75nm (Figure 6D).

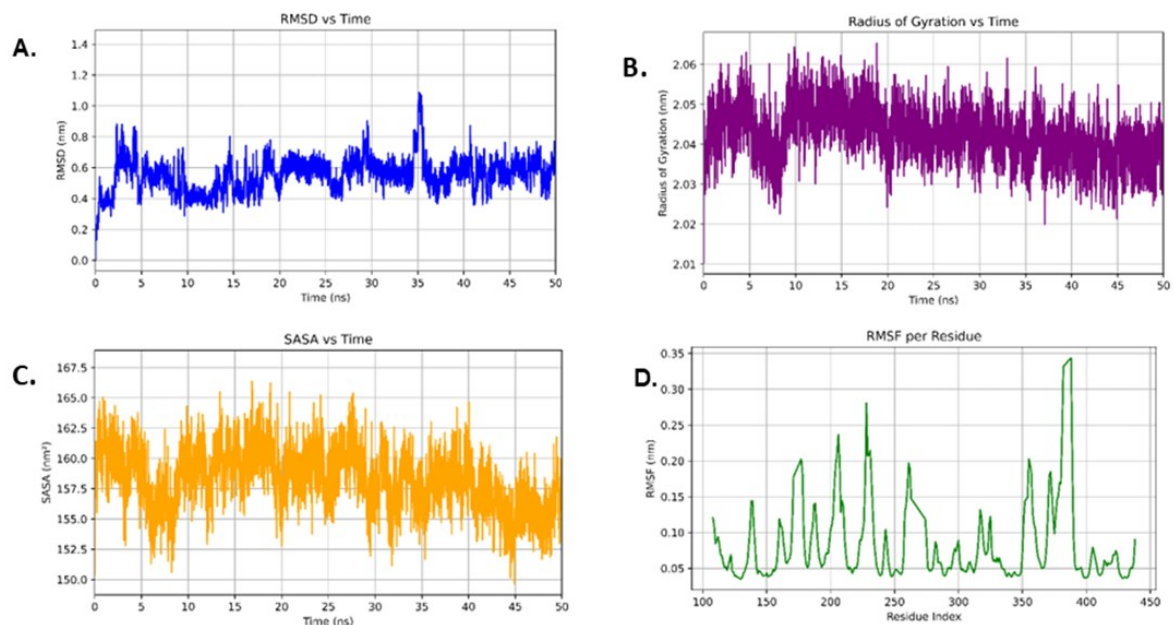


Figure 4. Molecular dynamics simulation analysis of the Cuminal-PfAMA1 complex. **A.** RMSD (Root Mean Square Deviation) vs Time plot indicates the overall stability of the protein-ligand complex across the 50 ns simulation, showing relatively stable fluctuations within an acceptable range. **B.** Radius of Gyration vs Time assesses the compactness of the protein structure, demonstrating minor variations and maintaining structural integrity. **C.** SASA (Solvent Accessible Surface Area) vs Time reveals slight fluctuations in the protein's surface exposure to solvent, suggesting consistent folding behaviour. **D.** RMSF (Root Mean Square Fluctuation) per Residue identifies flexible regions in the PfAMA-1 protein, with higher peaks corresponding to terminal or loop regions). Together, these results confirm the dynamic stability and structural compatibility of Cuminal with PfAMA-1

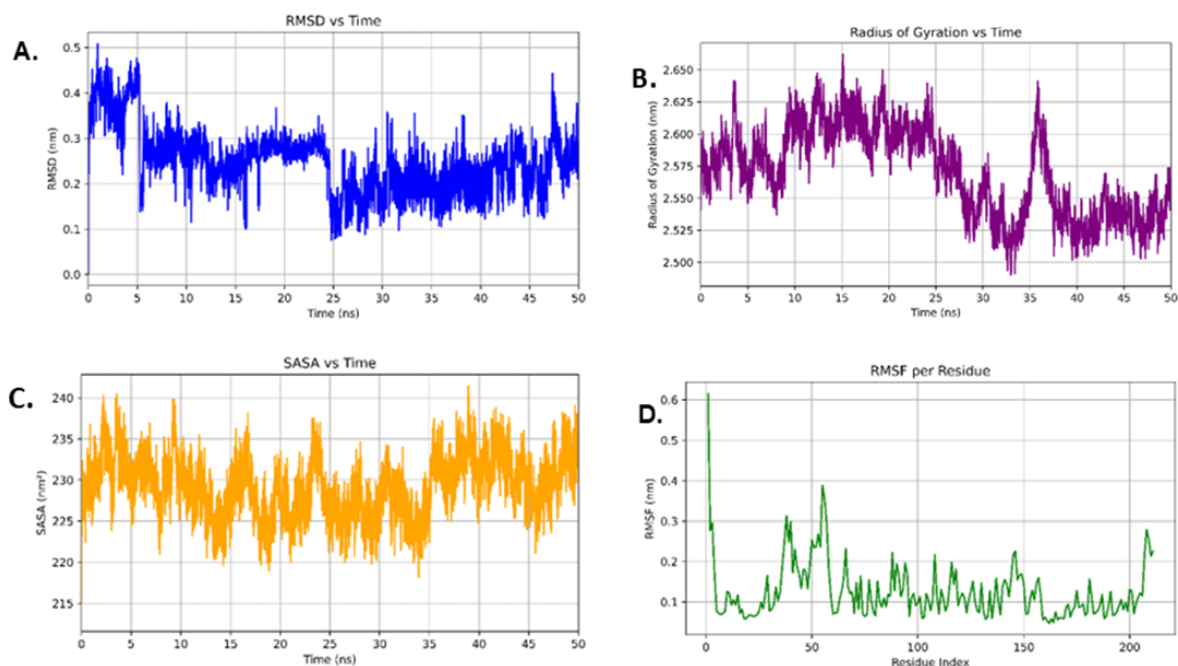


Figure 5. Molecular dynamics simulation analysis of the Sabinene-Pf GST complex. **A.** RMSD vs Time plot demonstrates the structural stability of the protein-ligand complex, with consistent fluctuations indicating a relatively stable interaction over the 50 ns simulation. **B.** Radius of Gyration vs Time reflects the compactness of the protein, indicating moderate conformational changes but overall maintenance of structural integrity. **C.** SASA vs Time reveals fluctuations in solvent-accessible surface area, signifying stable surface exposure over time. **D.** RMSF per Residue highlights residue-level flexibility, with higher peaks suggesting more dynamic regions, possibly at terminal loops or active sites. These results collectively suggest a stable binding of Sabinene to Pf GST, with acceptable dynamic behavior throughout the simulation.

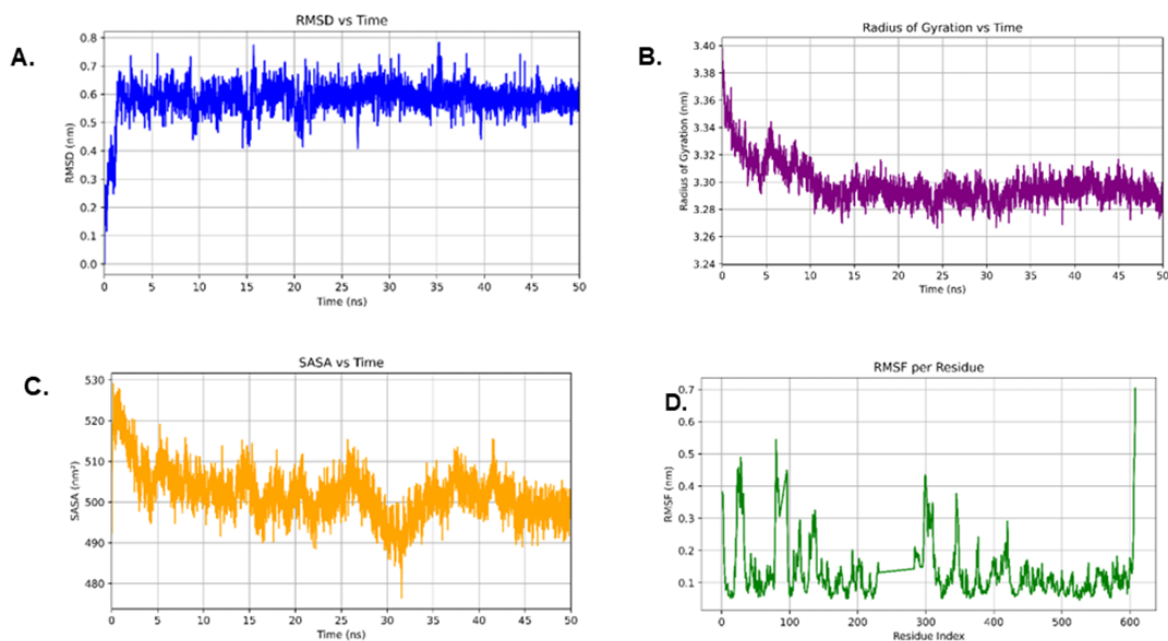


Figure 6. Molecular dynamics simulation analysis of the Phellandral-Pf DHFR-TS complex. **A.** RMSD vs Time plot shows the backbone stability of the protein-ligand complex, with the RMSD stabilizing around 0.4–0.6 nm after initial fluctuations, indicating conformational stability. **B.** Radius of Gyration vs Time indicates compactness of the protein structure, showing a steady decline and plateau, reflecting structural tightening upon ligand binding. **C.** SASA vs Time represents the solvent-accessible surface area, which remains consistent with moderate fluctuations, implying stable solvent exposure. **D.** RMSF per Residue depicts the flexibility of individual residues, with minor peaks indicating local flexibility in

terminal or loop regions. These parameters collectively suggest that Phellandral maintains a stable interaction with PfDHFR-TS throughout the simulation.

Network Pharmacology

Identification of Potential Targets Through Network Pharmacology Analysis

Network pharmacology is a multidisciplinary approach that explores the interactions of biological systems, drugs and diseases to provide a holistic view of drug action and identify potential therapeutic effects (Hopkins, 2007). We have found 5383 potential malaria targets from Gene card and DesGenet and, after elimination and integration, 240 were selected. Swiss target database revealed 99 malarial targets of the 3 selected phytochemicals (Cuminal, Sabinene, and Phellandral). On removal of duplicates and the addition of malarial-associated targets 23 common targets were found, that represent probable overlap between compounds and the disease targets (Supplementary table 7). These shared proteins were considered as potential targets of the chosen plant-based molecules against malaria (Figure 7A). A protein-protein interaction network identified the connections between various proteins within a cell that are crucial for insight into cellular functions and disease mechanisms (Wang et al., 2022; 2023).

Compound Target Network Interactions

The compound-target network was constructed using Cytoscape to analyze the interaction between the 3 active compounds and their potential targets (Figure B7). The interaction analysis showed that one active ingredient can affect multiple targets, while the same target may interact with more than one active compound. This reflects the multi-target and multi-components effects of the phytochemicals for the treatment of malaria.

Construction of Protein–Protein Interaction (PPI) Network and Hub Genes

The Protein–protein-Interaction network of 23 common target genes was constructed using STRING database (Figure 7C). After visualizing the PPI network in Cytoscape, 185 nodes with 300 edges were found (Figure 7D). The CytoHubba plugin was employed to identify hub genes and among the 12 available methods the degree method was chosen to identify the top ranked hub genes. The following genes, MPO, TNF, IL6, ALB, HMOX1, GSR, NOS2, IDO1, CXCL8, and PTPRC have emerged as the top-ranked genes due to their significantly high values. After the identification of the first stage node genes, Cuminal was found effective against ALB, MPO, PTPRC, CXCL8, NOS2, HMOX1 and GSR.

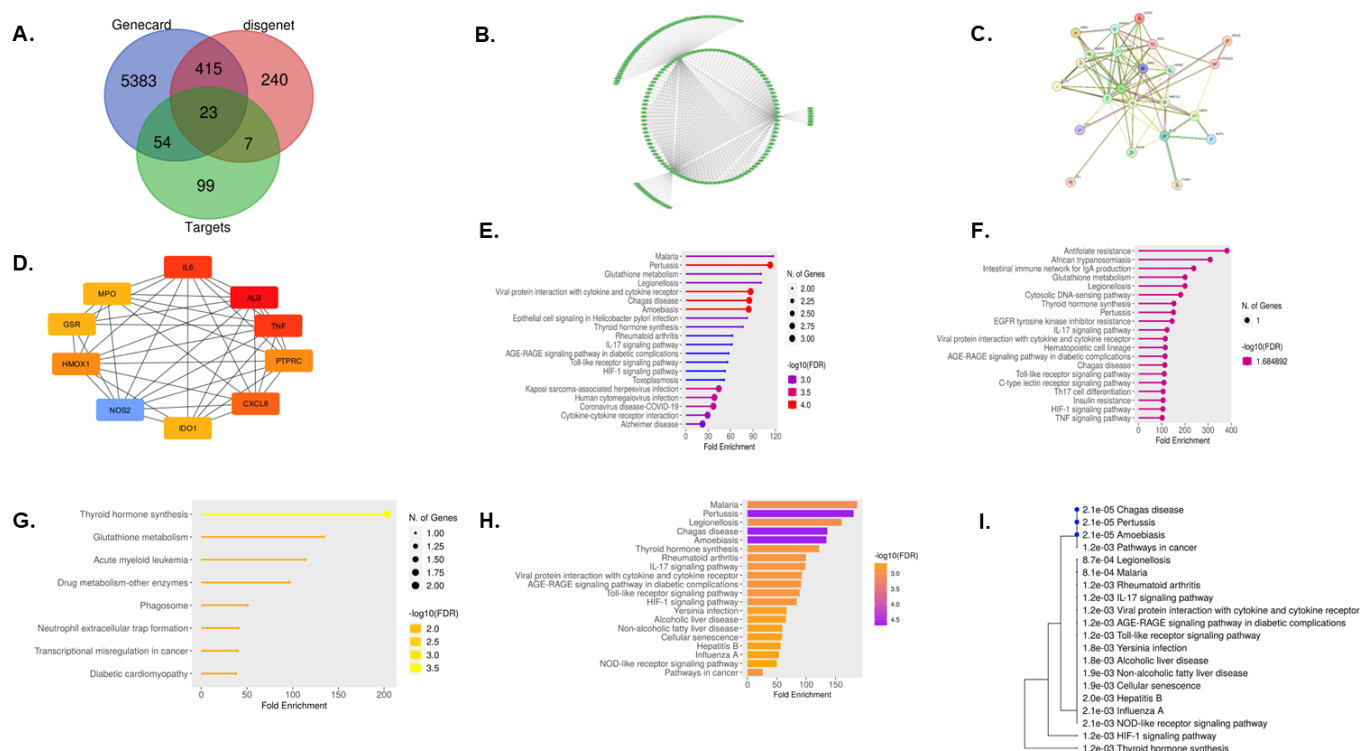


Figure 7. **A.** The diagrammatic representation of Venn diagram of target genes and 3 Drug targets from Genecards, DisGenet and Swisstar prediction data. **B.** The Cytoscape analysis representing compound target pathway network of related targets and genes. **C.** String Database representing protein-protein interactions of compound target. **D.** The top 10 hub genes from CytoHubba plugin of Cytoscape analysis. **E.** David Database represents Gene ontology plots of biological process of top 10 hub genes from Cytoscape analysis. **F.** David Database represents Gene ontology plots of Cellular Components of top 10 hub genes from Cytoscape analysis. **G.** David Database represents Gene ontology plots Molecular function of top 10 hub genes from Cytoscape analysis. **H.** Enrichment analyses of targeted genes including KEGG pathways. **I.** Enrichment analysis of targeted genes clustering between pathways.

Gene Ontology and Pathway Enrichment Analysis

Gene Ontology is a structured and standardized system used to categorize and describe the functions of genes and their products (Zhou et al., 2017) to identify the molecular mechanisms of active compounds, GO annotations and analysis of the KEGG pathway were carried out on a set of 99 targets associated with malaria. The GO analysis recognized various biological processes (BP) (Figure 7E), which include the regulation of malaria disease, the transport system, the positive regulation of cell communication, the positive regulation of signaling pathways, the regulation of programmed cell death and blood circulation; two cellular components (CC) (Figure 7F), including extracellular exosomes, membrane raft and one molecular function (MF) (Figure 7G) such as cytokine activity. The cutoff value of $P < 0.05$ was applied to select top 20 GO annotations (BP, CC, MF) and lollipop plots were drawn.

KEGG analysis predicted eight pathways associated with significant fold enrichment values and false discovery rate, such as malaria, pertussis and Chagas disease, indicating a strong involvement of these pathways in infectious diseases; other pathways include thyroid hormone synthesis, IL-17, and the AGE-RAGE signaling pathway involved in diabetic complications, metabolic regulations, and immune response (Figure 7H). A hierarchical clustering tree was constructed to

summarize the correlation among significant pathways. Pathways with many shared genes are clustered together (Figure 5I). The larger dots indicate more significant interactions. Furthermore, an interaction network was built to study the relationship between these enriched pathways (Figure 8A). The relationship between two pathways can be established if they possess 20% or more shared genes, with 20% being the default threshold. Nodes with a darker shade indicate more significantly enriched gene sets, whereas larger nodes signify larger gene sets. Thicker edges denote a higher degree of gene overlap (Figure 8B).

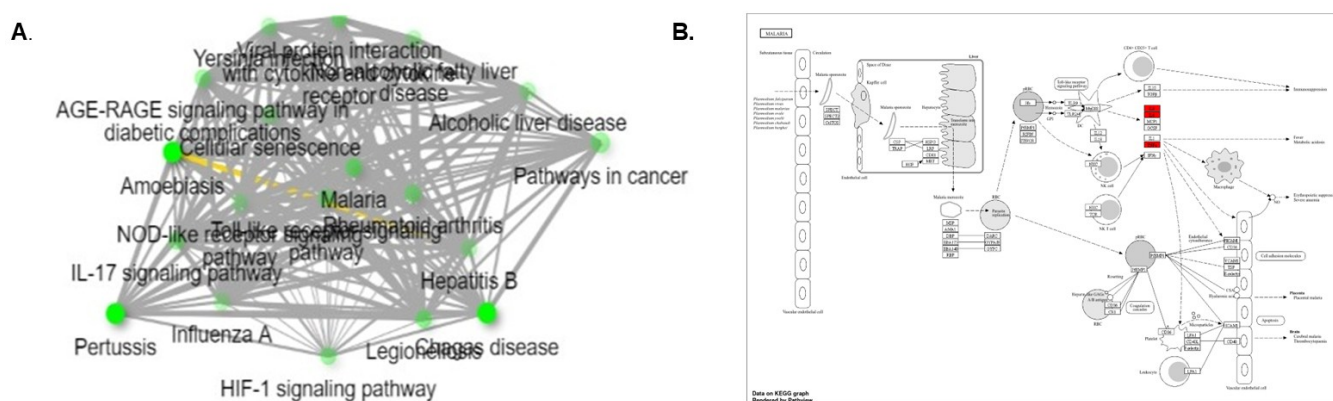


Figure 8. Enrichment analyses of targeted genes including **A.** signaling pathways and **B.** pathways interaction network

DISCUSSION

Malaria continues to be a significant global health concern, with *P. falciparum* responsible for 90% of infections and showing resistance to numerous existing medications (Muregi et al., 2011) (Kamaraj et al., 2023). Artemisinin-based combination therapies (ACTs) serve as the main treatment, merging rapidly acting ART derivatives with longer-acting partner medications. Investigating traditional medicinal plants presents a hopeful option to address the increasing issue of drug resistance (Eastman and Fidock, 2009). The combination of indigenous knowledge with rigorous scientific inquiry can pave the way for the development of novel and effective anti-malarial interventions. *Z. armatum* DC possesses a range of biological activities, including antibacterial, antifungal, antiviral, anthelmintic, cytotoxic, anti-inflammatory, mosquito larvicidal, hepatoprotective, antinociceptive, immunomodulatory, antioxidant, and cardiovascular effects (Mukthar and Kalsi, 2018). Therefore, the present study explored the protective effects of 40 phytochemicals from *Z. armatum* against malaria via molecular docking, molecular dynamic simulations and network pharmacology analysis. We have identified three important compounds (Cuminal, Sabinene and Phellandral) in *Z. armatum* with strong anti-malarial potential by inhibiting the activity of essential proteins involved in the pathogenesis of malaria. The 40 bioactive compounds were initially

screened using Lipinski's rule of five and only one compound (Tambuletin) showed three Lipinski violations while all the other compounds adhered to the rule. According to Lipinski's rule of five, Cuminal exhibited highest binding free energy that is an indicative of its excellent bioavailability and better function when taken orally as it will be able to cross the cell membrane (Singh and Verma, 2013).

Cuminal had the most favorable binding energy among the phytochemicals that were evaluated, suggesting a robust and long-lasting interaction with the target malarial proteins. This implies that Cuminal might have strong inhibitory effects, which would make it a viable medication option. In addition to having a higher binding affinity than other substances, Cuminal completely complied with Lipinski's Rule of Five, which indicates high oral bioavailability. Compounds with several Lipinski violations, such as Tambuletin, were deemed less appropriate for oral administration because of the possibility of poor permeability or absorption. Since their advantageous pharmacokinetic characteristics increase the likelihood of a successful medication development process, substances with zero or few violations were given priority. This criterion was crucial in reducing the number of potential candidates for additional molecular docking and pharmacokinetics prediction.

Prediction of the toxic potential of possible drug candidates is one of the most crucial elements in contemporary drug discovery. The toxicity analysis revealed that the LD50 values of the studied compounds ranged from 3 mg/kg (class 4 toxicity) to 70000 mg/kg (class 6 toxicity) (Ruiz et al., 2012). The α -Amyrins showed the highest LD50 value that indicated its low toxicity potential; however, it is proven highly immunotoxic. Cuminal, Sabinene and Phellandral showed nontoxic or mildly toxic effects whereas sulfadoxine exhibited slightly carcinogenic potential prompting serious safety concerns that resulted in its removal from further consideration. Following this toxicity assessment, only compounds categorized as nontoxic or mildly toxic were selected, culminating in the identification of 21 compounds for advanced molecular docking studies.

The results of molecular docking analysis revealed the strong binding of Pf AMA-1 (1Z40) with Cuminal (-5.9kcal/mol), Pf GST(1OKT) with Sabinene (-7.1 kcal/mol), and PfDHFR with Phellandral (-5.5 kcal/mol). In addition to the binding energy, the interacting amino acid residues of the target protein and the hydrogen bond energy play an important role in determining the effect of selected ligand on the protein target. Cuminal has been investigated to interact with 3 amino acid residues of the malarial antigen PfAMA-1, Sabinene to interact with 6 amino acid residues of Pf GST, and Phellandral interacts with 3 amino acids of the Pf DHFR which shows increased specificity and stability of molecular structures. The standard drug, sulfadoxine, did not demonstrate an interaction with Pf AMA-1 (1Z40) and Pf GST (1OKT) (Tables 4, 5 and 6) (Jha et al., 2010). Cuminal exhibits strong affinity and stability through interactions involving hydrogen bonding, Pi-alkyl, and Pi-anion, reinforcing its potential for further drug development. The numerous

hydrogen bonds of sabinene suggest favorable binding alignment and the possibility of significant inhibitory effects. The mixed bonding types of Phellandral indicate a level of moderate stability and specificity at the active site. Although sulfadoxine did not demonstrate significant binding to PfAMA-1 or PfGST in our docking study, it was used as a reference drug due to its established clinical use against *P. falciparum*. This further confirms the accuracy of the docking conducted in your research. The main target is dihydropteroate synthase (DHPS), involved in the folate synthesis pathway, rather than the proteins investigated in this study. Consequently, the lack of significant interactions with AMA-1 or GST does not undermine its role as a comparator; instead, it emphasizes the uniqueness of our target-based methodology. Artesunate, exhibiting robust binding affinities with all three targets and varied interactions, acts as a positive control, verifying that the proteins you selected are appropriate and that the docking approach effectively identifies significant interactions. In summary, these findings advocate for the potential repositioning or development of Cuminal, Sabinene, and Phellandral as innovative inhibitors, while underscoring the importance of multi-target binding in improving antimalarial effectiveness and decreasing the risk of resistance.

Molecular dynamics simulation studies are frequently used as a rational approach in predictive studies of potential ligand–receptor interactions to aid the process of drug design and development (Salmaso and Moro, 2018). A molecular dynamics simulation via GROMACS was performed and the RMSF, RMSD, and Rg values were measured to assess the stability and flexibility of docked complexes such as Cuminal and PfAMA-1, Sabinene and Pf GST, and Phellandral and Pf DHFR. All the three ligands showed small deviations in the binding energies which reveals the stable interactions. Sabinene shows the lowest deviations, suggesting a very strong and stable interaction, while the RMSD analysis validate structural consistency across all complexes. Phellandral notably causes a little compaction, which may contribute to increased structural rigidity, according to the Radius of Gyration (Rg) profiles, which indicate that ligand binding is not affecting the protein's overall folding. Likewise, the decreasing SASA for Phellandral suggests a tighter packing and less flexibility, whereas the constant SASA values for Cuminal and Sabinene show sustained solvent exposure. The RMSF data further show that, as a result of ligand-induced stability of key residues, fluctuations are mostly limited to the terminal and loop regions, with little movement seen near the active sites. These results collectively imply that all three ligands preserve the structural integrity of 3 proteins while producing complexes that are dynamically stable and energetically favorable, supporting their possible function in blocking parasite detoxification processes.

The main objective of this research was to create and evaluate potential compounds derived from *Z. armatum* that could imitate or enhance the therapeutic mechanisms of artemisinin, potentially aiming for the same pathways or producing multi-target effects. The isolated compounds from *Z. armatum* were assessed based

on their anticipated binding affinities, their ability to produce reactive oxygen species, and their interaction with crucial proteins involved in parasite metabolism and survival. These identified compounds might work similarly to artemisinin by inducing oxidative stress in the parasite or complement artemisinin by targeting other essential pathways for the parasite's survival, thus creating a synergistic effect in combined therapies (Omari et al., 2021).

One of the phytochemicals proven to be effective in our study was Cuminal. It is also known as Cuminaldehyde (CA) that regulates the formation of genes involved in synaptic plasticity and neurotrophic factors and thus modulates the process of memory and learning and causes neuroprotective effects (Omari et al., 2021). CA (25–100 mg/kg) helps to reduce inflammation by decreasing the serum concentration of IL-1 β and TNF- α in chronic constriction injury (CCI)-induced neuropathy (Koohsari et al., 2020). Recently, it has been implicated in the treatment of diabetes by affecting insulin levels, however, further research is warranted to identify its role in diabetes and other disorders such as malaria (Duraisamy et al., 2022).

Another important bioactive compound that has been shown to be effective against malaria in our study is Sabinene, which is known to reduce muscle atrophy by affecting the ROS-facilitated MAPK/MuRF-1 signaling pathway (Ryu et al., 2019). Sabinene treatment also decreased reactive oxygen species (ROS) and attenuated the levels of different antioxidant enzymes such as lipid peroxidase, GST and catalase in yeast cells (Sharma et al., 2019). As plasmodial GST is an important target of antimalarial drugs and in our study Sabinene also showed a stable interaction with Pf GST, so it may act as a promising agent for the treatment of malaria, however, further *in vivo* and clinical studies are required to validate its role in the treatment of malaria.

In the same study, exposure to Sabinene prevented the angiogenesis (Sharma et al., 2019). As angiogenesis is an important hallmark of cancer, it is postulated that Sabinene may cause beneficial effects in preventing cancer by inhibiting angiogenesis.

The essential oil of *Eucalyptus camaldulensis* is known to possess Phellandral and Cuminal in reasonable concentrations (1.4-4.7%) and this oil has been shown to be beneficial in preventing the growth of phytopathogenic fungi, so it is possible that Phellandral may act as an antifungal agent (Barra et al., 2010). Our results showed the inhibitory potential of Phellandral against Pf DHFR; however, the literature is scarce regarding the role of this very compound in disease treatment and prevention; therefore, it may serve as a novel research target for the management of malaria and other pathogenic conditions.

These phytochemicals target various important proteins that play a crucial role in malaria and these proteins are identified via network pharmacology method. The identified proteins are required for the normal functioning of the malarial parasite. According to the network pharmacology approach, Cuminal target proteins involve ALB, PTPRC, CXCL8, NOS2, HMOX1, MPO, and GSR, Sabinene targets for the

Sabinene include MPO, TNF, IDO1, and NOS2, whereas Phellandral mainly through MPO, IL6, PTPRC and NOS2. All compounds target nitric oxide synthase (NOS2) and myeloperoxidase (MPO). NOS2 modulated nitric oxide (NO) and NO helps fight against malarial parasite (Levesque et al., 2010) by producing oxidative stress and inhibiting its growth, and it also activates the immune system to produce pro-inflammatory cytokines and clear the parasite. However, increased production of NO can cause damaging effects in the host and cause ROS production, leading to oxidative stress and inflammation in the host; therefore, an intricate balance in NO production of NO is required to combat pathogens (Kotepui et al., 2023). The phytochemicals selected in this study can prevent the NOS2 thus regulating the production of NO in the event of malaria.

MPO is mainly produced by monocytes and is neutrophils and involved in ROS production; however, its exact role in malaria is still unknown. Increased levels of MPO are observed in malarial patients with increased parasitemia, and regional/altitudinal differences were observed between Santa, Bambili, and Bamenda (Ngum et al., 2023).

CONCLUSION

The present study explored the therapeutic potential of 40 natural bioactive compounds of *Z. armatum* against the *P. falciparum*. Molecular docking showed the stable complexes of three phytoconstituents such as Cuminal, Sabinene, and Phellandral against PfAMA-1, Pf GST, and Pf DHFR, respectively, which reveals their potential in mitigating malaria. Molecular dynamic simulations and network pharmacology analysis further confirmed the role of Cuminal, Sabinene, and Phellandral in inhibiting antigen and enzymes crucial for the pathogenesis of *P. falciparum*. Our study provides a strong basis for further *in vitro* and *in vivo* experimentation to validate the potential of these bioactive compounds in the treatment of malaria alone or in combination with available treatment options.

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