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Protective activity of biflavanones from *Garcinia kola* against *Plasmodium* infection

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ABSTRACT

Ethnopharmacological relevance: *Garcinia kola* is a medicinal plant traditionally used for malaria therapy in Central Africa.
Aim of the study: To evaluate the antimalarial potencies *in vitro* and *in vivo* of pure biflavanones from *G. kola*.
Materials and methods: The pure biflavanones were obtained by bioassay-guided fractionation of a 70% ethanol extract of *G. kola* seeds and their chemical structures were elucidated by comparison of their NMR (^{1}H and ^{13}C) and mass spectral data with those provided in the literature. *Plasmodium falciparum* (FCR-3, cycloguanil-resistant strain from Gambia) was used for *in vitro* assessments of antimalarial activities. Growth inhibition, intraerythrocytic development and parasite morphology were evaluated in culture by the microscopic observation of Giemsa-stained thin blood films. The cytotoxicity of the antimalarial compounds was evaluated against KB 3-1 (human epidermoid carcinoma) cells by MTT assay. *In vivo* antimalarial activities were determined in mice infected with *Plasmodium berghei* (ANKA strain) following a four-day suppressive test.
Results: The bioassay-guided fractionation of an extract of *G. kola* resulted in the isolation of three biflavanones (GB-1a, GB-1, and GB-2) as its active principles. These three biflavanones displayed not only potent inhibitory activity *in vitro* against *P. falciparum* proliferation but also antimalarial potency through oral administration in mice infected with *P. berghei* without signs of acute toxicity. GB-1 was found to exhibit the strongest *in vitro* antimalarial potency on *P. falciparum* with an IC_{50} of 0.16 μM, whereas it exhibited a very low *in vitro* cytotoxicity on KB 3-1 cells with an IC_{50} of greater than 150 μM. During an *in vivo* antimalarial assay in mice infected with *P. berghei*, GB-1 was found to exhibit biological potency with an approximate ED_{50} of 100 mg/kg following oral administration. GB-1 was also shown to increase the average life span of the infected mice significantly compared to that of control mice ($p < 0.01$ Student's *t*-test).
Conclusions: The antimalarial outcome of GB-1a, GB-1, and GB-2 may be related to the traditional utilization of this crude drug against malaria judging from their significant content in *G. kola* nuts. GB-1 showed the most potent antimalarial activity with a high selectivity index and, therefore could be exploited to identify the molecular target, which subsequently could be helpful to design novel therapeutics against malaria. GB-1 may be considered a promising antimalarial candidate for trial *in vivo* using higher animals infected with *P. falciparum*.

1. Introduction

Malaria remains endemic in more than 90 countries, principally in the tropical world. Annually, malaria afflicts 300–500 million people and kills approximately 600,000 people, primarily children in Africa. Dramatically, a child dies of malaria every 40 s, resulting in a daily loss of more than 2000 young lives worldwide. In addition to virtual worldwide resistance against chloroquine, resistance to alternative drugs such as amodiaquine, mefloquine, and sulphadoxine-pyrimethamine have been reported in most endemic areas (Beshir et al., 2010; Fidock et al., 2004; Matondo et al., 2014; Na-Bangchang et al., 2013). Parasite resistance to artemisinin derivatives in South-East Asia has also been reported (Dondorp et al., 2010; Yeung et al., 2009). The lack of a malaria vaccine and the emerging resistance of a deadly form of malaria parasite, *Plasmodium falciparum*, to commercially available antimalarial drugs make the search for new antimalarials a global

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demand (WHO, 2014). These circumstances have prompted us to explore some natural resources for new anti-malarial candidates with high selectivity indices between the parasite and mammalian cells.

Garcinia kola Heckel (Guttiferae) is a cultivated large forest tree found in most parts of West and Central Africa that is valued for its edible nuts. Extracts of various parts of the *G. kola* plant are extensively used in African traditional medicine, particularly for the preparation of remedies for treating laryngitis, cough, and liver diseases (Galam et al., 2013). The local people in Central Africa claim that chewing *G. kola* nuts in a small quantity daily has a chemopreventive effect against malaria infection (Tona et al., 1999). Based on this claim, the possible *in vitro* anti-malarial activity of *G. kola* nuts was examined and confirmed. In addition, we confirmed the inhibitory effect of a 70% EtOH extract of this medicinal plant on *P. falciparum* proliferation independently. These unambiguous biological outcomes and the traditional use of this plant encouraged us to perform systematic studies to determine the antimalarial constituents of this crude drug. In a preliminary examination, an EtOH extract of *G. kola* demonstrated potent *in vitro* antimalarial activity against *P. falciparum.* Previously, similar results have also been reported in the literature (Tona et al., 1999). As a result, the bioassay-guided fractionation of an extract of this crude drug yielded three biflavanones as the antimalarial principles: **1** (GB-1a), **2** (GB-1), and **3** (GB-2). This study describes the bioassay-guided isolation and antimalarial activities of biflavanones **1**–**3**.

2. Materials and methods

2.1. General

^{1}H NMR spectra in DMSO-d_6 were recorded using a JNM-GX-500 (JEOL, Tokyo, Japan) NMR spectrometer operating at 500 MHz. ^{13}C NMR spectra in DMSO-d_6 were recorded using a JNM-GX-500 (JEOL, Tokyo, Japan) NMR spectrometer operating at 125 MHz. Fast atom bombardment (FAB) mass was measured using a JMS SX-102 (JEOL, Tokyo, Japan) mass spectrometer in negative ion mode with nitrobenzyl alcohol (NOBA) as a matrix. For column chromatography, silica gel (60–230 mesh, Merck) and octadecyl silane (ODS) (Cosmosil 75C_{18} OPN, Nacalai Tesque) were used. TLC analysis was performed over pre-coated plates (Merck, Kiesel gel 60F_{254}, 0.25 mm) and reversed-phase HPTLC (RP-18 WF_{245s}, Merck). The spots were observed under u.v. light at 254 and 366 nm and visualized by either 1% $Ce(SO_4)_2$/10% H_2SO_4 or *p*-anisaldehyde/H_2SO_4 (5 mL of AcOH, 25 mL of *c*-H_2SO_4, 425 mL of EtOH, and 25 mL of water) spray reagents with subsequent heating.

Chloroquine (as diphosphate, Sigma) and quinine (Nacalai Tesque) were used as positive controls for *in vitro* antimalarial experiments, while artemisinin (Aldrich) was administered as a positive control for *in vivo* anti-malarial assays. The stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO) and diluted with complete medium. The DMSO concentration in the culture medium never exceeded 1% unless otherwise noted. The stock solution of chloroquine diphosphate was prepared in 2% DMSO in complete medium. Mitomycin C (Kyowa Hakko Kogyo) was used as a positive control for the cytotoxicy assay. Unless otherwise indicated, all bioassays were conducted in a PVC clean bench (Hitachi). KB 3-1 cells were incubated in a 5% CO_2 controlled incubator (Sanyo) at 37 °C and malaria parasites were incubated at a low temperature in a 5% O_2 and 5% CO_2 controlled incubator (Model-9200, Wakenyaku). Malaria parasites inspection was performed by oil immersion (100 × 1.25) using an Olympus BX51 microscope.

2.2. Plant materials

Fresh seeds of *G. kola* were collected in Kinshasa in the Democratic Republic of Congo. The seeds (vernacular name in Kinshasa: "Ngadiadia") were authenticated by the botanist S.M. Mambwana, and a voucher specimen (voucher number: IUK6050) was deposited at the Herbarium of the University of Kinshasa (Faculty of Sciences) for future reference. The seeds were dried in the sun, pulverized to a coarse powder and transported to Japan by air.

2.3. Isolation of antimalarial biflavanones **1–3** from G. kola

The coarse powder of sun-dried kola nuts (700 g) was extracted three times with 70% aq. EtOH (1.5 L) at room temperature for 24 h. The solvent was evaporated under reduced pressure to produce an EtOH extract (27 g), which showed 87% growth inhibitory activity against *P. falciparum* at a concentration of 5 μg/mL. The EtOH extract was suspended in H_2O (800 mL) and partitioned with CH_2Cl_2 (750 mL × 3), EtOAc (750 mL × 3) and *n*-BuOH (500 mL × 3) to produce a CH_2Cl_2 extract (7 g), EtOAc extract (10 g), *n*-BuOH extract (6 g) and H_2O extract (4 g). The antimalarial activity was observed in all organic layers, each showing nearly equal potency of approximately 70% growth inhibition at 0.5 μg/mL. The CH_2Cl_2 extract and EtOAc extract were combined, and an aliquot (3 g) of this combined extract was chromatographed on SiO_2 (80 g; CH_2Cl_2:EtOAc = 9:1 → 7:3 → 6:4 → 5:5 → 4:6 → EtOAc → MeOH) to provide five fractions: fr. A (900 mg), fr. B (445 mg), fr. C (a known biflavanone **2**, 655 mg, 1.2% from dried nuts) (Cotteril et al., 1978; Kabangu et al., 1987), fr. D (325 mg), and fr. E (340 mg). The antimalarial activity with a high selectivity index was confined to four successive fractions from fr. B to fr. E. Consequently, the purification of fr. B (445 mg) on ODS (15 g; MeOH:H_2O = 45: 55) column chromatography and reversed-phase HPLC (Cosmosil 5C18-AR; 10 mm i.d. × 250 mm, CH_3CN:H_2O:AcOH = 40:60:0.1; flow rate = 3.0 mL/min; detection: u.v. 254 nm) yielded active biflavanone **1** (Kabangu et al., 1987) (33 mg, 0.22%). Reversed-phase HPLC (Cosmosil 5C18-AR; 10 mm i.d. × 250 mm; flow rate = 2.5 mL/min; detection: u.v. 254 nm) of fr. D eluted with 45% aq. MeOH yielded known biflavanone **3** (Kabangu et al., 1987) (24 mg, 0.12%) as the antimalarial principle.

2.4. Parasites

P. falciparum (FCR-3, cycloguanil-resistant strain from Gambia) was cultured by standard methods and maintained in semi-automated continuous culture in RPMI 1640 medium containing HEPES buffer, heat-inactivated human serum (10% v/v) and gentamicin (Trager and Jensen, 1976). The parasites were synchronized at ring stage by sorbitol treatment (Lambros and Vanderberg, 1977). Initial parasitemia was adjusted to 0.5% with 2% haematocrit in all experiments. Malaria parasites were incubated at a low temperature in a 5% O_2 and 5% CO_2 controlled incubator (Model-9200, Wakenyaku).

2.5. Assessment of in vitro antimalarial activity

Growth inhibition, intraerythrocytic development and parasite morphology were evaluated in culture by the microscopic observation of Giemsa-stained thin blood films. After synchronization by sorbitol treatment, 50 μL of parasite culture at ring stage (0.5% parasitemia and 2% haematocrit) was added to each well of 96-well microculture plates. The test samples were dissolved in DMSO and diluted to the appropriate concentration using complete medium. Then, 50 μL of each sample solution was inoculated. The final concentration of DMSO in the culture was 1%.