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Anti-inflammatory, antimicrobial and antioxidant activities of *Diospyros bipindensis* (Gürke) extracts and its main constituents

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ABSTRACT

Ethnopharmacological relevance: *Diospyros bipindensis* (Gürke) stem bark is used in Cameroon by Baka Pygmies for the treatment of respiratory disorders.
Aim of the study: To assess the anti-inflammatory, antibacterial and antioxidant properties of constituents from the bark extracts through bioassay-guided fractionation.
Materials and methods: The anti-inflammatory activity of extracts, fractions and pure compounds was assessed through the inhibition of the pro-inflammatory mediator nuclear factor-kappa B (NF-κB) transcriptional activity and nitric oxide (NO) production. DPPH, ABTS and ORAC assays were used for determining the antioxidant properties. The activity against *Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Klebsiella pneumoniae*, was evaluated on the basis of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) by the macrodilution method.
Results: The water extract showed antimicrobial activity against *S. pneumoniae* (MIC: 300 μg/ml) and *S. pyogenes* (MIC: 300 μg/ml). The dichloromethane extract efficiently inhibited NF-κB transcriptional activity and NO production and exhibited significant antioxidant activity in the ORAC assay. An interesting activity was also found against *S. pneumoniae* (MIC: 200 μg/ml), *S. aureus* (MIC: 400 μg/ml) and *S. pyogenes* (MIC: 200 μg/ml). The phytochemical investigation of the dichloromethane extract afforded plumbagin, canaliculatin, ismailin, betulinic acid and 4-hydroxy-5-methyl-coumarin as the main constituents. Plumbagin and ismailin were found to be responsible for the main biological activities observed.
Conclusions: These results may provide a rational support for the traditional use of *Diospyros bipindensis* stem bark in the treatment of respiratory disorders, since the anti-inflammatory, antimicrobial and antioxidant compounds isolated from the dichloromethane extract were also present in the traditional water extract.

1. Introduction

The genus *Diospyros* (Syn: Persimmon, ebony), belonging to the Ebenaceae family, consists of 500 species distributed in tropical and temperate zones (Hotta et al., 1989). Many species are known to produce edible fruits, fine ebonies and timbers but have also been studied for their interesting biological properties. *Diospyros kaki L.*, commonly consumed as a fruit all over the world, is also used as a folk remedy against various diseases in Korea, Japan, and China (You Seon, et al., 2005). *D. peregrine* Gurka, known as Kalatendu in vernacular, is widely used in various parts of India as antibacterial and for the treatment of diarrhea, dysentery and diabetes mellitus (Pawan et al., 2011). The dichloromethane extract of the African plant *D. leucomelas* is known to possess an anti-inflammatory activity since it inhibits the carrageenan-induced paw-edema (Recio et al., 1995). Other African plants such as *D. usambarensis* and *D. zombensis* are reported to have both antifungal and molluscicidal activities (Hostettmann et al., 2000). *D. bipindensis* stem bark is traditionally used by Baka Pygmies in Cameroon as a remedy for respiratory diseases (Brisson, 1999). Several compounds were isolated and characterized from *Diospyros* species. These include triterpenes, coumarins, naphtoquinones and other phenolic compounds (Zhong

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et al., 1984; Tangmouo et al., 2009; Akak et al., 2010) that may be responsible for the biological activities reported.

The wide traditional use of several *Diospyros* species led to various biological and phytochemical investigations. To our knowledge, however, no studies have been carried out to validate the therapeutic use of *Diospyros bipindensis*, and to establish its phytochemical composition.

2. Materials and methods

2.1. Plant material

Samples of *D. bipindensis* stem bark were collected in Cameroon in July 2009 and July 2011 in the camps of Abing. The plant was identified at the Cameroon National Herbarium, Yaoundé by the Cameroonian botanist Mr. Nana; a voucher specimen (No. BWPV 08) was also deposited at the Department of Drug Sciences of the University of Pavia. Stem bark was dried in the dark, in a ventilated room at 25–30 °C, then grounded and the powder stored at −20 °C.

2.2. Bioassay-guided fractionation, isolation and identification of active compounds

2.2.1. Plant extraction and HPLC fingerprint analysis

A first extraction was carried out according to the traditional use. The stem bark dried powder (15 g) was macerated in distilled water (250 ml) for 12 h and the crude extract obtained was frozen and lyophilized (yield 4.3% on a dry mass). A new batch (50 g) was suspended in n-hexane (500 ml) in a round-bottom flask equipped with a condenser. The mixture was refluxed for 3 h, filtered, re-suspended in fresh *n*-hexane (500 ml) and refluxed for further 3 h. The procedure was repeated up to a constant weight of the dry extract (yield 1.3% on a dry mass basis). Further extractions with the same plant material were carried out with CH_2Cl_2, EtOAc and MeOH following the same procedure described above. The solvent removal afforded 1.3%, 2.4% and 13.2% of dried extracts on a dry mass basis, respectively. HPLC was used for the metabolite profiling of the extracts. Analyses were carried out on a HP 1100 system equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA, USA) using a X-Bridge (Waters, MA, USA) C18 column (5 μm, 150 × 4.9 mm i.d.). Solvent systems: MeOH–H_2O-0.02% TFA: gradient mode: 5–100% of MeOH in 50 min, 100% of MeOH for 10 min; flow rate: 1 ml/min; injection volume 20 μl; sample concentration 10 mg/ml in MeOH. The UV absorbance was measured at 217 and 254 nm, and UV spectra (DAD) were recorded between 190 and 600 nm (step 2 nm).

2.2.2. Fractionation of the CH_2Cl_2 extract

The dichloromethane extract (DME, 250 mg) was separated by solid phase extraction using a C18 Cartridge SEP-Pak 10 g (Waters, MA, USA) with a step gradient of MeOH–H_2O-0.02% TFA as follow: MeOH 30% afforded Fraction 1 (7.5 mg), MeOH 40% afforded Fraction 2 (38.6 mg), MeOH 50% afforded Fraction 3 (40.2 mg), MeOH 70% afforded Fraction 4 (120.6 mg) and MeOH 100% afforded Fraction 5 (15.2 mg). Fractions 3 and 4 were purified separately by flash chromatography using a C-18 cartridge Snap 12 g KP-C18-HS (Biotage, Uppsala, Sweden) on an Isolera Prime system (Biotage, Uppsala, Sweden). Fraction 3 was purified using a linear gradient MeOH–H_2O-0.02% TFA: 40% to 65% of MeOH over 30 min, flow rate of 30 ml/min, UV detection at 217 nm, yielding compounds (**1**) (24.5 mg) and (**2**) (9.2 mg). Fraction 4 was purified using a gradient MeOH–H_2O-0.02% TFA: 50–70% of MeOH over 15 min, 70–80% of MeOH over 10 min, 80–100% of MeOH over 15 min and 100% MeOH for 10 min, flow rate of 30 ml/min, UV detection at 217 nm, yielding (**3**) (93.3 mg) and. (**4**) (5.6 mg). Fraction 2 consisted of the pure compound (**5**) (38.6 mg).

2.2.3. Structure elucidation of compounds (*1*)–(*5*)

The structure of compounds (**1**)–(**5**) was elucidated by NMR and MS analyses. ^1H and ^{13}C NMR were recorded on a Varian (Palo Alto, CA, USA) Inova 500 spectrometer (499.87 and 125.70 MHz, respectively) in CD_3OD, CD_2Cl_2 and DMSO-d6 with TMS as internal standard. Complete assignment was performed on the basis of 2D experiments (DEPT, gradient COSY, gradient HSQC, gradient HMBC, NOESY). MS data were obtained with a Finnigan MAT LCQ (San Jose, CA, USA) ion trap mass spectrometer equipped with a Finnigan electrospray interface (ESI). MS conditions: capillary voltage 30 V, capillary temperature 250 °C, source voltage 5 kV, nitrogen as sheath gas flow, and positive-ion mode. Structures are reported in Fig. 1: plumbagin (**1**), canaliculatin, (**2**), ismailin (**3**), betulinic acid (**4**) and 4-hydroxy-5-methyl-coumarin (**5**).

2.2.4. Main spectroscopic data for compounds (*1*)–(*5*)

Plumbagin (**1**). ^1H NMR (CD_3OD, 500 MHz) δ 7.65 (1 H, dd, J=8.3, 7.4 Hz, H-7), 7.59 (1 H, d, J=7.4 Hz, H-8), 7.24 (1 H, d, J=8.3 Hz, H6), 6.85 (1 H, q, J=2.0 Hz, H-3), 2.15 (3 H, d, J=1.6 Hz, CH_3). ^{13}C NMR (CD_3OD, 126 MHz) δ 192.5 (C-4), 184.7 (C-1), 161.1 (C-5), 149.8 (C-2), 136.1 (C-7), 135.3 (C-3), 132.4 (C-9), 123.6 (C-6), 118.8 (C-8), 115.2 (C-10), 15.2 (C-11). LC-ESI-MS *m/z* 187.2 $[M-H]^-$.

Canaliculatin (**2**). ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.66 (1 H, d, J=7.6 Hz, H-8), 7.63 (1 H, dd, J=8.2, 7.6 Hz, H-7), 7.46 (1 H, dd, J=8.2, 7.6 Hz, H-7″), 7.26 (1 H, d, J=8.2 Hz, H6), 7.21 (1 H, d, J=8.2 Hz, H-8′), 7.12 (1 H, d, J=7.6 Hz, H-6′), 2.75 (3 H, s, CH_3-11′), 2.10 (3 H, s, CH_3-11). ^{13}C NMR (CD_2Cl_2, 126 MHz) δ 184.3 (C-1), 160.9 (C-5), 154.3 (C-9′), 150.2 (C-2), 138.4 (C-5′), 137.9 (C-3), 136.0 (C-7), 132.3 (C-9), 131.9 (C-7′), 127.8 (C-6′), 123.9 (C-6), 118.9 (C-8), 114.9 (C-8′), 114.5 (C-10′), 23.1 (CH_3-11′), 13.9 (CH_3-11). LC-ESI-MS *m/z* 361.1 $[M-H]^-$, 227.1, 175.2.

Ismalin (**3**). ^1H NMR (DMSO, 500 MHz) δ 11.92 (1 H, s, OH-5), 7.47 (1 H, s, H-8), 7.45 (2 H, t, J=7.9 Hz, H-7′), 7.24 (1 H, s, H-6), 7.11 (4 H, m, H-6′, 8′), 2.62 (6 H, s, CH3-5′), 2.47 (3 H, s, CH3-7). ^{13}C NMR (DMSO, 126 MHz) δ 187.4 (C-4), 181.5 (C-1), 165.8 (C-4′),

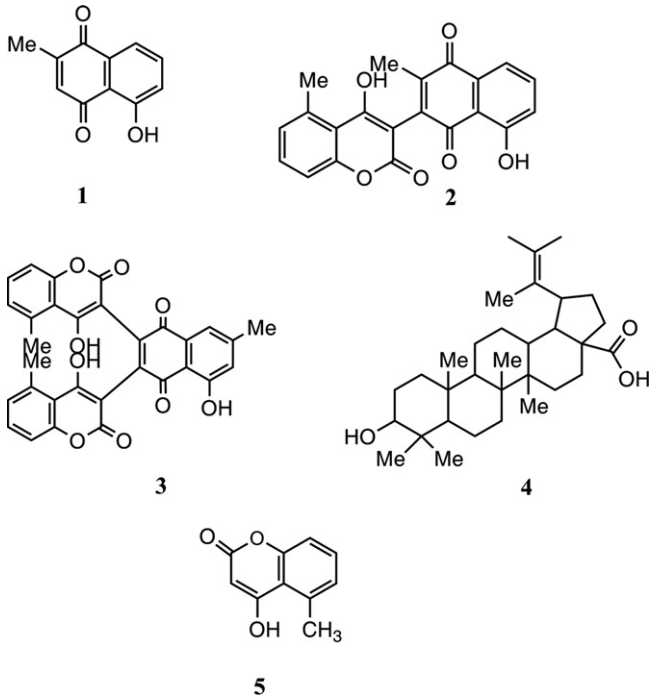

Fig. 1. Bioactive compounds isolated from *Diospyros bipindensis*.