Phytomedicine 30 (2017) 1–9

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original article

A phenolic glycoside from *Flacourtia indica* induces heme mediated oxidative stress in *Plasmodium falciparum* and attenuates malaria pathogenesis in mice

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ARTICLE INFO

Keywords:
Flacourtia indica
Plasmodium falciparum
Oxidative stress
Inflammation
Hemozoin

ABSTRACT

Background: *Flacourtia indica* is especially popular among the various communities of many African countries where it is being used traditionally for the treatment of malaria. In our previous report, we have identified some phenolic glycosides from the aerial parts of *F. indica* as promising antiplasmodial agents under *in vitro* conditions.
Purpose: Antimalarial bioprospection of *F. indica* derived phenolic glycoside in Swiss mice (*in vivo*) with special emphasis on its mode of action.
Methods: Chloroquine sensitive strain of *Plasmodium falciparum* was routinely cultured and used for the *in vitro* studies. The *in vivo* antimalarial potential of phenolic glycoside was evaluated against *P. berghei* in Swiss mice through an array of parameters *viz.*, hematological, biochemical, chemo-suppression and mean survival time.
Results: 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α, 2α, 6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (CPG), a phenolic glycoside isolated from the aerial parts of *F. indica* was found to exhibit promising antiplasmodial activity by arresting the *P. falciparum* growth at the trophozoite stage. Spectroscopic investigations reveal that CPG possesses a strong binding affinity with free heme moieties. In addition, these interactions lead to the inhibition of heme polymerization in malaria parasite, augmenting oxidative stress, and delaying the rapid growth of parasite. Under *in-vivo* condition, CPG exhibited significant antimalarial activity against *P. berghei* at 50 and 75 mg/kg body weight through chemo-suppression of parasitemia and ameliorating the parasite induced inflammatory and oxidative (hepatic) imbalance in the experimental mice.
Conclusion: CPG was found to be a potential antimalarial constituent of *F. indica* with an explored mechanism of action, which also offers the editing choices for developing CPG based antimalarial chemotypes.

Introduction

Every year, malaria globally threatens the lives of approximately 40% of the total world population with about 300 million clinical episodes and more than one million deaths (WHO malaria report, 2015). Young children living in the tropical and subtropical areas of the globe are the main victims of the disease (Ladhani et al., 2007). Due to the limitations associated with vaccine development and vector control programme, the management of malaria and related complications are thoroughly reliant on chemotherapy and chemoprophylaxis (Chia et al., 2014). *Plasmodium falciparum,* the main causative agent for the disease tidily develops resistance against most of the available antimalarials (Egan, 2015), raising strong interest for the search of bioactive agents specifically effective against resistant malaria (Choi et al., 2008).

As a part of its metabolic requirement, *P. falciparum* employs the heterophagy of host hemoglobin inside its digestive vacuole, resulting in an over deposition of redox active free heme in parasite (Ferreira et al., 2008). Furthermore, to preserve the intracellular redox balance, *P.*

Abbreviations: ANOVA, Analysis of variance; CC_{50}, 50% cytotoxic concentration; CPCSEA, Committee for the purpose of control and supervision of experiments on animals; CPG, 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α,2α,6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol; CMG, Cyanmethemoglobin; DCFDA, 2′,7′-dichlorofluorescin diacetate; DMSO, Dimethyl sulfoxide; H_2O_2, Hydrogen peroxide; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); IC_{50}, 50% inhibitory concentration; MDA, Malondialdehyde; MST, Mean survival time; RBCs, Red blood cells; ROS, Reactive oxygen species; RFUs, Relative fluorescence units; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid; WBCs, White blood cells; WHO, World health organization

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http://dx.doi.org/10.1016/j.phymed.2017.04.010
Received 15 November 2016; Received in revised form 6 April 2017; Accepted 20 April 2017

falciparum polymerizes the free heme units to hemozoin, where the causative mechanism(s) are still unclear (Dhangadamajhi et al., 2010). Inhibition of hemozoin formation leads to the free heme toxicity in parasite, which on succession, results into the parasite death. Thus, identification of the inhibitors of heme polymerization pathway or agents imposing oxidative burden (redox imbalance) in parasite by any means seems a valid and promising approach in malaria drug discovery programme (Nepveu and Turrini, 2013). Additionally, the chemotactic behavior of the released free heme moieties seems to be responsible for the activation of host neutrophils through a ROS dependent mechanism (Porto et al., 2007), where the clinical manifestations *viz.*, altered level of pro and anti-inflammatory cytokines is generally seen (Clark et al., 2006).

From ancient times, natural products were the source of effective antimalarial drugs, such as quinine and artemisinin (Kaur et al., 2009). Glycosides are the main phytoconstituents of various herbal products and in recent years have received special attention, as promising antiplasmodial agents (Marya et al., 2017). A novel stilbene glycoside (piceid-(1→6)-β-D-glucopyranoside) isolated from the leaves of *Parthenocissus tricuspidata* (Siebold & Zucc.) Planch. (Vitaceae) exhibited potential antiplasmodial behavior under both *in vitro* and *in vivo* conditions (Park et al., 2008). Moreover, some phenolic glycosides isolated from *Grevillea* genus (Proteaceae) also delayed the *P. berghei* growth significantly in rodent malaria model (Ovenden et al., 2011). These preliminary findings support the bioprospection of a variety of glycosides isolated from traditionally used antimalarial plants.

Flacourtia indica (Burm.f.) Merr. (Flacourtiaceae) is commonly known as 'Bilangra' or 'Baichi' in India and is traditionally globally used as a herbal antimalarial remedy (Kaou et al., 2008; Kota et al., 2012). In Madagascar and Comoro Islands, aerial parts of *F. indica* are very popular among the traditional healers for the management of malaria and related complications. In a recent report, pyrocatechol, homaloside D and poliothrysoside were isolated from the aerial parts of *F. indica*, among which poliothrysoside exhibited a strong antiplasmodial activity against chloroquine resistant strain (W2) of *P. falciparum* (Kaou et al., 2010). As a part of our ongoing project principally focused on the identification of effective natural products against malaria, we recently identified a phenolic glycoside from the aerial parts of *F. indica,* as promising antiplasmodial agent under *in vitro* conditions (Sashidhara et al., 2013). Chemical characterization and spectroscopic data identified the glycoside as 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α, 2α, 6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (CPG), Fig. 1 (Shaari and Waterman, 1995). The encouraging results of our previous study prompted us to go forward and test the CPG activity under *in vivo* conditions, with special emphasis on the possible mechanism of action.

Material and methods

Reagents and animals

Dichlorofluorescein diacetate (DCF-DA), hemin chloride, hypoxanthine, saponin, chloroquine diphosphate, reduced glutathione (GSH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), gentamycin and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Albumax-II, RPMI 1640 and fungizone were procured from Life Technologies (Thermo Fisher, Waltham, MA, USA). Dimethyl sulfoxide (DMSO) and *Giemsa* stain were purchased from TCI chemicals (Tokyo, Japan). All the chemicals used were of analytical grade.

For *in vivo* experiments, Swiss albino mice (20 ± 2 g) were used and maintained under hygienic conditions (22 ± 2 °C, 12:12 dark-to-light cycle with standard pellet diet and water) at institutional animal house. All the experiments were duly approved by the Institutional Animal Ethics Committee (Approval no.: AH-2012-18), under the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Govt. of India.

In vitro culture and stage specific activity

Chloroquine sensitive (3D7) strain of *P. falciparum* was initially procured from CSIR-Central Drug Research Institute, Lucknow, India and routinely cultured by following the method of Trager and Jensen (1976) at 5% hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 40 μg/ml gentamycin, 100 μM hypoxanthine and 0.5% (w/v) Albumax-II. The IC_{50} value of CPG and reference drug 'chloroquine' was evaluated by following the protocol earlier described in our previous report (Singh et al., 2011).

Furthermore, the stage specific activity of CPG was tested on ring synchronized culture at approx. 5% parasitemia. Briefly, *P. falciparum* culture was incubated in the presence and absence of CPG (3.2 ± 0.06 μM; IC_{50} concentration) for 60 h at the conditions mentioned above. Thin blood smears were prepared from each well at 12 h interval (till 60 h) from the incubation time and stained with 10% *Giemsa* stain for microscopic examination using light microscope (Leica, Tokio, Japan). The percentage of stage specific parasites was calculated by counting a total of 1000 erythrocytes of each smear.

Hemolytic assay

Additionally, the hemolytic behavior of CPG was also investigated using B^{+ve} healthy erythrocytes where hemolysis was considered as an

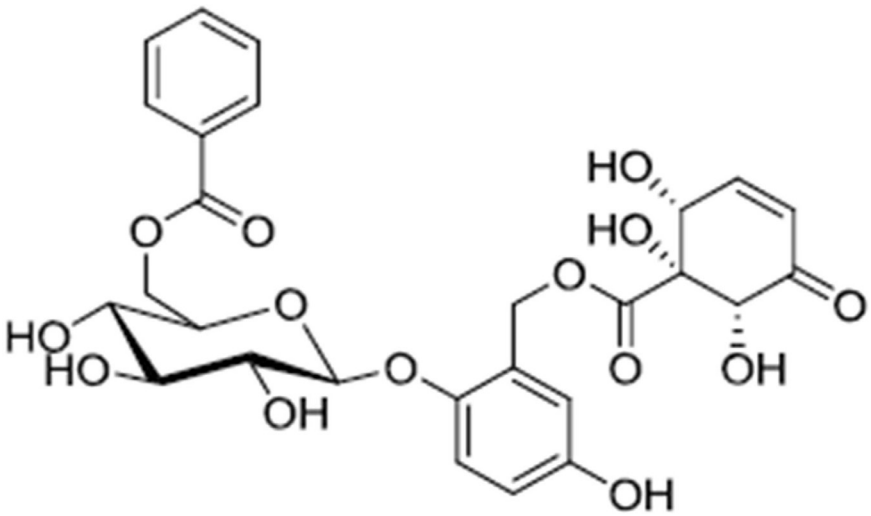

Fig. 1. Structure of 2-(6-benzoyl-β-D-glucopyranosyloxy)-7-(1α, 2α, 6α-trihydroxy-3-oxocyclohex-4-enoyl)-5-hydroxybenzyl alcohol (CPG).