

In Silico and In Vivo Anti-Malarial Studies of 18 β Glycyrrhetic Acid from *Glycyrrhiza glabra*

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Abstract

Malaria is one of the most prevailing fatal diseases causing between 1.2 and 2.7 million deaths all over the world each year. Further, development of resistance against the frontline anti-malarial drugs has created an alarming situation, which requires intensive drug discovery to develop new, more effective, affordable and accessible anti-malarial agents possessing novel modes of action. Over the past few years triterpenoids from higher plants have shown a wide range of anti-malarial activities. As a part of our drug discovery program for anti-malarial agents from Indian medicinal plants, roots of *Glycyrrhiza glabra* were chemically investigated, which resulted in the isolation and characterization of 18 β -glycyrrhetic acid (GA) as a major constituent. The *in vitro* studies against *P. falciparum* showed significant (IC₅₀ 1.69 μ g/ml) anti-malarial potential for GA. Similarly, the molecular docking studies showed adequate docking (LibDock) score of 71.18 for GA and 131.15 for standard anti-malarial drug chloroquine. Further, *in silico* pharmacokinetic and drug-likeness studies showed that GA possesses drug-like properties. Finally, *in vivo* evaluation showed a dose dependent anti-malarial activity ranging from 68–100% at doses of 62.5–250mg/kg on day 8. To the best of our knowledge this is the first ever report on the anti-malarial potential of GA. Further work on optimization of the anti-malarial lead is under progress.

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Introduction

Malaria is an endemic infection caused by the protozoan parasites belonging to the genus *Plasmodium* [1]. The disease has positioned about 3.3 billion people at risk. The recommended preventive drugs are a combination of sulfadoxine, pyramethamine and amodiaquine, while the therapeutic strategy includes use of artemisinin combinations in areas where *P. falciparum* is endemic and chloroquine (CQ) in the areas where it is still efficacious such as some American regions [2]. This enormous global health challenge is partly due to the development of resistance against the frontline drugs especially artemisinins, which has been recently detected in the four countries of the Asian sub-continent. This alarming situation calls for an intensive drug discovery to find out new natural leads. The resistance in malaria parasites is believed to have emerged through mutations in the active sites of drug targets or from the biochemical changes in the drug receptors [3]. After the successful elucidation of complete genome sequence of *P. falciparum* [4], it has opened a new prospect in

the drug discovery where the available databases and bio-informatics tools help us to determine potential receptors and targets within the pathogen [5]. Some of the new drug targets identified in *P. falciparum* are *Plasmodium falciparum* lactate dehydrogenase enzyme (*pfLDH*), type II fatty acid synthase (type II FAS) and plasmepsin. The *pfLDH* has been considered as a potential molecular target for anti-malarials due to this parasite's dependence on glycolysis for energy production. The *pfLDH* has significantly different structural and kinetic properties compared to human LDH iso-forms [6], but the LDH enzymes found in *P. vivax*, *P. malariae* and *P. ovale* (*pLDH*) all exhibit, ~ 90% identity to *pfLDH* and several selective inhibitors of *pLDH* have demonstrated anti-malarial activity *in-vitro* and *in-vivo*. Similarly, *plasmodium* fatty acids are synthesized in the apicoplast, utilizing a dissociable multi-enzyme system called type II fatty acid synthase (type II FAS), which differs significantly from human type I FAS [7]. On the other hand, plasmepsins are a class of at least 10 enzymes produced by the *P. falciparum* parasite. There are ten different isoforms of these proteins and ten genes coding them respectively in

Plasmodium. Through their hemoglobin-degrading activity, they are an important cause of symptoms in malaria sufferers. Hence, it would be desirable to use pLDH, type II FAS and plasmepsin as potential targets for the structure-based design of novel anti-malarial.

Recently, CoMFA, CoMSIA, QSAR and docking guided selection of type II FAS, plasmepsin and pLDH inhibitors have been used as important strategies in the discovery of new anti-malarials [3,8,9,10]. These approaches are inexpensive and more practical than discovering novel compounds.

Over the past few years, triterpenoids from higher plants have shown a wide range of biological activities, such as antitumor [11], antiviral [12], anti-inflammatory [13,14,15] and anti-HIV [16]. As a part of our drug discovery program on anti-malarial agents from Indian medicinal plants, the literature search revealed significant anti-malarial activity in pentacyclic triterpenes, betulinic, oleanolic and ursolic acids [17,18]. This prompted us to investigate anti-malarial activity in other triterpenoids, found in widely used Indian medicinal plants. For this purpose, roots of *Glycyrrhiza glabra* were taken for detailed chemical and biological investigations. *G. glabra* is one of the most important Indian medicinal plants regularly used by common masses from the ancient time. It is used to reduce the toxicity and the bitter taste of other drugs as well as it enhances the effectiveness of other gradients [19]. For more than 4000 years, it has been used against acute bronchitis, severe mouth ulcer, inflammation and other stomach related diseases [19,20].

The present study reports isolation and characterization of 18 β -glycyrrhetic acid (GA) as a major constituent from the roots of *G. glabra* followed by its *in-vitro*, *in-silico* and *in vivo* anti-malarial evaluation as shown in the Figure 1.

Experimental Section

Ethics statement

The animal experimentation was duly approved by the Institutional Animal Ethics Committee under the Committee for the Purpose of Control and Supervision of Experimentation on Animals, Govt. of India.

2.1: Isolation of 18 β -glycyrrhetic acid

Plant Material. The roots of *G. glabra* were collected after cultivation and harvesting from the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh, India during the month of January, 2008. A voucher specimen # 9900 was deposited in the Herbarium section of the Botany Department of CIMAP.

2.1.1: Extraction and Fractionation of *G. glabra* roots. The air dried powdered roots of *G. glabra* (2.04 kg) were extracted overnight with methanol (4 x 5L) at room temperature and the combined methanol extract was completely dried under vacuum at 40°C. This dried MeOH extract was dissolved in water and successively fractionated thrice with dichloromethane, ethyl acetate and saturated *n*-butanol, separately. The combined dichloromethane and ethyl acetate extracts were first washed with water and then dried over anhydrous Na₂SO₄. Finally, all the three extracts were

completely dried under vacuum, which yielded 99.0g of dichloromethane, 100.0g of ethyl acetate and 56.0g of *n*-butanol extracts (Figure 2).

2.1.2: Isolation and Characterization of glycyrrhizic acid (GL). The *n*-butanol extract (1.00 gm) was purified over the flash using Silica gel H (average particle size 10 μ m). Gradient elution of the flash was carried out with a mixture of CHCl₃: MeOH in increasing order (up to 50% MeOH). Fractions of 50ml each were collected and pooled on the basis of their TLC profile. A total of 149 fractions were collected. Fractions 42-58 eluted with CHCl₃: MeOH (85: 15) afforded a homogeneous compound (650mg), which was characterized as glycyrrhizic acid (GL) on the basis of its ¹H, ¹³C, DEPT-135 spectroscopic data. C₃₀H₄₆O₄, ESI-MS m/z 823.9 [M+H]⁺, ¹H NMR (300 MHz, CDCl₃): Δ 0.76-1.32 (3H each all s, 7 x tert.CH₃), 2.32 (s, 1 H; 9H), 1.97 (3H, s, C-32), 4.2 (1H, dd, J= 6.8 & 8.7 Hz, 3 α -H), 5.56 (1H, s, H-12), 5.20 (1H,d, H-1'), 3.23 (1H, m, H-2'), 3.62 (1H, m, H 3' & H-4'), 4.45 (1H, d, H-5'), 12.22 (1H, s, H-6'), 4.86 (1H,d, H-1''), 3.53 (1H, m, H-2'', H-3'' & H-4''), 4.5 (1H, d, H-5''), 12.3 (1H, s, H-6''). ¹³C NMR: 39.9 (C-1),27.0 (C-2),88.7 (C-3),37.8 (C-4),55.5 (C-5),18.1 (C-6),33.2 (C-7),43.7 (C-8), 62.4 (C-9),37.6 (C-10),199.9 (C-11),128.9 (C-12),169.9 (C-13), 45.8 (C-14),28.3 (C-15),26.8 (C-16),32.4 (C-17),48.9 (C-18), 41.9 (C-19),44.3 (C-20),31.8 (C-21),38.6 (C-22),28.0 (C-23), 16.8 (C-24),17.0 (C-25),19.0 (C-26),23.7 (C-27),28.7 (C-28), 28.9 (C-29),179.4 (C-30),104.1 (C-1'),83.1 (C-2'),75.8 (C-3'), 71.7 (C-4'),76.5 (C-5'),170.5 (C-6'),105.2 (C-1''),75.4 (C-2''), 76.9 (C-3''),71.8 (C-4''), 75.9 (C-5''), 170.6 (C-6'') (Figure 3).

2.1.3: Acid Hydrolysis of glycyrrhizic acid (GL). Further, GL (650mg) was dissolved in 25 ml of 10% H₂SO₄ solution in MeOH and was refluxed for 3-4 hrs. After completion of hydrolysis, the reaction mixture was diluted with water and neutralized with 10% NaOH solution, extracted with CHCl₃ and evaporated under vacuum till dryness (wt. = 450.0 mg).

2.1.4: Purification of aglycone 18 β -glycyrrhetic acid (GA). The aglycone (450 mg) was purified over the flash using Silica gel H. A total of 148 fractions were collected and pooled on the basis of their TLC profile. The fractions 29-46 eluted with CHCl₃: MeOH (99:1) afforded 18 β -glycyrrhetic acid (GA, 250mg); C₃₀H₄₆O₄, ESI-MS m/z 471 [M+H]⁺, ¹H NMR (300 MHz, CDCl₃): Δ 0.76-1.32 (3H each all s, 7 x tert.CH₃) 2.32 (s, 1 H, 9H), 3.36 (1H, dd, J= 6.8 & 8.5 Hz, 3 α -H) 5.62 (1H, m, H-12). ¹³C NMR: 39.9 (C-1), 27.0 (C-2), 78.1 (C-3), 37.8 (C-4), 55.5 (C-5), 18.1 (C-6), 33.2 (C-7), 43.7 (C-8), 62.4 (C-9), 37.5 (C-10), 199.1 (C-11), 128.9 (C-12), 169.9 (C-13), 45.8 (C-14), 28.3 (C-15), 26.8 (C-16), 32.4 (C-17), 48.9 (C-18), 41.9 (C-19), 44.3 (C-20), 31.8 (C-21), 37.5 (C-22), 27.8 (C-23), 16.8 (C-24), 17.0 (C-25), 19.0 (C-26), 23.7 (C-27), 28.8 (C-28), 28.9 (C-29), 179.4 (C-30) (Figure 3).

2.2: In-vitro, In-silico and In-vivo anti-malarial assay

2.2.1: In vitro anti-malarial activity. GA was initially evaluated for anti-malarial activity against *Plasmodium falciparum* (strain NF 54). The parasite was cultured on red blood cells (B+) suspended in RPMI 1640 medium under humidified conditions of 37°C and 5% CO₂. Before initiation of the assay, parasites were synchronized through 5% sorbitol treatment. GA was dissolved in DMSO and evaluated at three

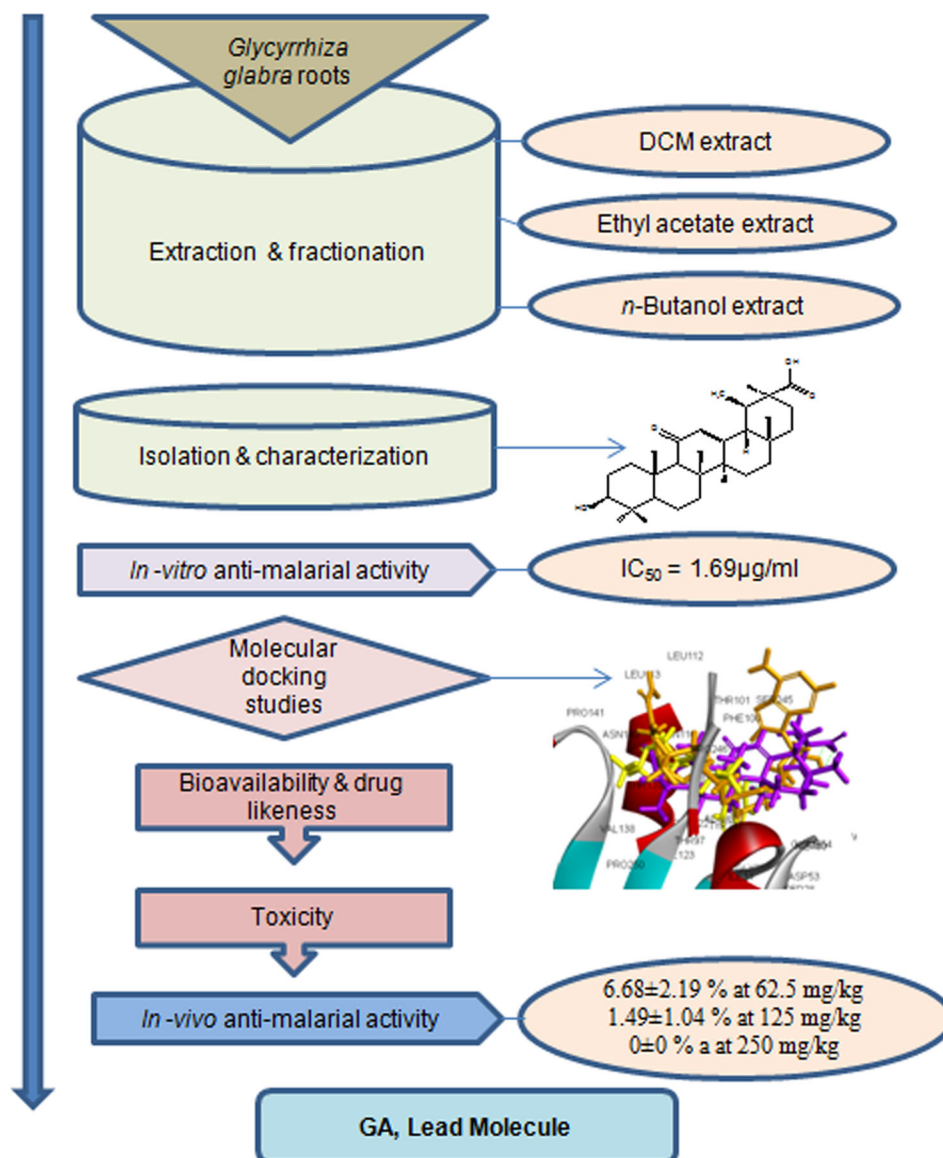


Figure 1. Schematic isolation, identification and bio-evaluation of anti-malarial lead, 18β-glycyrrhetic acid (GA).

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concentrations (100, 10 and 1 μg) in triplicate to derive IC_{50} values. The treatment was given for 24 hours and the parasitaemia from each treatment was microscopically evaluated through Giemsa staining of the blood smears.

2.2.2: *In-silico* molecular docking studies. The molecular docking and visualization studies were performed through Discovery Studio v3.5 (Accelrys Inc., USA, 2013) molecular modeling software. For target protein preparation, structure of *P. falciparum* lactate dehydrogenase (*pfLDH*) (PDB: 1CEQ) [21] was retrieved from a repository of experimentally elucidated crystal structure of biological macromolecules available at the Brookhaven Protein Data Bank (PDB), USA (<http://www.rcsb.org/pdb>). Initially the protein preparation protocol was used to perform tasks such as inserting missing

atoms in incomplete residues, deleting alternate conformations (disorder), removing water, standardizing names of the atoms, modeling missing loop regions and protonating titratable residues. The molecular docking studies were performed to generate the bioactive binding poses of inhibitors in the active site (i.e., NADH binding pocket) of the enzyme by using a LibDock program of Discovery Studio. LibDock uses protein site features referred to as hot spots, consisting of two types (polar and apolar). Then the ligand poses were placed into the polar and apolar receptor interactions site. For energy minimization of the ligands MMFF force field was used under parameterization step. In the present study, the NADH binding pocket was used to define the active site referred as hot spots (Figure 4A). The CAESAR (Conformer Algorithm based on

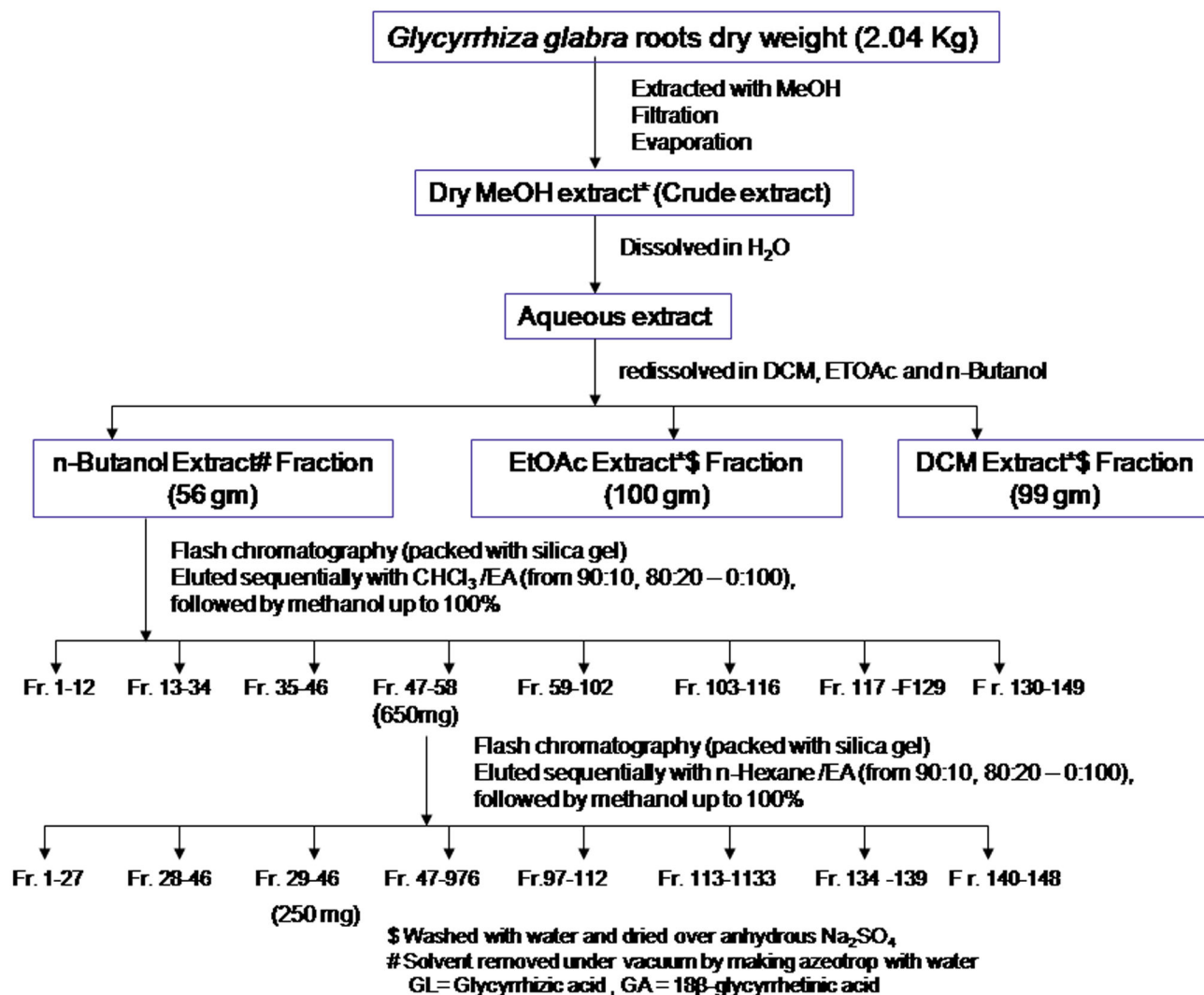


Figure 2. Schematic isolation of GA from *G. glabra* roots.

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Energy Screening and Recursive build-up) method was used for generating conformations. The Smart Minimiser was used for *in-situ* ligand minimization. All other docking and consequent scoring parameters used were kept at their default settings. The 2-D diagram of docking was derived to identify actual interacting receptor residues with bound ligand.

2.2.3: *In vivo* anti-malarial activity. *P. berghei* K173 (sensitive to CQ at 10 mg/Kg), was used to assess the *in-vivo* intrinsic anti-malarial activity. Parasites were maintained *in-vivo* by a weekly passage in mice as earlier reported [22]. The infection free female Swiss inbred mice, 6-8 weeks old, weighing 18–22g was maintained under standard environmental conditions of 22±3°C, 12:12 dark-to-light cycle with food and water. As reported earlier (Boniface and Pal 2013), mice were infected by intraperitoneal injection of 1×10⁶ infected erythrocytes, diluted in 0.2 ml of sterile acid citrate dextrose (citric acid 7.3g, sodium citrate 22.0g and dextrose 24.5g, dissolved in 1,000 ml of triple distilled water).

Parasitaemia was monitored through microscopic examination of Giemsa-stained thin blood smears. Parasitized erythrocytes were obtained from a donor infected mice with 8–10% parasitaemia from the retro orbital plexus in citrate saline (trisodium citrate 2%, NaCl 0.85%) and diluted further so as to contain one million (1×10⁶) parasitized erythrocytes, which was injected intraperitoneally to mice and divided into 5 groups of six mice each. Thin blood smears were drawn from the tip of the tail and stained with standard Giemsa stain. Parasitaemia was determined by counting RBCs comprising of both the parasitized and the normal RBCs. The blood smears were made every alternate day from day 4 till day 28. The results are presented as mean ± SEM. Significance between control and drug treated group were determined using one way analysis of variance (ANOVA) through InStat Software. Tukey's test was used to compare the means and p < 0.05 was considered significant.

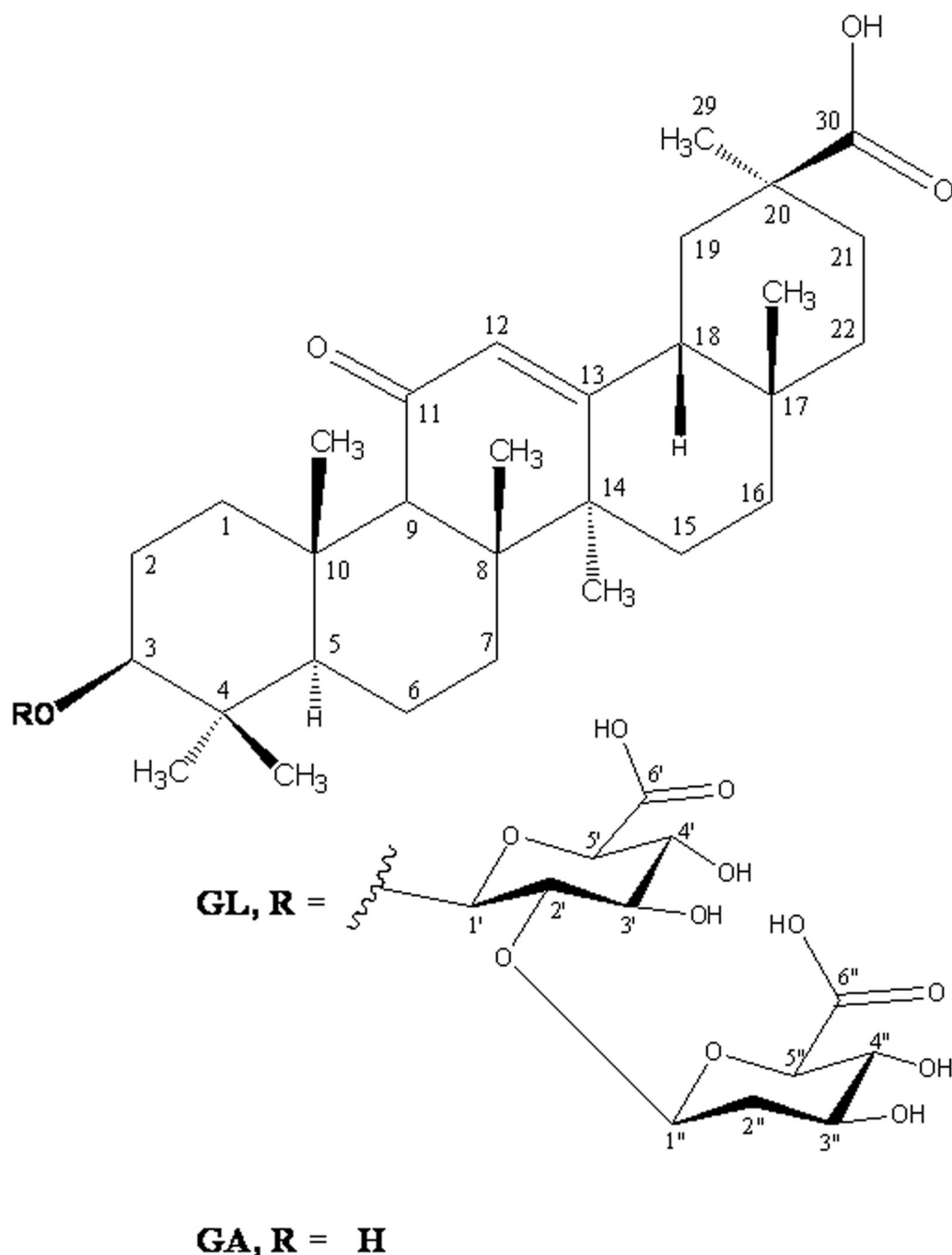


Figure 3. Chemical structures of glycyrrhizic acid (GL) and 18β-glycyrrhetic acid (GA).

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Results

3.1: Isolation and characterization of GA

The air dried powdered roots of *G. glabra* were cold extracted with MeOH and the completely dried MeOH extract was successively fractionated with dichloromethane, ethyl acetate and *n*-butanol as per the details given in the

experimental section. The *n*-butanol extract so obtained was purified over the flash using Silica gel H (average particle size 10 μM), which afforded a 650mg of homogeneous compound characterized as GL on the basis of its 1D and 2D NMR spectroscopic data [18]. Further, GL was acid hydrolyzed and the aglycone was again purified on flash using Silica gel H, which afforded a 250mg of a pure compound characterized as

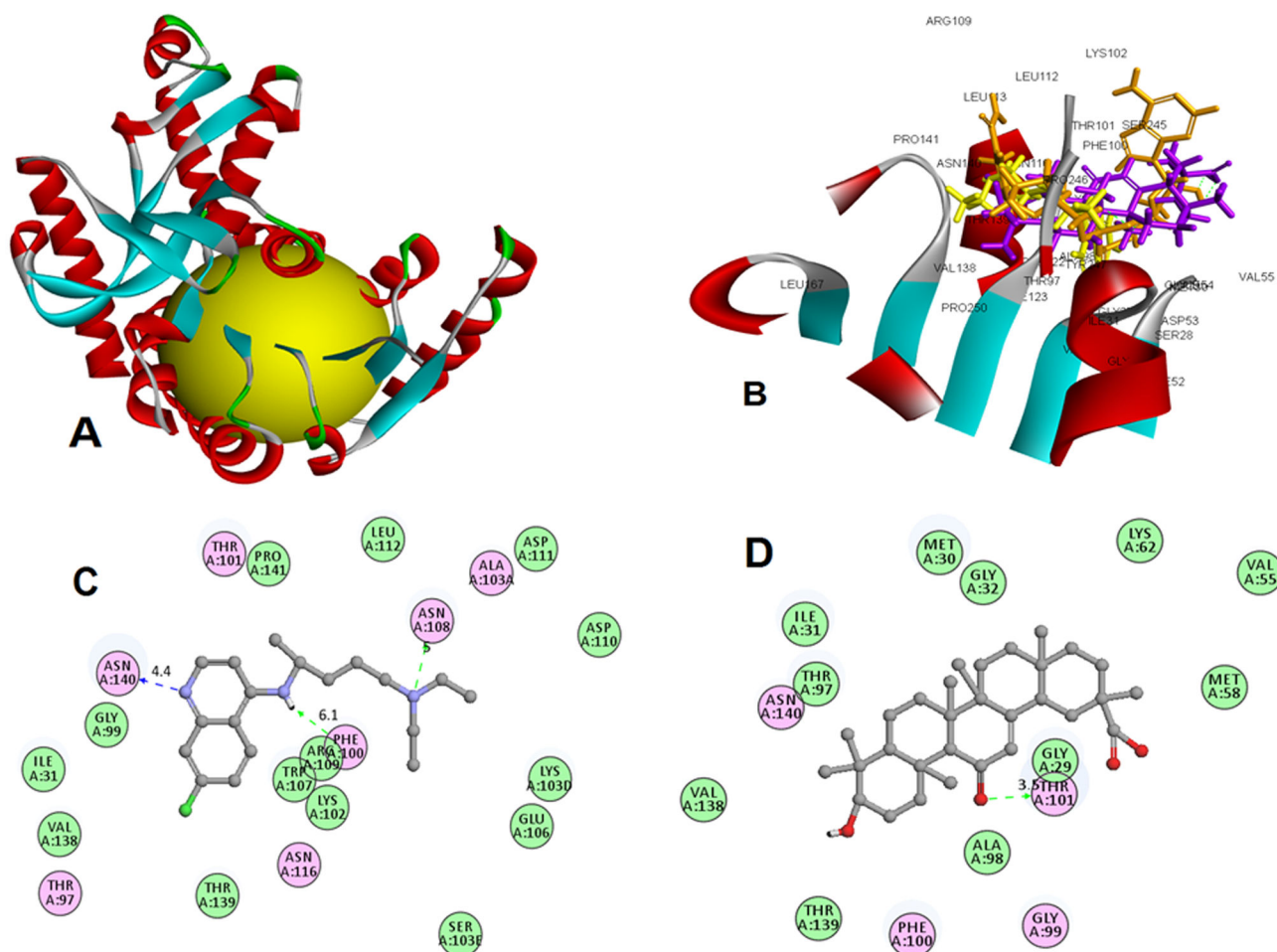


Figure 4. Structural model *Plasmodium falciparum* lactate dehydrogenase (pLDH) (PDB: 1CEQ) with NADH binding site (yellow color) (A). Superimposition of the best conformation of GA (in purple), CQ (in yellow) and NADH (in orange) in the active site pocket of *P. falciparum* enzyme lactate dehydrogenase pLDH (B). 2-D diagrams illustrating protein-ligand interactions: (C) Compound CQ; (D) Compound GA.

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GA on the basis of its 1D and 2D NMR spectroscopic data [23,24].

3.2: *In vitro* activity against *Plasmodium*

The GA was tested against *P. falciparum* NF 54 (*in vitro*) and *P. berghei* K173 (*in vivo*), which were CQ sensitive. When *P. falciparum* was subjected to GA in graded doses, an IC_{50} of 1.69 μ g/ml was derived as against 0.015 μ g/ml for CQ.

3.3: *In-silico* studies

3.3.1: Docking studies. The docking results are summarized in Table 1, which revealed that GA has moderate docking score (LibDock) of 71.18 for the target protein pLDH in comparison to the standard anti-malarial drug CQ (CQ) i.e., 131.15. Docking of GA on pLDH residues showed single hydrogen (H) bond formation with threonine (THR-101). Similarly, molecular binding of CQ exhibited three hydrogen (H)

bonds with phenylalanine (PHE-100) and asparagine (ASN-108, ASN-140). The residues of NADH binding site on pLDH interact with GA and CQ (Table 1). A similar binding site affinity was represented by the superimposition of co-crystallized NADH and docked compounds GA and CQ on pLDH (Figure 4B). The results of molecular docking and superimposition indicate that the candidate compound GA bound well within the NADH binding pocket of pLDH, but showed slightly lower binding affinity than standard drug CQ. The 2D diagram of molecular docking for compounds CQ (Figure 4C) and GA (Figure 4D) showed interaction of pLDH hydrophobic amino acid residues with bound ligands as represented by different colors e.g., pink indicates electrostatic interaction, purple indicates covalent bond and green color indicates Vander-Waals molecular interaction.

3.3.2: Bioavailability and drug likeness screening. Since docking studies showed promising results, the chemical descriptors for the pharmacokinetic properties were also

Table 1. Details of LibDock score, active site pocket residues and hydrogen bonds revealed through molecular docking of GA, CQ and NADH on Lactate dehydrogenase (PDB: 1CEQ) of *P. falciparum*.

Target	Ligand	LibDock score	Residues of the NADH binding	Residues involved in H-bond	No. of H-bond
pfLDH	NADH	159.04	GLY-27, SER-28, GLY-29, MET-30, ILE-31, GLY-32, ASP-53, VAL-55, THR-97, ALA-98, GLY-99, PHE-100, THR-101, LYS-102, TRP-107, ARG-109, VAL-138, THR-139, ASN-140, PRO-246, TYR-247, PRO-250	GLY-29, MET-30,	7
	(CQ)	131.2	ILE-31, THR-97, GLY-99, PHE-100, THR-101, LYS-102, TRP-107, ARG-109, VAL-138, THR-139, ASN-140	PHE-100, ASN-108, ASN-140,	3
	(GA)	71.18	GLY-29, MET-30, ILE-31, GLY-32, VAL-55, THR-97, ALA-98, GLY-99, PHE-100, THR-101, VAL-138, THR-139, ASN-140	THR-101	1

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calculated to check the compliance of GA with standard descriptors. For this aqueous solubility, blood brain barrier penetration, cytochrome P450 2D6 binding, hepatotoxicity, intestinal absorption and plasma protein binding were evaluated through Discovery Studio v3.5 (Accelrys, USA, 2013) molecular modeling software. A biplot (Figure 5) was also provided which shows the two analogous confidence ellipses i.e., 95% and 99% for the blood brain barrier penetration and human intestinal absorption models, respectively. The candidate compound GA showed water soluble nature, moderate intestinal absorption, and binding to plasma protein. Besides, no predictive hepatotoxicity was observed during ADME screening (Table 2). The predicted ADME results of GA were found comparable to standard range. Although drug likeness screening results showed that compound GA violate Lipinski's rule of five but within acceptable limit, in comparison to the control drug, CQ (Table 3). Moreover, to analyze the drug likeness score, GA was screened against the chemical fingerprints database 'World Drug Index' (<http://thomsonreuters.com/world-drug-index/>). The results showed similar drug likeness score i.e., 0.9 and 1.17 for GA and CQ respectively (Figure 6). Four important toxicity parameters i.e., mutagenicity, tumorigenicity, irritation, and reproductive/developmental toxicity were calculated for GA and CQ through the OSIRIS program (<http://www.organic-chemistry.org/prog/peo/>) (Table 4).

3.4: In vivo activity against Plasmodium

On the basis of *in-vitro* and *in-silico* results, GA was further evaluated in mice infected with *P. berghei*, which showed a dose dependent activity (6.68±2.19, 1.49±1.04 and 0±0% parasitemia at 62.5, 125 & 250mg/kg respectively) as against 20.57±3.13% parasitemia in infected but non-treated animals.

Discussion

4.1: Isolation and characterization of GA

The GA was isolated and discussed in the experimental section and characterized on the basis of their 1D and 2D NMR spectroscopic data which were in full agreement with the 1D and 2D NMR spectroscopic data recorded for an authentic sample of 18β-glycyrrhetic acid (ACROS ORGANICS).

4.2: Molecular interaction through docking

Recent studies have paved the way for identifying valuable leads [9], which could be useful for anti-malarial aspects as there is a persistent urge for novel compounds that can act at therapeutic targets. *P. falciparum* lactate dehydrogenase enzyme (pfLDH) represents one of such potential molecular target. Earlier studies (Read et al., 1999) suggest that CQ does not directly inhibit the enzyme but it competes with NADH for binding with the enzyme. Molecular docking results in the present study also revealed the similar molecular interaction and showed that bound compound GA interact with amino acid residues e.g., GLY-29, MET-30, ILE-31, GLY-32, VAL-55, THR-97, ALA-98, GLY-99, PHE-100, THR-101, VAL-138, THR-139, ASN-140, similar to NADH on the target enzyme pfLDH. In silico docking studies suggest that GA has similar binding site pocket residues as shown by NADH binding site on pfLDH, therefore suggesting a competitive inhibition similar to CQ, which resulted stability and potent activity.

4.3: Compliance with Bioavailability and ADMET parameters

The ADMET is one of the key measures which influence the drug levels and kinetics of drug and influence the pharmacological activity of the compound as a drug. In this study the aqueous solubility prediction (defined in water at 25°C) showed that GA is slightly less soluble. LogP value, which is a measure of lipophilicity and is the ratio of the solubility of the compound in octanol compared to its solubility in water was found to be quite high for GA in comparison to CQ, implicating low oral bioavailability (Table 2). Apart from the above, excretion process that eliminates the compound from human body also depends on LogP. The GA showed high (>= 90%) binding with the carrier proteins in the blood which depicts the efficiency of a drug. The standard drug CQ showed hepatotoxicity, while studied compound GA indicates no such toxicity (Table 2). It is important that the drugs which are administered orally must get absorbed in the intestine. In this regard, the predicted results showed that both the compounds can get easily absorbed from the intestine, as referred by the chemical descriptor TPSA. The topological polar surface area (TPSA) was calculated as a chemical descriptor for passive molecular transport through membranes. The results exhibited

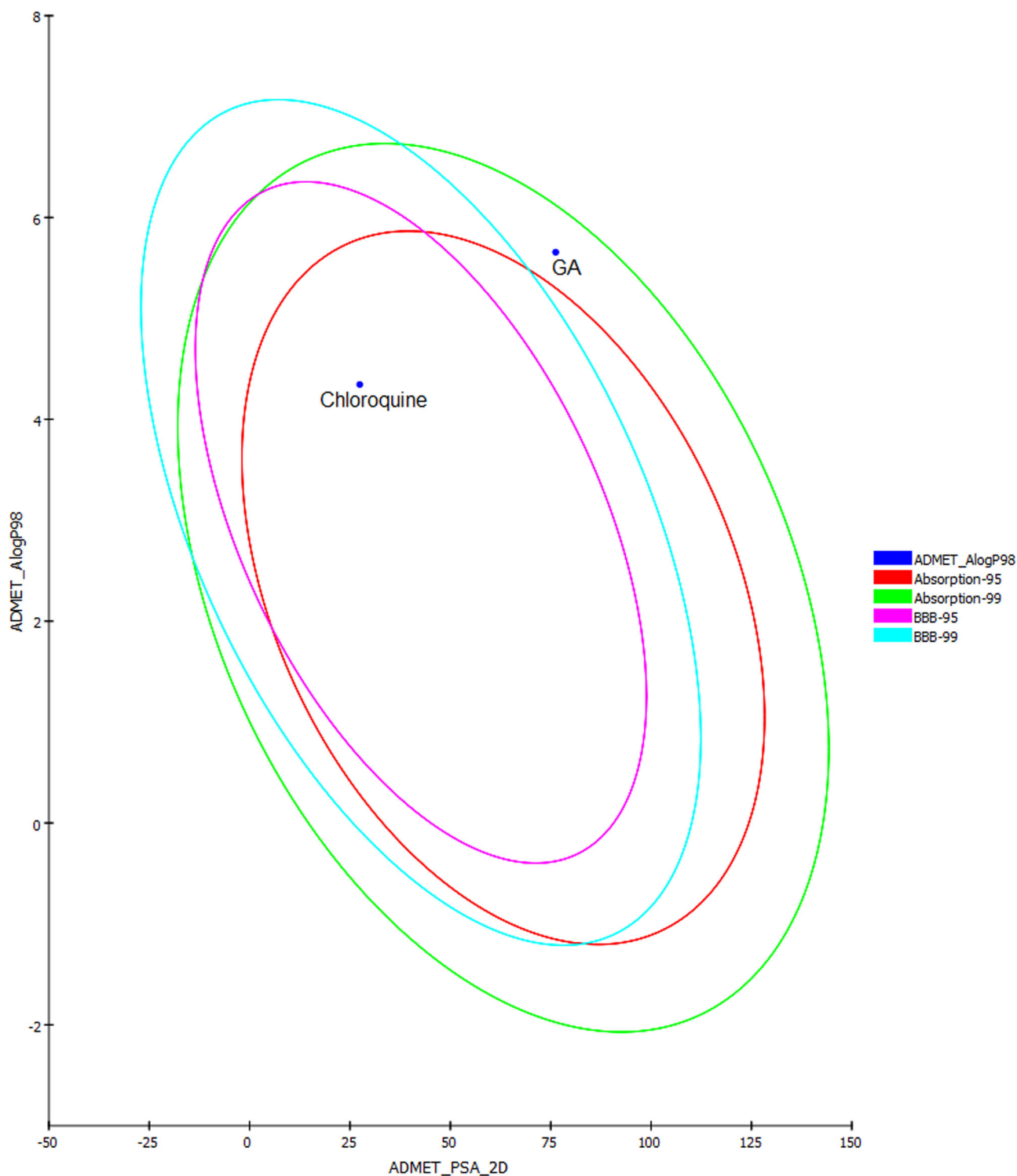


Figure 5. Adsorption model of the candidate compounds.

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a high TPSA value for GA in comparison to CQ but within acceptable limit i.e. $<140 \text{ \AA}^2$ (Table 3). Results of overall drug-likeness score against the chemical fingerprints database

(World Drug Index database) showed that both GA and CQ fall within the standard range of drugs (Figure 6). Similarly, results of toxicity risk assessment screening at high doses or long term

Table 2. Predicted ADME parameters (DS v3.5, Accelrys, USA).

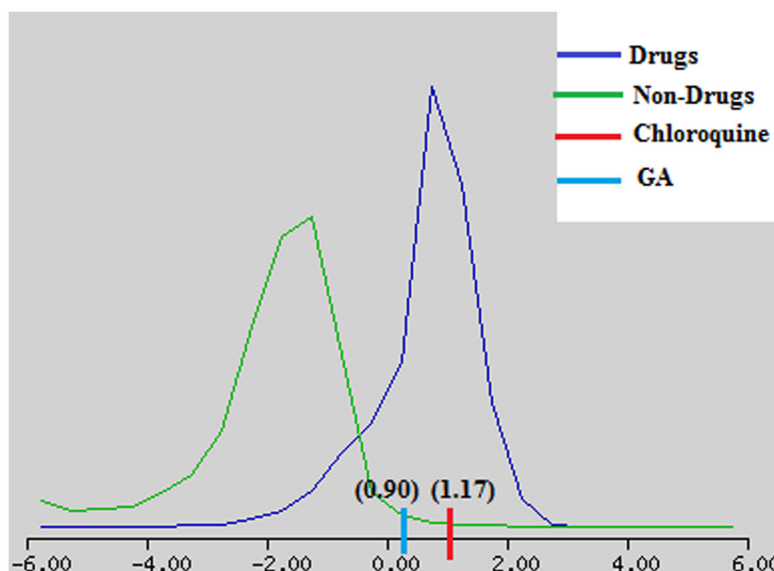
Compound Name	Aqueous solubility	Blood brain barrier penetration	CYP2D6 Binding	Hepatotoxicity	Intestinal absorption	Plasma protein binding
CQ	2 (low)	0 (very high)	True (inhibitor)	True (toxic)	0 (good)	True (highly bounded)
GA	1 (very low, but possible)	4 (underfined)	False (non inhibitor)	False (non-toxic)	1 (moderate)	True (highly bounded)

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Table 3. Compliance of compounds to the theoretical parameters of oral bioavailability and drug likeness properties.

Compound	Pharmacokinetic properties (ADME) dependent on chemical descriptors									Lipinski's rule of 5 violation
	ADM	AE	ADME	AD	H-bond acceptor			H-bond acceptor		
Oral bioavailability: TPSA (Å ²)		MW	Log P	H-bond acceptor			H-bond acceptor			
				NH ₂ group count	-NH group count	-OH group count	-N atom count	-O atom count		
CQ	28.157	319.88	3.808	0	1	0	3	0	0	
GA	74.598	470.69	6.101	0	0	1	0	3	1	

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**Figure 6.** Drug likeness score of 0.9 for GA and 1.17 for CQ lie under the drugs range.

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use, also suggest compliance of compound GA with standard drug CQ. The toxicity screening results showed that GA competes well with known drug CQ and indicates no toxicity (Table 4).

4.4: Anti-malarial activity of GA

The *in vitro* anti-malarial activity of GA against *P. falciparum* was comparatively lower than the standard drug CQ. This was also correlated with the docking studies which also revealed a moderate docking score for GA when compared to CQ. Further, *in vivo* studies on GA showed a dose dependent

activity when compared to infected but non-treated animals, though the cure rate was lower than the standard drug CQ. The likely reason for a comparative lower activity may be due to the low oral bioavailability of GA due to its higher logP value and low water solubility. GA being a natural compound has the possibility of further exploration and lead optimization by QSAR approach in resistant strains of *Plasmodium*. The triterpenoids from plants earlier showed *in-vitro* anti-plasmodial activity were rarely *in-vivo* evaluated for their anti-malarial potential [25,26].

Table 4. Details of computational toxicity risk parameters of GA and CQ calculated by OSIRIS.

Compound	Toxicity risk parameters			
	MUT	TUMO	IRRI	REP
CQ	No risk	No risk	No risk	No risk
GA	No risk	No risk	No risk	No risk

Note: MUT= Mutagenicity, TUMO= Tumorigenicity, IRRI= Irritation, REP= reproductively effective

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Conclusion

In view of potential anti-malarial activity, non-toxicity to the host, favourable pharmacokinetics and drug-like properties, GA was identified as a lead candidate for further detailed *in-silico*, biological and pharmaceutical investigations. This is the first ever report of GA showing *in-vitro*, *in-silico* and *in vivo* anti-malarial activity. Further, the effect of GA on the innate immune system in malaria infected mice and combination studies of GA with established anti-malarials can be carried out against the drug resistant strains to increase or revoke the efficacy of the

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resistant anti-malarial drugs. It is thus concluded that, the discovery of the novel lead molecule might hopefully bring advancement in the safe and effective treatment of malaria. Further, QSAR and docking guided anti-malarial lead optimization is under progress, which will assist in elucidating the precise mechanism of action.

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Author Contributions

Wrote the manuscript: AP SKS. Main design of work and chemistry: KK. Biological evaluation of sample: JA. In-silico and docking studies: SA. Operated the in-silico and docking studies; analysed and finalized the results: FK. Analysed the biological findings and finalized them for publication: AP. Conceived main concept of manuscript along with interpretation of data and making it suitable for publication: SKS. Contributed chemical reagents: KK. Contributed biological reagents: JA. Contributed software and tools: SA.

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