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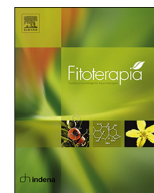

Indole alkaloids from the aerial parts of *Kopsia fruticosa* and their cytotoxic, antimicrobial and antifungal activities

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ABSTRACT

A chemical investigation on the 80% EtOH extract of the aerial parts of *Kopsia fruticosa* led to five new indole alkaloids, kopsifolines G-K (**1–5**), and one known alkaloid, kopsifoline A (**6**). Structural elucidation of all the compounds were performed by spectral methods such as 1D and 2D (^{1}H-^{1}H COSY, HMQC, and HMBC) NMR spectroscopy, in addition to high resolution mass spectrometry. The isolated components were evaluated *in vitro* for cytotoxic activities against seven tumor cell lines, antimicrobial activities against two Gram-positive bacteria and five Gram-negative bacteria, and antifungal activities against five pathogens. As a result, alkaloids **3–5** exhibited some cytotoxicity against all of seven tested tumor cell lines (HS-1, HS-4, SCL-1, A431, BGC-823, MCF-7, and W480) with IC_{50} values of 11.8–13.8, 10.3–12.5, and 7.3–9.5 μM, respectively. Alkaloids **3–5** also possessed significant antimicrobial and antifungal activities which was reported for the first time for the alkaloids isolated from *Kopsia* genus.

1. Introduction

The genus *Kopsia* (Apocynaceae) comprises some 30 species of shrubs and trees which are distributed widely in Southeast Asia, India, China, and Australia [1, 2]. The roots of several *Kopsia* species are known to be used for poulticing ulcerated noses in tertiary syphilis [3]. Several species of this genus have been reported to possess various biological activities, such as antileishmanial, antimanic, antitumor, and antitussive activities [4–6]. Plants belonging to this genus are prolific producers of a wide variety of indole and bisindole alkaloids, including many with intriguing carbon skeletons as well as interesting biological activities [7]. *Kopsia* alkaloids possess structurally intriguing molecular skeletons derived from known monoterpenoid indole precursors through pathways involving deep-seated rearrangements and/or loss of key fragments to form the cage [8], three-nitrogen pentacyclic [9], tetracyclic indole [10], tetracyclic quinolinic [11], and regioisomeric tetracyclic indole alkaloid [12]. Among them, aspidofractinine and methyl chanofruticosinate alkaloids are particular in the genus *Kopsia*, in which a methylene bridge connects carbon 16 and 20 of the aspidofractinine alkaloids whereas carbon-6 and 16 are joined by a ketonic carbonyl reminiscent of that in methyl chanofruticosinate alkaloids [13, 14]. One important member of this genus, *Kopsia fruticosa* naturally occurs in Indonesia, India, Philippines, and Malaysia and cultured in Guangdong province, China, which is used in folk medicine to treat respiratory infection and pharyngitis. Previous studies on *K. fruticosa* resulted into the isolation of pentacyclic quinoline alkaloid [15] and a series of indole alkaloids, including new venalstonine and dioxokopsan derivatives [14] as well as fruticosin und fruticosamin [16]. To find biologically active compounds from this medicinal plant, a phytochemical investigation on the 80% ethanol extract of the aerial parts of *K. fruticosa* afforded five new indole alkaloids, kopsiafrutines A–E (**1–5**), together with one known alkaloid kopsifoline A (6). This paper describes the isolation and structure elucidation of the new compounds, as well as the *in vitro* cytotoxic, antifungal and antimicrobial potential.

2. Experimental part

2.1. General

Optical rotations were determined with a JASCO P2000 digital polarimeter (JASCO Corporation, Tokyo, Japan). Ultraviolet (UV) and infrared (IR) spectra were obtained on JASCO V-650 and JASCO FT/IR-4100 spectrophotometers, respectively. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR spectrometer (Varian

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Medical Systems, Salt Lake City, UT, USA; 500 MHz for ^1H; 125 MHz for ^{13}C, respectively). High resolution EI-MS spectra were obtained on a Finnigan MAT 95 mass spectrometer (ThermoFinnigan, Fremont, California, USA). Silica gel 60 (Merck, Darmstadt, Germany, 230–400 mesh), LiChroprep RP-18 (Merck, 40–63 µm), and Sephadex LH-20 (Amersham Pharmacia Biotech., Roosendaal, The Netherlands) were used for column chromatography (CC). Precoated silica gel plates (Merck, Kieselgel 60 F254, 0.25 mm) and precoated RP-18 F_{254s} plates (Merck) were used for analytical thin-layer chromatography analyses. HPLC separation was performed on an instrument consisting of a Waters 600 controller, a Waters 600 pump, and a Waters 2487 dual λ absorbance detector, with a Prevail (250 × 10 mm i.d.) preparative column packed with C_{18} (5 µm).

2.2. Plant material

The aerial parts of *K. fruticosa* were collected from the Huizhou of Guangdong Province of China in July 2016. A specimen (201706001) was identified by one of the authors (Q.J. Zhao) and deposited at the Natural Product Laboratory, Captial Medical Univerisity, China.

2.3. Extraction and isolation

The air-dried aerial parts of *K. fruticosa* (8.6 kg) were cut into small pieces and were extracted with 80% ethanol (40 L) three times under reflux for 15 h. After removal of EtOH under reduced pressure at 55 °C, the aqueous brownish syrup (1 L) was suspended in H_2O (l L) and then partitioned with chloroform (1 L × 3) to afford chloroform soluble fraction (55.3 g). The chloroform soluble fraction was further fractionated through a silica gel column (200–300 mesh, 10 × 80 cm, 500 g) using increasing a proportion of acetone in petroleum ether (b.p. 60–90 °C) (100:1, 50:1, 30:1, 15:1, 10:1, 7:1, 5:1, 3:1, 1:1, v/v, each 2.5 L) as the eluent to give 6 fractions. Fraction 3 (petroleum ether-acetone 15:1, 3.1 g) was applied to an ODS MPLC column (8 × 30 cm,100 g) and eluted with MeOH-H_2O (20:80, 30:70, 40:60, each 500 mL) to yield four subfractions (Fr. 3–1 to Fr. 3–4). Subfraction 3–2 (MeOH-H_2O 70:30, 317 mg) was purified by preparative RP-HPLC (ODS column, 250 × 20 mm) using MeOH-H_2O (25% of MeOH-H_2O) as mobile phase to obtain **3** (61 mg, retention time: 13.5 min). Subfraction 3–3 (MeOH-H_2O, 50:50, 265 mg) was chromatographed by a Sephadex LH-20 column (2 × 200 cm, 150 g) eluted with MeOH-H_2O (50% of MeOH-H_2O), and purifed on preparative RP-HPLC (ODS column, 250 × 20 mm) using MeOH-H_2O (30:70) as mobile phase to yield **6** (78 mg, retention time: 15.0 min). Subfraction 3–4 (MeOH-H_2O 40:60, 210 mg) was purified by preparative RP-HPLC (ODS column, 250 × 20 mm) eluting with MeOH/H_2O (22% of MeOH-H_2O) to get **1** (55 mg, retention time: 14.6 min). Fraction 4 (petroleum ether-acetone 30:1, 1.2 g) was applied to an ODS column and eluted with MeOH-H_2O (30:70, 40:60, 50:50, each 500 mL) to provide three subfractions (Fr. 4–1 to Fr. 4–3), Subfraction 4–1 (MeOH-H_2O 10:90, 262 mg) was repeatedly chromatographed on silica gel (150 g, 60 × 2.8 cm, chloroform-methanol, 20:1 → 10:1, each 500 mL) and then purifed on a Sephadex LH-20 column (2 × 200 cm, 150 g) eluting with MeOH-H_2O (50:50) to afford **5** (78 mg). Subfraction 4–2 (MeOH-H_2O 20:80, 262 mg) was purified by preparative RP-HPLC (ODS column, 250 × 20 mm) eluting with MeOH/H_2O (20% of MeOH-H_2O) to get **4** (73 mg, retention time: 15.50 min). Subfraction 4–3 (MeOH-H_2O 25:75, 240 mg) was purified by preparative RP-HPLC (ODS column, 250 × 20 mm) using MeOH-H_2O (25% of MeOH-H_2O) as mobile phase to obtain **2** (61 mg, retention time: 13.7 min).

2.3.1. Kopsiafrutine a (1)

Colorless oil; $[\alpha]_D^{20}$–2.6 (*c* 0.10, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 213 (4.28), 249 (3.94), 295 (3.52) nm; IR (KBr) ν_{max}: 3370, 1711 cm^{-1}; ^1H and ^{13}C NMR: Tables 1 and 2; HREIMS *m/z*: 396.1681 ($C_{22}H_{24}N_2O_5$ $[M]^+$, calc. 396.1685).

2.3.2. Kopsiafrutine B (2)

Colorless oil; $[\alpha]_D^{20}$–5.3 (*c* 0.20, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 214 (4.26), 248 (3.94), 296 (3.50) nm; IR (KBr) ν_{max}: 3368, 1710 cm^{-1}; ^1H and ^{13}C NMR: Tables 1 and 2; HREIMS *m/z*: 398.1837 ($C_{22}H_{26}N_2O_5$ $[M]^+$, calc. 398.1842).

2.3.3. Kopsiafrutine C (3)

Colorless oil; $[\alpha]_D^{20}$–29.5 (*c* = 0.10, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 219 (4.30), 250 (3.89), 294 (3.46) nm; IR (KBr) ν_{max}: 3376, 1708 cm^{-1}; ^1H and ^{13}C NMR: Tables 1 and 2; HREIMS *m/z*: 414.1795 ($C_{22}H_{26}N_2O_6$ $[M]^+$, calc. 414.1791).

2.3.4. Kopsiafrutine D (4)

Colorless oil; $[\alpha]_D^{20}$–13.9 (*c* 0.05, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 221 (4.33), 247 (3.92), 293 (3.55) nm; IR (KBr) ν_{max}: 3367, 1713 cm^{-1}; ^1H and ^{13}C NMR: Tables 1 and 2; HREIMS *m/z*: 416.1945 ($C_{22}H_{28}N_2O_6$ $[M]^+$, calc. 416.1947).

2.3.5. Kopsiafrutine E (5)

Colorless oil; $[\alpha]_D^{20}$ + 102.1 (*c* 0.10, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 217 (4.29), 245 (3.91), 290 (3.47) nm; IR (KBr) ν_{max}: 3380, 1712 cm^{-1}; ^1H and ^{13}C NMR: Tables 1 and 2; HREIMS *m/z*: 402.1787 ($C_{21}H_{26}N_2O_6$ $[M]^+$, calc. 402.1791).

2.4. Cytotoxicity assay in vitro

The isolated alkaloids (**1**–**6**) were subjected to cytotoxic evaluation against HS-1 (dermatoma), HS-4 (dermatoma), SCL-1 (dermatoma), A431 cells (dermatoma), BGC-823 cells (human gastric carcinoma), MCF-7 cells (human breast cancer), and W480 (colon cancer) by employing the revised MTT [3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide] method in literature [17]. Adriamycin was used as the positive control. All tumor cell lines were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in 25-cm^2 culture flasks at 37 °C in humidified atmosphere with 5% CO_2. For the cytotoxicity tests, cells in exponential growth stage were harvested from culture by trypsin digestion and centrifuging at 180 × g for 3 min, then resuspended in fresh medium at a cell density of 5×10^4 cells per ml. The cell suspension was dispensed into a 96-well microplate at 100 µL per well, and incubated in humidified atmosphere with 5% CO_2 at 37 °C for 24 h, and then treated with the compounds at various concentrations (0, 1, 10, 100 µM). After 48 h of treatment, 50 µL of 1 mg/mL MTT solution was added to each well, and further incubated for 4 h. The cells in each well were then solubilized with DMSO (100 µL for each well) and the optical density (OD) was recorded at 570 nm. All drug doses were tested in triplicate. The IC_{50} values were derived from the mean OD values of the triplicate tests *versus* drug concentration curves and was used as criteria to judge the cytotoxicity. All cell lines were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences.

2.5. Antimicrobial and antifungal assays in vitro

A total of 12 microorganisms were assayed among which two Gram-positive bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis*, five Gram-negative bacteria: *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Shigella dysenteriae*, as well as three pathogen fungi: *Candida albicans*, *Candida tropicalis* and *Candida glabrata*. In addition, the tests were performed against the oral pathogens *Streptococcus mutans* and *Streptococcus viridans*. Antimicrobial and Antifungal activity was evaluated by the disc diffusion method by measuring the zone of inhibitions [18]. Standard antibiotic netilmicin and 5-flucytocine were used in order to control the sensitivity of the tested bacteria and fungi respectively, while standard intraconazole was used especially for pathogen fungi and sanguinarine

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