Antimalarial and Antimycobacterial Activities of Dimeric naphthoquinone from *Diospyros glandulosa* and *Diospyros rhodocalyx*

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ABSTRACT

Lupeol (1), β-sitosterol (2), stigmasterol (3) and diospyrin (4) were isolated from the woods of Diospyros glandulosa and Diospyros rhodocalyx (Ebenaceae), while betulinaldehyde (5) was obtained only from D. rhodocalyx. Compounds 4 and 5 showed in vitro antimalarial activity against Plasmodium falciparum K1 with respective IC_{50} values of 3.29 and 6.25 µg/mL. In addition, compounds 4 and 5 also showed antimycobacterial activity with MIC values of 6.25 and 25 µg/mL, respectively.

Key words: *Diospyros glandulosa*, *Diospyros rhodocalyx*, Ebenaceae, Diospyrin, Antimalarial and antimycobacterial activities

INTRODUCTION

Malaria continues to be a growing health problem of global concern. According to the World Health Organization estimations, the number of clinical cases has reached 300-500 millions per year, most of which are in Africa. Increased geographical spreading of the disease is observed, mainly in Asia. In addition to a mortality rate of 1.1 to 2.7 million deaths per year (WHO, 1998), mostly among children, malaria puts a heavy economic burden on the developing countries by exhausting health system resources and by associated loss of economic activity. Only a limited number of chemotherapeutic agents for the treatment of malaria is available, and the growing problem of drug resistance makes adequate treatment of malaria increasingly difficult (WHO, 1998). In the absence of a functional, safe and widely-available malarial vaccine, efforts to develop new antimalarial drugs are profoundly important. Since the majority of the existing antimalarial chemotherapeutic agents are based on natural products (Ekthawatchai et al., 1999), biological chemodiversity continues to play an important role in the search that would lead to antimalarial drugs. Therefore, it is necessary to search for new compounds as back-up antimalarials. As a part of our continuous search for novel bioactive compounds from plant source, plants in the genus Diospyros are the rich sources of biologically-active metabolites (Kuo et al., 1997; Costa et al., 1998; Li et al., 1998; Mallavadhani et al., 1998; Likhitwitayawuid

et al., 1999; Duan et al., 2004; Ganapaty et al., 2004; Gu et al., 2004; Higa et al., 2004). Several plant extracts have been routinely screened for biological activities, and among the screened extracts, it was found that the crude extracts from dichloromethane (CH_2Cl_2) of *Diospyros glandulosa* and *Diospyros rhodocalyx* exhibited antimalarial activity and therefore, were chosen for further investigation.

Diospyros glandulosa is a timber tree, belonging to the family Ebenaceae (Smitinand, 2001). This plant is known in Thai as "Kluai ruesi". It has never been chemically investigated, and this is the first report on bioactive compounds from this plant.

D. rhodocalyx (Family Ebenaceae) is known in Thai as "Tako Na". The fruits are used for treatments of diarrhea, bleeding, abdominal discomfort, parasitic infestation abscess and renal disease; the bark is used for symptomatic relief of leucorrhea and as antidiuretic (Sutthivaiyakit et al., 1995).

Detailed characterizations of bioactive compounds 4 and 5 and biological activities of compounds 1- 5 are reported in this paper.

MATERIALS AND METHODS

General

^1H, ^{13}C NMR spectra were recorded on a Bruker-AM 400 (400 and 100 MHz for ^1H and ^{13}C, respectively), using $CDCl_3$ as a solvent with $Si(CH_3)4$ as an internal standard. IR spectra were obtained on Perkin Elmer System 2000 FT-IR and 1760 X FT-IR spectrometers. Mass spectra were recorded with a Finnigan Mat GCQ spectrometer for EIMS and ESITOFMS spectra were obtained from a Bruker Micro TOFLC spectrometer. UV spectra were recorded on a Shimadzu UV-vis 2001s spectrophotometer.

Plant Material

Woods of *D. glandulosa* were collected from Khao Yai National Park, Nakhon Ratchasima province in November 2005, and woods of *D. rhodocalyx* were collected from Nakhon Sawan province in September 2006, and both plants were identified by Miss Panarat Charoenchai in the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University. The voucher specimens are deposited at the Laboratory of Natural Products, Chulabhorn Research Institute.

Extraction and Isolation

Woods of *D. glandulosa*

Woods of *D. glandulosa* (3.3 kg) were ground and extracted with CH_2Cl_2 at room temperature for 3 days. Evaporation of a CH_2Cl_2 extract under reduced pressure gave a dark solid (55.5 g) which was subjected to column chromatography on *Sephadex LH-20* column (eluted with methanol, MeOH), yielding twenty fractions (A1-A20). Fractions A9-A10 were purified by *Sephadex* LH-20 column (eluted with MeOH) to yield ten fractions (B1-B10), and fractions B6-B10 were also re-chromatographed on *Sephadex LH-20* column (eluted with MeOH) to afford diospyrin (4)(159 mg).

Woods of *D. rhodocalyx*

Woods of *D. rhodocalyx* (2.5 kg) were ground and extracted with CH_2Cl_2 at room temperature for 3 days. Evaporation of a CH_2Cl_2 extract under reduced pressure gave a brown solid (17.15 g) which was subjected to column chromatography on *silica gel*, using hexane with increasing CH_2Cl_2, CH_2Cl_2 with increasing proportions of MeOH, and finally with MeOH, to provide thirty-six fractions (A1-A36). Fractions A26-A36 were chromatographed on *Sephadex LH-20* (eluted with MeOH) to yield twelve fractions (B1-B12). Fractions B1-B2 were chromatographed on *Silica gel* column (acetone: hexane, 10:90) to afford lupeol (1)(48 mg) and betulinaldehyde (5)(18.2 mg). Fractions B11-B12 were purified by *Sephadex LH-20* (eluted with MeOH) to afford diospyrin (4)(235 mg) together with β-sitosterol (2) and stigmasterol (3).

Structure elucidation of compound 4

The chemical structure of compound 4 was elucidated by analysis of ^1H and ^{13}C NMR spectra. These spectra were obtained from a Bruker-AM400 (400 and 100 MHz) spectrometer. The solvent for these spectra was deuterated chloroform, using tetramethylsilane as the internal reference. The chemical shifts were reported in ppm scale : ^1H NMR ($CDCl_3$, 400 MHz) δ 2.24(3H, s, 7-CH_3), and 2.39(3H, s, 7′-CH_3), 6.83(^1H, s, H-3′), 6.89(1H, s, H-2), 6.89(1H, s, H-3), 7.06(1H, br, H-6′), 7.44(^1H, brs, H-8′), 7.49(^1H, s, H-8), 11.81(^1H, s, H-5′) and 12.07(^1H, s, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.0(7-Me), 22.2(7′-Me), 112.9(C-4a), 113.1(C-4′a), 120.7(C-8), 121.2(C-8′), 124.2(C-6′), 128.8(C-6), 131.3(C-8a), 131.6(C-8′a), 138.7(C-3′), 138.8(C-3), 139.4(C-2), 145.7(C-2′), 159.1(C-5), 161.7(C-5′), 182.5(C-1′), 184.1(C-1), 188.9(C-4′) and 189.7(C-4).

Structure elucidation of compound 5

^1H NMR (CDCl3, 400 MHz) δ 0.68(3H, s, 24-CH_3), 0.75(3H, s, 25-CH_3), 0.84(3H, s, 23-CH_3), 0.90(3H, s, 26-CH_3), 1.19(3H, s, 27-CH_3), 1.62(3H, s, 30- CH_3), 2.80(1H, m, H-19), 3.2(1H, q, H-3), 4.56(1H, d, *J*= 2.0 Hz, H-29), 4.69(1H, d, J= 2.0 Hz, H-29) and 9.6(1H, s, H-28); ^{13}C NMR($CDCl_3$, 100 MHz) δ 14.2(C-27), 15.3(C-24), 15.8(C-25), 16.1(C-26), 18.2(C-6), 19.0(C-30), 20.7(C-11), 25.5(C-12), 27.3(C-2), 27.9(C-23), 28.8(C-16), 29.2(C-15), 29.8(C-21), 33.2(C-22), 34.3(C-7), 37.1(C-10), 38.6(C-1), 38.7(C-13), 38.8(C-4), 40.8(C-8), 42.5(C-14), 47.5(C-19), 48.0(C-18), 50.4(C-9), 55.2(C-5), 59.3(C-17), 78.9(C-3), 110.1(C-29), 149.7(C-20) and 206.7(C-28).

Bioassays

Antimalarial activity. The parasite *Plasmodium falciparum* (K1, multidrug resistant strain) was cultured continuously according to the method of Trager and Jensen (1976). Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microculture radioisotope technique, based upon the method described by Desjardins et al., (1979). Briefly, a mixture of 200 µl of 1.5% of erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µl of the medium containing a test sample dissolved in DMSO (0.1% final concentration) for

24 h, employing the incubation conditions described above. Subsequently, 25 µl of [^{3}H] hypoxanthine (Amersham, USA) in culture medium (10 µCi) was added to each well, and plates were incubated for an additional 24 h. Levels of incorporated radioactively-labeled hypoxanthine indicating parasite growth were determined, using the TopCount microplate scintillation counter (Packard, USA). The standard drug was dihydroartemisinin (Table 1).

Antitubercular activity. The antitubercular activity was assessed against Mycobacterium tuberculosis H_{37}Ra, using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). *M. tuberculosis* H_{37}Ra was grown in 100 ml of 7H9GC containing 0.005% Tween 80. Culture was incubated in 500 mL plastic flask on a rotary shaker at 200 rpm and 37°C until it reached an optical density of 0.4-0.5 at 550 nm. Bacteria were washed and suspended in 20 mL of phosphate-buffered saline and passed through an 8-µm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at -80°C. Antimicrobial susceptibility test was performed in a 96-well microplate reader. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial screened-sample dilutions were prepared in either DMSO or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media, containing 0.2% v/v glycerol and 1.0 g/l casitone (7H9GC), and subsequent two-fold dilutions were performed in 0.1 mL of 7H9GC in the microplates. Frozen inocula were diluted 1:100 in 7H9GC. Addition of 0.1 mL to the well resulted in final bacterial titers of about $5x10^4$ CFU/mL. Wells containing sample only were used to determine whether the tested samples themselves could reduce the dye or not. Additional control wells consisting of bacteria (B) or medium (M) were included. Plates were incubated at 37°C. Starting at day 6 of incubation, 20 µl of Alamar Blue solution and 12.5 µl of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37°C. Wells were observed at 24 h for a colour change from blue to pink. If the B wells became pink by 24 h, Alamar Blue solution was added to all testing plates. However, if a colour (blue) of M and B wells did not change, both wells were tested daily until a colour of B wells changed from blue to pink. After the change of B well colour, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37°C for 24 h, and the results were recorded with a fluorescence multi-well reader (CytoFluor, Series 4000) at the excitation and emission wavelengths of 530 and 590 nm, respectively. The standard drugs were isoniazid and kanamycin sulfate (Table 1).

RESULTS AND DISCUSSION

Extraction, isolation and *in vitro* antimalarial and antimycobacterial activities

The CH_2Cl_2 wood extracts of *Diospyros glandulosa* and *Diospyros rhodocalyx* were sequentially subjected to column chromatography on *Sephadex LH-20* and *Silica gel* to obtain lupeol (1), β-sitosterol (2) and stigmasterol (3), diospyrin (4) and betulinaldehyde (5). Diospyrin (4) had the most active antimalarial activity

against *P. falciparum* K1 with the IC_{50} value of 3.29 µg/mL, as shown in Table 1. Compound 5 showed antimycobacterial activity with the MIC value at 25 µg/mL. However, β-sitosterol (2) did not dissolve in DMSO, therefore, its antimalarial and antimycobacterial activities could not be evaluated.

Structure elucidation of compound 4

Compound 4 was obtained as orange-red powder, and its molecular formula was established by ESITOFMS as $C_{22}H_{14}O_6$. Compound 4 could be assigned as a known naphthoquinone, diospyrin (4), through analysis of its ^1H and ^{13}C NMR spectra. The ^1H NMR ($CDCl_3$, 400 MHz) spectrum showed signals at δ_H 2.39 and 2.25 ppm which were the signals of methyl protons of a naphthoquinone compound. The signals at δ_H 6.89, 6.89, 7.06, 7.44 and 7.50 ppm were of aromatic methine protons. The signals at δ_H 11.80 and 12.10 ppm were of chelated hydroxyl groups at C-5 and C-5′. The ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum showed the signals of fourteen quaternary, six methine and two methyl carbons.

Structure elucidation of compound 5

Compound 5 was obtained as white solid, and its molecular formula was established by ESITOFMS as $C_{30}H_{48}O_2$. Compound 5 could be assigned as a known triterpene, betulinaldehyde (5), through analysis of its ^1H and ^{13}C NMR spectra. The ^1H NMR ($CDCl_3$, 400 MHz) spectrum showed signals at δ_H 0.68, 0.75, 0.84, 0.9, 1.19 and 1.62, respectively, which were six singlet methyl groups of a lupane triterpene, an aldehyde group [δ_H 9.6 ppm], a typical lupene Hβ-19 proton [δ_H 2.8 ppm] and the 3α geminal proton [δ_H 3.2 ppm]. The ^{13}C NMR ($CDCl_3$, 400 MHz) spectrum showed signals of ten methylene, six methyl, a formyl and an exomethylene carbons. Spectral data of known compounds 1-5 were identical in all respects to those reported in the literature (Tezuka et al., 1973; Kouam et al., 1993; Ravindranath et al., 2004). Biological activities of these isolated compounds are summarized in Table 1.

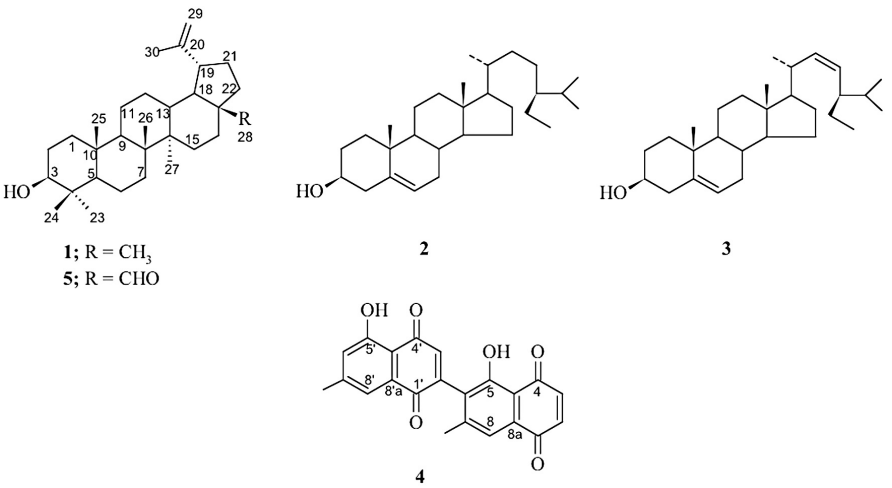

Figure 1. Compounds isolated from Diospyros glandulosa and Diospyros rhodocalyx.

Table 1. Biological activities of compounds 1-5; IC_{50} and MIC values are expressed in µg/mL.

Compound	Antimalarial activity (IC_{50}, µg/mL)	Antimycobacterial activitya (MIC, µg/mL)
1	inactive	inactive
2	Not dissolved in DMSO	Not dissolved in DMSO
3	Not determined	Not determined
4	3.29	6.25
5	6.25	25
isoniazid	-	0.06
kanamycin sulfate	-	2.5
dihydroartemisinine	0.004±0.001	-
(n=3)		

[a]) MIC values were obtained from the two-fold dilution technique, and triplicate experiments were performed showing the same MIC value for each compound.

CONCLUSION

The antimalarial and antimycobacterial activities and chemical constituents of *D. rhodocalyx* and *D. glandulosa* were investigated. The results demonstrated that diospyrin (4) of the CH_2Cl_2 extract of woods of *D. rhodocalyx* and *D. glandulosa* showed the highest level of *in vitro* antimalarial activity against *P. falciparum* K1. Betulinaldehyde (5) also showed *in vitro* antimalarial activity against *P. falciparum* K1. Both compounds 4 and 5 exhibited antimycobacterial activity.

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