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EXTRACT OF ULMUS DAVIDIANA PLANCH BARKS INDUCED APOPTOSIS IN HUMAN HEPATOMA CELL LINE HEPG2

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ABSTRACT

The cytotoxicity and mechanism of cell death induced by an extract of *Ulmus davidiana* Planch (UDP) were investigated. A 70 % ethanol extract of UDP exerted cytotoxic effects on HepG2 cells in a time- and dose-dependent manner caused a significant dose-dependent increase in the number of apoptotic cells. In addition, obvious shrinkage and destruction of the monolayer were observed in UDP-treated cells, but not in untreated cells. RT-PCR analysis revealed that the mRNA expression of *p53* and *c-myc* was markedly increased in cells treated with the plant extract. In contrast, *caspase*-3 expression was induced after 1 h of incubation and then decreased at 3 h; however, it subsequently increased and remained at a relatively high level thereafter. Our data suggest the presence of a bioactive compound capable of killing liver carcinoma cells by apoptosis in a 70 % ethanol extract of UDP, and that the mechanism of apoptosis in the HepG2 cells included *p53* and *c-myc* signaling.

Keywords: *Ulmus davidiana* Planch, 70 % ethanol extract, apoptosis, HepG2

INTRODUCTION

Cancer is a serious clinical problem that poses significant social and economic challenges to the healthcare system. Despite improved imaging and molecular diagnostic techniques, cancer continues to affect millions of people globally (Eisenberg et al., 1998). Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death (Parkin et al., 2001). In most cases, the recovery rate from HCC is low, and current conventional and modified therapies are rarely beneficial (Sheu, 1997; Thomas and Zhu, 2005). Thus, there is an urgent need for new therapeutic agents for HCC patients. A common means of drug discovery is the ethnomedical data approach, in which the selection of a plant is based on information related to its use as a folk medicine. A number of anti-cancer drugs have been extracted from plants, including paclitaxel (Matsuhashi et al., 2005), adriamycin (Hirao et al., 2004), etoposide (Baldwin and Osheroff, 2005), and camptothecin (Legarza and Yang, 2006). Apoptosis, as a physiological mode of cell death, may be mediated by the excess production of reactive oxygen species (ROS) caused by exogenous and endogenous sources (Slater et al., 1963). Therefore, the search for agents that can trigger apoptosis in tumor cells has become a major goal in anti-cancer drug discovery (Reed, 2003).

Ulmus davidiana Planch (UDP) is a deciduous tree that is widely distributed throughout Korea. Its bark and roots are used in traditional oriental medicine to treat edema, mastitis, gastric cancer, and in-

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flammation (Kim, 2000). A number of biologically active compounds has been isolated from UDP, and their biological actions have been reported (Lee et al., 2001; Guo and Wang, 2007). For example, a glycoprotein isolated from UDP was shown to have strong antioxidant and anti-apoptotic effects (Lee et al., 2004).

The aims of this study were to evaluate the cytotoxic properties of an extract prepared from the bark of UDP and to determine the mechanism of cell death elicited by the extract in HCC cells. The human hepatoma HepG2 cell line is widely used as an experimental model for *in vitro* studies of HCC (Cheng et al., 2008). Thus, we investigated the effects of an ethanolic extract of UDP bark on HepG2 cells.

MATERIALS AND METHODS

Plant material and extract preparation

Stems and bark from UDP plants were purchased from the Herbal Medicine Co-operative Association in Chuncheon, Korea. The bark samples were air-dried under shade at room temperature and powdered. About 10 g of each powdered sample were then treated with 200 mL of water, ethanol, 70 % ethanol, methanol, 70 % methanol, or chloroform for 72 h. The extracts were then filtered, and the solvents were evaporated at 50 °C using a rotary evaporator. The recovery weight from the dried material was about 10 %. The extracts were dissolved in distilled water and subsequently diluted to the appropriate working concentrations.

Cell line and culture medium

Human liver cancer HepG2 cells were maintained in RPMI 1640 medium containing 10 % (v/v) heat-inactivated fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C under 5 % CO_2 in air.

MTT assay

HepG2 cells were seeded in a 96-well plate at a concentration of 2×10^4 cells/mL using RPMI. After 16 h at 37 °C in a humidified atmosphere of 5 % CO_2, the extracts were applied to the wells at different concentrations. The cells were then incubated for an additional 72 h at 37 °C. An MTT stock solution (50 µL of 2 mg/mL in PBS) was then added to each well for a total reaction volume of 250 µL. After 4 h of incubation at 37 °C in a humidified atmosphere of 5 % CO_2, the plate was centrifuged at 800 x *g* for 5 min, and the supernatants were aspirated to remove all untransformed MTT. The formazan crystals in each well were then dissolved in 150 µL of DMSO. The amount of purple formazan was determined by measuring the absorbance at 550 nm. For the treated cells, viability is expressed as the percentage of control cells.

Trypan blue exclusion assay for cell viability

After 24, 48, or 72 h of exposure, the medium was removed from the wells, and the cells were washed with 2 mL of sterile PBS. Next, 100 µL of 0.25 % trypsin were added, and the plates were incubated at 37 °C for 5-10 min. Full growth medium (50 µL) and 0.4 % trypan blue (50 µL) were added to each well, and the plates were returned to the incubator for another 5 min. Next, a 20 µL aliquot was removed and placed under a cover slip on a hemacytometer, and the numbers of viable and nonviable cells were counted under a microscope. The number of viable cells was calculated using the following equation: UC $\times$ D $\times$ 10^4 / #SQ, where UC = the unstained cell count (viable cells), D = the dilution of the cell suspension, and #SQ = the number of squares on the hemacytometer that were counted. The percent viability for the population on each Petri dish was calculated as: UC/TC $\times$ 100, where UC = the unstained cell count (viable cells) and TC = the total cell count (stained plus unstained cells). Each experiment was repeated four times.

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Flow cytometry

An Annexin V-FITC/PI Apoptosis Detection Kit (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to detect apoptosis. Cells (5×10^5 per well) were seeded into six-well plates and then treated with varying concentrations of the 70 % ethanol extract. After 24 h of incubation, the cells were washed twice with ice-cold PBS (0.01 M, pH 7.2), and 100 µL of each sample were centrifuged at 200 x *g* for 5 min. The supernatant was then removed, and the cells were resuspended in 100 µL of Annexin V/FITC as per the manufacturer's instructions. Apoptosis was monitored by FACScan flow cytometry (Becton-Dickinson). All Annexin V-positive, PI-negative cells were scored as apoptotic, while all double-stained cells were considered to be necrotic or late apoptotic (Wang et al., 2006).

Electron microscopy

About 5×10^5 HepG2 cells were grown in 35-mm sterile Petri dishes in complete medium with or without 500 µg/mL 70 % ethanol extract of UDP for 48 h. The cells were then fixed for 5 min in 10 % methanol/PBS before being analyzed for morphological changes under an inverted microscope (Axiophot, Jena, Germany).

Determination of the expression level of apoptosis-related genes

The mRNA expression of three established apoptosis-related genes (*c-myc*, *p53*, and *caspase-3*) was analyzed by semi-quantitative RT-PCR. The cells were cultured in T-25 flasks and then starved in medium containing 0.5 % (v/v) FCS for 4 h prior to stimulation. Total RNA was extracted from the untreated and treated cells using TRI-reagent® according to the manufacturer's instructions (MRC, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 µg of the DNase-treated total RNA using AccuPower® PCR PreMix containing oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA, USA). The reactions were carried out as follows: 5 min of denaturation at 94 °C followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final extension for 7 min at 72 °C. The sequences of the primers used herein were designed based on the human mRNAs encoded by the respective genes (Table 1). An aliquot of each RNA sample and 12 µL of each PCR product were separated on 1 % agarose gels and visualized by staining with ethidium bromide. All experiments were performed in triplicate.

Calculations and statistics

Cytotoxicity was analyzed in triplicate; the results are expressed as the percent growth inhibition with respect to the control. The data are given as the mean ± SEM. Gene expression at each time point was determined by densitometric scanning using PhotocaptMw gel analysis software. The signals from *c-myc*, *p53*, and *caspase-3* were normalized to that from *β-actin*; the ratio in the unstimulated samples was defined as 1.

Table 1: The sequences of primers used in RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
c-myc	GAACAAGAAGATGAGGAAGA	AGTTTGTGTTTCAACTGTTC
p53	TGTGGAGTATTTGGATGACA	GAACATGAGTTTTTTATGGC
Caspase-3	TCACAGCAAAAGGAGCAGTTT	CGTCAAAGGAAAAGGACTCAA
β-Actin	TCACCCTGAAGTACCCCATC	CCATCTCTTGCTGCAAGTCC

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RESULTS AND DISCUSSION

Antiproliferative activity in various extracts of UDP

In this study, the human liver cancer cell line HepG2 was used to examine the antiproliferative activity of UDP extracts. Cultured HepG2 cells were treated with different extracts of UDP at a concentration of 0.5 mg/mL (Fig. 1). All extracts exerted an inhibitory effect on the cells; however, the 70 % ethanol and ethanol extracts had the most significant effect.

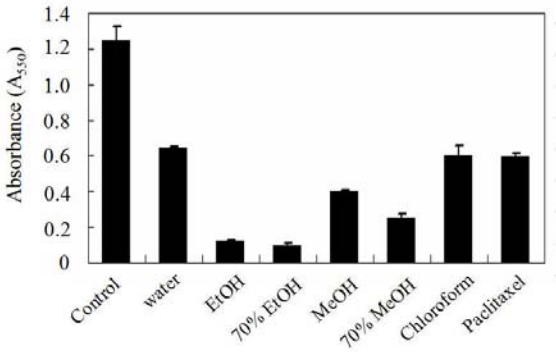

Figure 1: MTT assay of various extract of UDP on HepG2 liver cancer cells at the concentration of 500 μg/mL. Cells were treated with water, ethanol, 70 % ethanol, methanol, 70 % methanol and chloroform extract of UDP for 48 h. Each data is expressed as the mean ± SD obtained from triplicate experiments.

The MTT assay is a method for quantifying metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to blue-purple formazan crystals (Mossman, 1983). By using 96-well microtiter plates and a multi-well spectrophotometer (enzyme-linked immunosorbent assay plate reader), the assay can be semiautomated in order to process a large number of samples and provide a rapid, objective measurement of cell number.

Assessing cellular viability by trypan blue exclusion

To determine the inhibitory effect of the 70 % ethanol extract of UDP on human HepG2 cell growth, trypan blue exclusion analysis was used to assess the effect of UDP on cell viability (Fig. 2). Cell viability was evaluated by counting with a hemacytometer. As shown in Fig. 1, UDP dramatically inhibited HepG2 cell viability in a dose- and time-dependent manner. However, the cells showed varying levels of sensitivity to different concentrations of the extract, with increased susceptibility at a concentration of 200 μM. Incubation with 50 to 100 μM UDP did not have an obvious inhibitory effect on HepG2 cell proliferation.

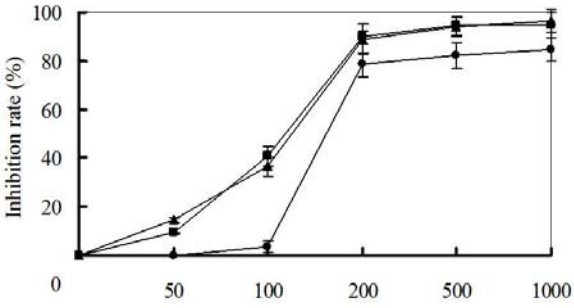

Figure 2: Dose- and time-dependent cytotoxicity of 70 % ethanol extract of UDP to HepG2 cells. Cells were treated with 50, 100, 200, 500, and 1000 μg/ml of 70 % ethanol extract of UDP, respectively, for 24 (black circles), 48 (black squares) and 72 h (black triangles). Inhibition rate was expressed as cell death versus reference (cell viability = 100 %). Each data is expressed as the mean ± SD obtained from triplicate experiments.

There is evidence that most anti-cancer agents exert their cytotoxic effects by inducing apoptosis in tumor cells (Kaufman, 1989). Impairments in apoptosis are related to cell immortality, carcinogenesis, and the induction of apoptosis in neoplastic cells (Shinomiya et al., 1994); therefore, induction of apoptosis is vital in cancer treatment. Several chemotherapeutic drugs have been shown to induce apoptosis *in vitro*, including etoposide, camptothecin, VM26, vincristine, *cis*-platinum, cyclophosphamide, paclitaxel, 5-fluorouracil, and doxorubicin (Huschtscha et al., 1996).

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Apoptotic effect of the UDP extract

To further quantify the apoptotic effect of the 70 % ethanol extract of UDP, HepG2 cells were stained with Annexin V-FITC and PI and subsequently analyzed by flow cytometry. The Annexin V assay monitors the turnover of phospholipids from the inner to the outer layer of the plasma membrane, an event typically associated with apoptosis. The proportion of Annexin V-stained cells was increased in the sample-treated cells (Fig. 3). After 48 h of treatment, the percentage of Annexin V-positive cells was 0.31 % for the controls (0.2 % DMSO), 18.56 % for the cells treated with 100 µg/mL 70 % ethanol extract, and 25.05 % for the cells treated with 200 µg/mL 70 % ethanol extract. A significant dose-dependent increase in the number of apoptotic HepG2 cells was detected for the cells treated with the 70 % ethanol extract. The quantitative analysis of phosphatidylserine externalization through Annexin V/FITC and PI staining indicated that the percentage of double-positive cells in late apoptosis was increased in a concentration-dependent manner after 48 h of treatment with the 70 % ethanol extract. Our data indicate that the 70 % ethanol extract of UDP evoked apoptosis in the HepG2 cells.

Light microscopic observation

To examine the effect of the 70 % ethanol extract on cellular morphology during cell death, the morphological changes in untreated HepG2 cells and cells treated with 100, 200, or 500 µg/mL extract for 24 h were analyzed by light microscopy after trypan blue staining (Fig. 4). Compared to the untreated cells, the UDP-treated cells showed obvious cell shrinkage and destruction of the monolayer, which was not seen in the untreated cells. UDP treatment caused swelling in the HepG2 cells, resulting in condensed chromatin and a crooked membrane. A dose-dependent reduction in population size was also noted in the UDP-treated cells.

Expression of apoptosis-related genes in the 70 % ethanol extract-treated cells

To investigate the mechanism by which the 70 % extract induced apoptosis in HepG2 cells, the expression of several apoptosis-related genes, including *p53*, *caspase-3*, and *c-myc*, was evaluated by RT-PCR with *β-actin* as an internal control. The steady-state level of *p53* mRNA was increased drastically in the extract-treated cells after 1 h; however, it decreased thereafter (Fig. 5). The expression of *caspase-3* was induced after 1 h of incubation and decreased at 3 h; however, it later increased again and was maintained at a relatively high level thereafter. The level of *c-myc* mRNA was significantly increased at 1 h and reached its maximum at 3 h; it then decreased slightly but remained at a high level. Our results strongly indicate that the extract killed the HepG2 cells through apoptosis, mainly via the activation of *p53* and *c-myc*.

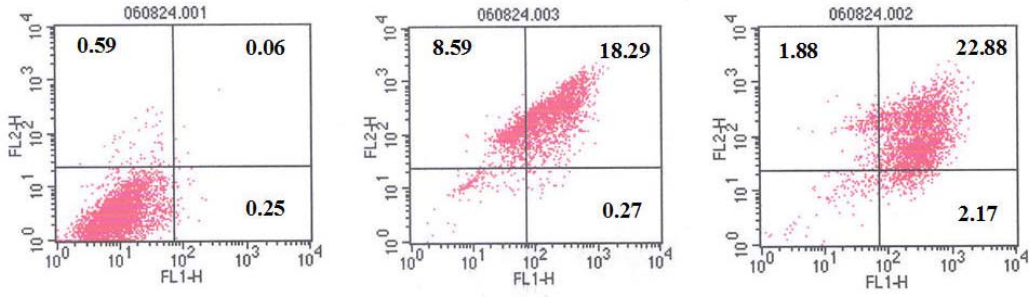

Figure 3: Flow cytometry analysis of 70 % ethanol extract of UDP induced apoptosis. Cells treated with 100 and 500 µg/mL of samples, respectively, for 48 h. (A), control; (B-C) represent 100 and 500 µg/mL samples treated HepG2 cells, respectively. Results are representative of triplicate experiments.

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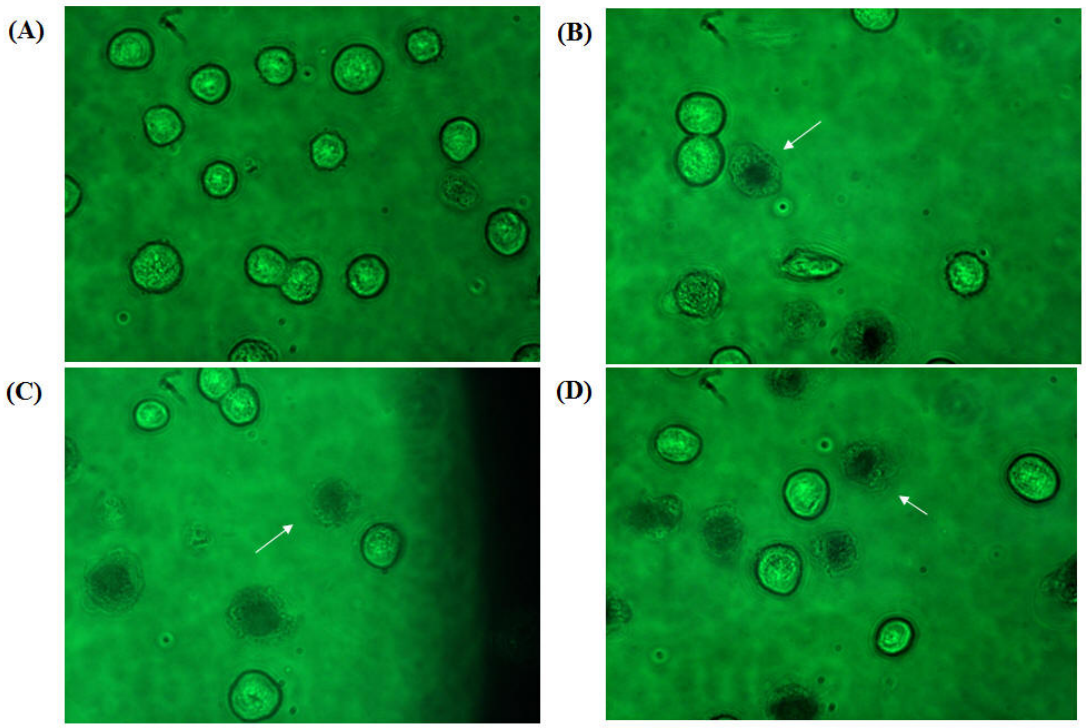

Figure 4: Morphological changes of HepG2 cells under light and fluorescence microscope. HepG2 cells were incubated for 48 h with 70 % ethanol extract of UDP. The medium was removed and the cells were rinsed and visualized under light microscope in control (**A**), 100 mg/L (**B**), 200 mg/L (**C**) and 500 mg/L (**D**) of 70 % ethanol extract of UDP.

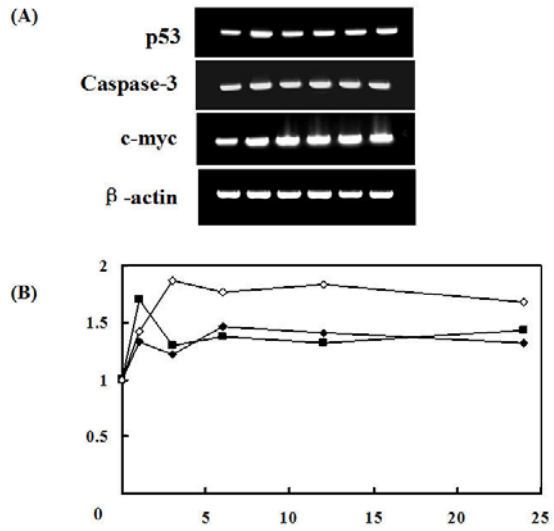

Figure 5: (A) Time dependency effects of the *p53*, *caspase-3* and *c-myc* mRNA levels in human liver cancer cell line, HepG2, incubated in the absence or presence of 70 % ethanol extract of UDP. *β-Actin* was used as an internal control for integrity and equal amount of cDNA used in each PCR reaction. (B) Graphical representation of the expression profile of *p53* (black squares), *caspase-3* (black diamonds) and *c-myc* (white diamonds) mRNA in HepG2 cells following treatment with the extract.

p53 is a major inhibitor of tumorigenesis. It stimulates a broad network of signals that act through two major apoptotic pathways: the extrinsic death receptor pathway, which triggers the activation of a caspase cascade, and the intrinsic mitochondrial pathway, which shifts the balance in the Bcl-2 family towards the pro-apoptotic members and, consequently, toward caspase-mediated apoptosis (Haupt et al., 2003). Myc is a co-activator of p53 that has been suggested to be an important determinant of the choice between p53-induced growth arrest or apoptosis (Hu and Kavanagh, 2003). Myc inhibits the growth arrest caused by UV light, γ-irradiation, and ROS-induced DNA damage (Sheen and Dickson, 2002).

Caspases play an important role in apoptotic cell death. Caspase-3 is the most commonly activated caspase during apoptosis (Janicke et al., 1998). Caspase-3 has been widely shown to mediate the limited proteolysis of the structural protein gelsolin, p21-activated kinase 2 (PAK2), focal adhe-

sion kinase (FAK), and rabaptin 5 (Tan et al., 2005). C-myc, a member of the Myc family of transcription factors, is a regulator of cell cycle progression and can induce apoptosis (Askew et al., 1991).

In conclusion, our results strongly suggest that a 70 % ethanol extract of UDP exhibited significant cytotoxicity toward HepG2 liver cancer cells and that it may contain a bioactive compound capable of killing liver carcinoma cells by apoptosis through the activation of the *p53* and *c-myc* pathways. Thus, *U. davidiana* should be considered as a functional food ingredient and pharmaceutical. However, the identification of the individual compounds and *in vivo* experiments are needed to understand the mechanism of action.

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