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Research paper

Isolation and characterization of a new cytotoxic dihydrophenanthrene from *Dioscorea membranacea* rhizomes and its activity against five human cancer cell lines

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ABSTRACT

Ethnopharmacological relevance: The rhizomes of *Dioscorea membranacea* Pierre (DM) have been used as ingredients in anticancer herbal formulations in Thai traditional medicine (TTM). Thus, the aim of this study was to investigate the active constituents of DM for cytotoxic activity in order to support its TTM use.

Materials and methods: A bioassay-guided isolation procedure was used to separate the cytotoxic constituents from ethanolic extract of *Dioscorea membranacea* rhizomes by testing against five human cancer cell lines, i.e. large cell lung carcinoma, COR-L23; liver cancer cells, HepG2; prostate cancer cells, PC3; breast cancer cells MCF-7; cervical cancer cells, Hela; and one normal human lung cell line (MRC 5) using the SRB assay.

Results: Two known dihydrophenanthrene compounds [2,4 dimethoxy-5,6-dihydroxy-9,10-dihydrophenanthrene (**1**) and 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene (**2**)], and a new dihydrophenanthrene compound, 5,6,2 -trihydroxy 3,4-methoxy, 9,10-dihydrophenanthrene (**3**) were isolated and fully characterized. **1** showed the highest cytotoxic activity against COR-L23, MCF-7 and PC3 cell lines (IC_{50} = 14.89, 17.49 and 19.04 μM, respectively), and **2** showed selective cytotoxic activity against PC3 (IC_{50} = 23.54 μM). The new compound **3** showed selective cytotoxic activity against only MCF-7 cells (IC_{50} = 31.41 μM). Interestingly the crude extract of DM was much less toxic to the normal cell line (MRC-5) (IC_{50} > 50 μg/ml) compared to the five cancer cell lines, (IC_{50} value ranged between 6 and 29 μg/ml).

Conclusion: The phytochemicals isolated from DM may serve as lead compounds for the design of new anticancer agents with better selective cytotoxic indices.

1. Introduction

Cancer is the largest contributor of death in Thailand (The Bureau of Policy and Strategy, 2013). Whereas some cancer patients can afford treatment with plethora of orthodox cancer treatment procedures, a large percentage still relies on Thai traditional medicines (TTMs). In some case TTMs are used as alternative medicines, but in most they are used as complimentary treatments prior to cancer chemotherapy to stimulate the immune function to reduce the side effects of the drugs used in chemotherapy (Jiradhammo, 2008). In many cases, use of such herbal medicines has no scientific rationale, although there is now an increased interest in carrying out systematic phytochemical, safety, cytotoxicity and clinical efficacy studies of a variety of Thai traditional medicines. The rhizomes of *Dioscorea membranacea* Pierre (DM) (Dioscoreaceae), called Hua Khao Yen Tai in Thai, are commonly used as ingredients in Thai traditional medicine drug formulations for the treatment of cancer. These formulations are used to treat various types of cancer, especially liver, lung, prostate, cervical and breast cancer. DM is the main ingredient of cancer drug formulations at the famous Arocayasala, Kumpramong Temple, Sakonnakorn Province, Thailand (http://www.khampramong.org/cancer2.html), and an important center for treatment of cancer patients.

The ethanolic extract of the rhizome of DM showed high and selective cytotoxicity against three human cancer cell lines using the SRB assay (Itharat et al., 2004). A subsequent study has described the bioassay-guided isolation of its active ingredients from rhizomes by testing cytotoxic activity against three human

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cancer cell lines: large cell lung carcinoma (COR-L23), colon cell line (LS-174T) and breast cancer cell line (MCF-7), using the SRB assay (Itharat et al., 2007). Eight compounds were isolated; two naphthofuranoxepins dioscorealides A (**1**) and B (**2**), a 1,4-phenanthraquinone, dioscoreanone (**3**), three steroids β-sitosterol (**4**), stigmasterol (**5**) and β-*D*-sitosterol glucoside (**8**) and two steroid saponins (diosgenin 3-*O*-α-*L*-rhamnopyranosyl (1→2)-β-*D*-glucopyranoside (**6**) and diosgenin 3-*O*-β-*D*-glucopyranosyl (1→3)-β-*D*-glucopyranoside (**7**)). In a preliminary cytotoxicity screening study **2**, **3** and **6** showed activity against three cancer cell lines, and **2** showed selective cytotoxic activity against lung and breast cancer, but was less active against the two normal cells and had no toxicity on cell membranes in the LDH assay (Itharat et al., 2003, 2007).

In the present study we describe our further detailed investigations which led to the isolation of 3 phenanthrenes compounds (**1–3**), one of which is a new cytotoxic compound (**3**), and the cytotoxic activity of compounds **1–3** against five cancer cells and one type of normal cells.

2. Material and methods

2.1. Reagents and chemicals

RPMI Medium 1640 (RPMI 1640), Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Penicillin Streptomycin (PS) and 0.5% trypsin-EDTA were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Phosphate buffer saline (PBS) was purchased from Amresco (Ohio, USA). Sulforhodamine B (SRB), MEM non-essential amino acid solution (NEAA), HEPES buffer solution, vincristine sulfate, Trisma base, and Trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Munich, Germany). Acetic acid was purchased from Merck (Darmstadt, Germany). Analytical grade reagent; hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) were purchased from RCI Labscan (Thailand). Anisaldehyde reagent was purchased from Sigma-Aldrich (St Galen, Switzerland). Silica gel 60 (0.063–0.200 mm) for Vacuum Liquid Chromatography (VLC), silica gel 60 (0.040–0.063 mm) for Column Chromatography (CC) and TLC silica gel 60 F_{254} were purchased from Merck (Darmstadt, Germany). CO_2 humidified incubator was purchased from Shellab (OR, USA). Laminar air flow cabinet was purchased from Boss tech (Bangkok, Thailand). Microplate reader was purchased from Bio Tek (VT, USA).

2.2. Plant material

The rhizomes of *Dioscorea membranacea* Pierre (Dioscoreaceae) were collected from Amphor Pa-tue, Chumporn province, Thailand in February 2011. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where plant specimen samples have been lodged. Specimens have also been keeping in the herbarium of the Southern Center of Thai Medicinal Plants, at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand (voucher number, SKP A062041305). The collected specimens were wash, cut into small pieces, dried (50 °C) and milled to obtain powder (mesh size 40 μm), and stored in refrigerator (4 °C).

2.3. In vitro assay for cytotoxic activity

Five different kinds of human cancerous cell lines, and one normal cell line were used, and the technique was similar to that described earlier (Itharat et al., 2004, 2007). The human cancer cell lines were caucasian lung, large cell carcinoma, COR-L23 (ECACC no. 92031919), prostate adenocarcinoma, PC-3 (ATCC no. CRL-1435), breast adenocarcinoma, MCF-7 (ECACC no. 86012803), the human cervical cancer cell, Hela (ATCC no. CCL-2), Hepatocellular carcinoma, HepG2 (ATTC no. HB-8065). The one normal cell line was human lung fibroblast cell, MRC-5 (ATCC no. CCL-171). COR-L23 and PC-3 cells were cultured in RPMI 1640 supplemented with 10% heated-inactivated FBS, 1% PS. MCF-7 cells were cultured in MEM supplemented with 10% heated-inactivated FBS, 1% PS and 1% NEAA. Hela cells were cultured in MEM supplemented with 10% heated-inactivated FBS, 1% PS. HepG2 cells were cultured in MEM supplemented with 10% heated-inactivated FBS, 1% PS and 1% HEPES. MRC-5 cells were cultured in DMEM supplemented with 10% heated-inactivated FBS, 1% PS. All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity (Itharat et al., 2004; Ruangnoo et al., 2012). According to their growth profiles, the optimal plating densities of each cell line were determined (1×10^3, 1×10^3, 3×10^3, 3×10^3, 3×10^3 and 5×10^3 cells/well for COR-L23, PC-3, MCF-7, Hela, HepG2, and MRC-5, respectively). The plates were incubated for 72 h, then incubated for a recovery period of 6 days and cell numbers were analyzed by SRB assay (Skehan et al., 1990; Itharat et al., 2004). Vincristine sulfate was use as the positive control.

According to National Cancer Institute guidelines (Boyed, 1997) compound with IC_{50} values <4 μg/ml was considered active.

2.4. Isolation and purification of compounds

Dried powder of rhizome of *Dioscorea membranacea* (1 kg) was macerated with 95% ethanol (3 L) for 3 days and filtered. The residue after filtering was macerated a further 2 times. The combine extracts were concentrated to dryness under reduced pressure (room temperature), to give 31 g of crude extract. This was further dried to constant weight in vacuum desicator (yield 3.1% w/w). The crude ethanolic extract (10 g, dissolved in methanol 10 ml) of *Dioscorea membranacea* was subjected to silica gel 60 (0.063–0.200 mm) (300 g). Vacuum liquid chromatography (VLC) using a glass column (5 in. × 4.5 in.). The column was eluted in sequence with increasingly polar solvents: hexane (1000 ml), hexane:$CHCl_3$ (1:1, 1000 ml), $CHCl_3$ (500 ml), $CHCl_3$ (500 ml), $CHCl_3$ (500 ml), $CHCl_3$:MeOH (1:1, 1000 ml) and MeOH (1000 ml). Evaporation of each fraction yielded residues of 0.029 g (F1), 0.024 g (F2), 0.034 g (F3), 0.239 g (F4), 0.339 g (F5), 6.00 g (F6) and 2.00 g (F7). These seven fractions were tested for cytotoxic activity against only the lung cancer cell line (COR-L23) by the SRB assay because the crude ethanolic extract showed the highest cytotoxicity against this cancer cell line. The IC_{50} of F1–F7 for these fraction were, >50, >50, 3.9 ± 0.23, 10.2 ± 0.83, 11.4 ± 0.92, 20.4 ± 1.12 and 27.2 ± 1.90 μg/ml. Three of the fractions (F3, F4 and F5) exhibited high cytotoxicity toward the lung cancer cell line; these were therefore subjected to further purification by normal, gravity feed column chromatography (CC).

Fraction F3 (34.0 mg) was subjected to further separation using CC on silica gel 60 (0.040–0.063 mm) (20 g), using glass column (1 in. × 40 in.), eluting with hexane:$CHCl_3$ (1:4, 300 ml) and collecting 2 ml fractions of the eluting solvent. Following TLC of each collected fraction (silica gel 60 F_{254}/hexane:$CHCl_3$ (1:4) and detection with acidic anisaldehyde spraying reagent), fractions containing the same compound were pooled and evaporated to dryness (at room temperature) to yield compound **1** (10.7 mg, 31.47% w/w of F3 weight).

Fraction F4 after VLC (238.9 mg) was similarly separated by CC on the silica gel 60 (size 0.040–0.063 mm) (100 g), glass column (1.5 in. × 45 in.) eluting with hexane:$CHCl_3$ (1:4, 1000 ml), collecting 5 ml fractions, to give five fractions (F4A–F4E). Fraction F4D (70.8 mg) was further purified by CC on silica gel using hexane: EtOAc (4:1, 300 ml) as eluting solvent, collecting 2 ml fractions, to