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Anticancer effect of the extracts from *Polyalthia evecta* against human hepatoma cell line (HepG2)

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ABSTRACT

Objective: To investigate the anticancer activity of *Polyalthia evecta* (*P. evecta*) (Pierre) Finet & Gagnep against human hepatoma cell line (HepG2). **Methods:** The anticancer activity was based on (a) the cytotoxicity against human hepatoma cells (HepG2) assessed using a neutral red assay and (b) apoptosis induction determined by evaluation of nuclei morphological changes after DAPI staining. Preliminary phytochemical analysis of the crude extract was assessed by HPLC analysis. **Results:** The 50% ethanol-water crude leaf extract of *P. evecta* (EW-L) showed greater potential anticancer activity with high cytotoxicity [IC_{50} = (62.8 ± 7.3) μg/mL] and higher selectivity in HepG2 cells than normal Vero cells [selective index (SI) = 7.9]. The SI of EW-L was higher than the positive control, melphalan (SI = 1.6) and the apoptotic cells (46.4 ± 2.6) % induced by EW-L was higher than the melphalan (41.6 ± 2.1)% (P<0.05). The HPLC chromatogram of the EW-L revealed the presence of various kinds of polyphenolics and flavonoids in it. **Conclusions:** *P. evecta* is a potential plant with anticancer activity. The isolation of pure compounds and determination of the bioactivity of individual compounds will be further performed.

1. Introduction

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) are the two major forms of primary liver cancers (PLC), accounting for approximately 90% and 5% respectively[1,2]. The incidence of each is the most common widespread cancer in the world. HCC causes high annual mortality rates, particularly in Thailand, Cambodia and Laos, where viral hepatitis is endemic[3]. The induction of HCC is preceded by the occurrence of hepatocellular damage via reactive oxygen species (ROS) and the generation of chronic inflammation related to hepatocarcinogenesis[4]. Adjunctive therapies such as tumor necrosis factor and melphalan; or cisplatin, epirubicin and 5-FU; doxorubicin, interferon alpha and 5-FU have been used to overcome the HCC[5]. However, the main problem of chemotherapy to treat HCC is the cancer resistance mechanism, due to up-regulation of the multi-drug resistance protein (MDR) and a decrease of apoptotic proteins[6]. Thus, more effective chemotherapy is needed to control cancer and apoptosis induction, which is the desired effect for successful cancer treatment[7].

Polyalthia evecta (*P. evecta*) (Pierre) Finet & Gagnep belongs to the family Annonaceae and it is widely distributed in the semi-deciduous forests of southern Indochina. The root of *P. evecta* has long been used, based on Thai local wisdom, as a carminative and as a galactagogue for inducing milk secretion in breastfeeding mothers[8,9]. The phytochemicals found in the hexane extract of the root of *P. evecta* are evectic acid and furans[8,9]. The respective bioactive constituents from the hexane and dichloromethane extracts of the air-dried roots were shown to be active against *Plasmodium falciparum* and *Mycobacterium tuberculosis*[8,9]. High tannic acid content

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was reported in 50% ethanol-water leaf extract of *P. evecta* (EW-L), which was found to contribute to the antioxidant activity while the EW-L crude extract showed strong antimutagenicity in TA98[10].

The plants in the genus *Polyalthia* showed varieties of biological activities[11]. The extracts of *P. longifolia* is currently reported to have a cytotoxic effect on cancer cell lines while the extract of *P. jucunda* was found to have a growth inhibitory effect on tumor cell lines, possibly via apoptosis induction in NCI-H460 cells[12,13]. Our study has formerly reported the potential anticancer effect of the extract from the EW-L screened plants based on its selective cytotoxic activity in HepG2 cells[14].

The mechanism(s) of anticancer activity of this EW-L extract has, however, not been reported; therefore, the anticancer mechanism of the EW-L crude extract via apoptosis induction was investigated in the current study.

2. Materials and methods

2.1. Chemicals and reagents

The organic solvents used for extraction were of analytical grade from Fisher Scientific (UK) and Labscan (Thailand). Acetonitrile (HPLC grade, Fisher Scientific, UK), Ortho-phosphoric acid (analytical grade, BHD, England). Ultrapure water from Milli-Q system (Millipore, Bedford, USA) were used for the mobile phase preparation. The standard agents and melphalan were provided by Sigma-Aldrich Chemie GmbH (Germany). The reagents used in the cell assay were of molecular biological grade. Dimethylsulfoxide (DMSO) was bought from United States Biological (USA). The reagent and culture media Dulbecco's modified eagle's medium (DMEM) were bought from GIBCO®, Invitrogen Corporation (USA). Sodium bicarbonate ($NaHCO_3$) and the fluorescence dye 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Chemie GmbH (Germany). Neutral red and a standard anticancer drug (melphalan) were purchased from Sigma Chemical Co. (USA). Flexi Gene DNA kit was obtained from QIAGEN GmbH (Germany). Agarose molecular grade was purchased from Bio-Rad (USA). 100 bp + 1.5 Kb DNA ladder with stain was purchased from SibEnzyme Ltd. (Russia).

2.2. Plant material

Leaves of *P. evecta* (Pierre) Finet & Gagnep were collected from Khon Kaen Province in 2009. A voucher specimen (CRD-HHP-011L) was kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University.

2.3. Extraction and isolation

After drying, 1 kg of dried leaves was pulverized then macerated in 8 L of 50% ethanol-water for seven days, and then filtered. The solvent was removed using a rotary evaporator at 40-50 ℃. The residue (EW-L) was further freeze-dried (8.4% yield) and kept in amber in an air-tight container at 4 ℃ until being used.

2.4. High performance liquid chromatography (HPLC) analysis

The HPLC system comprised an 1 100 Agilent series, (Minnesota, USA) with a pumping system model G1310A, a manual injection models G1328B and a variable wavelength detector model G1314A. The chromatographic determination was performed following the method of Prayong *et al*[15] with minor modifications. The stationary phase was a HiQ Sil C18W reverse phase column 4.6 mm I.D. × 250 mm with a 5 μm particle sizes (KYA TECH Corporation, Japan). Reverse phase HPLC was conducted using an isocratic mobile phase of 20% acetonitrile in 80% Milli-Q water and 0.1% o-phosphoric acid with a flow rate of 0.7 mL/min. The detector wavelength was set at 213 and 280 nm. Gallic acid, chlorogenic acid, caffeic acid, ferulic acid, catechin and epicatechin were used as the reference standards at a final concentration of 1 mg/mL in dimethylsulfoxide (DMSO) to confirm their presence in the extract fraction.

2.5. Cell culture

The human hepatoma cell line HepG2 and the African green monkey kidney cell line Vero were maintained at the Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University. The HepG2 passage number 25-30 and the Vero passage number 37-40 were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2.

2.6. Cytotoxicity assay

Both the HepG2 and Vero cell lines were separately cultured in a T25 flask with medium DMEM (supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin) and incubated at 37 ℃ in a humidified 5% CO_2 atmosphere.

To determine the cytotoxicity of the samples in the cell model, neutral red (NR) uptake assay was used for identification of vital cells[16]. Briefly, cells were seeded at a density of 3×10^5 cells in 96-well plates with medium and incubated for 24 h. The cells were then treated with the crude extracts at various concentrations for a 24-h exposure time. Cells were centrifuged at 550 × g for 5 min and the supernatant were removed. Then, 50 μg/mL NR was added to each well and incubated for another 1 h. After NR incubation, cells were washed using media. The viable cells that accumulated NR were lyzed with 0.33% HCl in