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Original article:

NEW BIOACTIVE TRITERPENOIDS AND ANTIMALARIAL ACTIVITY OF *DIOSPYROS RUBRA* LEC.

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ABSTRACT

The first investigation of the chemical constituents and bioactivities of *Diospyros rubra* Lec. is reported. *D. rubra* extracts were screened for antimicrobial, antimalarial and cytotoxic activities. They were only shown to be active antimalarials. The extracts with good antimalarial activity were isolated and extensively purified to give lupeol (**1**), lupenone (**2**), betulin (**3**), lupeol acetate (**4**), 28-*O*-acetylbetulin (**5**), *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside (**6**) and a mixture of *β*-sitosterol and stigmasterol. Some of the isolates were tested for antimicrobial and cytotoxic actions. Betulin (**3**) displayed antimicrobial activity against *Streptococcus pyogenes* with a minimum inhibitory concentration (MIC) of 85 μg/mL. Interestingly, bioactive fractions all selectively exerted some antimicrobial activity against *Corynebacterium diphtheriae* NCTC 10356 with the MIC range of 64–256 μg/mL. The study provides data to support the medicinal importance of the *D. rubra*.

Keywords: *Diospyros rubra* Lec.; triterpenoids; antimicrobial; antimalarial and cytotoxic activities

INTRODUCTION

Previously, many species of *Diospyros* were studied and a diverse group of compounds such as triterpenoids, steroids, naphthaquinones, hydrocarbons and lipids were found (Jain et al., 1994; Kuo et al., 1997). However, no chemical and pharmacological studies of *Diospyros rubra* Lec. (Ebenaceae) have been reported in the literature to date. *D. rubra* is a medicinal plant known in Thai as "Phaya-raak-dam". It has been used as an indigenous medicine for treatment of pain and tuberculosis (Bunyapraphatsara and Chokechareunporn, 1999). In continuation of our investigations of medicinal plants, the components of *D. rubra* were isolated and tested for antimicrobial, antimalarial and cytotoxic activities. The present study reports some bioactive triterpenes from this plant species.

MATERIALS AND METHODS

General

Melting points were determined on an Electrothermal 9100 melting point apparatus and were uncorrected. ^{1}H- and ^{13}C-NMR

spectra were recorded on a Bruker AVANCE 300 NMR spectrometer (operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Mass spectra were recorded on a Finnigan INCOS 50. Column chromatography was carried out using silica gel 60 (0.063–0.200 mm). Analytical thin layer chromatography (TLC) was performed on silica gel 60 PF_{254} aluminium sheets (cat. No. 7747 E., Merck). Solvents were distilled prior to use. Chemicals for cell culture and assays were RPMI-1640 (Gibco and Hyclone laboratories, USA), HEPES, L-glutamine, penicillin, streptomycin, sodium pyruvate and glucose (Sigma, USA), Ham's/F12, fetal bovine serum (FBS, Hyclone laboratories, USA), gentamicin sulfate (Government Pharmaceutical Organization, Thailand), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA).

Plant materials

Stems of *D. rubra* were collected from Thap Sakae, Prachuap Khiri Khan Province, Thailand. It has been identified (BKF 151270) by The Forest Herbarium, Royal Forestry Department, Bangkok. A voucher specimen has been deposited at Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.

Extraction

The milled air dried *D. rubra* (7 kg) was extracted with hexane 10 L (3 × 5 days), followed by filtration. The filtrates were combined and evaporated *in vacuo* to give a hexane extract (22 g). Similar extraction was conducted using dichloromethane, ethyl acetate and methanol to give dichloromethane (17 g), ethyl acetate (16 g) and methanol (95 g) extracts, respectively.

Isolation

The extracts were isolated by silica gel column chromatography using gradient elution with solvent mixtures of increasing polarity. Fractions were combined based on TLC and evaporated to dryness *in vacuo*.

The dichloromethane extract (17 g) was subjected to a silica gel (800 g) column chromatography, then eluted by hexane-dichloromethane and dichloromethane-ethyl acetate to give five fractions: D1-D5. Three selected fractions D2, D3 and D4 were reseparated. D2 (1.0 g of yellow oily liquid from 7:3 hexane-CH_2Cl_2) was placed onto a silica gel (40 g) column. Elution with hexane-CH_2Cl_2 gave four fractions D2.1–D2.4; D2.2, a red oil, gave lupenone (**2**; 12.2 mg as oily liquid) from 85:15 hexane-CH_2Cl_2 elution.

D2.3 was obtained as pale violet solid (from 1:9 hexane-CH_2Cl_2 elution) of lupeol (**1**; 25.8 mg); mp 220–222 °C (lit. mp 221–223 °C, Yoshihira et al., 1971).

Fraction D3 (1.5 g of brown solid eluted with 2:8 hexane-CH_2Cl_2) was recrystallized from hexane to give additional compound **1** (800.6 mg).

Fraction D4, a yellow solid (5.3 g) obtained by elution with 9:1 CH_2Cl_2-EtOAc was rechromatographed on a silica gel (200 g) column to provide five fractions D4.1–D4.5. Fraction D4.1 (0.1 g of green solid from 7:3 hexane-CH_2Cl_2 elution) was recrystallized from hexane to give lupeol (**1**; 74.3 mg). D4.2 (0.2 g of red brown solid eluted with 1:9 hexane-CH_2Cl_2) was placed onto a silica gel (8 g) column and elution with 3:7 hexane-CH_2Cl_2 gave fraction D4.2.1 (light green solid 49.8 mg) which was recrystallized from methanol to afford a mixture of β-sitosterol and stigmasterol (40.2 mg). Fraction D4.3 (2.5 g of yellow solid from 1:9 hexane-CH_2Cl_2 elution) was applied to a silica gel (100 g) column and eluted with 3:7 hexane-CH_2Cl_2 to give fraction D4.3.1, a mixture of β-sitosterol and stigmasterol (3.3 mg). Next, elution with 2:8 hexane-CH_2Cl_2 gave fraction D4.3.2, a pale yellow solid (57.2 mg) of long chain hydrocarbon which was not identified. Further elution with CH_2Cl_2 provided fraction D4.3.3; a yellow–green solid (140 mg) which was rechromatographed on a silica gel (6 g) column. Elution with CH_2Cl_2 afforded fraction D4.3.3.1 of a green solid (34.4 mg), followed by 95:5 CH_2Cl_2-EtOAc

elution to give fraction D4.3.3.2 as a yellow solid (50.6 mg). Recrystallization of D4.3.3.2 from methanol provided a white solid of betulin (**3**); 39.1 mg; mp 237–240 °C (lit mp 236–238 °C, Tinto et al., 1992).

The ethyl acetate extract (16 g) was separated as described on a silica gel (800 g) column to provide seven fractions E1–E7. Fractions E1-E4 and E6-E7 were reisolated. E1 (0.4 g of light green solid from 1:1 hexane-CH_2Cl_2 elution) was rechromatographed on a silica gel (8 g) to give fraction E1.1 (10 mg of oily liquid from 9:1 hexane- CH_2Cl_2). Further elution with 8:2 hexane-CH_2Cl_2 gave fraction E1.2 (81.7 mg of yellow solid) which after recrystallization from MeOH afforded lupeol acetate (**4**; 50.9 mg; mp 190–192 °C).

Elution with 4:6 hexane-CH_2Cl_2 gave yellow solids of E2 (0.1 g) and E3 (1.4 g) which were recrystallized from MeOH to give 60.4 and 73.5 mg of lupeol (**1**), respectively. Fraction E4 (1.1 g of oily brown liquid from 9:1 CH_2Cl_2-EtOAc elution) was placed onto a silica gel (35 g) and eluted with CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH to give four subfractions E4.1–E4.4. E4.1 was a pale yellow solid (7.8 mg). E4.2 (15.0 mg as light-brown solid from CH_2Cl_2 elution) was recrystallized from MeOH to give a mixture of *β*-sitosterol and stigmasterol. E4.3, a yellow solid (169.7 mg from 95:5 CH_2Cl_2-EtOAc elution) was recrystallized from MeOH to afford a white solid of 28-*O*-acetylbetulin (**5**; 81.4 mg; mp 209–210 °C). Fraction E4.4 (360 mg of oily brown liquid from 1:1 EtOAc-MeOH elution) was rechromatographed on a silica gel (15 g) to provide a green solid (51.6 mg from hexane-CH_2Cl_2, 4:6) which was recrystallized from MeOH to give white solid (14.6 mg) of long chain hydrocarbon. Fraction E6 (1.5 g of brown solid from 85:15 CH_2Cl_2-EtOAc elution) was placed onto silica gel (50 g) to give five fractions E6.1–E6.5; E6.2 (28.8 mg of pale yellow solid from 9:1 CH_2Cl_2-EtOAc elution) was recrystallized from MeOH to provide white solid of long chain hydrocarbon (9.1 mg). E6.3, as yellow solid (101.9 mg from 95:5 CH_2Cl_2-EtOAc) was recrystallized from MeOH to give betulin (**3**; 22.6 mg). Fraction E7 (2.1 g of brown solid from 1:1 CH_2Cl_2-EtOAc) was recrystallized from MeOH to afford *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside (**6**; 5.1 mg); mp 285–287 °C (lit. mp 286 °C, Mitra and Misra, 1965).

Spectral data

Lupeol (**1**); IR ($CHCl_3$): υ_{max} 3,345, 2,944, 2,862, 1,638, 1,453, 1,380, 1,043 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.66 (d, 1H, J = 9.1 Hz, H-5), 0.73 (s, 3H, H-24), 0.76 (s, 3H, H-28), 0.80 (s, 3H, H-25), 0.92 (s, 3H, H-27), 0.94 (s, 3H, H-23), 1.00 (s, 3H, H-26), 1.65 (s, 3H, H-30), 1.82–1.96 (m, H-21), 2.35 (dt, 1H, J = 10.9, 5.5 Hz, H-19), 3.16 (dd, 1H, J = 10.8, 5.1 Hz, H-3), 4.55 (br s, 1H, H-29), 4.65 (br s, 1H, H-29); ^{13}C-NMR ($CDCl_3$): δ 14.5 (C-27), 15.3 (C-24), 15.9 (C-25), 16.1 (C-26), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.1 (C-12), 27.4 (C-2, C-15), 28.0 (C-23), 29.7 (C-21), 34.3 (C-7), 35.6 (C-16), 37.1 (C-10), 38.0 (C-13), 38.7 (C-1), 38.8 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 43.0 (C-17), 48.0 (C-18), 48.3 (C-19), 50.4 (C-9), 55.3 (C-5), 79.0 (C-3), 109.3 (C-29), 150.9 (C-20); LRMS (EI): *m/z* (%) = 426 (34)$[M]^+$, 411 (30), 218 (23), 189 (34), 91 (100), 77 (65).

Lupenone (**2**); IR ($CHCl_3$): υ_{max} 2,927, 2,860, 1,708, 1,459, 1,382 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.77 (s, 3H, H-28), 0.90 (s, 3H, H-25), 0.93 (s, 3H, H-27), 1.00 (s, 3H, H-24), 1.04 (s, 3H, H-23), 1.22 (s, 3H, H-26), 1.66 (s, 3H, H-30), 1.84-1.97 (m, H-21), 2.24-2.52 (m, H-19), 4.55 (br s, 1H, H-29), 4.66 (br s, 1H, H-29); ^{13}C-NMR ($CDCl_3$): δ 14.4 (C-27), 15.7 (C-26), 15.9 (C-25), 17.9 (C-28), 19.2 (C-6), 19.6 (C-30), 21.0 (C-24), 21.4 (C-11), 25.1 (C-12), 26.6 (C-23), 27.4 (C-15), 29.6 (C-21), 33.5 (C-7), 34.1 (C-2), 35.5 (C-16), 36.8 (C-10), 38.1 (C-13), 39.6 (C-1), 39.39 (C-22), 40.7 (C-8), 42.8 (C-14), 42.9 (C-17), 47.3 (C-4), 47.9 (C-19), 48.2 (C-18), 49.7 (C-9), 54.9 (C-5), 109.3 (C-29), 150.8 (C-20), 218.2 (C-3); LRMS (EI): *m/z* (%) = 424(10) $[M]^+$, 218 (7), 189 (13), 91 (100), 77 (66).

Betulin (**3**); IR ($CHCl_3$): υ_{max} 3,396, 2,941, 2,862, 1,643, 1,545, 1,375, 1,027 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.65 (d, 1H, *J* = 9.4 Hz, H-5), 0.73 (s, 3H, H-24), 0.79 (s, 3H, H-25), 0.94 (s, 3H, H-27), 0.95 (s, 3H, H-23), 0.99 (s, 3H, H-26), 1.65 (s, 3H, H-30), 2.35 (dt, 1H, *J* = 10.5, 6.2 Hz, H-19), 3.16 (dd, 1H, *J* = 10.8, 4.9 Hz, H-3), 3.30 (d, 1H, *J* = 10.8, H-28), 3.77 (d, 1H, *J* = 10.8 Hz, H-28), 4.55 (br s, 1H, H-29), 4.65 (br s, 1H, H-29); ^{13}C-NMR ($CDCl_3$) : δ 14.7 (C-27), 15.3 (C-24), 15.9 (C-26), 16.0 (C-25), 18.2 (C-6), 19.0 (C-30), 20.8 (C-11), 25.2 (C-12), 27.0 (C-15), 27.3 (C-2), 27.9 (C-23), 29.1 (C-16), 29.7 (C-21), 33.9 (C-22), 34.2 (C-7), 37.1 (C-13), 37.3 (C-10), 38.6 (C-1), 39.3 (C-4), 40.9 (C-8), 42.7 (C-14), 47.7 (C-17, C-18), 48.7 (C-19), 50.3 (C-9), 55.2 (C-5), 60.5 (C-28), 78.9 (C-3), 109.6 (C-29), 150.4 (C-20); LRMS (EI): *m/z* (%) = 442 (18)$[M]^+$, 411 (63), 203 (48), 189 (21), 91 (79), 77 (100).

Lupeol acetate (**4**): IR ($CHCl_3$): υ_{max} 2,942, 2,866, 1,735, 1,451, 1,379, 1,243, 1,027 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.76 (dd, 1H, *J* = 10.8, 5.8 Hz, H-5), 0.81 (s, 3H, H-28), 0.82 (s, 9H, H-23, H-24, H-25), 0.91 (s, 3H, H-27), 1.00 (s, 3H, H-26), 1.66 (s, 3H, H-30), 1.82-1.93 (m, 2H, H-21), 2.01 (s, 3H, H-2′), 2.33 (dt, 1H, *J* = 11.1, 5.6 Hz, H-19), 4.44 (dd, 1H, *J* = 10.8, 5.8 Hz, H-3), 4.54 (br s, 1H, H-29), 4.66 (br s, 1H, H-29); ^{13}C-NMR ($CDCl_3$): δ 14.5 (C-27), 15.9 (C-24), 16.1 (C-25), 16.4 (C-26), 17.9 (C-28), 18.1 (C-6), 19.0 (C-30), 21.3 (C-2′), 20.9 (C-11), 23.7 (C-2), 25.0 (C-12), 27.4 (C-15), 28.2 (C-23), 29.8 (C-21), 34.2 (C-7), 35.5 (C-16), 37.0 (C-10), 37.7 (C-4), 38.0 (C-13), 38.3 (C-1), 39.9 (C-22), 40.8 (C-8), 42.8 (C-14), 42.9 (C-17), 48.0 (C-18), 48.2 (C-19), 50.3 (C-9), 55.3 (C-5), 80.9 (C-3), 109.3 (C-29), 150.9 (C-20), 171.0 (C-1′); LRMS (EI): *m/z* (%) = 468 (28)$[M]^+$, 396 (100), 218 (24), 189 (22), 91 (49).

28-O-Acetylbetulin (**5**); IR ($CHCl_3$): υ_{max} 3,587, 2,942, 2,868, 1,739, 1,458, 1,388, 1,239, 1,033; ^{1}H-NMR ($CDCl_3$): δ 0.65 (d, 1H, *J* = 9.0, H-5), 2.04 (s, 3H, H-2″), 1.00 (s, 3H, H-24), 0.73 (s, 3H, H-25), 0.94 (s, 3H, H-23), 0.95 (s, 3H, H-27), 0.80 (s, 3H, H-26), 1.65 (s, 3H, H-30), 2.42 (dt, 1H, *J* = 10.8, 5.8 Hz, H-19), 3.16 (dd, 1H, *J* = 10.3, 4.8 Hz, H-3), 3.83 (d, 1H, *J* = 11.0 Hz, H-28), 4.22 (d, 1H, *J* = 11.0 Hz, H-28), 4.56 (br s, 1H, H-29), 4.66 (br s, 1H, H-29); ^{13}C-NMR ($CDCl_3$): δ 14.7 (C-27), 15.3 (C-24), 16.0 (C-25, C-26), 18.2 (C-6), 19.1 (C-30), 20.7 (C-11), 21.0 (C-2″), 25.1 (C-12), 27.0 (C-15), 27.3 (C-2), 27.9 (C-23), 29.5 (C-16), 29.7 (C-21), 34.1 (C-22), 34.5 (C-7), 37.5 (C-13), 38.7 (C-1, C-10), 38.8 (C-4), 40.8 (C-8), 42.6 (C-14), 46.3 (C-17), 47.6 (C-18), 48.7 (C-19), 50.3 (C-9), 55.2 (C-5), 62.8 (C-28), 78.9 (C-3), 109.8 (C-29), 150.1 (C-20), 171.6 (C-1″); LRMS (EI): *m/z* (%) = 484 (36)$[M]^+$, 466 (84), 411 (63), 216 (52), 189 (84), 91 (100).

β-Sitosteryl-3-O-β-D-glucopyranoside (**6**): IR ($CHCl_3$): υ_{max} 3,385, 2,938, 1,458, 1,436, 1,030; ^{1}H-NMR ($CDCl_3$): δ 0.61-0.94 (m, 6CH_3), 1.18-2.30 (m, CH, CH_2), 4.34 (d, H, *J* = 7.5 Hz, H-1′); 3.22 (m, H-3), 3.22-3.76 (glycosidic H), 5.29 (br s, 1H, H-6); ^{13}C-NMR ($CDCl_3$): δ 11.7 (C-18), 11.8 (C-29), 18.7 (C-26), 18.9 (C-19), 19.2 (C-27), 19.7 (C-21), 20.9 (C-11), 22.9 (C-15), 24.2 (C-28), 25.9 (C-23), 28.1 (C-16), 29.0 (C-25), 29.5 (C-2), 31.8 (C-7), 33.8 (C-22), 36.0 (C-8), 36.5 (C-10), 37.1 (C-1), 39.6 (C-20), 42.2 (C-12), 45.7 (C-4, C-13), 49.9 (C-9), 55.9 (C-17, C-24), 56.6 (C-14), 61.9 (C-6′), 70.0 (C-3, C-4′), 76.2 (C-5′), 73.4 (C-2′), 75.5 (C-3′), 101.0 (C-1′), 122.2 (C-6), 140.1 (C-5).

Cell cultures

Chloroquine Resistant Plasmodium falciparum (T9.94)

Human erythrocytes (type O) infected with chloroquine resistant *P. falciparum* (T9.94) were maintained in continuous culture, according to the method described previously (Satayavivad et al., 2004). RPMI-1640 culture medium supplemented with 25 mM of HEPES, 40 mg/L gentamicin sulfate and 10 mL of human serum was used in continuous culture.

Cancer cells

Cancer cell lines were human cholangiocarcinoma cancer cells (HuCCA-1) and human epidermoid carcinoma of the mouth (KB). The cells were grown in Ham's/F12 medium containing 2 mM L-glutamine supplemented with 100 U/mL penicillin, streptomycin and 10 % fetal bovine serum.

Antimicrobial assay

The antimicrobial activity of the tested compounds was carried out using the agar dilution method (Prachayasittikul et al., 2008a). Briefly, the tested compounds dissolved in DMSO were individually mixed with Müller Hinton (MH) broth (1 mL) while the negative control was the MH broth plus DMSO without the tested compounds. The solution was then transferred to the MH agar solution to yield the final concentrations of 32–256 μg/mL. Twenty seven strains of microorganisms, cultured in MH broth at 37 °C for 24 h, were diluted with 0.9 % normal saline solution to adjust the cell density of 3×10^9 cell/mL. The organisms were inoculated onto each plate and further incubated at 37 °C for 18–48 h. Compounds which showed high efficacy to inhibit bacterial cell growth were identified. The tested microorganisms were **gram negative bacteria**: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 15442, *Edwardsiella tarda*, *Shigella dysenteriae*, *Citrobacter freundii*, *Morganella morganii, Vibrio cholera*, *Vibrio mimicus*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Xanthomonas maltophilia*, *Neisseria mucosa*, *Branhamella catarrhalis*; **gram positive bacteria**: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus lutens* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus cereus*, *Micrococcus flavas* and a **diploid fungus (yeast):** *Candida albicans*.

Antimalarial assay

Antimalarial activity of the tested compounds was evaluated against chloroquine resistant *P. falciparum* (T9.94) using the literature method (Trager and Jensen, 1976). Before performing the experiment, *P. falciparum* culture was synchronized by using sorbitol induced hemolysis according to the method of Lambros and Vanderberg (Lambros and Vanderberg, 1979) to obtain only ring stage-infected red blood cells and then incubated for 48 h prior to the drug testing to avoid the influence of sorbitol. The experiments were started with synchronized suspension of 0.5 % to 1 % infected red blood cell during ring stage. Parasites were suspended with culture medium supplemented with 15 % human serum to obtain 10 % cell suspension. The parasite suspension was put into 96-well microculture plate; 50 μL in each well and then 50 μL of various tested drug concentrations were added. These parasite suspensions were incubated for 48 h in the atmosphere of 5 % CO_2 at 37 °C. The percents parasitemia of control and tested compounds were examined by microscopic technique using methanol-fixed Giemsa stained of thin smear blood preparation. The bioactivity of the compounds was evaluated by determining the concentration that reduced parasite growth by 50 % (IC_{50}).

Cytotoxic assay

Cytotoxic assay was performed using the modified method as previously described (Tengchaisri et al., 1998). In brief, cell lines suspended in RPMI-1640 containing 10 % FBS were seeded at 1×10^4 cells (100 μL) per well in 96-well plate and incubated in humidified atmosphere, 95 % air, 5 % CO_2 at 37 °C. After 24 h, additional medium (100 μL) containing the tested compound and vehicle was added to a final concentration of 50 μg/mL, 0.2 % DMSO, and further incubated for 3 days. Cells were subsequently fixed with 95 % EtOH, stained with

crystal violet solution, and lysed with a solution of 0.1 N HCl in MeOH, after which absorbance was measured at 550 nm. Whereas HuCCA-1 cell was stained by MTT. IC_{50} values were calculated as the drug and sample concentrations at 50 % inhibition of the cell growth.

RESULTS AND DISCUSSIONS

Isolation

D. rubra stem solvent extracts (hexane, dichloromethane, ethyl acetate and methanol) were tested for antimicrobial, antimalarial and cytotoxic activities. Two active antimalarial extracts (dichloromethane and ethyl acetate) were isolated by repeated silica gel column chromatography. The dichloromethane extract gave lupeol (**1**, 826.4 mg) from fractions D2.3 and D3. Lupenone (**2,** 12.2 mg) was isolated from fraction D2.2. Fraction D4 afforded betulin (**3**, 39.1 mg from D4.3.3.2) and lupeol (74.3 mg from D4.1) together with a mixture of *β*-sitosterol and stigmasterol (from D4.2.1 and D4.3.1). The ethyl acetate extract provided lupeol acetate (**4**, 50.9 mg from fraction E1.2), 28-*O*-acetylbetulin (**5**, 81.4 mg from fraction E4.3) and *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside (**6**, 5.1 mg from fraction E7). Lupeol was also isolated from fractions E2 and E3 (133.9 mg). Fraction E6.3 also afforded betulin (22.6 mg). The major constituent, however, is lupeol, with a total amount of 1,034.6 mg. Structures of the isolates **1**-**6** (Figure 1) were deduced by comparison of their IR, ^{1}H- and ^{13}C-NMR with the literature data. 2D-NMR; COSY, HMQC, HMBC, DEPT 90 and DEPT 135 studies were also performed. Our results represent the first report of isolation of constituents **1**-**6** from *D. rubra*. These compounds have been found in other *Diospyros* species, e.g., *D. maritima* Blume (Higa et al., 1998; Mallavadhani et al., 1998) and *D. rhodocalyx* (Sutthivaiyakit et al., 1995) and other species. Lupeol (**1**), lupeol acetate (**4**) and glycoside **6** were previously isolated from the root of *Minusops elengi* (Misra and Mitra, 1968), lupenone (**2**) from the stem bark of *Salacia beddomei* (Hisham et al., 1995), betulin (**3**) from the leaves of *Salacia cordata* (Tinto et al., 1992). In addition, *β*-sitosteryl glycoside **6** was also found in the leaves of *Enkiathus cernuss* (Sakakibara et al., 1983).

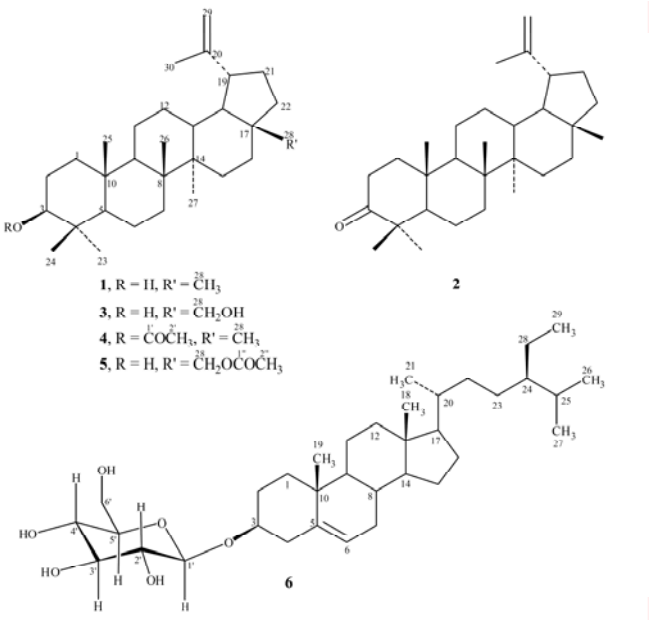

Figure 1: Chemical structures of compounds **1**-**6**

Bioactivities

Extracts (hexane, dichloromethane, ethyl acetate and methanol) and fractions of *D. rubra* were screened for antimicrobial (against 27 strains of microorganisms), antimalarial (*P. falciparum*) and cytotoxic (HuCCA-1 and KB cell lines) activities. They all were inactive antimicrobials at 256 μg/mL (Table 1), fair to good antimalarials (Table 2) and inactive cytotoxic agents (Table 3) that exhibited ED_{50} > 100 μg/mL. However, selected semi-purified fractions (D2, D3, D4 and D5) of the dichloromethane extract did not show antimalarial activity. This is presumably due to some synergistic effects of the compounds in the dichloromethane extract. Interestingly, semi-purified fractions of the dichloromethane and ethyl acetate extracts exerted antimicrobial activity with minimum inhibitory concentrations (MICs) in the 64–256 μg/mL range. In particular, the active fractions (D4.4, D4.5, E6, E6.2, E6.4 and E7) all selectively inhibited the growth of *Corynebacterium diphtheriae* NCTC 10356; D4.4 and E6.2 were the most active fractions with MIC of 64 μg/mL.

Additionally, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus*, as well as *Micrococcus lutens* ATCC 10240, were also inhibited by E6 and E7, respectively. The isolated compounds; lupeol (**1**), lupeol acetate (**4**) and betulin (**3**) were evaluated for antimicrobial action. It was shown (Table 1) that **3** exhibited the activity against *Streptococcus pyogenes*, with a MIC of 85 μg/mL, but the compounds **1** and **4** were inactive toward all the tested organisms at 256 μg/mL. Betulin was previously reported to be active against *Fusarium oxysporum* (Cota et al., 2003), but no significant antimicrobial activity of betulin was observed against *Streptococcus pneumoniae* R6 and *Staphylococcus aureus* OM481 and OM 584 (MICs >128 μg/mL) (Horiuchi et al., 2007). Betulin also displayed anticancer activity against Walker carcinoma-256 (Mallavadhani et al., 1998) as well as antiviral activity against herpes simplex virus (Pavlova et al., 2003) and antiinflammatory activity (Mallavadhani et al., 1998; Recio et al., 1995). An acetate of **3**, 28-*O*-acetylbetulin (**5**), was reported to show cytotoxic effects on many tumor cell lines (Kvasnica et al., 2005). The cytotoxic effects of lupeol (**1**) and its acetate **4** were tested against the HuCCA-1 and KB cell lines. Results (Table 3) showed that both triterpenoids were inactive (ED_{50} > 100 μg/mL). Lupeol was reported to show anticancer activity against Walker carcinoma-256 (Mallavadhani et al., 1998) and antiarthritic action (Argay et al., 1997). Recently, lupeol and lupenone have been reported to inhibit protein tyrosine phosphatase 1B (PTP 1B) which appears to be an attractive target of new drugs development for type 2 diabetes and obesity (Na et al., 2009). Lupeol acetate was documented to exert antiarthitic and antiurolithiatic activities (Argay et al., 1997) including inhibition against stress induced ulcers in rat (Mallavadhani et al., 1998). In addition, stigmasterol and *β*-sitosteryl glucoside are strong antioxidants (Prachayasittikul et al., 2008b; Prachayasittikul et al., 2009).

CONCLUSION

The investigation of *D. rubra* extracts with good antimalarial action reveals the presence of some bioactive triterpenes in this species for the first time. They were lupeol (**1**), lupenone (**2**), betulin (**3**), lupeol acetate (**4**), 28-*O*-acetylbetulin (**5**), *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside (**6**) and a mixture of *β*-sitosterol and stigmasterol. These isolates **1**–**5** are lupane type triterpenes which were reported to possess antimicrobial, antiviral, anticancer, and antiinflammatory activities, as well as inhibitory effects on PTP 1B. However, the isolated betulin **3** from *D. rubra* is shown to be an active antimicrobial agent. In addition, the *D. rubra* extracts (dichloromethane and ethyl acetate) exhibited good antimalarial activity. The most active fractions from such extracts selectively inhibit the growth of *C. diphtheriae* NCTC 10356

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with MIC of 64 μg/mL. The results provide experimental data to support the use of *D. rubra* for medicinal applications.

Table 1: Antimicrobial activity* of *D. rubra*

Compound[a,b,d,e]	Microorganism	MIC[f] (μg/mL)
D4.4	*Corynebacterium diphtheriae* NCTC 10356	64
D4.5	*Corynebacterium diphtheriae* NCTC 10356	256
E6[c]	*Corynebacterium diphtheriae* NCTC 10356	128
	Bacillus cereus	128
	Bacillus subtilis ATCC 6633	256
E6.2[c]	*Corynebacterium diphtheriae* NCTC 10356	64
E6.4	*Corynebacterium diphtheriae* NCTC 10356	256
E7[c]	*Corynebacterium diphtheriae* NCTC 10356	128
	Micrococcus lutens ATCC 10240	256
Betulin (**3**)	*Streptococcus pyogenes*	85

*Ampicillin at 10 μg/mL was used as a control of the testing system; it showed 100 % inhibition against *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *B. subtilis* ATCC 6633, *Neisseria mucosa*, *Branhamella catarrhalis*, *Edwardsiella tarda* and *S. pyogenes*. Partial inhibition: [a] against *C. diphtheriae* NCTC 10356, 50 % (D5 at 128 μg/mL) and 25 % (E1, E4 and E5 at 256 μg/mL), [b] against *N. mucosa* (50 %, E4.4 at 128 μg/mL), [c] against *M. lutens* ATCC 10240 (75 %, E6 and E7 at 128 μg/mL; E6.2 at 256 μg/mL), [d] against *B. subtilis* ATCC 6633 and *B. cereus* at 128 μg/mL (25 %, D4.4, D4.5 and E7). [e] Fractions D4.2, E1.2, extracts (hexane, CH_2Cl_2, EtOAc, MeOH) and isolates; lupeol (**1**) and lupeol acetate (**4**) were tested at 256 μg/mL, but found to be inactive. [f] MIC is a minimum inhibitory concentration.

Table 2: Antimalarial activity of *D. rubra*

Compound[a]	Activity	IC_{50} (μg/mL)[b]
Hexane extract	Fair	176.20
Dichloromethane extract	Good	23.94
Ethyl acetate extract	Good	33.58
Methanol extract	Fair	135.05
D2	Inactive	45.05
D3	Inactive	215.26
D4	Inactive	473.50
D5	Inactive	413.95

[a] Chloroquine hydrochloride was used as a reference drug; [b] IC_{50}: for the extract, 100–1,000 μg/mL denotes fair activity and 10 – <100 μg/mL for good activity; for partial purified fractions, >1 μg/mL for inactive antimalarials.

Table 3: Cytotoxic activity of *D. rubra*

Compound	ED_{50} (μg/mL)[a,b]	
	HuCCA-1	KB
Hexane extract	>182	>182
Dichloromethane extract	163	163
Ethyl acetate extract	>144	>144
Methanol extract	>173	>173
Lupeol (**1**)	>100	>100
Lupeol acetate (**4**)	>100	>100
Etoposide	4.0	0.25

[a] ED_{50} > 100 μg/mL denotes inactive cytotoxic activity; [b] The assays were performed in triplicate, using etoposide as a reference drug.

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