Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: Y. Liu, N. Murakami, H. Ji, Pedro Abreu & S. Zhang (2007) Antimalarial Flavonol Glycosides from *Euphorbia hirta*., Pharmaceutical Biology, 45:4, 278-281, DOI: 10.1080/13880200701214748

To link to this article: https://doi.org/10.1080/13880200701214748

Published online: 07 Oct 2008.

Pharmaceutical Biology
2007, Vol. 45, No. 4, pp. 278–281

informa
healthcare

Antimalarial Flavonol Glycosides from *Euphorbia hirta*

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Abstract

Bioassay-guided fractionation of the MeOH extracts of *Euphorbia hirta* Linn (Euphorbiaceae) aerial parts led to the isolation of flavonol glycosides afzelin (**1**), quercitrin (**2**), and myricitrin (**3**), whose structures were established by MS and NMR analysis. Compounds **1–3** showed proliferation inhibition of *Plasmodium falciparum*, with IC_{50} values of 1.1, 4.1, 5.4 μg/mL, repectively. On the other hand, they exhibited little cytotoxic property against human epidermoid carcinoma KB 3-1 cells.

Keywords: Afzelin, antimalarial, cytotoxicity, *euphorbia hirta*, myricitrin, quercitrin.

Introduction

Malaria is one of the three killers among communicable diseases in the world today, infecting approximately 300–500 million people every year. Mortality exceeds 1–3 million people per year, most of them being children in Africa under the age of 4 years, a number that is continuously increasing due to the rapid spread of drug-resistant *Plasmodium* parasites (Greenwood & Mutabingwa, 2002; Schwikkard & van Heeden, 2002; Wright, 2005). After an antimalarial screening of Congolese medicinal plants, we have observed that the MeOH extract of *Euphorbia hirta* Linn (Euphorbiaceae) (Oliver-Bever, 1986; Iwu, 1993) demonstrated not only potent *in vitro* antimalarial activity against *P. falciparum* but also little cytotoxic property against human epidermoid carcinoma KB 3-1 cells. The antimalarial activity of *E. hirta* extracts has been previously reported (Tona et al., 1999, 2004; Köhler et al., 2002; Koli et al., 2002), in addition to other healing properties, such as antiulcer (Lin & Hsu, 1988), antimicrobial (Oyewale et al., 2002; Sudhakar et al., 2006), antibacterial (Satyanarayana & Singhai, 1979; Vijaya et al., 1995), anti-*Helicobacter pylori* (Wang & Huang, 2005), sedative and anxiolytic (Lanhers et al., 1990), neurophysiologic (Lanhers et al., 1996), antihypertensive (Williams et al., 1997; Johnson et al., 1999), antihistaminic and immunosuppressive (Singh et al., 2006), molluscicidal (Singh et al., 2005), antiamebic (Tona et al., 2000), antifungal (Masood & Rajan, 1991), spasmolytic (Tona et al., 2000), antidiarrheic (Galvez et al., 1993a, 1993b; Mallavadhani et al., 2002; Hore et al., 2006), analgesic and antipyretic (Lanhers et al., 1991), and anti-inflammatory (Lanhers et al., 1991; Martinez-Vazquez et al., 1999; Singh et al., 2006). Phytochemical investigation on this species led to the isolation of tannins (Lanhers et al., 1991; Yoshida et al., 1988, 1990a, 1990b), flavonoids (Lin & Hsu, 1988; Lanhers et al., 1991; Galvez et al., 1993a, 1993b; Aquiland & Zhan, 1999; Koli et al., 2002; Oyewale et al., 2002; Tona et al., 2004), triterpenes (Martinez-Vazquez et al., 1999), phenolic acids, saponins, and amino acids (Lanhers et al., 1991). In this communication, we report the bioassay-guided isolation of the antimalarial principles of *E. hirta* MeOH extract, which were identified by spectroscopic methods as afzelin (**1**), quercitrin (**2**), and myricitrin (**3**) (Fig.1) (Zhang et al., 2003).

Materials and Methods

General experimental procedures

^1H and ^{13}C NMR spectra were recorded on a JNM-GX-500 (JEOL, Tokyo, Japan) spectrometer. Chemical shifts were reported with reference to the respective residual solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD). Fast Atom

Accepted: November 17, 2006.

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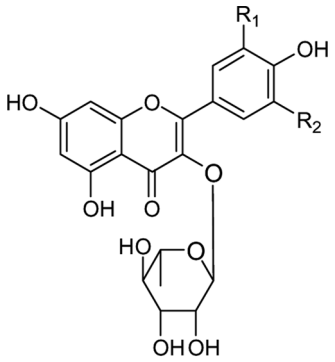
DOI: 10.1080/13880200701214748



1 $R_1 = R_2 = H$
2 $R_1 = OH, R_2 = H$
3 $R_1 = R_2 = OH$

Figure. 1. Flavonol Glycosides 1–3.

Bombardment Mass Spectometry (FABMS) data were obtained on a JMS SX-102 (JEOL) instrument using *m*-nitrobenzyl alcohol as the matrix. For flash column chromatography, silica gel (BW-200, 400-500 mesh, Fuji Sylisia) was used, whereas thin-layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) analyses were carried out over precoated plates (Merck, Kiesel gel $60F_{254}$, 0.25 mm, and RP-18 WF_{254}, respectively). Spots were visualized under UV 254 and 366 nm, and 1% $Ce(SO_4)_2$/10% H_2SO_4, *p*-anisaldehyde/H_2SO_4 (AcOH 5 mL, *c*-H_2SO_4 25 mL, EtOH 425 mL, water 25 mL) spray reagents. Reversed-phase HPLC was performed on a semipreparative Cosmosil C18-AR-II column (250×10 mm, 4 μm 80 Å), using a Shimadzu SPD-10A vp UV-Vis detector.

Plant material

Euphorbia hirta was collected in Kinshasa, Congo, in July 2001. A voucher specimen was deposited in the Medicinal Plants Source Exploration Lab, Graduate School of Pharmaceutical Science, Osaka University, Japan.

Extraction

The air-dried aerial parts of *Euphorbia hirta* (300 g) were cut into small pieces and successively extracted one-time with MeOH at room temperature and three-times at 75°C under reflux. The extracts were combined and concentrated in vacuum to obtain a residue that showed growth inhibition of *P. falciparum*. A part of this extract (3 g) was partitioned between water and EtOAc to yield an active organic fraction (1 g) that was then subjected to normal-phase flash chromatography (hexane:EtOAc, 10:1, hexane:EtOAc, 3:1, and MeOH elution) to afford four fractions (E1–E4). Fraction E-4 (647 mg) showed 90% growth inhibition of *P. falciparum* at a concentration of 5 μg/mL and little cytotoxicity against KB3-1 cells up to a concentration of 50 μg/mL. Flash chromatography of this fraction, eluted with $CHCl_3$:MeOH:H_2O, 30:3:1 (lower layer), $CHCl_3$:MeOH:H_2O, 10:3:1 (lower layer), and MeOH, afforded five fractions (E41–E45), which were monitored against*P. falciparum* and KB3-1 cells. The active fraction (E44, 204 mg) was submitted to RP-18 HPLC (MeCN:H_2O, 25:75) to yield three fractions (E443, E442, and E444), containing compounds **1** (2.9 mg), **2** (6.5 mg), and **3** (2.7 mg), respectively, which were further purified by HPLC.

In vitro antimalarial bioassays

Quinine (Nacalai, Tokyo, Japan) was used as a positive control for *in vitro* antimalarial experiments. The stock solutions of drugs were prepared in DMSO and diluted with complete medium. DMSO concentration in culture medium never exceeded 1%, unless otherwise noted. Colchicine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was used as a positive control in cytotoxic assay. Bioassays were carried out in a PVC clean bench (Hitachi, Tokyo, Japan). Parasites were incubated in a low-temperature 5% O_2 and 5% CO_2 controlled incubator (Model-9200, Wakenyaku, Co. Ltd., Kyoto, Japan), and the microscopic inspection of malaria parasites was done over under oil emersion (Olympus BX51, 100×1.25, Tokyo, Japan). Two strains of *P. falciparum* (CDC1, chloroquine-sensitive; and FCR-3, cycloguanil-resistant from Gambia) were maintained in semiautomated continuous culture in medium RPMI 1640 containing HEPES buffer, heat-inactivated human serum (10%v/v), and gentamicin and cultured by standard methods.The parasites were synchronized at ring stage by sorbitol treatment. Initial parasitemia was adjusted to 0.5% with 2% hematocrit in all experiments (Krishna & Ganapaty, 1983). Growth inhibition, intraerythrocytic development, and parasite morphology were evaluated in culture by microscopic observation of Giemsa-stained thin blood films. Drug-free cultures were always used as controls. To evaluate growth inhibition, parasitemia was measured by counting 10,000 erythrocytes and is reported as the percentage of parasitized erythrocytes. Intraerythrocytic development was monitored by examining a minimum of 1000 parasitized cells on each film, for differential counting of rings, trophozoites, and schizonts. The proportion of each group was calculated as a percentage of the total parasitized cells. Repetitive dosing effect was evaluated against FCR-3 strain following a model proposed in literature (Bwijo et al., 1997). Parasites were exposed to daily dosing of drugs by replacing the culture medium with the same volume of fresh medium containing the drug for 6 consecutive days. The parasites treated with drug-free growth medium containing 0.1% DMSO was considered as control. Thin smears were prepared every 24 h for the assessment of parasitemia and parasite replication.

Evaluation of cytotoxicities

Human epidermoid carcinoma KB cells were cultured in RPMI 1640 medium with 0.58 mg/mL glutamine, 50 μg/mL kanamycin sulfate, supplemented with 10% fetal bovine serum, and incubated in 5% CO_2 controlled incubator (Sensor; Sanyo, Nakashina, Japan) at 37°C. Cytotoxic activity was measured by means of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) formazan colorimetric assay performed in 96-well plates (Nunclon, Roskilde, Denmark). Equal numbers of cells were inoculated into each well with 100 μL of the culture medium, and then a 100 μL solution of each tested compound was added to each well. After 72 h incubation under 5% CO_2 atmosphere at 37°C, 25 μL of MTT solution (2 mg/mL in PBS) was added to each well and incubated for further 3 h. The percentage of cell growth inhibition was calculated from the absorbance at 540 nm, recorded on a microplate reader (BIO RAD 450, Hercules, CA, USA) (Krishna & Ganapaty, 1983). Inhibitory concentration 50% (IC_{50}) values were determined by linear interpolation from the inhibition curve.

Results and Discussion

Bioassay-guided fractionation of the MeOH extract of *E. hirta* aerial parts, monitored against *P. falciparum* parasites, yielded a main active chromatographic fraction showing 90% growth inhibition of *P. falciparum* at a concentration of 5 μg/mL and, in addition, little cytotoxic property against human epidermoid carcinoma KB 3-1 cells up to a concentration of 50 μg/mL. The active constituents were isolated by flash chromatography and semipreparative reversed-phase HPLC and identified as the flavonol glycosides afzelin (**1**), quercitrin (**2**), and myricitrin (**3**). Their structures were established by HRFABMS and NMR, whose data corresponded with that published in literature (Zhang et al., 2003). Compounds **1–3** inhibited the proliferation of *P. falciparum* (CDC1); IC_{50} values 1.1, 4.1, 5.4 μg/mL, repectively. On the other hand, these flavonol glycosides have little influence on the growth of KB 3-1 representing the host cell, showing cytotoxic activity; IC_{50} values 276.1, 88.2, 156.4 μg/mL, respectively. This effect was previously demonstrated for quercitrin and other naturally occurring flavonol glycosides isolated from a Japanese traditional crude drug (Murakami et al., 2001). Quercitrin is also a recognized antidiarrheic agent of *E. hirta* (Galvez et al., 1993b; Mallavadhani et al., 2002; Hore et al., 2006). Although afzelin and myricetin have been previously isolated from an *E. hirta* specimen collected in Taiwan (Lin & Hsu, 1988), their characterization as antimalarial principles is here reported for the first time.

On the other hand, the CDC1 and FCR-3 strains showed similar IC_{50} results. The effects of flavonol glycosides on the life cycle of *P. falciparum* were investigated. It was shown that monoglycoside **2** arrested the life cycle of the parasite irreversibly at the trophozoite stage. *P. falciparum* possesses a 48-h intraerythrocytic growth cycle, which is morphologically defined as ring, trophozoite, and schizont in a chronological order. A mature parasite can produce around 20 merozoites, with each merozoite able to invade other erythrocytes (Miller et al., 2002). As a result, the infection ratio increases many-fold after a single cycle. This cyclical way of invasion to new erythrocytes progresses until the death of the host. The effect of flavonol glycosides on the life cycle of malaria parasites was examined against the synchronized culture of ring stage parasite with inoculation of samples at 0 h. It was shown that flavonol monoglycoside **2** irreversibly arrested the life cycle of the parasites beyond the transition from trophozoite stage (24 h) to schizont stage (28–32 h). However, **2** was shown to kill the parasite dose-dependently between 0.5 and 5 μg/mL and exhibited complete growth inhibition of *P. falciparum* at a dose of 10 μg/mL.

Acknowledgments

This study was supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan; the K. C. Wong Education Foundation, Hong Kong; and by a grant from the Hundred Talents Project of the Chinese Academy of Sciences.

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