Sesquiterpenes from *Curcuma zedoaria* rhizomes and their cytotoxicity against human gastric cancer AGS cells

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ABSTRACT

*Curcuma zedoaria* rhizome (Zingiberaceae) is a well-known traditional medicinal plant used in Ayurvedic and traditional Chinese medicine to treat various cancers. This study aimed to identify the cytotoxic components from *C. zedoaria* rhizomes that act against gastric cancer, which is the third leading cause of death from cancer worldwide because the MeOH extract of *C. zedoaria* rhizome was found to show a cytotoxic effect against gastric cancer AGS cells. Repeated column chromatography and semi-preparative HPLC purification were used to separate the components from the *C. zedoaria* MeOH extract. Two new sesquiterpenes, curcumenol-9,10-epoxide (1) and curcuzedoalide B (2), and 12 known related sesquiterpenes (3–14) were isolated from the *C. zedoaria* MeOH extract. The structures of new compounds were determined by 1D and 2D NMR spectroscopic experiments and HR-ESIMS, and quantum chemical ECD calculations. The cytotoxic effects of the isolated compounds were measured in human gastric cancer AGS cells using an MTT cell viability assay. Compounds 9, 10, and 12 exhibited cytotoxic effects against gastric cancer AGS cells, with IC50 values in the range of 212–392μM. These findings provide further experimental scientific evidence to support the traditional use of *C. zedoaria* rhizomes for the treatment of cancer. Curcumenol (9), 4,8-dioxo-6β-methoxy-7α,11-epoxycarabrane (10), and zedoarofuran (12) were identified as the main cytotoxic components in *C. zedoaria* rhizomes.

1. Introduction

Cancer is a group of diseases characterized by uncontrolled proliferation and growth of abnormal cells, their invasion into neighboring tissues, and their metastasis to distant organs. It is now the second leading cause of global mortality, estimated to have caused 8.7 million deaths worldwide in 2015 [1,2]. Since potent anticancer activity was observed in alkaloids isolated from *Vinca rosea* Linn in 1959, natural products have been demonstrated to be important sources of plentiful lead compounds for novel anticancer drug discovery [3–6]. Indeed, a number of drugs based on leads isolated from natural products, such as vincristine, paclitaxel, and doxorubicin, are currently used as therapeutic interventions in cancer or are being evaluated in preclinical trials [4–6].

As part of our continuing efforts to discover traditional medicinal plants exhibiting pharmacological potential and to characterize the responsible compounds [7–15], we found that the MeOH extract from rhizomes of *Curcuma zedoaria* Roscoe displayed a cytotoxic effect against gastric cancer AGS cells, which was well matched to the ethnopharmacological evidence of the *C. zedoaria* rhizome as an anticancer agent [16,17]. *C. zedoaria* (Zingiberaceae), also known as white turmeric or zedoaria, is a well-known traditional medicinal plant used in Ayurvedic and traditional Chinese medicine to treat various cancers [16,17]. According to phytochemical studies, *C. zedoaria* rhizome is a rich natural source of sesquiterpenes, which have demonstrated various biological activities including anti-inflammatory [18], cytotoxic [19], and anti-fungal properties [20]. However, the compounds having anticancer activity in the *C. zedoaria* rhizome have not yet been explored in detail. Based on the bioactivity-guided fractionation of the *C. zedoaria* MeOH extract for cytotoxic effects on gastric cancer AGS cells, five sesquiterpenes were isolated from the cytotoxic hexane fraction [21]. Among the isolates, curcuzedoalide was identified as an active compound, which inhibited AGS human gastric cancer cells by activating cleavage of caspases and PARP, which are representative markers for...
apoptosis [21]. These findings led us to further investigate potential cytotoxic components from the C. zedoaria rhizome. Additional phytochemical analysis led to the isolation of 14 sesquiterpenes, including two new sesquiterpenes, curcumenol-9,10-epoxide (1) and cucurzeadoalide B (2). Here, we describe the isolation and structural elucidation of these sesquiterpenes (1–14) and their cytotoxicity against human gastric cancer AGS cells.

2. Experimental

2.1. General experimental procedures

Optical rotations were calculated on a Jasco P-2000 polarimeter in MeOH (Jasco Inc, Easton, MD, USA). UV spectra were acquired on an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). IR spectra were acquired with a BrukerIFS 66/S FT-IR spectrometer (Bruker AXS GmbH, Karlsruhe, Germany). Electronic circular dichroism (ECD) spectra were measured on a Jasco J-1500 spectropolarimeter (Jasco Inc). ESI and HR-ESI mass spectra were recorded using a Waters Micromass Q-ToF Ultima ESI-TOF mass spectrometer (Waters Corporation, Milford, CT, USA). LC/MS/MS analysis was performed using an Agilent 1200 HPLC with a diode array detector system and an analytical Kinex C18 100 Å column (100 mm × 2.1 mm i.d., 5 μm) (Phenomenex Inc, Torrance, CA) coupled to an Agilent ESI 6130 mass spectrometer. NMR spectra were recorded using a Bruker Avance III HD 850 MHz NMR spectrometer with a 5 mm TCI CryoProbe operating at 850MHz(1H) and 212.5 MHz(13C) (Bruker AXS GmbH), with chemical shifts given in ppm (δ) for 1H and 13C NMR analyses. Preparative HPLC and semi-preparative HPLC were performed with a Waters 1525 binary HPLC pump with a Waters 996 photodiode array detector using an Agilent Eclipse XDB-C18 column (250 × 21.2 mm, 7 μm) (Waters Corporation), a Phenomenex Luna C18 100 Å column (250 × 10 mm, 10 μm) and a Phenomenex Luna C18 100 Å column (250 × 10 mm, 10 μm), respectively. Column chromatography was performed with silica gel 60 (Merck KGaC, Darmstadt, Germany; 70–230 mesh and 230–400 mesh), and RP-C18 silica gel (Merck, 230–400 mesh). Merck precoated silica gel F254 plates and RP-C18 F254A plates were used for thin-layer chromatography (TLC). Spots were detected after TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid. The 3D molecular modeling was performed by using ChemBioDraw Ultra and Avogadro.

2.2. Plant material

The dried rhizomes of C. zedoaria were purchased from Kyongdong Herbal Market, Seoul, in July 2015. The plant material was identified by one of the authors, Ki Hyun Kim. A voucher specimen (BC-2016) was deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University (Suwon, Korea).

2.3. Extraction and isolation

The dried rhizomes of C. zedoaria (1.5 kg) were extracted three times with MeOH at 65°C and filtered. The filtrate was concentrated under reduced pressure using a rotovapor to obtain a crude MeOH extract (69.1 g), which was sequentially solvent partitioned with hexane, CHCl3, EtOAc, and n-BuOH to give hexane (19.4 g), CHCl3 (8.6 g), EtOAc (4.1 g), and n-BuOH (1.7 g)-soluble fractions. Of these, the hexane and CHCl3 fractions exhibited cytotoxic effects against gastric cancer AGS cells [21]. The hexane-soluble fraction (2.0 g) was further fractionated via silica gel column chromatography using a hexane:EtOAc gradient (from 15:1 to 1:1, v/v) to afford 15 fractions (H1–H15). Fractions H5 (241.1 mg) and H7 (33.0 mg) were separately subjected to semi-preparative HPLC (flow rate: 2 mL/min; Phenomenex Luna C18, 250 × 10.0 mm, 10 μm) using an isocratic solvent system of 70% MeOH, which yielded compounds 3 (tR 36.5 min, 7.2 mg) and 4 (tR 42.0 min, 4.0 mg) from fractions H5, and compound 5 (tR 30.3 min, 0.9 mg) from fractions H7. Fraction H8 (347.0 mg) was further subjected to RP-C18 silica gel column chromatography (eluted with 50%/75% MeOH, gradient system) to afford 8 subfractions (H81–H88). Subfraction H84 (7.8 mg) was separated by semi-preparative HPLC (60% MeOH) to yield compound 6 (tR 27.0 min, 1.8 mg). Subfractions H87 (30.5 mg) and H88 (191.3 mg) were consolidated and purified using semi-preparative HPLC (70% MeOH) to furnish compounds 1 (tR 24.2 min, 2.5 mg), 7 (tR 27.9 min, 5.1 mg), 8 (tR 17.0 min, 4.4 mg), and 9 (tR 21.8 min, 103.6 mg). Fraction H9 (115.5 mg) was separated by semi-preparative HPLC (55% MeOH) to yield compounds 10 (tR 14.5 min, 2.2 mg) and 11 (tR 24.3 min, 3.5 mg). Compound 2 (tR 19.0 min, 2.7 mg) was obtained from fraction H10 (73.0 mg) by semi-preparative HPLC (65% MeOH). Fraction H12 (110.0 mg) was separated by semi-preparative HPLC (60% MeOH) to yield compound 12 (tR 28.4 min, 3.5 mg). The CHCl3-soluble fraction (0.6 g) was subjected to column chromatography performed with silica gel 60 (Merck KGaC, Darmstadt, Germany) using a CH2Cl2/EtOAc gradient (from 15:1 to 1:1, v/v) to afford 15 fractions (M1–M15). Fractions M7 (187.3 mg) and M8 (185.3 mg) were separated by semi-preparative HPLC with 55% MeOH to yield compounds 13 (tR 12.5 min, 2.4 mg) and 14 (tR 12.4 min, 0.7 mg).

2.3.1. Curcumenol-9,10-epoxide (1)

Colorless gum. [α]D +40.3 (c 0.05, MeOH); UV (MeOH) λmax (log ε): 205 (3.6) nm; IR (KBr) νmax: 3485, 3356, 2982, 2929, 1709, 1451, 1397, 1116, 972 cm−1; ECD (MeOH) λmax (Δε): 200 (+13.2) nm; 1H NMR (CDCl3, 600 MHz) and 13C NMR (CDCl3, 121.5 MHz) spectroscopic data, see Table 1; HR-ESIMS (positive-ion mode) m/z: 251.1624 [M + H]+ (calcld. for C13H23O3Na 251.1647).

2.3.2. Curcuzedoalide B (2)

Colorless gum. [α]D +3.9 (c 0.001, MeOH); UV (MeOH) λmax (log ε): 220 (3.6) nm; IR (KBr) νmax: 2843, 1705, 1640, 1054, 1032 cm−1; 1H NMR (MeOH) λmax (Δε): 251 (± 10.3) nm; 1H NMR (CD3OD, 580 MHz) and 13C NMR (CDCl3, 121.5 MHz) spectroscopic data, see Table 1; HR-ESIMS (positive-ion mode) m/z: 273.1469 [M + Na]⁺ (calcld. for C10H13O2Na 273.1467).

2.4. Computational analysis

To acquire the conformational optimization of 1a/1b and 2a/2b,
computational DFT calculations were carried out. The first structural energy minimizations of 1a/1b and 2a/2b were performed by utilizing Avogadro 1.2.0 with a UFF force field. The ground-state geometries of 1a/1b and 2a/2b were then established by TmoleX 4.3.1 with the DFT settings (B3-LYP functional/M3 grid size), geometry optimization options (energy $10^{-6}$ hartree, gradient norm $|dE/dxyz|=10^{-3}$ hartree/bohr), and the basis set def-SV(P) for all atoms [22,23]. The calculated ECD spectra of optimized structures were acquired at the B3LYP/DFT functional settings with the basis set def2-TZVPP for all atoms [22,23].
AGS cells were seeded at $1 \times 10^4$ cells/100 μL in 96-well plates and treated with varying concentrations of the test samples for 24 h. The cells were cultured in cell culture medium with or without samples. After incubation for 24 h, cell viability was determined using the Ez-Cytox cell viability assay kit (DaiLab Service Co., Seoul, Korea) according to the instructions. Ten microliters of kit reagent was added to each well, and the cells were incubated for 30 min. The absorbance at 450 nm (absorbance for live cells) was measured using a microplate reader (PowerWave XS; BioTek Instruments, Winooski, VT, USA).

3. Results and discussion

3.1. Isolation of compounds from the MeOH extract of C. Zedoaria

The MeOH extract of C. zedoaria was solvent partitioned to provide hexane, CHCl₃, EtOAc, and n-BuOH-soluble fractions. Among them, the hexane and CHCl₃-soluble fractions inhibited gastric cancer cell viability, with IC₅₀ values of 136.88 ± 2.59 μg/mL and 155.65 ± 1.37 μg/mL, respectively [21]. Based on this result, phytochemical investigation of the hexane and CHCl₃-soluble fractions, combined with LC/MS analysis, using successive column chromatography and HPLC purification resulted in the isolation and identification of 14 sesquiterpenes (1–14), including two new sesquiterpenes (1–2) (Fig. 1).

3.2. Structural elucidation of the isolated compounds

Compound 1 was obtained as a colorless gum. Its molecular formula was determined to be C₁₅H₂₂O₃ based on positive-ion mode HR-ESI-MS data at m/z 251.1624 [M + H]⁺ (calcd for C₁₅H₂₃O₀₂, 251.1647) and ¹³C NMR data. Detailed analysis of the NMR data (Table 1) of 1 revealed that the NMR spectra were typical of sesquiterpene and similar to those of curcumenol (9) [24]. The major differences in ¹³C NMR data between compound 1 and curcumenol (9) were the absence of two sp² carbon signals and the presence of two sp³ signals at δC 60.1 and 59.4 in 1 [24]. In the ¹H NMR spectrum of 1 (Table 1), the olefinic proton at δH 5.76 for curcumenol (9) was replaced by an oxygenated proton at δH 3.11 (1H, s), which is correlated to the carbon signal at δC 60.1 based on the HSQC spectrum. Analysis of hydrogen deficiency of 1 and the chemical shifts allowed us to deduce the presence of an epoxy group at C-9/C-10 in 1, instead of the Δ⁹,10 double bond in curcumenol (9) [25]. This deduction was corroborated by the HMBC correlations of CH₃-15 with C-1/C-9/C-10 (Fig. 2) [25,26]. The relative configuration of 1 was established by analysis of NOEY experiments (Fig. 3), where the NOEY correlations from H-1 with H-4/H-6 α-orientations of CH₃-14, the β-orientations of CH₃-14, the epoxy ring, and the oxygen bridge (Fig. 3). In order to confirm the absolute configuration, quantum chemical ECD calculations were performed by the comparison of the experimental ECD spectrum of 1 with the calculated ECD data of two possible enantiomers, 1a (1R,4S,5S,8S,9R,10R) and 1b (1S,4R,5R,8R,9S,10S) (Fig. 4). The experimental ECD data of 1 matched well with the calculated ECD curve of 1a. Thus, the chemical structure of 1 including its absolute configuration (1R,4S,5S,8S,9R,10R) was elucidated as drawn in Fig. 1, and we named compound 1 as curcumenol-9,10-epoxide.

Compound 2 was isolated as a colorless gum. Its molecular formula was determined as C₁₈H₂₂O₂ based on positive-ion mode HR-ESI-MS data at m/z 273.1469 [M + Na]⁺ (calcd for C₁₈H₂₃O₀₃Na, 273.1467). The ¹H NMR data (Table 1) of 2 showed the signals for four methyl doublets at δH 1.03 (3H, d, J = 7.0 Hz), 1.05 (3H, d, J = 7.0 Hz), 1.07 pyrrole, 100 units/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

2.6. MTT cell viability assay

AGS cells were seeded at $1 \times 10^4$ cells/100 μL in 96-well plates and treated with varying concentrations of the test samples for 24 h. The cells were incubated in cell culture medium with or without samples. After incubation for 24 h, cell viability was determined using the Ez-Cytox cell viability assay kit (DaiLab Service Co., Seoul, Korea) according to the instructions. Ten microliters of kit reagent was added to each well, and the cells were incubated for 30 min. The absorbance at 450 nm (absorbance for live cells) was measured using a microplate reader (PowerWave XS; BioTek Instruments, Winooski, VT, USA).

The human gastric cancer AGS cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute 1640 medium (Corning, New York, NY, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 1% sodium pyruvate, 100 units/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

2.5. Cell culture

The 3D molecular model of compound 2 showing key NOESY (ρ++) correlations.
(3H, d, J = 7.0 Hz), and 1.98 (3H, d, J = 1.0 Hz); methylene proton doublets at δH 2.85 (1H, d, J = 16.0 Hz) and 3.00 (1H, d, J = 16.0 Hz); and one olefinic proton at δH 5.67 (1H, d, J = 1.0 Hz); and the 13C NMR data (Table 1) indicated the presence of 15 carbon resonances, including a keto-carbonyl, an ester carbonyl, and two olefinic carbons. The NMR data of 2 were almost identical to those of curcuzedoalide, a rare 7,8-seco-guaiane-type sesquiterpene [24,27]. However, the NMR data assigned to the Δ11,12 double bond in curcuzedoalide were absent, instead, methyl and methine signals were observed in the reduction of the Δ11,12 double bond in 1/C-4/C-5/C-7/C-11, H-11 with C-7/C-12/C-13, and CH3-12/CH3-13 at C-5 via a methylene bridge by the HMBC correlations of H-6 with C-2b. Because the isolated sesquiterpenes originated from the fractions of the C. zedoaria MeOH extract showing cytotoxicity against gastric cancer AGS cells, all the isolates were evaluated for in vitro cytotoxicity against AGS gastric cancer cells by the MTT cell viability assay [36–39], with cisplatin as the positive control with an IC50 value of 68.29 ± 2.70 μM. As shown in Fig. 8, compounds 9, 10, and 12 exhibited inhibition of proliferation in a dose-dependent manner in AGS gastric cancer cells with IC50 values of 9 (IC50: 263.34 ± 2.97 μM), 10 (IC50: 392.95 ± 3.19 μM) and 12 (IC50: 212.50 ± 2.37 μM), respectively. These findings demonstrated that sesquiterpenes were identified to be the main cytotoxic components in C. zedoaria rhizomes.

4. Conclusions

In this study, we provide further experimental scientific evidence to support the traditional use of C. zedoaria rhizomes for the treatment of cancer. Phytochemical analysis of the cytotoxic fractions of the C. zedoaria MeOH extract led to the isolation of two new sesquiterpenes, namely curcumeneol-9,10-epoxide (1) and curcuzedoalide B (2), and 12 known related sesquiterpenes (3–14). Compounds 9, 10, and 12 exhibited cytotoxic effects against gastric cancer AGS cells, with IC50 values in the range of 212–392 μM. Future studies, including Western blotting analysis, will need to be conducted to confirm the molecular mechanisms of the cytotoxic sesquiterpenes.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary material

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