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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

isopropanol. The absorbance of viable cells was measured as 520 nm and 650 nm (reference wavelength) using a spectro microplate reader. The maximum concentration of the compound used in the study was 500 μg/mL in order to maintain 1% v/v DMSO with a cytotoxicity of less than 10% compared to the untreated cells.

The Vero cells were used as a normal cell model for a comparison to the HepG2 cell model. The anticancer drug melphalan was used as a positive control. The IC_{50} value or the concentration causing 50% cell death (or 50% cytotoxicity) was determined from the linear equation obtained from the plot between the percentages of cell cytotoxicity vs. the concentrations. The selectivity index (SI) was defined as the ratio of the IC_{50} obtained from the experiment on normal cells vs. cancer cells. High selectivity was achieved when the SI was $\geqslant 3$[14].

2.7. Apoptosis induction assay

2.7.1. Nuclei morphology determined by DAPI staining assay

Apoptosis induction was determined by fluorescence dye staining, using 4′,6-diamidino-2-phenylindole (DAPI) to identify the condensation and fragmentation of nucleic DNA of the apoptotic cells. The cancer cells were treated with 2 × IC_{50} concentration of the plant extracts or melphalan for 24 h unless otherwise indicated. The culture medium was removed and rinsed by using 500 μL of fresh medium without FBS and the cells were fixed by using 50 μL methanol. Then 50 μL of DAPI was added to cells with a final concentration 1 μg/mL and exposed for another 1 h. The excess dye was removed and 20 μL of PBS: Glycerin (1:1) was added. The stained nucleic DNA was captured in 10 views under inverted fluorescence microscopy at a magnitude of 40 ×. The percentage of apoptotic nuclei was calculated by the formula: % Apoptotic cells = ($Cell_{apoptosis}$/$Cell_{total}$) × 100.

2.7.2. DNA fragmentation detection assay

The DNA fragmentation was used to determine apoptosis induction by observation of biochemical changes. Briefly, after cancer cells were treated with the 2 × IC_{50} of crude extracts and melphalan for 24 h, cancer cells were collected and washed with media. Then cells suspension were transferred to microcentrifuge tube (1.5 mL) and centrifuged at 300 × g for 5 min to collect the cell pellet. The DNA in the cell pellet was extracted using QIAGEN kit and 2 μg of DNA was analyzed by electrophoresis on 2% agarose gels containing 0.1 mg/mL ethidium bromide. After electrophoresis, DNA fragment was analyzed by using UV-luminated camera.

2.8. Statistical analysis

All of the tests were done in triplicate. The results were expressed as Mean ± SD. A probability level less than 5% ($P < 0.05$) was considered significant.

3. Results

3.1. HPLC fingerprints

The HPLC chromatograms at 213 nm for 1 mg/mL of the standard phenolic compounds (*e.g.*, gallic acid, chlorogenic acid, caffeic acid, ferulic acid) and the standard flavonoids (*e.g.*, catechin and epicatechin) showed peaks at retention times 3.67, 4.88, 6.74 and 14.53; 5.07 and 5.95 min, respectively (Figure 1). The retention time for DMSO appeared at 2.84 min. The HPLC chromatograms for the same set of standard compounds at 280 nm showed no DMSO peak while the respective peak for gallic acid, chlorogenic acid, caffeic acid, ferulic acid, catechin and epicatechin was 3.67, 4.87, 6.74, 14.54, 5.08 and 5.95 minutes (Figure 1). The HPLC fingerprint detected at 213 nm and 280 nm for the EW-L crude extract showed major peaks for catechin, caffeic acid and ferulic acid as well as two unknowns [*i.e.*, peak A (at 4.28 min) and unknown peak B (at 22.24 min)] (Figure 1A).

Table 1

Percent of the cytotoxicity and selectivity in malignant human hepatoma (HepG2) with 24-h exposure times to *P. evecta* crude leaf extract compared to melphalan.

Samples	IC_{50}±SD (μg/mL)		Selectivity index
	HepG2	Vero	
P. evecta crude extract (EW-L)	62.8 ± 7.3	>500	>7.9
Melphalan	37.7 ± 9.8	59.9 ± 3.2	1.6

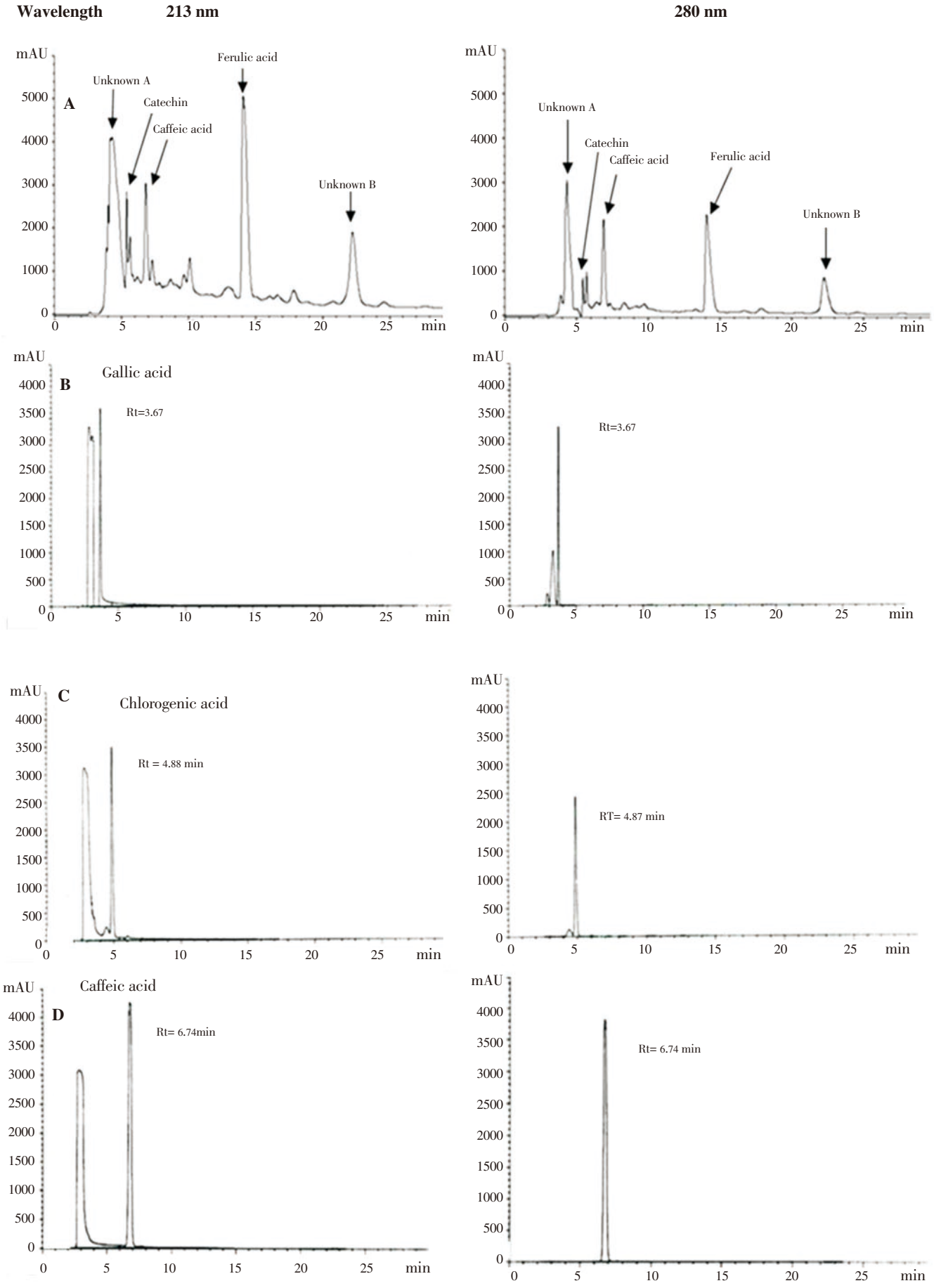
3.2. Cytotoxicity effects of P. evecta extracts in HepG2 cells

The cytotoxicity of the crude extract of *P. evecta* (EW-L) was determined in comparison to the anticancer drug melphalan (Table 1). The results showed that the EW-L crude extract of *P. evecta* exhibited significant high cytotoxicity to HepG2 cells ($P < 0.05$) [IC_{50} (62.8 ± 7.3) μg/mL] with a high selectivity index (SI > 7.9) (Table 1). Melphalan was stronger

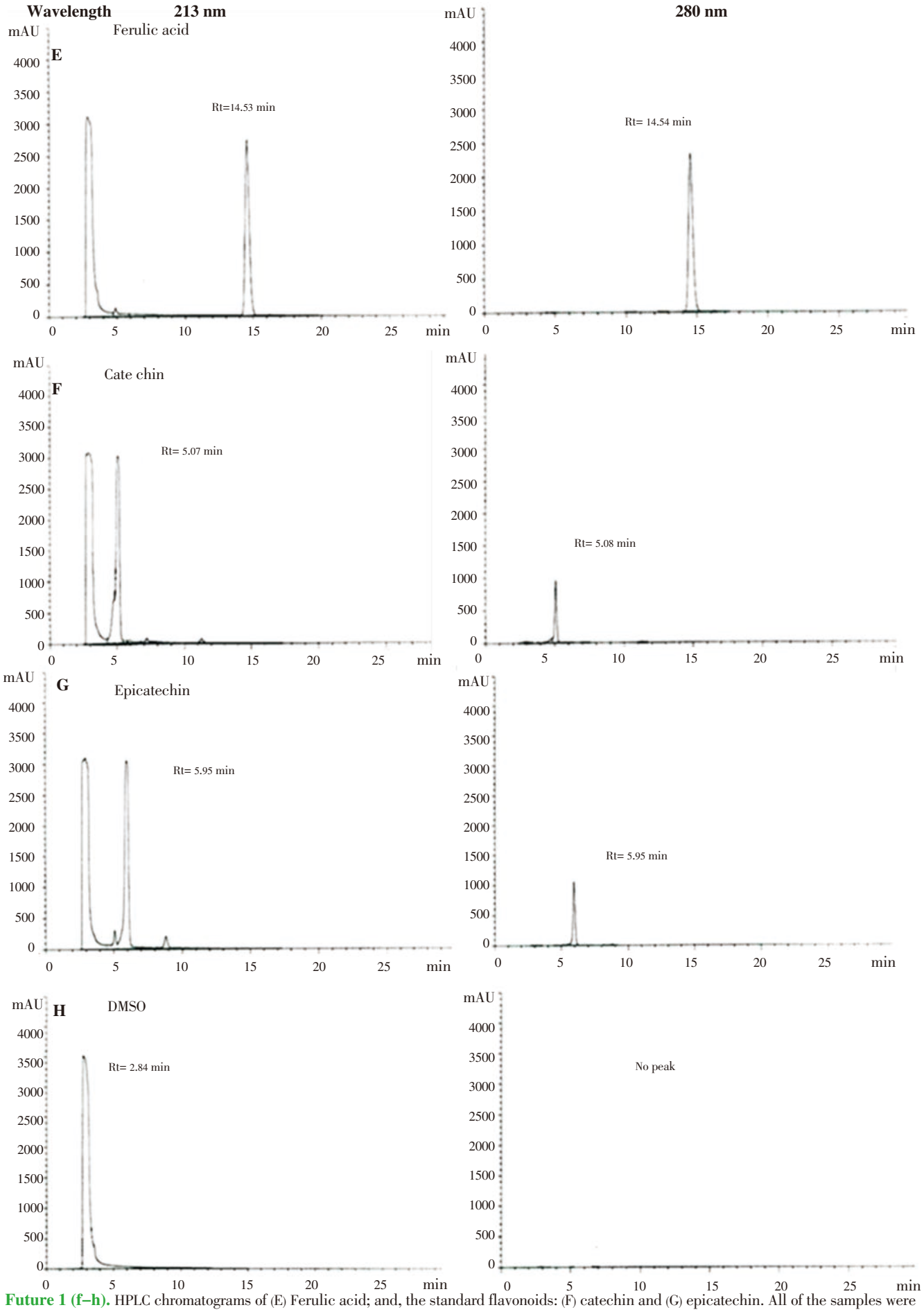
Table 2

Percentage of apoptotic cells induced in malignant human hepatoma (HepG2) by *P. evecta* extract compared to melphalan.

Samples	Final concentration (μg/mL)	% Cytotoxicity		% Apoptotic cells in HepG2
		HepG2	Vero	
P. evecta crude extract (EW-L)	140	60.0 ± 4.8	30.2 ± 9.3	46.4 ± 2.6
P. evecta crude extract (EW-L)	500	100.0 ± 4.1	37.2 ± 4.2	92.8 ± 10.8
Melphalan	76	67.2 ± 3.1	70.3 ± 3.1	41.6 ± 2.1



Future 1 (a–d). HPLC chromatograms of (A) the EW–L crude extract and the standard phenolic compounds: (B) Gallic acid; (C) Chlorogenic acid; (D) Caffeic acid;



Future 1 (f–h). HPLC chromatograms of (E) Ferulic acid; and, the standard flavonoids: (F) catechin and (G) epicatechin. All of the samples were prepared in (H) DMSO and detected at 213 nm (left column) and 280 nm (right column).

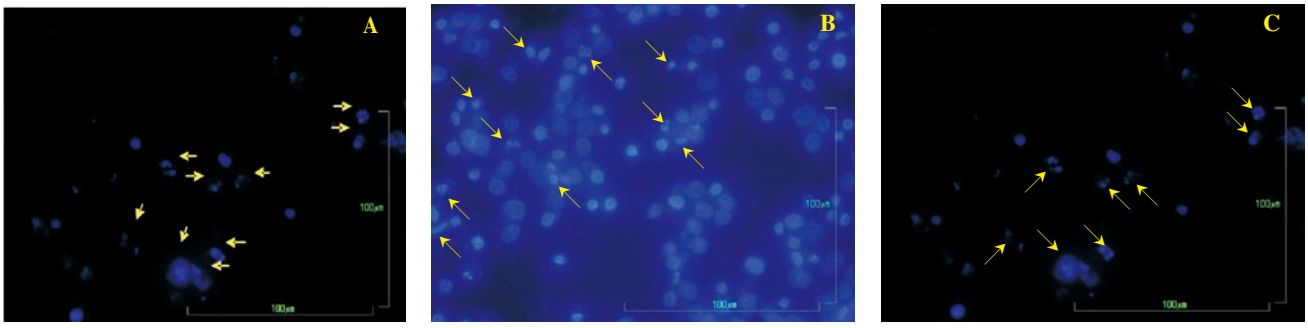


Figure 2. Nuclei morphological changes based on apoptosis induction in HepG2 cells after staining with DAPI and observed under inverted fluorescent microscopy with 40 × magnification. The HepG2 cells were incubated as: (A) untreated HepG2 cells (control); (B) 2×IC_{50} of EW-L extract 140 μg/mL; (C) 2 × IC_{50} of melphalan alone (76 μg/mL). Arrows indicate the cells undergoing apoptosis and nuclear fragmentation.

cytotoxic compound (IC_{50} 37.7 ± 9.83 μg/mL) than EW-L but with the lowest SI = 1.6. The EW-L crude extract was inactive in the normal Vero cells; since a concentration up to 500 μg/mL showed a cytotoxicity rate less than 50%. This extract concentration was the maximum concentration that could be used in the study and contained as high as 1% v/v DMSO with a toxicity less than 10%.

3.3. Apoptosis effects of the extract from P. evecta in HepG2 cells

To confirm the anticancer potential of the EW-L crude extract, apoptosis induction in cancer cells was tested with 2 × IC_{50} concentrations. The morphological changes of the nuclei DNA—after being treated with EW-L extract of *P. evecta*—are shown in Figure 2. The results showed that EW-L crude extract exhibited a moderate percentage of apoptotic cells [(46.4±2.6)%] but higher than melphalan (41.6±2.1)% (P<0.05) (Table 2). In addition, the HepG2 cells treated with EW-L exerted characteristic DNA ladder as same as melphalan (Figure 3).

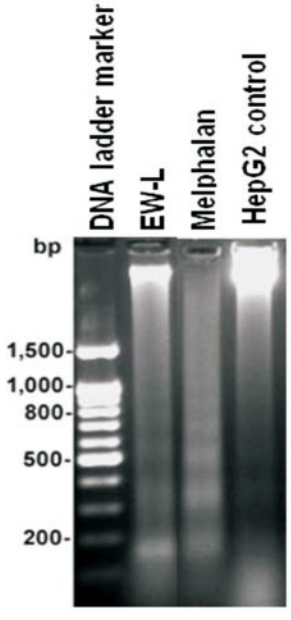


Figure 3. The latter stage of apoptosis (DNA laddering) in HepG2 cells induced by EW-L and melphalan.

4. Discussion

HPLC fingerprinting provides the chemical characterization of the crude extract. It is known that the polyphenols and flavonoids, being secondary metabolites, are present in several plants and can serve as markers of the crude extract. Therefore, phytochemical analysis of the crude extract was assessed by HPLC analysis. Since the retention time can be shifted due to the different polarity of the extract environment; therefore, the HPLC detection wavelengths were determined at two wavelengths (*i.e.*, 213 and 280 nm) to confirm peak identity. The HPLC fingerprint was detected at 213 nm and 280 nm for the EW-L crude extract might comprise both phenolic and flavonoid compounds. Unknown peak A seemed to comprise more than one peak, as there appeared to be poor separation under the studied conditions.

The anticancer activity of *P. evecta* (EW-L) was determined based on the cytotoxicity against human hepatoma cells (HepG2) and apoptosis induction. Programmed cell death or apoptosis performs an essential role for balancing cell proliferation and cell death and contributes to an effective cancer therapy[17-24]. Several anticancer activity studies have defined apoptosis as the pharmaco-dynamic endpoint for anti-cancer treatment[25]. Interestingly, the EW-L crude extract exhibited a higher selectivity than the melphalan and showed significant cytotoxicity and a high selectivity against HepG2 cells after 24 h exposure. When the concentration of EW-L increased, both cytotoxicity and the rate of apoptotic cells was also increased. It was indicated that the anticancer activity of EW-L was in a concentration dependent manner. The characteristic of apoptosis was also further confirmed by determination of the DNA ladder which is a result of DNA fragmentation and indicative of late stage of apoptosis.

In conclusion, our results clearly show a selective cytotoxicity and apoptosis inductive effect indicative of an anticancer activity of the 50% ethanol-water leaf extract of *P. evecta* on the human hepatoma (HepG2) cell line. The isolation of the active chemical constituents from the EW-L and determination of their individual anticancer activity will

be further performed.

Conflict of interest statement

We declare that we have no conflict of interest.

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