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Original article

Isolation and identification of β-hematin inhibitors from *Flacourtia indica* as promising antiplasmodial agents☆

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Dedicated to Prof. William Fenical (SIO, UCSD, USA) for his successful efforts in the discovery of anticancer lead compounds marizomib and plinibulin.

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ABSTRACT

An ethanolic extract (A001) of the leaves and twigs of *Flacourtia indica* (Burm.f.) Merr., was purified to give a new phenolic glycoside, 2-(2-benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (**1**) together with poliothrysoside (**2**), catechin-[5,6-*e*]-4β-(3,4-dihydroxyphenyl)dihydro-2(3*H*)-pyranone (**3**), 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (**4**), chrysoeriol-7-O-β-D-glucopyranoside (**5**), and mururin A (**6**). Compound **6** significantly inhibited the *in vitro* growth of both a chloroquine-sensitive (3D7) and a chloroquine-resistant (K1) strain of *Plasmodium falciparum*. It forms a complex with hematin and inhibits β-hematin formation, suggesting that this compound act on a heme polymerization target.

1. Introduction

Malaria is a devastating infectious disease, causing great suffering and loss of human life. The global incidence of malaria is around 120 million clinical cases annually, with some 300 million people infected and 1–2 million dying from the disease each year [1]. Out of the five malaria species infecting humans, *Plasmodium falciparum* is responsible for the majority of deaths [2,3].

Quinine, an alkaloid isolated from the bark of *Cinchona officinalis*, was the drug of choice for malaria caused by *P. falciparum* until the 1940's [4,5] when other more effective drugs replaced it that have less unpleasant side effects. Chloroquine, a synthetic analog of quinine has long been used in the control of acute uncomplicated malaria caused by parasite of genus *Plasmodium* as the first line treatment until recently. The effectiveness of chloroquine against *P. falciparum* has declined as resistant strains of the parasite evolved [6]. Another natural product, a sesquiterpene lactone artemisinin and its semi-synthetic derivatives remain the most effective remedy for malaria for more than 20 years; however, their use also seems to be limited by recent cases of emerging resistance [7,8].

Presently, artemisinin-based combination therapies (ACT) are the recommended first line of treatment for falciparum malaria in all countries with endemic disease [9,10]. However, with the growing incidents of drug resistance threatening the effectiveness of currently available malaria therapies makes their long term use doubtful [11]. The global fight to control malaria requires a multi-faceted approach, one of them is to develop and identify new

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scaffolds with different targets and mechanism of action that could sustain the dreaded falciparum. Hence, taking into account the pivotal role of plant-derived compounds in drug discovery and the development of malaria chemotherapy, the isolation of new bioactive compounds or leads from medicinal plants seems to be a promising approach.

Flacourtia indica (Burm.f.) Merr., belonging to the family Flacourtiaceae, is a small deciduous tree indigenous to the Indian Peninsula. It is commonly known as 'Bilangra' or 'Baichi' in Hindi and 'Madagascar plum' in English [12]. Traditionally, the fruits of *F. indica* are used for the treatment of jaundice and enlarged spleen, while its gum resin is administered in the treatment of cholera [13]. The stems and leaves are used in traditional medicine against malaria in Madagascar and the Comoro Islands [14]. Previous phytochemical work on *F. indica* had led to the isolation of phenolic glycosides [15–19], a butyrolactone lignan, sterols [20], and flavonoids [17]. In a recent study, poliothrysoside isolated from the ethyl acetate extract of the plant showed promising *in vitro* antiplasmodial activity against the chloroquine-resistant strain (W2) of *P. falciparum* [21]. This prompted us to further explore this plant for new antimalarial chemotypes.

As part of a drug discovery programme from Indian medicinal plants [22,23], a new phenolic glycoside (**1**), together with five known compounds (**2**–**6**) [24–29], were isolated from the leaves and twigs of *F. indica*. Compounds **3**, **5**, and **6** are reported for the first time from this natural source. In this paper, we report the isolation and structure elucidation of the new compound, along with the antiplasmodial evaluation of the isolated phytoconstituents.

2. Results and discussion

2.1. Chemistry

The 95% ethanolic extract (A001) of the leaves and twigs of *F. indica* was suspended in water and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate soluble fraction (F003) was subjected to a series of chromatographic techniques, leading to the isolation of a new phenolic glycoside (**1**), together with five known compounds (**2**–**6**). The known compounds were identified as poliothrysoside (**2**), catechin-[5,6-*e*]-4β-(3,4-dihydroxyphenyl)dihydro-2(3*H*)-pyranone (**3**), 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (**4**), chrysoeriol-7-O-β-D-glucopyranoside (**5**), and mururin A (**6**) (Fig. 1), by comparison of their spectroscopic data (see Supplementary material) with the reported literature values.

Compound **1** was obtained as a colorless gum. Its molecular formula was deduced to be $C_{27}H_{28}O_{14}$ by HRMS, having an index of hydrogen deficiency of 14. The IR spectrum displayed absorption bands indicating hydroxy (3378 cm^{-1}), carbonyl (1708 cm^{-1}) and phenyl ring (1627 cm^{-1}) functionalities. The ^1H NMR spectrum exhibited peaks characteristic of benzoyl, gentisyl, and glucose moieties suggesting a partial structure similar to that of poliothrysoside. A set of peaks at δ_H 8.08 (2H, d, J = 7.2 Hz), 7.46 (2H, m) and 7.58 (1H, t, J = 6.7 Hz) represented a benzoyl group, and another set of peaks at δ_H 7.04 (1H, d, J = 8.8 Hz), 6.66 (1H, m) and 6.78 (1H, d, J = 2.7 Hz) represented a 1,2,5-trisubtituted gentisyl alcohol moiety. A doublet appearing at δ_H 5.15 (J = 6.2 Hz) corresponding to the anomeric proton was consistent with a β-oriented glucose. Acid hydrolysis of **1** afforded D-glucose ($[\alpha]_D$ +50.5) [30]. Two mutually coupled protons ($^1H-^1H$ COSY) appearing at δ_H 6.03 (1H, dd, J = 10.3, 2.5 Hz) and 6.70 (1H, dd, J = 10.3, 1.7 Hz) were designated to *cis* olefinic protons. Both the olefinic protons coupled with another proton at δ_H 4.89 (1H, m), indicative of an isolated allylic system (−CH=CH−CH).

The ^{13}C NMR spectrum, combined with the DEPT data, revealed the presence of two oxygenated methylene, 10 olefinic methine, seven oxymethine, two esterified carbonyl, one α,β-unsaturated carbonyl, one aliphatic quaternary, and four aromatic quaternary carbons, accounting for 27 carbon signals (Table 1). Fig. 2 shows selected COSY and HMBC correlations which permitted assignment of the relative positions of the atoms and the overall structure of compound. The resonances appearing at δ_C 85.9 (C-1‴), 77.2 (C-2‴), and 71.7 (C-6‴) represented three oxygenated carbons which showed HMBC correlations with the olefinic protons (H-4‴ and H-5‴). The olefinic protons showed correlation with the carbonyl carbon at δ_C 198.4 (C-3‴). Additional correlation between the proton at δ_H 4.42 (1H, s, H-2‴) with the ester carbonyl at δ_C 172.1 (C-7‴), indicated a 1, 2, 6-trihydroxycyclohexenoyl moiety linked to an ester functionality.

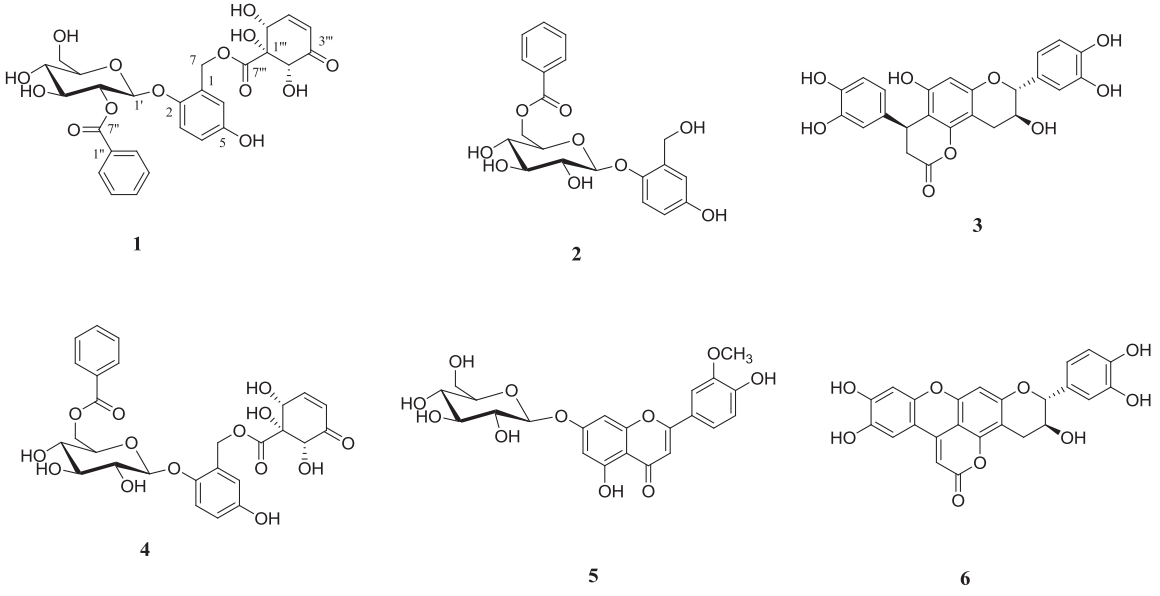

Fig. 1. Chemical structure of the isolated compounds from the leaves and twigs of *F. indica*.

Table 1
NMR spectroscopic data (300 MHz, CD_3OD) for phenolic glycoside (**1**).

Position	δ_C, type[a]	δ_H (*J* in Hz)	HMBC[b]
Gentisyl alcohol			
1	127.7, C		
2	149.3, C		
3	118.7, CH	7.04, d (8.8)	1, 2, 5
4	116.6, CH	6.66, m	2, 6
5	154.0, C		
6	116.6, CH	6.78, d (2.7)	2, 4, 7
7	63.8, CH_2	5.01, d (13.1) 5.19, d (13.2)	1, 2, 6, 7‴
Glucosyl			
1′	101.8, CH	5.15, d (6.2)	2
2′	75.7, CH	5.24, m	1′, 3′, 7‴
3′	75.9, CH	3.81, m	4′
4′	71.5, CH	3.56, m	5′
5′	78.3, CH	3.53, m	6′
6′	62.4, CH_2	3.75, m 3.92, d (11.8)	
Benzoyl			
1″	131.1, C		
2″/6″	130.8, CH	8.08, d (7.2)	1″, 4″
3″/5″	129.6, CH	7.46, m	2″/6″
4″	134.4, CH	7.58, t (6.7)	2″
7″	167.4, C		
Cyclohexenoyl			
1‴	85.9, C		
2‴	77.2, CH	4.42, s	1‴, 3‴, 6‴, 7‴
3‴	198.4, C		
4‴	127.1, CH	6.03, dd (10.3, 2.5)	6‴
5‴	151.0, CH	6.70, dd (10.3, 1.7)	1‴, 3‴
6‴	71.7, CH	4.89, m	5‴
7‴	172.1, C		

[a] Multiplicity of signals were determined by DEPT (Distortionless Enhancement by Polarization Transfer) experiments.
[b] HMBC (Heteronuclear multiple bond correlation) correlations are from proton(s) stated to the indicated carbon.

The HMBC spectrum revealed correlation between the proton at δ_H 5.24 (H-2′) with the carbonyl at δ_C 167.4 (C-7″) that assigned the benzoyl group to C-2′ of glucose. A three bond correlation between the anomeric proton H-1′ and C-2 (δ_C 149.3) established the glycosidic linkage to the gentisyl alcohol moiety, while the correlation between H-7 and C-7‴ verified the linkage between the cyclohexenoyl and gentisyl alcohol moieties.

The chemical shift values and the coupling constants of the protons of the cyclohexenoyl moiety in **1** were in good agreement with known 5-hydroxybenzyl alcohol derivatives like xylosmin (relative configuration assigned on the basis of X-ray crystallographic data) [31] and compound **4**. Compounds **1** and **4** were subjected to alkaline hydrolysis, and the specific rotation values of the substituted cyclohexenones thus obtained were in good accord ($[\alpha]_D$ +13.2 and +12.6, respectively); suggesting that the relative orientation of the three hydroxy substituents in the cyclohexenoyl moiety of **1** was axial/equatorial/equatorial (all 'α'), as in xylosmin and **4**. Thus, structure of **1** was established as 2-(2-benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol.

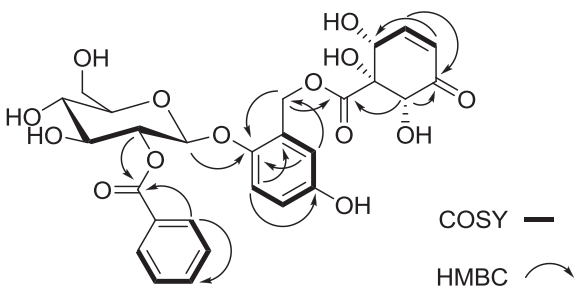

Fig. 2. Selected COSY and HMBC correlations of compound **1**.

2.2. In vitro antimalarial evaluation

The traditional use of *F. indica* in malaria chemotherapy is well known in Madagascar and the Comoro Islands [14]. Thus, the crude extracts and the isolated compounds were tested against a chloroquine-sensitive (3D7) and a chloroquine-resistant (K1) strain of *P. falciparum* [32,33]. The extract A001 was found to be active against the sensitive 3D7 strain with an IC_{50} value of 0.5 μg/mL, and inactive against the resistant K1 strain (tested up to 10 μg/mL). In comparison, the antiplasmodial activity shown by fraction F003 against the sensitive 3D7 strain was marginal (IC_{50} 10 μg/mL). However, the purified compounds (**1**–**6**) showed promising antiplasmodial activity with IC_{50} values ranging between 1.1 and 8.1 μM against the 3D7 strain, validating the traditional use of this plant against malaria. The results for all the tested isolates are detailed in Table 2. The new phenolic glycoside **1** revealed an IC_{50} of 4.4 μM, while related glycosides **2** and **4** gave IC_{50} values of 8.1 and 3.6 μM, respectively. Chrysoeriol glycoside **5**, has been reported as the major antiplasmodial principle of *Phlomis brunneogaleata* [34], showed moderate activity in our assay. Compound **3** was found to be the most active constituent against the sensitive 3D7 strain (IC_{50} 1.1 μM), while the related compound **6** was the only isolate that significantly inhibited both the strains (IC_{50} 1.2 and 1.3 μM against the 3D7 and K1 strains, respectively).

It is interesting to note that, these compounds (**3** and **6**) showing significant activity against *P. falciparum*, belong to the same phenylpropanoid catechin class of natural products. Compound **6** derived its trivial name mururin A from its first natural source *Brosimum acutifolium* (Murure) [35], and has never been reported for antiplasmodial activity. Same is the case with the compound **3** which is closely related to cinchonains [36]. Thus, it is the first report of antiplasmodial activity for both the compounds **3** and **6**. The isolated compounds were also tested for their cytotoxicity against Vero cell line (monkey kidney fibroblast) as described previously [37]. They were considered selective (SI values ranging from 16.8 to 170.8) and non-toxic ($CC_{50} > 50$ μM).

2.2.1. In vitro inhibition of β-hematin formation

Malaria parasite *Plasmodium* requires hemoglobin as the chief source of nutrition for its feeding and development. The degradation of hemoglobin occurs inside the digestive vacuole of parasite

Table 2
In vitro antimalarial activity of phytoconstituents from *F. indica* against a chloroquine-sensitive (3D7) strain and a chloroquine-resistant (K1) strain of *P. falciparum* and their cytotoxicity against Vero cell line.[a]

Compound/ fraction	IC_{50} (μM)		Cytotoxicity CC_{50} (μM)	Selectivity index (SI)
	3D7	K1		
A001[b]	0.5 ± 0.0	>10	85.4 ± 5.2	170.8
F003[b]	10 ± 0.0	>10	>100	>10
1	4.4 ± 0.2	>5	77.3 ± 5.5	17.6
2	8.1 ± 0.3	>25	136.5 ± 9.0	16.8
3	1.1 ± 0.1	>5	200 ± 10.0	181.8
4	3.6 ± 0.3	>5	118.5 ± 9.0	32.9
5	4.4 ± 0.2	>5	200 ± 10.9	45.4
6	1.2 ± 0.1	1.3 ± 0.1	58.5 ± 3.2	48.7
Chloroquine	5.5 ± 0.9[c]	254 ± 20.6[c]	125.9 ± 10.5	22,890

SD denotes standard deviation, IC_{50} denotes 50% inhibitory concentration, and CC_{50} denotes 50% cytotoxic concentration.
[a] Results are expressed as mean ± SD ($n = 3$).
[b] IC_{50} and CC_{50} values are in μg/mL.
[c] Values are in nM.

Table 3
In vitro inhibition of β-hematin formation by the phytoconstituents of *F. indica*.

Compound/fraction	Inhibition of β-hematin formation IC_{50} (μM)[a]
A001	>50[b]
F003	35.6 ± 3.6[b]
1	>50
2	>50
3	10.1 ± 1.3
4	>50
5	>50
6	8.3 ± 1.2
Chloroquine	7.7 ± 0.8

[a] Data are the mean ± SD of three different experiments.
[b] IC_{50} values are in μg/mL.

where excess of toxic heme is released as by-product which could cause extensive damage to the membranes and inhibit a variety of enzymes resulting in the death of the parasite [38]. However, there are several detoxification pathways through which parasite removes the excess heme, one of the prominent way is polymerization of heme into non-toxic crystalline compound hemozoin [39]. Thus, the inhibition of hemozoin formation is one of the main targets for several antimalarial drugs, such as chloroquine, which is known to be a potent inhibitor of β-hematin (*in vitro* analog of hemozoin) formation [40]. To explore the mechanism of action of the isolated compounds, they were subjected to *in vitro* assay for the inhibition of β-hematin formation. The fraction F003 exhibited marginal effects on inhibition (IC_{50} 35.6 μg/mL) as expected, while compounds **3** and **6** were found to be the potent inhibitors of β-hematin formation showing IC_{50} values of 10.1 and 8.3 μM, respectively. The inhibitory activity of these two compounds was comparable to the reference drug chloroquine (IC_{50} 7.7 μM), while the remaining compounds were considered inactive (tested up to 50 μM) (Table 3).

2.2.2. Hydrogen peroxide-mediated hemin degradation

Besides polymerization, heme undergoes peroxidative degradation by reacting with hydrogen peroxide (H_2O_2) produced during the hemoglobin degradation within the digestive vacuole of the *Plasmodium* parasite. Thus, hydrogen peroxide mediated degradation process provides an alternate secondary detoxification route for toxic heme [41,42]. To further understand the additional mechanism of action, the two most active compounds **3** and **6** were analyzed for protection against *in vitro* hydrogen peroxide-mediated hemin (oxidized form of heme) degradation. In this additional study, compounds **3** and **6** showed 23.0% and 37.5% protection against the hemin degradation, while for chloroquine, 53.5% protection was observed with respect to the positive control (Table 4).

Thus, the *in vitro* analysis of the inhibition and protection assays implied that the antiplasmodial activity shown by the isolated constituents may be partly attributable to these two detoxification pathways. Although, **3** and **6** were good at inhibition of β-hematin formation but their antiplasmodial activity was not comparable to that of chloroquine. This might be due to the involvement of other factors essential for the activity, such as the degree of accumulation within the parasite's digestive vacuole which is structure and pH dependent [32,43–45].

Table 4
Inhibition of hydrogen peroxide-mediated hemin degradation by compounds **3** and **6**.

Test sample	Remaining hemin (%)[a]	Protection value (%)[b]
Negative control	98 ± 5.8	
Positive control	42 ± 4.6	
Chloroquine	72 ± 5.2	53.5
3	55 ± 3.4	23.0
6	63 ± 5.6	37.5

[a] Data are the mean ± SD of three different experiments.
[b] Protection values were calculated with respect to positive control.

3. Conclusion

In conclusion, we have isolated and characterized a new phenolic glycoside **1** together with five known compounds from the leaves and twigs of *F. indica*, which were evaluated for their antiplasmodial activity against a chloroquine-sensitive (3D7) and a chloroquine-resistant (K1) strain of *P. falciparum*. Interestingly, the phenylpropanoid catechin derivatives **3** and **6** emerged as a new class of antiplasmodial agents which may act through the inhibition of β-hematin formation and may also provide additional protection against hydrogen peroxide mediated hemin degradation, as suggested by the *in vitro* results.

4. Experimental

4.1. General experimental information

Optical rotations were measured on an Autopol III polarimeter (Rudolph Research, USA) at room temperature. UV spectra were obtained on a Thermo Evolution 500 UV–visible spectrophotometer. CD spectra were obtained using a JASCO single beam spectropolarimeter. IR spectra were recorded on a Perkin Elmer 399B spectrophotometer. All 1D and 2D NMR experiments were performed on a Bruker Avance DRX 300 MHz spectrometer, using CD_3OD or acetone-d_6 or DMSO-d_6 as solvent. ESIMS and Tandem mass spectra were recorded on a Thermo LCQ Advantage Max-IT. HRMS data were acquired using an Agilent 6500 Q-TOF mass spectrometer. Column chromatography was carried out on silica gel (Merck) and reversed-phase C_{18} silica gel (Sigma–Aldrich), while Sephadex LH-20 (Sigma–Aldrich) was used for gel filtration. Analytical and preparative TLC were performed on precoated silica gel $60F_{254}$ aluminum and glass plates (Merck, 0.25 and 1 mm), respectively. Spots were detected on TLC under UV light or by heating after spraying with 0.1% vanillin (w/v) in 10% H_2SO_4 (v/v) solution. Organic solvents were distilled prior use.

4.2. Plant material

The leaves and twigs of *F. indica* were collected from Bhubaneswar, India, in February 2006. Plant material was authenticated and a voucher specimen (No. 8506) was deposited in the herbarium of Botany Division, Central Drug Research Institute, Lucknow, India.

4.3. Extraction and isolation

The air-dried plant material (18 kg) was extracted with 95% aqueous ethanol (3 × 50 L; each 24 h) at room temperature. The combined ethanolic extract was evaporated to dryness under reduced pressure ($t < 50$ °C) to obtain crude ethanolic extract A001 (733 g). A part of A001 (500 g) was suspended in water (1 L), and successively partitioned with *n*-hexane (3 × 1 L), ethyl acetate (3 × 1 L), and *n*-butanol (3 × 1 L), to afford hexane fraction F002 (0.5 g), ethyl acetate fraction F003 (170 g), and butanol fraction F004 (80 g), respectively. The ethyl acetate fraction F003 (160 g) was subjected to normal phase silica gel column chromatography (CC). Successive elution with a gradient of methanol–chloroform (0:1 to 1:0) afforded fourteen fractions (F005 to F018) based on

the TLC analysis. Fraction F012 (35.0 g) was rechromatographed over silica gel with the gradient of methanol–chloroform (1:99 to 20:80) to obtain five major subfractions (F019-F023). Subfraction F019 (5.0 g) was purified over Sephadex LH-20 (25 g) eluted with methanol–chloroform (5:95 to 20:80) to obtain crude solid, which was recrystallized from methanol to afford white solid **2** (0.84 g). Subfraction F021 (10 g) was subjected to gel filtration over Sephadex LH-20 (50 g) eluted with the gradient of methanol–chloroform (0:1 to 3:7) as mobile phase to obtain two major fractions. First fraction (1.8 g) was subsequently purified by silica gel flash CC using ethyl acetate as the eluting solvent to give **4** (0.55 g) and the second fraction (0.6 g) was purified over reversed-phase C_{18} silica gel CC eluted with isocratic system of methanol–water (20:80) to afford off-white amorphous solid **3** (0.04 g). Subfraction F022 (6.0 g) was repeatedly purified by normal and reverse phase CC, followed by gel filtration over Sephadex LH-20 with methanol–water (30:70) as the mobile phase to afford yellow solid **5** (0.02 g). Subfraction F023 (4.0 g) was similarly chromatographed to afford major seven subfractions. Fourth subfraction (0.3 g) on purification over Sephadex LH-20 gave light yellow solid **6** (0.02 g), while fifth subfraction (0.68 g) was subjected to reversed-phase C_{18} silica gel CC with gradient of methanol–water (20:80 to 50:50) to give two fractions (A and B). Subsequently, fraction B was purified by preparative TLC in methanol–ethyl aceate–water (5:95:5) to afford **1** (0.04 g).

4.3.1. 2-(2-Benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (*1*)

Gum; $[\alpha]_D^{25}$ −34.6 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.06), 258 (4.03), 270 (3.83) nm; IR (KBr) ν_{max} 3378, 2948, 1708, 1627, 1440, 1221, 1076, and 771 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRMS *m/z* 599.1369 $[M + Na]^+$ (calcd for $C_{27}H_{28}O_{14}Na$, 599.1377).

4.4. Acid hydrolysis of compounds *1*, *4*, and *5*

Compounds **1**, **4**, and **5** (5 mg each) in 10% HCl-dioxane (1:1, 5 mL) were heated separately at 80 °C for 6 h. After the reaction was completed, the solvent was removed under *vacuo*, and the residue was diluted with water. The aqueous layer was successively extracted with ethyl acetate (3 × 5 mL), neutralized with $NaHCO_3$ and then concentrated to give a white solid. The solid was dissolved in minimum amount of methanol–water (1:1) and was purified over preparative TLC, eluted with methanol–chloroform–water (10:90:1) to get pure sugar. The sugar obtained in each case was analyzed for optical rotation. The values of specific rotation were $[\alpha]_D^{25}$ +50.5 (c 0.10, water), +48.6 (c 0.11, water), and +46.7 (c 0.10, water) for compounds **1**, **4**, and **5**, respectively. These values corresponded to that of D-(+)-glucose.

4.5. Alkaline hydrolysis of compounds *1* and *4*

Compounds **1** and **4** (5 mg each) were refluxed with $Ba(OH)_2 \cdot 8H_2O$ (8 mg) in water (5 mL) for 2 h. The resultant solution was acidified with H_2SO_4 to precipitate $BaSO_4$. It was then filtered off and the filtrate was extracted with diethylether (3 × 5 mL) to yield a residue. The residue showed two spots on TLC, one was benzoic acid (co-TLC with authentic sample) and the other belonged to substituted cyclohexenone. Identical spots were obtained in both the cases. The residue obtained for each compound was purified over preparative TLC eluted with methanol–ethyl aceate–water (10:90:5) to afford respective pure substituted cyclohexenones (1.4 mg for **1** and 1.2 mg for **4**). The specific rotation values obtained for them were +13.2 (c 0.07, MeOH) and +12.6 (c 0.06, MeOH), respectively.

4.6. In vitro antimalarial assay

The compounds were dissolved in DMSO at 5 mg/mL or 10 mM concentration. Two-fold serial dilutions of test samples were made in 96 well plates and incubated with 1.0% parasitized cell suspension containing 0.8% parasitemia (Asynchronous culture with more than 80% ring stages). The plates were incubated at 37 °C in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. 72 h later 100 μL of lysis buffer containing 2× concentration of SYBR Green-I (Invitrogen) was added to each well and incubated for 1 h at 37 °C. The plates were examined at 485 ± 20 nm of excitation and 530 ± 20 nm of emission for relative fluorescence units (RFUs) per well using the fluorescence plate reader (FLX800, BIOTEK). Data was transferred into a graphic program (EXCEL) and 50% inhibitory concentration (IC_{50}) was obtained by Logistic regression analysis of dose response curves [46]. Three replicates were carried out to assess antimalarial activity. Chloroquine was used as the standard reference drug.

4.6.1. Cytotoxicity assay

Cytotoxicity of the compounds was carried out using Vero cell line (C1008; monkey kidney fibroblast) as described previously [37]. The 1×10^4 cells were incubated with different dilutions of test agents for 72 h and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as reagent for the detection of cytotoxicity. Three replicates were carried out to assess cytotoxic activity. 50% cytotoxic concentration (CC_{50}) was determined using non-linear regression analysis of dose response curves. Selectivity Index (SI) was calculated as $SI = CC_{50}/IC_{50}$.

4.6.2. In vitro inhibition of β-hematin formation

Male swiss mice, weighing 15–20 g were inoculated with 1×10^5 *Plasmodium yoelii* infected RBCs. Blood of infected animal at 50% parasitemia was collected by cardiac puncture in 2.0% citrate buffer and centrifuged at 5000 rpm for 10 min at 4 °C. The plasma was used in assay of β-hematin formation. The assay mixture contained 100 mM sodium acetate buffer pH (5.1), 50 μL plasma, 100 μM hemin as the substrate and 1–50 μg compound/drug in a total volume of 1.0 mL. The control tube contained all reagents except compound. The reaction mixture in triplicate was incubated at 37 °C for 16 h in a rotary shaker. The reaction was stopped by centrifugation at 10,000 rpm for 10 min at 30 °C. The pellet was suspended in 100 mM Tris–HCl buffer pH (7.4) containing 2.5% SDS. The pellet obtained after centrifugation was washed thrice with triple distilled water (TDW) to remove free hemin attached to β-hematin. The pellet was solubilized in 50 μL of 2 N NaOH and volume was made up to 1.0 mL with TDW. Absorbance was measured at 400 nm [47]. The IC_{50} values were determined using non-linear regression analysis of dose response curves.

4.6.3. Measurement of hydrogen peroxide-mediated hemin degradation

Hydrogen peroxide-mediated hemin degradation was also evaluated in the presence/absence of compounds by using the method of Loria et al. (1999) with some modifications [41]. The inhibition was monitored in 96 well ELISA plate with a total reaction volume of 200 μL for each well consisting 25 μM hemin (in 0.1 N NaOH), 180 μg bovine serum albumin (in 0.2 M sodium acetate buffer pH 5.1), 20 mM of H_2O_2 and 20 μM of chloroquine/compounds. The plates were incubated to equilibrate for 10 min at room temperature after addition of hemin and the bovine serum albumin. The peroxidative reaction was initiated by the addition of H_2O_2, and followed by measuring the decrease in absorption at the Soret band (405 nm), after 30 min incubation at room temperature. Negative control groups (TDW instead of H_2O_2) and positive control groups (H_2O_2) were also included in each experiment. Results were

expressed as the percentage of remaining hemin in the reaction mixture. Also, the protection against hemin degradation was measured with respect to the positive control. Three independent experiments were carried out to evaluate the results.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.12.019.

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