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Isolation and validation of antimalarial compounds from *Phyllanthus emblica* leaves for new antimalarial drug development

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Natural products can serve as an alternative source of novel therapies that are required to address the problem of malarial infection resistance. Indian gooseberry (*Phyllanthus emblica* L.) leaves are often used in traditional medicine to treat fevers, but there isn't enough scientific proof that they contain antimalarial and effective phytochemicals. This study's primary goal was to investigate the antimalarial efficacy of *P. emblica* leaf ethyl acetate extract against *Plasmodium falciparum* (3D7) and its cytotoxicity against the HeLa cell line. The active compounds from *P. emblica* were isolated using a bioassay-guided fractionation of column chromatography technique, and NMR spectroscopy was employed to identify their structures. The drug's anti-malarial efficacy was assessed by estimating its growth-inhibitory activities employing the SYBR Green I asexual parasite drug assay. The cytotoxic effect was evaluated using the MTT assay. *P. falciparum* dihydroorotate dehydrogenase protein (*Pf*-DDP) was used as a drug target to investigate molecular docking. *P. emblica* crude extract and two fractions exhibited > 90% inhibition of 3D7 parasite proliferation, indicating good antimalarial activity at 100 and 10 µg/mL, respectively. Subsequently, the column chromatography study of each fraction, a targeted purification, contributed to the separation of six active compounds designated as 9-hydroxy isolongifolene (C1), Hexadecanoic acid (C2), Phenol, 2,6-Bis(1,1-Dimethylethyl) (C3), Furan, tetrahydro-3-methyl-4-methylene (C4), Octadecanoic acid (C5), and Beta-Sitosterol (C6). Compound C4 showed stronger bioactivity against *P. falciparum* (3D7) (IC_{50} 4.32 µg/mL) parasites than other constituents, equivalent to the drug-sensitive strains (100 µg/mL). Considering the IC_{50} levels of the two compounds, 90.56 and > 100 µg/mL, respectively, both demonstrated low cytotoxicity against HeLa cell lines. This research offers scientific support for the historical application of *P. emblica* in combating malaria. Building on existing knowledge, this study represents a groundbreaking effort to isolate and identify antimalarial compounds from *P. emblica* leaves for the first time. Moreover, our research underscores the potential of *P. emblica* in the development of antimalarial agents, encouraging further investigation of different species to suppress the growth of *P. falciparum*. This plant species could be a valuable source for developing new anti-malarial drugs.

Keywords *Phyllanthus emblica*, Malaria, *Plasmodium falciparum*, Parasite inhibition, Cytotoxicity, Compounds

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Malaria in humans is caused by five protozoan parasites, one of which is the most prevalent, *Plasmodium falciparum*[1]. It is causing the highest rate of mortality in the African region[2] and is the most infectious and significant reason for patients' serious cases of malaria. Both humans and female *Anopheles* mosquitoes serve as hosts to the *Plasmodium* parasite, which has a multistage, highly intricate life cycle[3]. The World Malaria Report shows 227 million malaria cases occurred in 2019, and then rose to 241 million cases in 2020[4]. Malaria stands as one of the major health threats worldwide since it triggered 249 million cases and 608,000 deaths in 2022, and 94% of cases and 95% of deaths occurred within Africa. In 2021 the number of malaria victims reached 750,000. The substantial disease burden has not prevented global malaria incidence and mortality rates from substantially decreasing during the past three decades[5]. Malaria is a pertinent public health concern, as evidenced by the 2021 global malaria burden data, which indicates 247 million cases and 619,000 fatalities[6]. According to WHO[7,8], pregnant women and children are the most infested groups. The primary factor contributing to the rising prevalence of malaria worldwide is the documented resistance of *P. falciparum* to antimalarial medications[9,10]. The current data points to a pattern of both lack of financial growth and non-advancement in outcomes[11]. Therefore, medicinal plant compounds that also have the capacity to prevent the parasite from spreading from person to mosquito are given priority when developing novel antimalarial candidates[12,13]. To effectively interrupt the malaria transmission cycle, new drug candidates should ideally target both the sexual gametocyte forms (TCP-5) and the asexual stage of the parasite (TCP-1)[14–17].

Phyllanthus emblica L. (also known as *Emblica officinalis*) has consistently been regarded for both its medicinal benefits and as a nutritive plant. While all parts of the plant offer therapeutic benefits, its fruit, in particular, is widely used in Ayurveda as a powerful Ras Ayana (rejuvenating agent) to encourage longevity and treat conditions such as inflammation, jaundice, and diarrhoea[18]. The fruits of *P. emblica* can be used on their own or in conjunction with other plants within the Indian traditional medical system. They are utilized to treat conditions such as fever and the common cold, alongside avoiding dyspepsia and ulcers. Additionally, they have laxative, diuretic, cooling, stomachic, restorative, liver-tonic, fever-reducing, and hair-growth-promoting properties[19–22]. According to[23], *P. emblica* fruits are abundant in nutrients and comprise vital vitamins and minerals. A commonly used herbal formulation, Triphala, is made by combining equal parts of *P. emblica*, *Terminalia chebula*, and *T. bellerica* fruits. Triphala is known for its effectiveness in treating conditions such as an enlarged liver, hemorrhoids, biliousness, and chronic diarrhoea[24,25]. The fruits are believed to enhance strength, promote overall well-being, boost the immune system, and revitalize the body's organ systems.

Pharmacological studies of *P. emblica* have proven its antiplasmodial effects against *P. falciparum*, and *P. berghei*[11,26–29]. Additionally, the plant displays various other therapeutic properties, like antimicrobial[30], antioxidant[31,32], anti-inflammatory[33], cardioprotective[34], anti-tussive[35], gastroprotective[36], anti-atherogenic[37], adaptogenic[38], and nephroprotective[39] effects. *P. emblica* has also demonstrated anticancer activity[40,41], neuroprotective effects[42], and anti-diabetic properties[43]. Furthermore, it has been shown to exhibit immunomodulatory[40], radioprotective[44], and chemopreventive[45] effects.

The study sought to ascertain the ethyl acetate extract of *P. emblica* leaves, which underwent column chromatography-based bioassay-guided fractionation, followed by structure elucidation confirmed through various spectroscopic analyses. The extract and its isolated pure compounds were tested against *P falciparum* (3D7). The results revealed that fractions containing acetone and ethyl acetate exhibited the lowest parasite survival rates, indicating the highest activity. This study presents the first report on the, antiparasitic effects, cytotoxicity and in silico drug screening of antimalarial compounds from the active fraction of *P. emblica*. Which highlights the novelty.

Materials and methods

Plant collection

The plant materials were obtained from Nagavathi Dam Reservoir, Dharmapuri district (11° 45′ 0 N to 12° 15′ 0 N latitudes and 77° 30′ 0 E to 78° 30′ 0 E longitudes), Tamil Nadu, India (Fig. 1), in April and May 2024. Figure 1a and b satellite imagery was generated from QGIS Software 3.40.4 Bratislava. Figure 1C is generated through Google Earth Pro 7.1.8. Dr. P. Jayaraman, Director of the medicinal plants research unit at the Plant Anatomy Research Centre, Tambaram, Chennai-45, Tamil Nadu, India, verified the authenticity of the collected plants. Sustainable harvesting practices were carefully employed during the collection of raw materials to preserve the natural habitat. Information in regards to the vernacular name, used plant parts, preparation methods, administration, and dosage of each medicinal plant was gathered. The plant specimen was prepared and kept in herbarium repositories at IIISM (IIISM/DDT/2024003), Drug Testing Laboratory, SRM IST, Kattankulathur, Chengalpattu-603,203, Tamil Nadu, India.

Extraction and bioassay-guided fractionation

Fresh leaves of *P. emblica* were thoroughly washed and allowed to dry in the shade and milled into a fine powder. About 5 kg of plant powder aerial parts were macerated thrice with ethyl acetate (7 L each for 48 h) and filtered. A Soxhlet apparatus (boiling point: 60–80 °C) was used to concentrate the infusions for eight hours per cycle till exhaustion. The concentrated extract was further reduced at high pressure (22–26 mmHg at 45 °C), yielding a residue (70.6 g, 5.85%) stored at 4 °C.

The residue was subjected to gravity column chromatography, and obtained fractions were tested on *P. falciparum* (3D7) at different concentrations (100, 50, and 25 µg/mL). Fractions deemed highly active and chosen for additional fractionation have IC_{50} values less than 15 µg/mL. Five fractions (A–E) were separated from the crude ethyl acetate extract using column chromatography (50 × 5 cm, gravity). The stationary phase consisted of a 1:2 (150:300 g) mixture of charcoal and silica gel (60–120 mesh), and a stepwise gradient of hexane and ethyl acetate (100:0 to 0:100) was used for elution at a flow rate of 2 mL/min[13,46]. Three additional fractions (F, G, and H) were collected by further eluting the column with chloroform and methanol in different ratios:

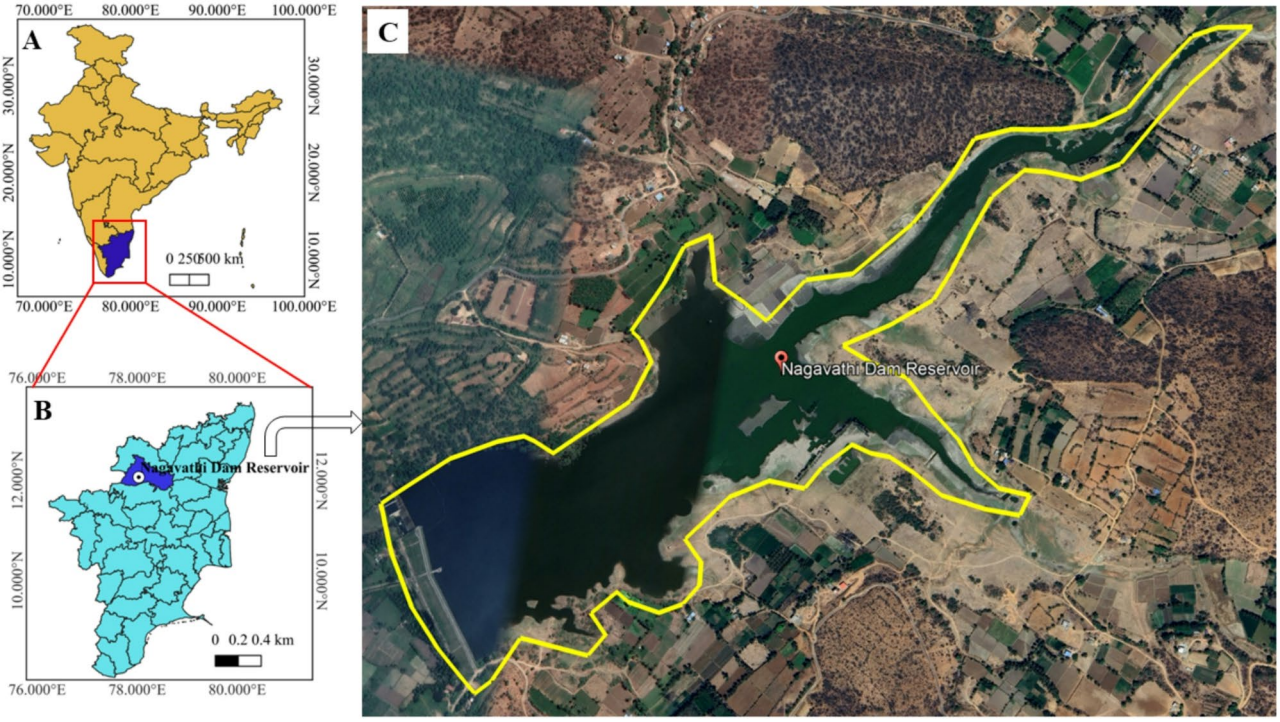

Fig. 1. Maps showing the *P. emblica* collection site from Nagavathi Dam Reservoir (**C**), Dharmapuri district (11° 45 0 N to 12° 15 0 N latitudes and 77° 30 0 E to 78° 30 E longitudes), Tamil Nadu (**B**), India (**A**).

100:0 (5 × 200 mL), 50:50 (12 × 200 mL), and 0:100 (3 × 200 mL). Among these, fractions FC and FE displayed the most promising activity, with IC_{50} values of 6.50 and 8.25 µg/mL, respectively. Fractions FC (16.32 g), FC2 (10.43 g), FC2C (8.12 g), FE (4.84 g), FE3 (2.67 g), and FE3B (1.97 g) were purified through repeated gravity column chromatography. Fractions FC (16.32 g), FC2 (10.43 g), FC2C (8.12 g), FE (4.84 g), FE3 (2.67 g), and FE3B (1.97 g) were purified through repeated gravity column chromatography. Silica gel with mesh sizes ranging from 230 to 400 mesh (180 g) to 70–320 mesh (240 g) was used as the stationary phase in the column of size (30 × 1.5 cm, dimensions). Hexane and ethyl acetate mixtures were employed as eluents, resulting in the isolation of several sub-fractions from each of the original fractions. Biological activity-directed separation resulted in the isolation of pure compounds: FC2A (2.78 g), FC2C2 (2.84 g), FC2C4 (2.43 g), FC2C5 (1.10 g), FE3A (1.43 g), and FE3B (1.97 g) using the same sub-column procedure as mentioned above. These were obtained using elution gradients of 97:3 (6 × 50 mL), 95:5 (22 × 50 mL), 90:10 (12 × 50 mL), 85:15 (8 × 50 mL), 75:25 (6 × 50 mL), and 70:30 (8 × 50 mL) at a flow rate of 1.0 mL/min (Supplementary Figure S1).

The results of Thin-Layer Chromatography (TLC) were used to pool the fractions. TLC plates (pre-coated, 0.02 mm, E. Merck, Germany 60 F254) were monitored until single spots were observed, visualized in an iodine vapor chamber. The Rf values of the purified compounds were as follows: FC2A (0.36), FC2C2 (0.42), FC2C4 (0.38), FC2C5 (0.64), FE3A (0.94), and FE3B (0.52), and they were reassigned as C1, C2, C3, C4, C5, and C6, respectively, for easy flow. Identification and characterization of these purified compounds were completed using various spectroscopic techniques.

Parasite culture

The *P. falciparum* (3D7) strain that is sensitive to chloroquine (CQ) was cultured *in-vitro* using the following protocol (Trager and Jensen, 1976). The parasite was cultured in RPMI-1640 medium, which was supplemented with several components to support its growth and survival. These supplements included 25 mM HEPES for pH buffering, 0.2% sodium bicarbonate to maintain the pH of the culture, and 100 µM hypoxanthine to support purine metabolism. Additionally, 0.5% (w/v) AlbuMAX-II, a lipid-rich supplement, was added to provide essential nutrients, and 50 µg/L gentamycin was included as an antibiotic to prevent bacterial contamination. The cultures were maintained at a 4% hematocrit using fresh human type O^+ red blood cells (RBCs) to mimic the natural environment of the parasite. The cultures were kept in a CO_2 incubator at 37 °C, and the medium was replaced daily to ensure optimal growth conditions.

Drug dilutions

All compounds were diluted with full RPMI medium to achieve a concentration of 2 mg/mL after being dissolved in dimethyl sulfoxide (20 mg/mL). For primary screening, all stocks were used in three concentrations i.e. 100, 50, and 1 µg/mL. Thereafter, leading hits were serially diluted from 100 µg/mL to 1.56 µg/mL for evaluation of IC_{50} values. The final concentration of DMSO was always kept lower than 0.5% which has no side effect on parasite growth.

Assessment of antimalarial potential using SYBR green I assay

The culture was synchronised at the ring stage by 5% (w/v) sorbitol treatment. Synchronised iRBCs were diluted with washed uninfected RBCs to get a final parasitemia of 0.5% and hematocrit level of 2% with culture medium. Afterward, 95 µl of this parasite culture suspension was introduced to each well of culture 96-well assay plate containing 5 µl of each drug concentration. All microplates were maintained at 37 °C in a CO_2 incubator for 48 h. Each test was run repeatedly. Positive and negative controls were iRBCs with and without CQ. Plates were incubated, then frozen and thawed, and 100 µL of buffered SYBR Green was added. Fluorescence was measured at 490 nm excitation and 530 nm emission after the plates were incubated for an hour at 37 °C in the dark. CQ served as the reference drug, with infected erythrocytes as the positive control and uninfected erythrocytes as the negative control[13,47–49].

Spectroscopic analysis of purified compounds

A Bruker-500 MHz AVANCE equipment was used to identify the structure of the six compounds using ^{1}H and ^{13}C NMR spectroscopy in DMSO-d6 and $CDCl_3$ solvents. Measurements were made of ^{13}C NMR at 125.8 MHz and ^{1}H NMR at 500 MHz. This analysis confirmed the molecular structures of the compounds. The LC/MS experiments were performed using the QTOF-ESI source on Bruker Daltonik GmbH equipment, and the FT-IR spectrum was obtained on the Bruker A250/D Alpha spectrophotometer in the region of 4000–400 cm^{-1}. The isolated compounds were analyzed in an LC-MS/MS system (LCMS 2000 Sigle-Quadrupole-LC-MS system; Shimadzu). 20 µL of the sample was injected onto a ProntoSIL® column (C18; 5 µm, 50 × 2 mm, Bischoff, Germany) attached to a precolumn (C18, 4 × 2 mm, Phenomenex, USA).

Cytotoxicity

The in vitro cytotoxic activity of *P. emblica*, its fractions, and pure identified compounds was studied by MTT assay on the MDA human breast cancer cell line as described by[50]. The cells were cultured in full DMEM with 10 mg/mL penicillin-streptomycin and 10% fetal bovine serum. For cell adhesion, 96-well plates were seeded with approximately 10,000 cells per well in 200 µL of medium and incubated for 24 h. After that, the cells were subjected to variable concentrations (10–100 µg/mL) of *P. emblica* and its fractions in triplicate for 48 h at 37 °C in a CO_2 incubator. Each well was then filled with 10 µL of MTT reagent (1 mg/mL), and the wells were incubated for an hour. After dissolving the formazan crystals in 100 µL of DMSO, an ELISA reader was used to determine the optical density at 450 nm.

Molecular docking analysis

The candidate compounds (C1-C6) were analyzed for interactions with *P. falciparum* dihydroorotate dehydrogenase protein (*Pf*-DDP), a potential drug target, interference of which will inhibit *Plasmodium* infection[51]. The XRD (2.15 A˚) 3D assembly of pf-DDP was acquired from Protein Data Bank (PDB ID: 5TBO). All the co-crystallized ligands were removed using Chimera 1.17.3. The .pdb structures of candidate compounds (C1-C6) were prepared using Maestro 2D sketcher (Student version), and 3D files were generated and exported as .pdb files. Protein was prepared initially by removal of water atoms, followed by addition of polar hydrogen and Kollman charges. The grid box was generated as center_x = 21.602, center_y = − 14.336, and center_z = − 0.218. The prepared protein and ligand were exported as. pdbqt files. Both protein and ligand were prepared with Autodock MGL Tools 1.5.7 software. Then, molecular docking analysis was carried out in AutoDock Vina v1.2.x. 10 poses were generated; from that, the pose with the lowest binding energy and low RMSD was selected for visualization. The 3D and 2D protein-ligand complexes were analysed and visualized using Discovery Studio Visualizer 2021[52].

Validation of docking results

For validation of docking results, the co-crystallized ligand and standard drug chloroquine were redocked in the protein[53,54]. In Chimera 1.17.3, the .pdb file of *Pf*-DDP downloaded from the PDB website was loaded. Then the redocked co-crystallized ligand was opened. All the other structures were hidden for better visualization and the ligands were superimposed. RMSD values were calculated using the command 'match #0:1001.A & ~@H= #1:333.het & ~@H='. The pose with the lowest RMSD was selected.

Statistical analysis and interpretation

GraphPad 8.0 and Microsoft Excel were used to evaluate the data. Each inter-group was also characterized by mean ± SD. Analysis of variance between groups was done using Students 'Unpaired t-test. *P* values less than 0.05 were regarded as statistically significant. Additional survival analysis using the Kaplan–Meier estimator was also conducted to determine survival rates.

Results

Assessment of antiplasmodial efficacy of isolated compounds

In light of the initial screening result, the leaves methanol extract of *P. emblica* exhibited promising antiparasitic inhibitory to *P. falciparum* (3D7). Following the selection of the *P. emblica* leaf methanol extract for bioactivity-directed separation, six pure fractions were isolated: C2A (C1), C2C2 (C2), C2C4 (C3), C2C5 (C4), E3A (C5), and E3B (C6). The above fractions were tested on *P. falciparum* (Fig. 2a, b; Table 1). *P. emblica* exhibited promising antiparasitic inhibition with IC_{50} value of 3.13 ± 1.64 µg/mL (Fig. 2a). The purified fractions (**C1-C6**) exhibited activity with IC_{50} values of 10.30 ± 3.00, 35.80 ± 3.10, 23.42 ± 2.95, 4.32 ± 2.34, 7.66 ± 3.00, and 9.29 ± 2.26 µg/mL, respectively (Fig. 3a–c).

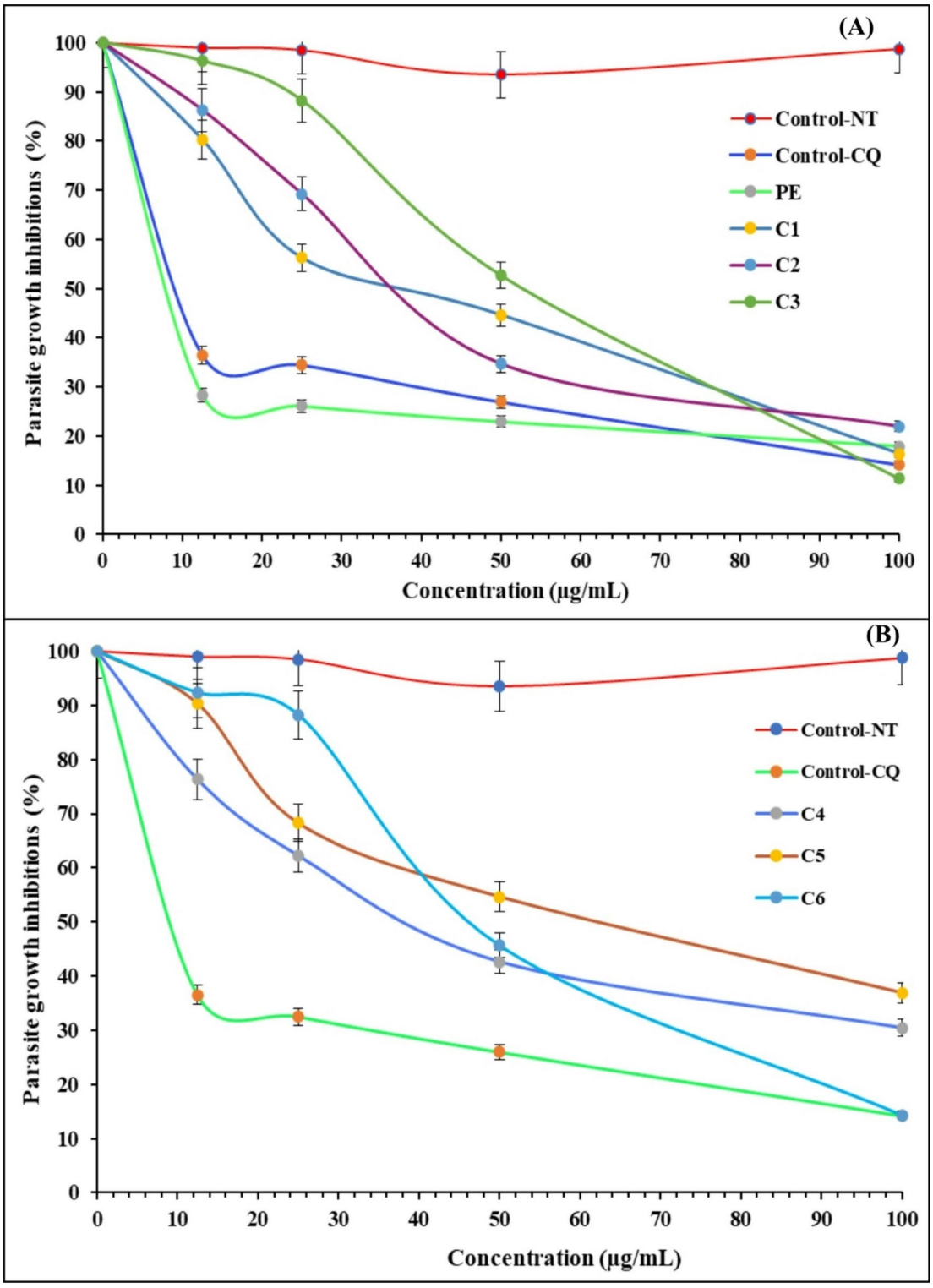

Fig. 2. Leaves ethyl acetate extract of *P. emblica* and its purified fractions inhibit the growth (%) against *P. falciparum* (3D7) parasite. (**A**) *P. emblica* (PE), non-treated (NT), chloroquine treated (CQ), and purified fractions (C1-C3) tested at different concentrations. (**B**) purified fractions (C4-C6) tested against *P. falciparum* (3D7).

Cytotoxicity of isolated fractions

Results from cytotoxicity assays are presented in Fig. 4a–g with percent viability values of the HeLa cells incubated for 48 h with leaves ethyl acetate extract of *Phyllanthus emblica* (Fig. 3A), isolated fraction **C1** (B), **C2** (C), **C3** (D), **C4** (E), **C5** (F), and **C6** (G). The percentage of cell viability decreased progressively with increasing

S. No	Compounds	*P. falciparum* 3D7 ± SD (IC_{50} µg/mL)[a]	Cytotoxicity ± SD (CC_{50} µg/mL)[a] HeLa cell line	*Therapeutic index CC_{50}/IC_{50} HeLa/3D7
1	*P. emblica*	3.15 ± 1.64	> 100 ± 0.0	31.74
2	C2A (**C1**)	10.30 ± 3.70	47.5 ± 1.42	4.16
3	C2C2(**C2**)	35.80 ± 3.10	> 100 ± 0.0	2.79
4	C2C4(**C3**)	23.42 ± 2.95	96.45 ± 2.0	4.11
5	C2C5(**C4**)	4.32 ± 2.34	> 100 ± 0.0	23.15
6	E3A(**C5**)	7.66 ± 3.00	90.56 ± 0.0	11.82
7	E3B(**C6**)	9.29 ± 2.26	> 100 ± 0.0	10.76
8	CQ	6.24 ± 3.14	> 100 ± 0.00	10.76

Table 1. In vitro antimalarial activity and cytotoxicity of purified compounds. [a]Mean values of three replicates ± standard deviation. *Therapeutic index: CC_{50} HeLa/IC_{50}*Pf3D7*.

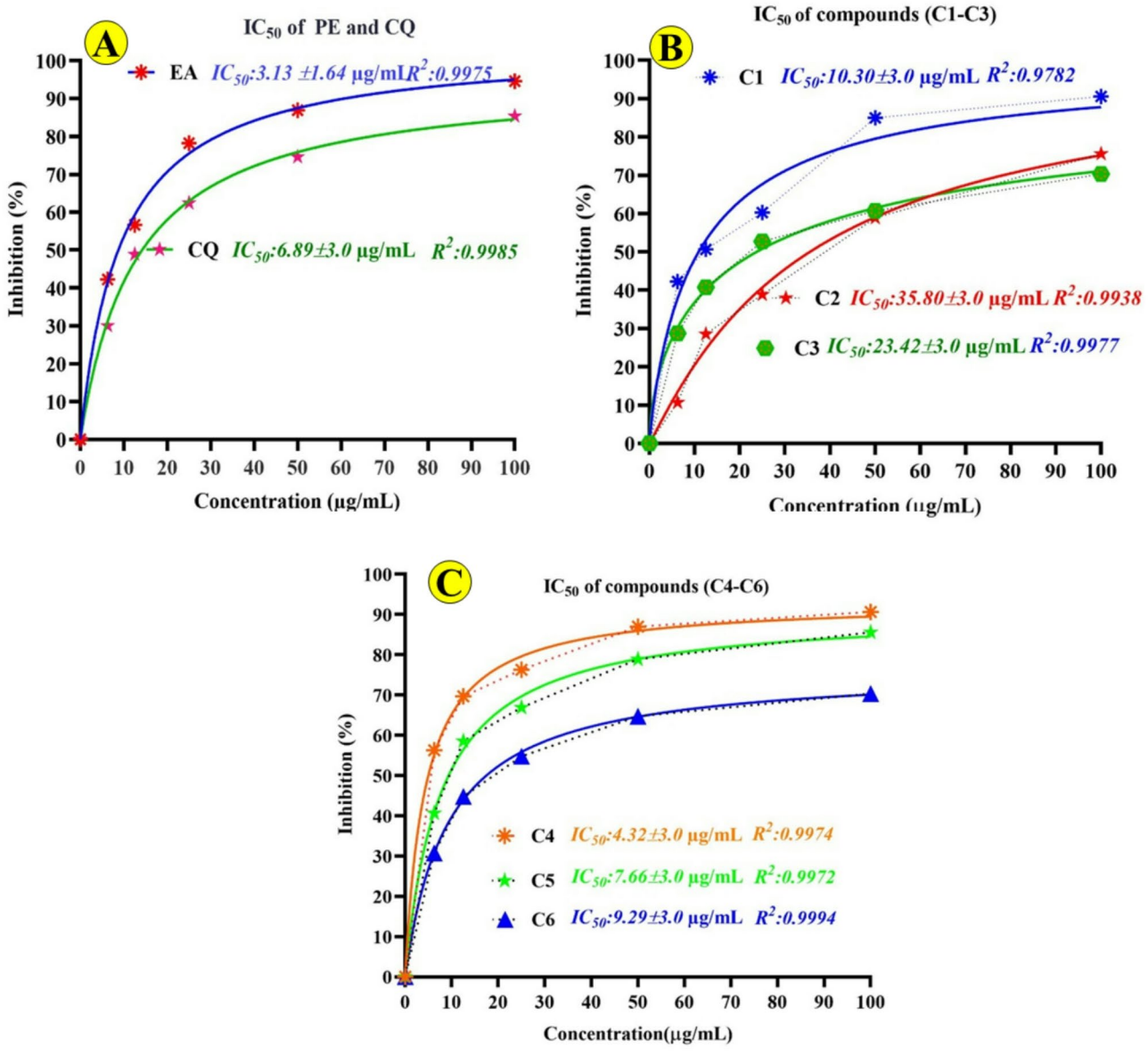

Fig. 3. Antiplasmodial activity on *P. falciparum* IC_{50} (µg/mL) (**A**) *P. emblica* (PE), chloroquine treated (CQ), and (**B**) purified fractions (C1-C3) tested at different concentrations. (**C**) purified fractions (C4-C6) tested against *P. falciparum* (3D7).

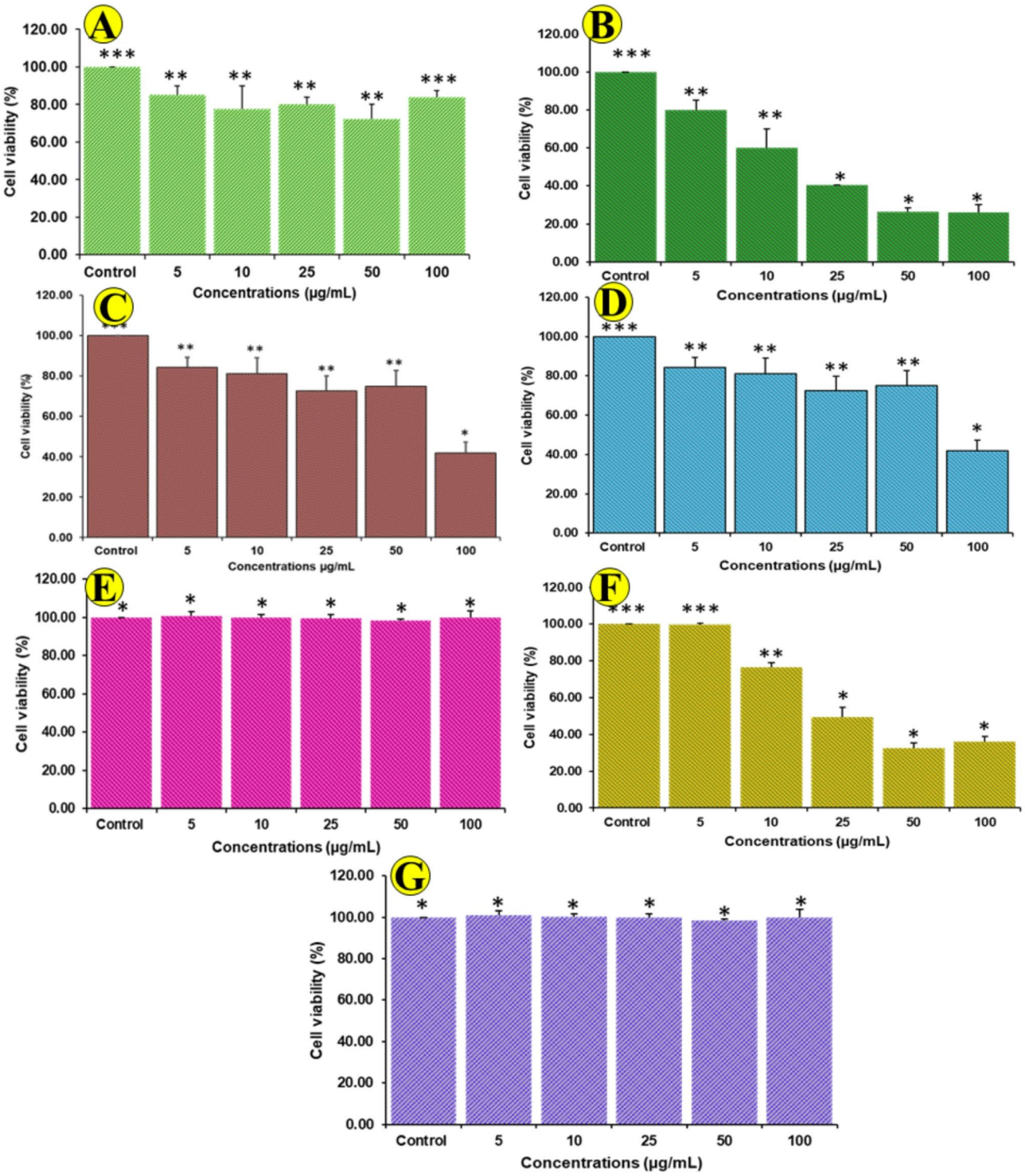

Fig. 4. Cell viability of HeLa cells after 48 h of treatment with leaves ethyl acetate extract of *P. emblica* (**A**), isolated fraction **C1** (**B**), C2 (**C**), C3 (**D**), C4 (**E**), C5 (**F**), and C6 (**G**) using the MTT test at different concentrations. (***, **, *) indicates the significant difference between the treated and control $P < 0.005$.

concentrations. Fractions **C1** and **C5** showed in vitro cytotoxic effects after 48 h of incubation, resulting in reduced cell viability compared to untreated controls. Figure 4c and d, isolated fractions revealed the increase in viability values of cells with a decrease in concentration. At extract concentrations of 5 µg/mL–100 µg/mL, cell viability increased from 84.22 to 41.80%. The fractions **C4**, **C5**, and **C6** (Fig. 4e–g) were found non-toxic (> 100%) to HeLa cells, as indicated by the therapeutic index values of 23.15, 11.82, and 10.76, respectively (Table 1).

Spectral characterizations of isolated compounds

FT-IR analysis of isolated fractions

Figure 5a displays the FTIR spectrum for C1. The absorbance frequencies were acquired at 3439.33, 2923.67, 1737.25, 1458.01, 1173.93, 809.45, and 723.13 cm^{-1} (O–H, C–H, C=O, C–H (alkane), C–O (ester), and C–H). The compound **C2** showed different functional groups and absorbance frequencies were revealed at 2921.10, 2855.74, 1703.95, 1458.93, 1297.10, 944.83, and 724.03 cm^{-1} (O–H, C–H, C=O, C–H (alkane), C–O, and C=C) (Fig. 5b). The Fig. 5c, represents the compound **C3** functional groups such as 3436.99, 2925.49, 2857.64, 1715.89,

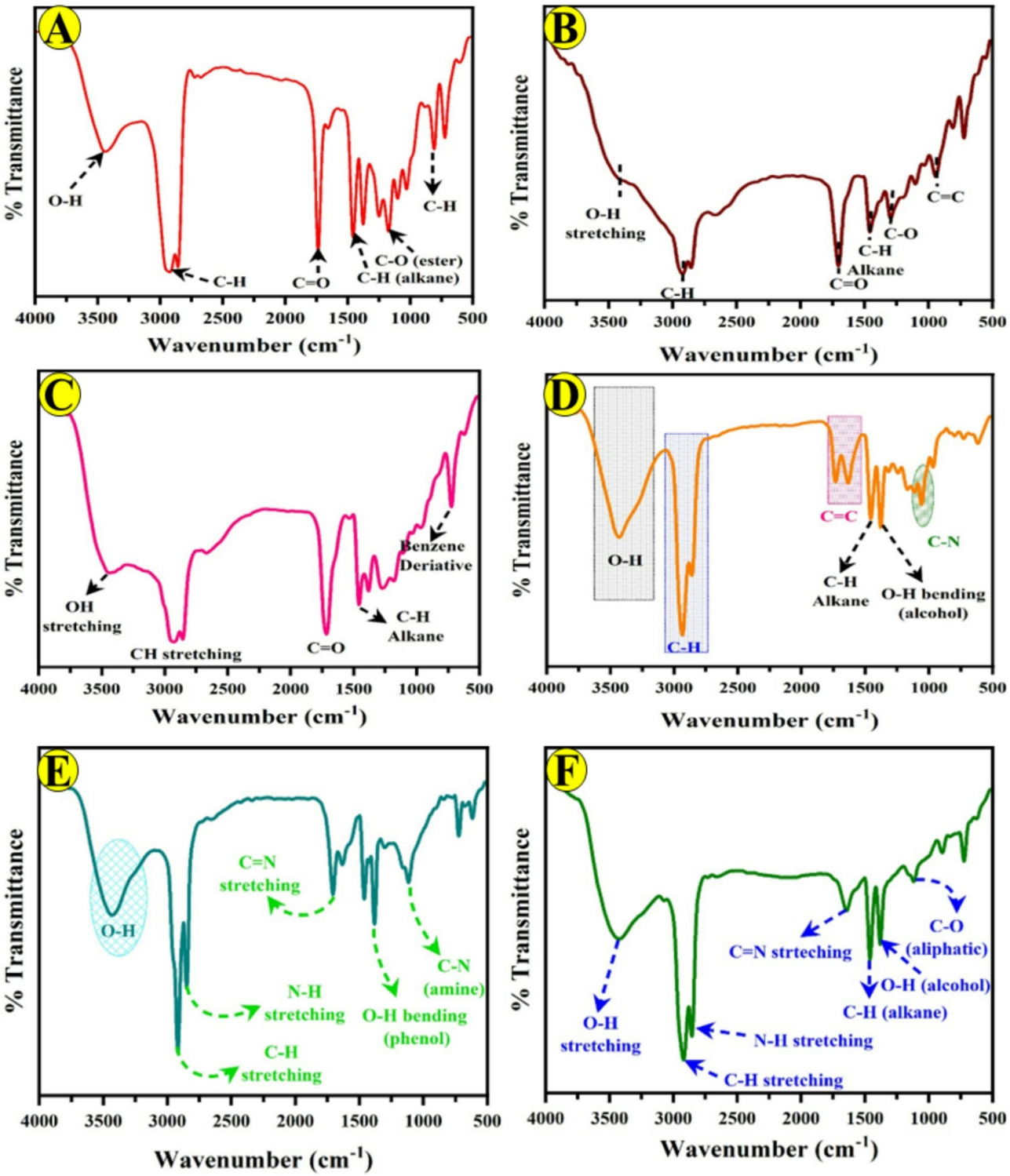

Fig. 5. Represent the FT-IR spectrum of isolated compounds **C1** (**5a**), **C2** (**5b**), **C3** (**5c**), **C4** (**5d**), **C5** (**5e**), and **C6 (5f)** from the leaf ethyl acetate extract of *P. emblica*.

1457.50, and 622.58 cm^{-1} (O–H stretching, C–H stretching, C=O, C–H (alkane), and Benzene derivative). The isolated fraction **C4** exhibited absorbance frequencies at 3431.07, 2935.19, 2861.24, 1733.35, 1633.83, 1456.75, 1379.49, 968.65, and 616.76 cm^{-1} (O–H, C–H stretching, C=O, C–H (alkane), O-H bending (alcohol), and C-N) (Fig. 5d). The compound **C5** obtained various absorbance frequencies at 3432.04, 2918.81, 2850.24, 1706.84, 1631.99, 1462.50, 1382.48, and 1116.45 cm^{-1} (O–H, C–H stretching, N–H stretching, C=N stretching, and C-N (amine)) (Fig. 5e). The functional groups of isolated fractions **C6** revealed at 3425.33, 2920.34, 2855.12, 1642.97, 1460.41, 1381.30, 1118.11, and 724.34 cm^{-1} (O–H stretching, C–H stretching, N–H stretching, C=N stretching, O–H bending (alcohol), C–O (aliphatic), and C–N (amine)) (Fig. 5f), respectively.

NMR spectral characterization of isolated compounds

Compound C1

9-hydroxy isolongifolene; Yellow color transparent crystal; Molecular formula: $C_{15}H_{24}O$; Molecular weight: 220.35 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.5 to 2.5 (CH, Aliphatic carbons),), 4.0 (OH, Hydroxy); ^{13}C-NMR (100 MHz, $CDCl_3$): 28 (C–H, Aliphatic carbon) 77 (CDCl3 Peak). FT-IR (KBr): 3431 (OH-Hydroxy), 2935, 2861 (CH Stretch, Alkanes), 1633 (C=C Stretch, Alkene), 1456 cm^{-1} (CH bend, Alkane). From the spectral analysis, it can be concluded that the isolated compound is identified as 9-hydroxy isolongifolene.

Compound C2

Hexadecanoic acid, ethyl ester; White color solid; Molecular formula: $C_{18}H_{34}O_2$; Molecular weight: 284 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.5 to 3 (CH, Aliphatic), 5.4 (C=O, ester); ^{13}C-NMR (100 MHz, $CDCl_3$): 0.5 to 3 (CH, Aliphatic), 5.4 (C=O, ester); FT-IR (KBr): 3439, 2923, 2858 (CH Stretch, Aliphatic),1715 (C=O, Ester) 1658 (C=C Stretch, Alkene), 1458 cm^{-1} (CH bend, Alkane). The spectral analysis above leads to the conclusion that the isolated component is Hexadecanoic acid.

Compound C3

Phenol, 2,6-Bis(1,1-Dimethylethyl); Lemon yellow color powder; Molecular formula: $C_{14}H_{22}O$; Molecular weight: 206.32 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.5 to 2.5 (CH, Aliphatic), 0.9 (CH_3, Aliphatic), 5.4 (OH), 7.3 (CH, Aromatic); ^{13}C-NMR (100 MHz, $CDCl_3$): 0.5 to 2.5 (CH, Aliphatic), 0.9 (CH_3, Aliphatic), 5.4 (O–H), 7.3 (C-H, Aromatic); FT-IR (KBr): 2921 (O–H-Phenolic), 2857, 2669 (C–H Stretch, Aliphatic), 1103 cm^{-1} (CO stretch). According to the spectral study above, the isolated component was Phenol, 2,6-Bis(1,1-Dimethylethyl).

Compound C4

Furan, tetrahydro-3-methyl-4-methylene; White color solid; Molecular formula: $C_6H_{10}O$; Molecular weight: 98.145 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.5 to 2.5 (CH, Aliphatic carbons); ^{13}C-NMR (100 MHz, $CDCl_3$): 10 to 40 (C–H, Aliphatic carbons) 117 (CO); FT-IR (KBr): 3425 (CH-Alkyl), 2920, 2855 (C–H Stretch, Alkanes), 1642 (C=C Stretch, Alkene), 1460 cm^{-1} (C–H bend, Alkane). According to the spectral study above, the isolated component was Furan, tetrahydro-3-methyl-4-methylene.

Compound C5

Octadecanoic acid; Lemon yellow color solid; Molecular formula: $C_{18}H_{34}O_2$; Molecular weight: 282.5 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.1 to 3 (CH, Aliphatic), 5.4 (COOH, acidic); ^{13}C-NMR (100 MHz, $CDCl_3$): 10 to 40 (CH, Aliphatic), 179 (COOH, acidic); FT-IR (KBr): 3436 (O-H, Acidic), 2925 (C-H, Aliphatic),1715 (C=O, Acidic), 1457 cm^{-1} (CH bend, Alkane). The spectral analysis above leads to the conclusion that the isolated component is Octadecanoic acid.

Compound C6

Beta-sitosterol; White powder; Molecular formula: $C_{29}H_{50}O$; Molecular weight: 414.72 Da; ^{1}H-NMR (500 MHz, $CDCl_3$): 0.5 to 2.5 (multiplet, CH-Aliphatic carbons), 5.0 (OH stretch); ^{13}C-NMR (100 MHz, $CDCl_3$): 10 to 30 (C-H, Aliphatic carbons), 62 (C-OH stretch, Hydroxy carbon) 44 (C = C, Alkyne carbon); FT-IR (KBr): 3432 (OH-Hydroxy), 2918, 2850 (CH Stretch, Alkanes), 1631 (C=C Stretch, Alkene), 1462 cm^{-1} (CH bend, Alkane). The spectrum analysis above leads to the conclusion that the isolated molecule is 9-hydroxy Beta-sitosterol. The present results of purified compounds names, codes, molecular structures, and IC_{50} were presented in Fig. 6. All the six compounds ^{13}C and ^{1}H NMR spectra are presented in supplementary information (Supplementary Figure S2 to S7). LC profiles and MS spectra for purified fractions C1 (a), C2 (b), C3 (c), C4 (d), C5 (e), and C6 (f) from the leaf extract of *P. emblica* showed in the supplementary information (Supplementary Figure S8). The TLC chromatogram of *P. emblica* compounds represented in the supplementary information (a) FC2A (C1) eluted with ethyl acetate and hexane (4:1); (b) FC2C2 (C2) eluted with ethyl acetate and hexane (4:1); (c) FC2C4 (C3) eluted with ethyl acetate and hexane (1.5:3.5); (d) FC2C5 (C4) eluted with ethyl acetate and hexane (2:3); (e) FE3A (C5) eluted with ethyl acetate and hexane (3.5:1.5); (f) FE3B (C6) eluted with ethyl acetate and hexane (1:1) (Supplementary Figure S9).

Molecular docking studies

These studies play a pivotal role in exploring protein-ligand interactions, avoiding unproductive in vitro and in vivo experiments, and significantly reducing costs. In this study, the interactions between the isolated compounds and the *P. falciparum* dihydroorotate dehydrogenase protein (*Pf*-DDP) were investigated using molecular docking. The 3D crystallographic assembly of *Pf*-DDP (PDB ID: 5TBO) was obtained from the Protein Data Bank, resolved using X-ray diffraction at 2.15 Å. The coordinates of the grid box center for 5TBO were arranged as center_x = 21.602, center_y = − 4.336, and center_z = − 0.218 and all the generated poses along with the docking score is given in Supplementary Figure S10. The docking scores of compounds **C1** (− 7.1 kcal/mol), **C3**

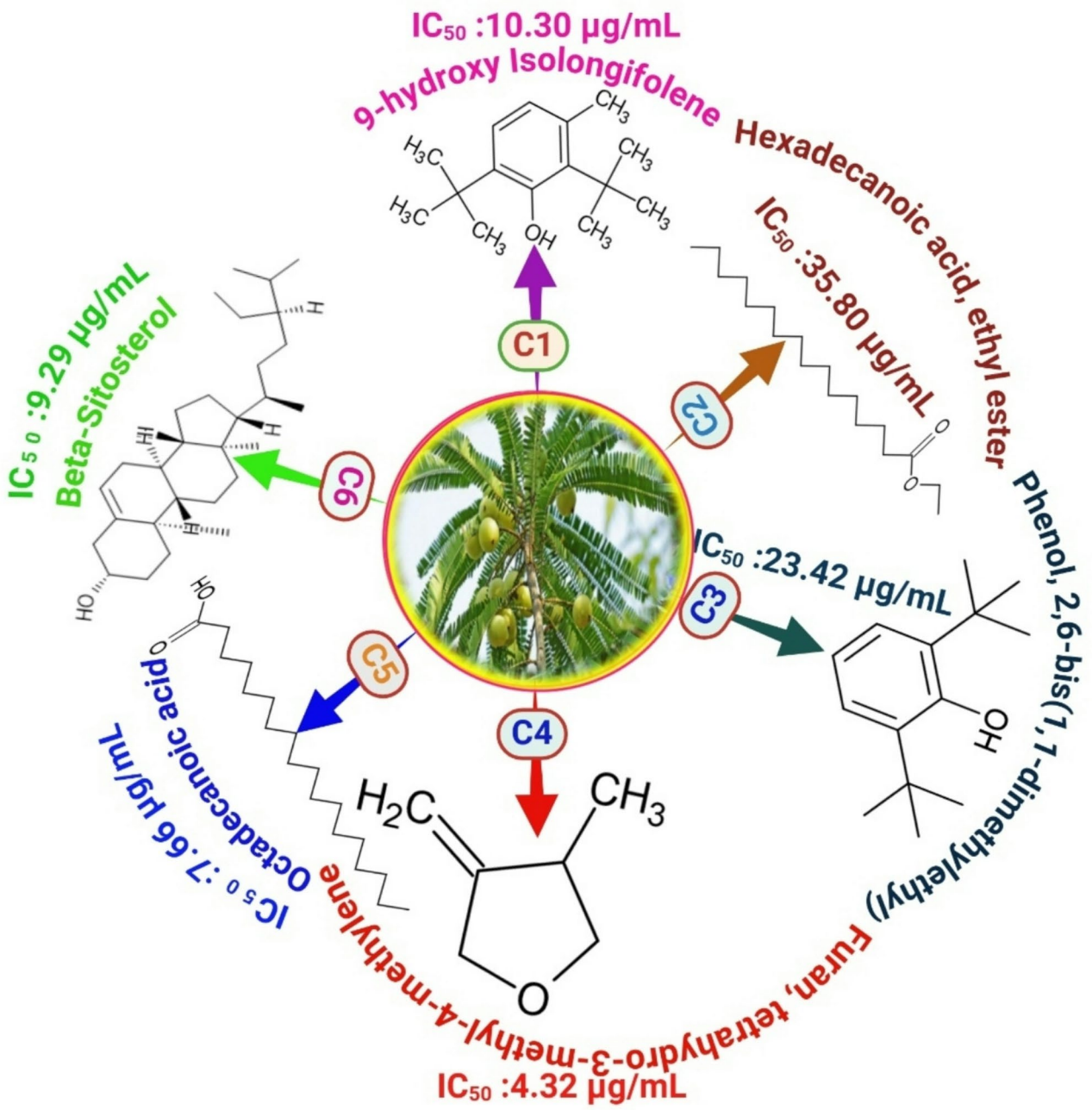

Fig. 6. Displayed *P. emblica* isolated compounds name, code, molecular structure, and IC_{50} values on *P. falciparum*.

(– 7.0 kcal/mol), and **C6** (– 7.9 kcal/mol) were greater than that of the standard drug Chloroquine (Fig. 7). None of the candidate compounds were greater than co-crystallized ligand (– 9.0 kcal/mol). The compound **C1** exhibits a binding affinity of – 7.1 kcal/mol, forming a Pi-sigma bond with Phe A:278, alkyl bond with Cys A:276, and conventional H-bond with Asn A:274, Thr A:459. Compound **C2** shows a binding affinity of – 6.5 kcal/mol with *Pf*-DDP, forming alkyl and pi-alkyl interactions with Leu A:531, Ile A:237, Phe A:188, Met A:536, Leu A:172, Leu A:189, Phe A:227, Leu A:240, Cys A:233, Val A:532, His A:185, and Cys A:184. Compound **C3** demonstrates a binding affinity of – 7.0 kcal/mol with *Pf*-DDP, forming a Pi-Pi T-shaped interaction with Phe A:227, and alkyl and Pi-alkyl interaction with Leu A:240, Leu A:197, Ile A:237, Leu A:189, His A:185, Phe A:188. Compound **C4** shows a binding affinity of – 4.4 kcal/mol with *Pf*-DDP, forming a carbon-hydrogen bond with His A:185. Compound **C5** demonstrates a binding affinity of – 5.3 kcal/mol with *Pf*-DDP, forming a conventional-hydrogen bond with Ser A:529, Gly A:478, Gly A:507 and Pi-Alkyl interactions with Phe A:278, Tyr A:528 and Carbon H-bond with Gly A:506. The compound **C6** exhibits an excellent binding affinity of – 7.9 kcal/mol with *Pf*-DDP, involving Pi-sigma interactions with TYR A:528, and alkyl and Pi-alkyl interactions with Cys A:276, Phe A:278.

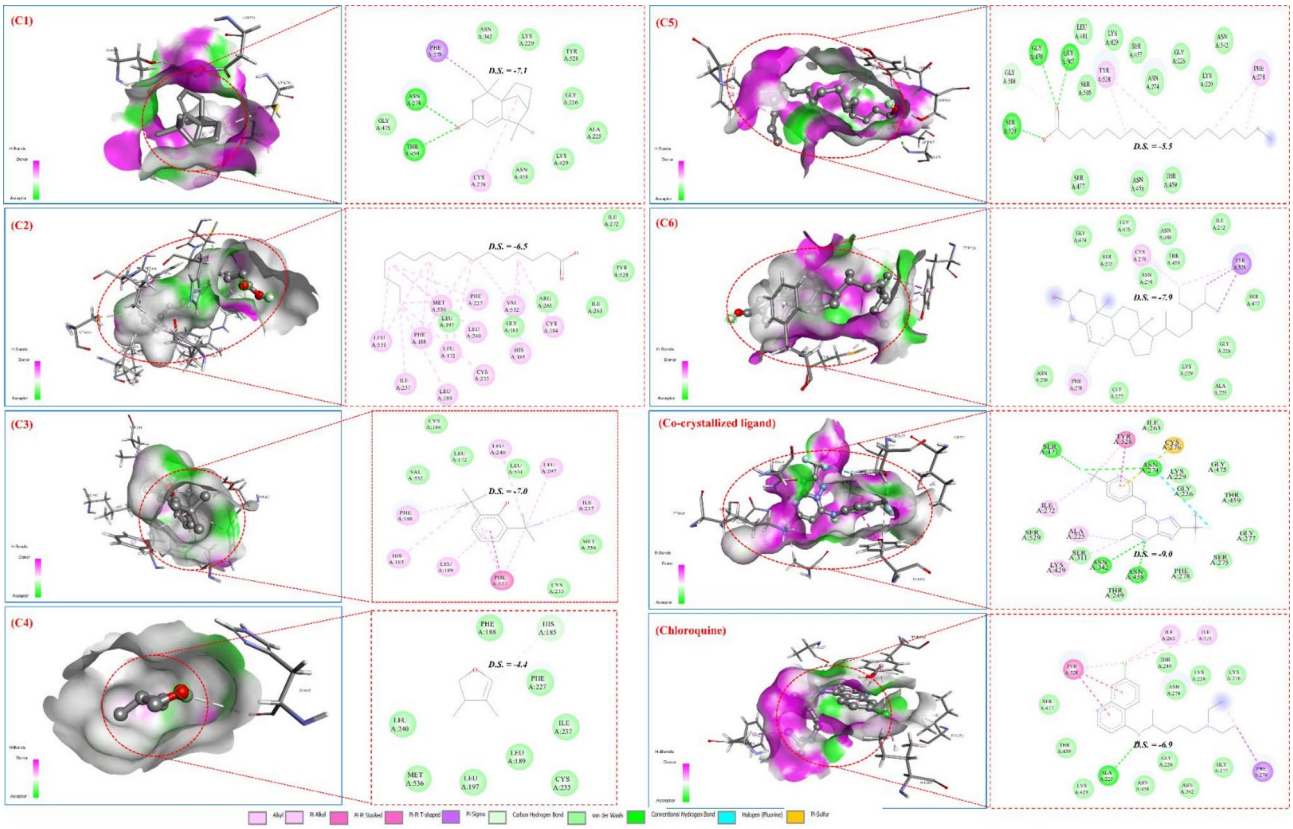

Fig. 7. Showing the 3D and 2D molecular interaction of isolated compounds (C1-C6) with *Plasmodium falciparum* dihydroorotate dehydrogenase protein.

Chloroquine binds to *Pf*-DDP with a binding affinity of – 6.9 kcal/mol, forming conventional hydrogen bonds with Ala A:225, Pi-Pi stacked interactions with Tyr A:528, and alkyl and Pi-alkyl interactions with Ile A: 263, Ile A:272. Co-crystallized ligand 78Z showed binding affinity of -9.0 kcal/mol with Pi sulfur (Cys A:276), Pi-Pi stacked (Tyr A:528), conventional hydrogen bond (Ser A:477, Asn A:274, Asn A:342, Asn A:458), alkyl and Pi-alkyl interactions (Ile A:272, Ala A:225, Lys A: 429) with a very low RMSD of 0.977 when superimposed with the XRD structure of *Pf*-DDP (Supplementary Figure S11). Further, non-bonded interactions like van der Waals were present between all the ligands and *Pf*-DDP (Table 2).

Discussion

The progress made through public health initiatives to combat malaria is being undermined by the increasing resistance of parasites to insecticides, the increasing resistance of malaria parasites to artemisinin-based combination therapies (ACTs) and their partner drugs[55]. To achieve rapid malaria eradication, the World Health Organization recommends speeding up the study and creation of new technologies for both prevention as well as treatment[8]. As a result, investigating traditional medicinal plants, such as *P. emblica*, could provide insightful information about the identification of effective bioactive compounds for combating the disease. *P. emblica* contains various secondary metabolites with numerous health benefits. Tannins have anti-inflammatory effects, polyphenols provide antimicrobial activity, and flavonoids act as antioxidants and antimicrobial agents. Saponins possess antibacterial properties, while alkaloids show anticancer and antidiabetic effects. Together, these compounds make *P. emblica* effective for managing inflammation, infections, oxidative damage, cancer, and diabetes[56,32]. found that the aqueous extract of *P. emblica* fruit showed significant antimalarial activity, with an IC_{50} of 14.37 ± 0.17 µg/mL against the multidrug-resistant *P. falciparum* K1 strain in an in vitro test. For comparison, dihydroartemisinin, the positive control, had an IC_{50} of 0.0035 ± 0.0007 µg/mL. The in vitro findings were further validated using in vivo testing on the chloroquine-sensitive *P. berghei* (CQ-sensitive strain) rodent malaria model. At a dose of 250 mg/kg/day, the aqueous extract of *P. emblica* fruit exhibited strong anti-plasmodial activity in all mouse groups. The treated mice showed a mean parasitemia value of 11.85, representing a 69.46% suppression of parasitemia. This value was significantly lower compared to the group that received normal saline treatment ($p < 0.05$), indicating the extract's effective reduction of malaria parasite load. Artesunate (5 mg/kg/day) achieved complete parasite suppression (100%). However, unlike the artesunate-treated mice, which survived for over 15 days, the fruit extract administered mice had a survival period of 6.8 ± 0.97 days. Additionally, the isolated compounds also displayed significant growth inhibition of parasite in the in vitro studies.

S. No	Compound code	Docking score (kcal/mol)	Amino acid interactions
1.	C-1	− 7.1	Pi-Sigma: Phe A:278;
			Alkyl: Cys A:276;
			Conventional H-bond: Asn A:274, Thr A:459;
			van der Waals: Gly A:475, Asn A:342, Lys A:229, Tyr A:528, Gly A:226; Ala A;225, Lys A:429, Asn A:458
2.	C-2	− 6.5	Alkyl and Pi-alkyl: Leu A:531, Ile A:237, Phe A:188, Met A:536, Leu A:172, Leu A:189, Phe A:227, Leu A:240, Cys A:233, Val A:532, His A:185, Cys A:184
			van der Waals: Ile A:272, Tyr A:528, Ile A:263, Arg A:265, Gly A:181, Leu A:197
3.	C-3	− 7.0	Pi-Pi T-shaped: Phe A:227;
			van der Waals: Cys A:184, Leu A:172, Leu A:531, Val A:532, Met A:536, Cys A:233
			Alkyl and Pi-alkyl: Leu A:240, Leu A:197, Ile A:237, Leu A:189, His A:185, Phe A:188
4.	C-4	− 4.4	Carbon H-bond: His A:185
			van der Waals: Phe A:188, Phe A:227, Ile A:237, Leu A:189, Cys A:233, Leu A:197, Met A:536, Leu A:240
5.	C-5	− 5.3	Pi-alkyl: Phe A:278, Tyr A:528
			Conventional H-bond: Ser A:529, Gly A:478, Gly A:507
			Pi-alkyl: Tyr A:528, Phe A:278
			Carbon H-bond: Gly A:506
			van der Waals: Leu A:481, Ser A:505, Leu A:481, Lys A:429, Ser A:457, Asn A:274, Gly A:226, Lys A:229; Asn A:342, Thr A:459, Asn A:458, Ser A:477
6.	C-6	− 7.9	Pi-sigma: Tyr A:528
			Alkyl and Pi-alkyl: Cys A:276, Phe A:278
			van der Waals: Gly A:474, Ser A:275, Gly A:475, Asn A:458, Asn A:274, Thr A:459, Ile A:272, Ser A:477, Gly A:226, Ala A:225, Lys A:229, Gly A:277, Asn A:258
7.	Chloroquine	− 6.9	Pi-Pi stacked: Tyr A:528
			Pi-sigma: Phe A:278
			Conventional H-bond: Ala A:225
			Alkyl and Pi-alkyl: Ile A: 263, Ile A:272
			van der Waals: Ser A:477, Thr A:459, Lys A:429, Asn A:458, Gly A:226, Asn A:342, Gly A:277, Cys A:276, Lys A:229, Asn A:274, Thr A:249
8.	Co-crystallized ligand	− 9.0	Pi-sulfur: Cys A:276
			Pi-Pi stacked: Tyr A:528
			Conventional H-bond: Ser A:477, Asn A:274, Asn A:342, Asn A:458
			Alkyl and Pi-alkyl: Ile A:272, Ala A:225, Lys A: 429
			van der Waals: Ile A:263, Lys A:229, Gly A:475, Gly A:226, Thr A:459, Gly A:277, Ser A:275, Phe A:278, Thr A:249, Ser A:311, Ser A:529

Table 2. List of isolated compounds and its Docking score on *Pf*-DDP.

Found that the ethyl acetate extract of *P. emblica* leaves exhibited strong antiplasmodial activity (IC_{50} 17.849 µg/mL), indicating its effectiveness in inhibiting malaria parasite growth[26]. Similarly[57], reported that aqueous extracts of the entire *P. urinaria* demonstrated strong in vitro efficacy against the W2 strain of *P. falciparum*. Furthermore[58], discovered that *P. niruri* extract had the highest in vivo activity, with an ED_{50} of 9.1 mg/kg/day[59]. Found that *P. niruri* extract inhibited *P. falciparum* parasitemia by 72.73% when provided at a dose of 200 mg/kg, demonstrating its great antimalarial potential. These findings highlight *P. niruri* significant antimalarial activity in vitro and in vivo, indicating its potential for therapeutic usage in malaria treatment at 1000 mg/kg, *P. acuminatus* leaf extract showed 52% inhibition, indicating moderate efficacy[60]. In mice infected with *Plasmodium berghei*, an ethanolic extract of *P. emblica* leaves was found to reduce liver damage. Various doses have been tested (100, 300, and 1200 mg/kg body weight), and the most effective dose for reducing liver damage was 600 mg/kg body weight[61]. Due to its abundant physiologically active secondary metabolites, *P. emblica* has been extensively used in traditional medicine for a range of therapeutic purposes. These metabolites contribute significantly to its health benefits[62,63].

Numerous metabolite groups, including terpenoids, glycosides, phenolic compounds and flavonoids have been detected in this plant through phytochemical studies[64,65]. Fraction-based fractionation has already been used to report chemical substances such as geranin, furosin, phyllemblin, corilagin, and gallic acid[66]. *P. emblica* also contains alkaloids including phyllantine and phyllantidine, as well as flavonoids like quercetin. Polyphenols and flavonoids, which are abundantly present in *P. emblica*, are renowned for their strong anti-inflammatory and antioxidant qualities and are essential in halting the development of oxidative stress[67]. These compounds, such as flavonoids, utilize a radical scavenging mechanism by donating an electron to free radicals' unpaired electrons, thereby preventing oxidation reactions and mitigating their harmful effects[68]. Flavonoids are believed to protect the liver by binding to free radicals, which otherwise compromise the stability of hepatocyte membranes and allow the release of various enzymes from the hepatocytes. Flavonoids and steroids help fight malaria by blocking the nutrients *Plasmodium* needs to survive, like L-glutamine and myoinositol, during the stage when the parasite is inside red blood cells[69]. They also disrupt the parasite's ability to produce fatty acids, which are essential for

its growth and reproduction[70]. These actions make flavonoids and steroids potential antimalarial agents[71]. In addition to interacting with cellular components of the *Plasmodium* parasite, they can also form complexes with soluble and extracellular proteins. Highly lipophilic flavonoids may block specific enzymes, disrupt malaria parasite membranes, and neutralize toxins[69,72].

Evaluating the in vitro antimalarial activity of an extract or compound is a crucial step in antimalarial drug discovery. These tests are also valuable for assessing *Plasmodium*'s susceptibility to novel and purified natural compounds[73]. However, it is equally important to assess the toxicity of any compound, extract, or drug that shows strong antiplasmodial activity during the drug development process. A successful drug candidate must demonstrate high efficacy against malaria while maintaining an acceptable toxicity profile. The MTT test was utilized in this research to evaluate the in vitro cytotoxicity using HeLa cell lines. The isolated compounds 9-hydroxy isolongifolene (**C1**), Hexadecanoic acid (**C2**), Phenol, 2,6-Bis(1,1-Dimethylethyl) (**C3**), Furan, tetrahydro-3-methyl-4-methylene (**C4**), Octadecanoic acid (**C5**), and Beta-Sitosterol (**C6**) showed moderate and low toxicity to HeLa cells (TC_{50} 41.80 to > 100 µg/mL), indicating their safety for human use. Additionally, the ratio of a compound's or drug's cytotoxicity (TC_{50}) to antiparasitic efficacy (IC_{50}) was calculated as the selectivity index. SI measures the specificity of a drug or treatment's action, determining whether its antiplasmodial efficacy is due to targeted action against the parasite or if the observed activity results from its inherent toxicity to host cells. A higher SI indicates a more selective and effective antimalarial agent[49]. A chemical, medication, or extract with a SI above 10 is regarded as having strong antimalarial efficacy and a high potential for safe use, according to the standards established by[74]. Purified fractions and compounds can therefore be classified as active antimalarials against strains of *P. falciparum*. Whereas, the current drug Artemisinin showed decreased viability in HeLa cells at approximately 600.19 nmol/mL on 24 h of incubation[75].

Molecular docking analyses were achieved to investigate the binding interactions of the extracted 9-hydroxy isolongifolene (**C1**), Hexadecanoic acid (**C2**), Phenol, 2,6-Bis(1,1-Dimethylethyl) (**C3**), Furan, tetrahydro-3-methyl-4-methylene (**C4**), Octadecanoic acid (**C5**), and Beta-Sitosterol (**C6**) on *P. falciparum* dihydroorotate dehydrogenase (*Pf*-DDH) is an enzyme involved in the biosynthesis pathway of de novo pyrimidine. It is necessary for the *Plasmodium* parasite to thrive and survive. A comparative analysis with established drugs like CQ was conducted to explore potential combination therapies. Among these compounds, compound C1 exhibited a binding affinity of − 7.1 kcal/mol, indicating a strong interaction with the target protein. This binding is facilitated through a Pi-sigma bond with Phe A:278, alkyl bond with Cys A:276 and a conventional H-bond with Asn A:274, Thr A:459. These interactions suggest that compound C1 binds effectively to the *Pf*-DDH protein, potentially inhibiting its function and offering a promising lead for further antimalarial drug development. Compound **C2** shows a binding affinity of − 6.5 kcal/mol with *Pf*-DDP, forming alkyl and pi-alkyl interactions with Leu A:531, Ile A:237, Phe A:188, Met A:536, Leu A:172, Leu A:189, Phe A:227, Leu A:240, Cys A:233, Val A:532, His A:185, and Cys A:184. In addition, with a Pi-Pi T-shaped interaction with Phe A:227, and alkyl and Pi-alkyl interaction with Leu A:240, Leu A:197, Ile A:237, Leu A:189, His A:185, Phe A:188, compound **C3** shows a binding affinity of − 7.0 kcal/mol with *Pf*-DDH. Compound C4 forms a carbon-hydrogen bond with His A:185 and exhibits a binding affinity of − 4.4 kcal/mol with *Pf*-DDH. Docking interaction profile suggested the compound **C6** exhibits an excellent binding affinity of − 7.9 kcal/mol with *Pf*-DDP, involving Pi-sigma interactions with TYR A:528, and alkyl and Pi-alkyl interactions with Cys A:276, Phe A:278. Chloroquine binds to *Pf*-DDP with a binding affinity of − 6.9 kcal/mol. Further, these results were validated by redocking of co-crystallized ligand which showed a binding affinity of -9.0 kcal/mol. However, the active compounds, the underlying mechanism of action require further investigation, and in vivo studies are necessary to confirm these findings.

Conclusion

The ethyl acetate extract of *P. emblica* and its pure compounds-9-hydroxy isolongifolene (**C1**), Hexadecanoic acid (**C2**), Phenol, 2,6-Bis(1,1-Dimethylethyl) (**C3**), Furan, tetrahydro-3-methyl-4-methylene (**C4**), Octadecanoic acid (**C5**), and Beta-Sitosterol (**C6**)- demonstrated promising antimalarial activity towards *P. falciparum* 3D7 strain without causing toxicity to normal or cancer cells. This work is the initial source for disclosing the antimalarial potential of *P. emblica* compounds. Additionally, this is the first documentation of the isolation of these six compounds from *P. emblica* extract. This study is the first to provide comprehensive insights into the antimalarial potential of these drugs, supported by molecular docking studies targeting the parasite's essential metabolic enzyme, *P. falciparum* dihydroorotate dehydrogenase (*Pf*-DDH). These findings lend scientific validation to the traditional use of *P. emblica* in the treatment of malaria and suggest that it holds promise as an effective antimalarial agent. The study not only highlights the importance of *P. emblica* compounds in combating malaria but also lays the groundwork for further exploration into the specific bioactive components within the plant extract. Ongoing research aims to identify additional bioactive molecules in the extract and evaluate the in vivo antiplasmodial efficacy of the isolated pure compounds, both individually and in combination with existing antimalarial drugs, to assess their potential for enhancing therapeutic outcomes and overcoming resistance. In the future, we will deploy as in-detailed assessments such as antimalarial activity towards resistant strains, non-cancerous cell lines, molecular mechanisms of action, and combined effect of these potential compounds which will be contrasted with prevailing antimalarial drugs to make these available as antimalarial drugs.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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