Apoptosis Induction Effect of Three Jujube Cultivars in HepG2 and Jurkat Cell Lines

Natthanan Taechakulwanijya, Natthida Weerapreeyakul, Sahapat Barusrux, and Sirithorn Siriamornpun

***Abstract*—The potential cancer preventive effects of water and ethanolic extracts of three new jujube cultivars were examined in HepG2 and Jurkat cancer cell lines. Apoptosis induction was detected from alteration of nuclei morphology via DAPI staining, and apoptosis death mode was detected via Annexin V-FITC and propidium iodide fluorescence flow cytometry. Results showed that ethanolic jujube seed extracts exhibited antiproliferation only in the Jurkat cell line. Cultivar Taiwan exerted high antiproliferation effect (IC_{50}=232.4±7.8 μg/ml) > Jumbo (IC_{50}=312.0±18.3 μg/ml) > Rianthong (IC_{50}=401.6±9.9 μg/ml), respectively (p<0.05). Melphalan (a positive control) exhibited significant antiproliferation in both HepG2 (IC_{50}=37.5±3.9 μg/ml), and Jurkat cell lines (IC_{50}=119.1±10.4 μg/ml) but also had cytotoxic effect on the normal Vero cell line (IC_{50}=75.0±2.3 μg/ml). The early and late stages of apoptotic cell death as well as necrosis were detected in Jurkat cells, in both a cultivar and a concentration dependent manner. Jumbo induced more late stage apoptosis > Rianthong > Taiwan > melphalan. The order of test compounds that caused necrosis were Taiwan > Jumbo > Rianthong > melphalan. Rianthong was the most promising cancer preventive cultivar based on antiproliferation and apoptosis induction activity with corresponding less necrosis. However, as all jujube seed extracts were not toxic to Vero cells, all jujubes cultivars tested are promising candidates for more elaborate study of their anticancer mechanisms.**

***Index Terms*—Apoptosis, jujube, cytotoxicity, cancer.**

I. Introduction

Jujubes came from two major genuses which are *Ziziphus mauritiana* Lam. and *Ziziphus jujuba* Mill [1]. Jujube belong to RHAMNACEAE family. It has long been recognized as an edible fruit [2]. In addition, various parts of jujubes were reported to be useful for treatment of many symptoms and diseases, for example; diabetes (96% ethanolic extract of *Z. jujuba* leaves) [3], diarrhea (methanolic extract of *Z. mauritiana* roots) [4], inhibition of hepatic lipid peroxidation (aqueous extract of *Z. mauritania* leaves) [5], anti-fungal activity (ethanolic extract of *Z. jujuba* stone) [6] enhancing choline acetyltransferase associated with Alzheimer's disease (methanolic extract of *Z. jujuba* fruits) [7], anxiolytic effect (methanolic extract of *Z. jujuba* seed) [8], and enhanced permeability in cell culture monolayer (aqueous extract of *Z. jujuba* seed) [9]. The active compounds found in jujube were triterpeonic acid (roots) [10], 6'''-feruloylspinosin, 6'''-sinapoylspinosin, jujuboside A, B (seeds) [11], linoleic, oleic and stearic acids (seeds) [12]. Previously, *Ziziphus* species were reported to induce apoptosis programmed cell death in many cancer cell lines [13]-[15] and in tumor cells of Swiss albino mice [13].

Cancer is the severe chronic disease that is found worldwide with increasingly high rate of morbidity and mortality [16]. Therefore, it is of interest to search for cancer preventive agent from natural source. One preferable pharmacodynamic endpoint for cancer treatment is via inducing apoptotic cell death [17]. Hence, apoptosis induction is the primary goal of chemotherapy. Apoptosis is the cell mechanism that balances between cancer cell proliferation and damage irreparable cell including damage DNA. Then, dead cells are phagocytosed by macrophages. The advantage of this death mode does not lead to inflammation in neighboring cells as same as necrosis [17].

According to the reports of biological activity of jujubes and new cultivars of Thai jujubes have been cross-bred, this study was aimed to investigate the apoptosis induction effect of the jujube seed extract of three jujube cultivars (i.e., Rianthong, Jumbo and Taiwan) against the human hepatocellular carcinoma HepG2 and human leukemic Jurkat cell lines compared to the normal African green monkey kidney Vero cells.

II. Materials and Methods

A. Plant Materials and Reagents

The jujube fruits cultivars Rianthong (R), Taiwan (T) and Jumbo (J) were purchased from local markets in Khon Kaen, Thailand in 2012. The Jumbo and Taiwan cultivars are the result of a cross between *Z. mauritiana* and Z. *jujuba*, but cultivar (Rianthong) remains unidentified. The cell culture media and fetal bovine serum were purchased from GIBCO, Invitrogen Corporation (USA.). Neutral red dye was purchased from Sigma Chemicals Co. (USA), and melphalan (mel; an apoptosis induction positive control) was bought from Sigma-Aldrich Chemie GmbH (Germany). Apoptotic detection kit was bought from eBiosciences (USA).

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B. Preparation of Jujube Seed Extracts

The ground dry jujube seeds were macerated in deionized water (1:3 w:v) overnight. The water filtrate was collected and freeze-drying yielded crude water extract. The left over residue from water extraction was macerated with ethanol. Ethanol was removed by rotary evaporation to yield the ethanolic extract. Percentage yield was calculated and compared to the weight of dry seed. The extracts were stored at−20 ℃.

C. Cell Culture

Human hepatocellular carcinoma (HepG2) and African green monkey kidney (Vero) cell lines were cultured in DMEM medium with 10% fetal bovine serum. The human acute T cells leukemia cell line (Jurkat) was cultured in RPMI medium with 10% fetal bovine serum. All cells were maintained under 5% CO_2 at 37 ℃.

D. Cell Viability Assay

The cell viability assay was based on neutral red (NR) uptake [18]. The cationic NR dye penetrates cell membranes and binds in the lysosomal matrix of viable cells. After washing and fixation, the NR can be detected by spectrophotometer [19]. Briefly, Vero cells at 3×10^5 cells/ml or HepG2 cells at 4×10^5 cells/ml or Jurkat at 5×10^5 cells/ml were independently seeded into 96 well plates and incubated for 24 h under 5% CO_2 at 37 ℃. Various concentrations of the extracts from each cultivar of jujube seed (10-500 µg/ml) were added to the cells and incubated for 24 h. After washing cells, 100 µl of 50 µg/ml NR was added to each well and plates were incubated for another 2 h. The supernatant was aspirated and 0.33% HCl in isopropanol was added. The absorbance of NR was measured at 537 nm with reference wavelength of 650 nm. The IC_{50} value was calculated and compared to the untreated cells.

E. Determination of Nuclei Morphological Change

DAPI or 4',6-diamidino-2-phenylindole is a cell-permeable fluorescent dye which binds to DNA and is commonly used to detect nuclei [20]. When cancer cells undergo apoptosis, the nuclei undergo morphological changes, therefore apoptotic cells stained with DAPI could be identified using fluorescence microscopy. The DAPI staining assay was performed following the method of Machana *et al.*, [21] with minor modification. Jurkat cells (3×10^5 cells/ml) were incubated with 250 µg/ml or 500 µg/ml of jujube seed extracts for 24 h. The cells were fixed with cold methanol for 15 min, and then incubated in the dark with 0.3 µg/ml DAPI for 30 min. Then the supernatant was removed and the cells were mounted in 15 µl of PBS: glycerin, 1:1.

F. Flow Cytometric Analysis

The flow cytometric analysis of modes of cell death was conducted using Annexin V-FITC and propidium iodide (PI) staining. Annexin V-FITC binds phosphatidylserine in the outer membrane, whereas PI binds nuclei in dead cells. Cells positively stained with Annexin V and PI (+/+) are considered to be in the late stages of apoptosis. Annexin V positive but PI negative cells (+/-) indicate cells in the early stages of apoptosis, while cells not stained with Annexin V but PI positive (-/+) are considered necrotic [22]. The method for this assay was adapted from Nasri *et al.*, with some modification [22]. Briefly, Jurkat cells at 3×10^5 cells/ml were treated with the seed extracts at $1\times IC_{50}$ and $2\times IC_{50}$ concentrations (400 µg/ml and 800 µg/ml for Rianthong; 300 µg/ml and 600 µg/ml for Jumbo; and 200 µg/ml and 400 µg/ml for Taiwan) at different incubation times (12 and 24 h). Cells were washed in 200 µl 1x binding buffer and centrifuged at 1677 ×g for 5 min. Then 5 µl Annexin V in 95 µl binding buffer was added and the cells were incubated for 15 min at 25 ℃. Then 5 µl propidium iodide in 95 µl binding buffer was added and the cells were incubated for another 15 min. All of the procedure was kept from light. Cells were washed and resuspended with 200 µl binding buffer. The cell dot plot was analyzed using flow cytometry (FACSDiva software version 6.1.3).

G. Statistical Analysis

The data are shown as means ±standard deviation (n=3). The statistical analysis was performed by using one-way ANOVA test (IBM SPSS Statistic 19) with 95% confidence interval.

III. Results and Discussion

A. Antiproliferation

The %yield and IC_{50} values of water (H_2O) and ethanolic (EtOH) extracts of jujube seeds from the three cultivars are shown in Table I. The ethanolic extracts of all three jujube cultivars possessed significant cytotoxic effects in the human acute T cell leukemia cell line (Jurkat, p<0.001, one-way ANOVA). Cultivar Taiwan exerted a significantly greater antiproliferation effect than Jumbo and Rianthong, respectively (p<0.001, one-way ANOVA). Melphalan exhibited an antiproliferation effect on both cancer cell lines and the non-cancer cell line, Vero. Interestingly, the ethanolic seed extract of all three jujube cultivars did not exert any cytotoxic effect on the normal Vero cell line, indicating the cancer selectivity and safety of ethanolic jujube extracts.

TABLE I: Percent Yield and Antiproliferation Represented as the IC50 Values of Jujube Seed Extracts in Various Cell Lines at 24 Hours

Jujube Cultivar	%Yield		IC_{50} (µg/ml) (N= 3)					
			HepG2		Jurkat		Vero	
	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH
R	1.3	2.8	in	in	in	401.6±9.9	in	in
J	0.5	1.2	in	in	in	312.0±18.3	in	in
T	1.7	1.1	in	in	in	232.4±7.8	in	in
Mel			37.5±3.9		119.1±10.4		75.0±2.3	

in = inactive, IC_{50} > 500 µg/ml

R = Rianthong; J = Jumbo; T = Taiwan; Mel = Melphalan

A. Apoptotic Cell Staining

The apoptotic activity of the ethanolic extracts in Jurkat cells was observed and scored as morphological changes to the nuclei in the dead cells: chromatin condensation, nuclear-fragmentation, and appearance of apoptotic bodies (Fig. 1). The results showed that Rianthong extract induced the highest % of apoptotic cells among the jujube cultivars (54.4 ±4.2%). The order of % apoptotic Jurkat cells from high to low were melphalan (70.7 ± 4%) > Rianthong (54.4 ± 4.2%) > Taiwan (45.0 ± 4.6%) > Jumbo (41.5 ± 5.5%). The amount of apoptosis induced by the jujube seed ethanolic extracts were concentration-dependent (see Fig. 1).

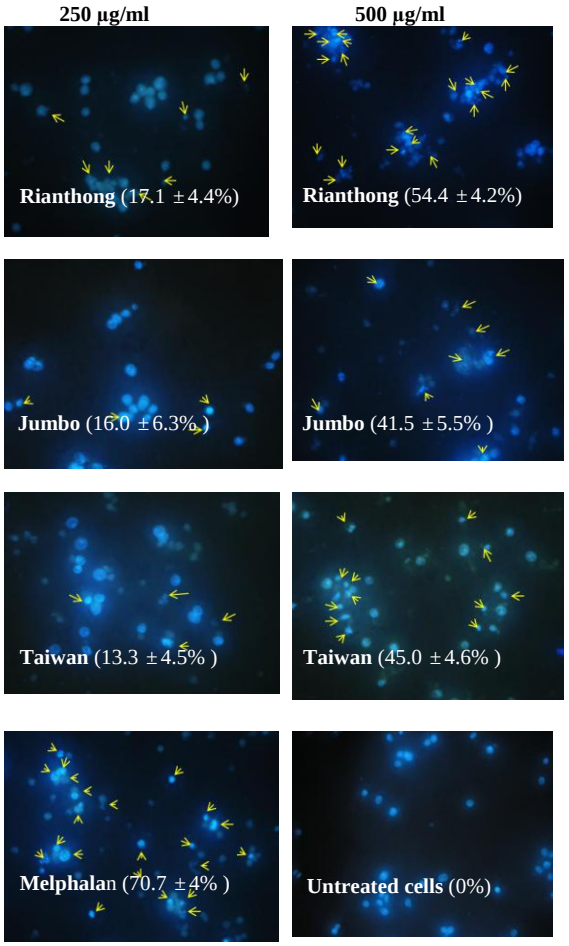

Fig. 1. Nuclei morphological changes and %apoptotic Jurkat cells treated with jujube seed extracts at 250 and 500 µg/ml for 24 h (40 × magnification). Arrows indicate apoptotic nuclei.

B. Detection of Induced Death Mode by Flow Cytometry

Annexin V - FITC staining was used to differentiate apoptotic cell death from necrotic cell death. The increases in apoptotic cell death induced by the extracts and melphalan were both concentration- and time-dependent (Fig. 2). The ethanolic extract of Jumbo seed caused more apoptotic cell death (early and late stage) than other cultivars. However, more necrosis was observed at higher concentration and longer incubation times for all jujube extracts.

The ethanolic extracts, but not the water extracts, of the three jujube cultivar seeds showed potential anti-cancer activity in the Jurkat cell line based on antiproliferation and apoptosis induction effects. No cytotoxicity was observed in the HepG2 cancer cell line or the normal Vero cells. It should be noted that melphalan was cytotoxic in normal Vero cells. Cultivar Taiwan caused the most cell death in Jurkat cells as measured by NR assay (Taiwan > Jumbo > Rianthong). Additional study of the cancer death mode revealed that the ethanolic extracts from the three cultivars of jujube seed induced late stage apoptotic cell death after 24 h at both $1 \times IC_{50}$ and $2 \times IC_{50}$ concentrations. Jumbo induced the most late stage apoptosis (Jumbo > Rianthong > Taiwan > melphalan), while, Taiwan induced more undesirable necrotic cell death (Taiwan > Jumbo > Rianthong > melphalan). Taking the antiproliferation and apoptosis induction effect into consideration with necrotic effects, the ethanolic extract of Rianthong seeds showed the most potential as an anti-cancer therapeutic.

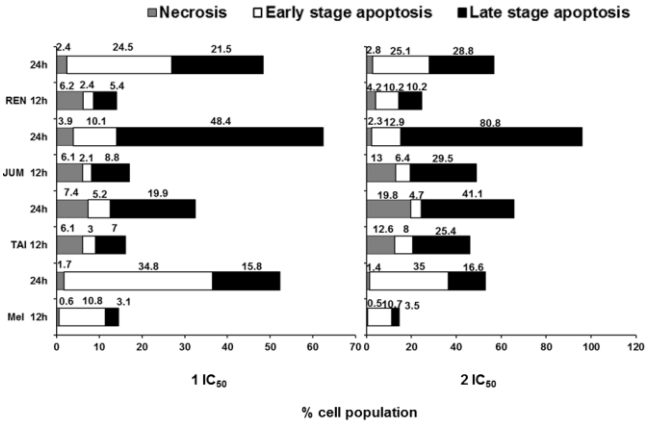

Fig. 2. %Cell population of Jurkat cells treated with the jujube seed extracts at 1 ×IC50 and 2 ×IC50 concentrations for 12 and 24 h.

Previous studies have shown *Ziziphus* species anti-cancer activity mediated via induction of apoptotic cell death in cancer cell lines [13]-[15]. The chloroform extract of *Z. jujuba* fruit has been reported to cause apoptosis induction effect against hepatocellular carcinoma cells (HepG2) [15], which is in contrast to our findings where both the water and ethanolic seed extracts of all jujube cultivars tested did not affect HepG2 cells. This suggests a different mechanism of anti-cancer activity in our jujube seed extracts, presumably due to the isolation of different active compounds by the different extraction methods. Another study showed that extracts from *Z. jujuba* fruit induced apoptosis in estrogen receptor alpha (ERα) positive (MCF-7) and ERα negative (SKBR3) breast cancer cells [14]. Similar to our study, the ethanolic extract from seed of *Z. mauritiana* was demonstrated to cause apoptotic cell death in human promyelocytic leukemia cells (HL-60), human acute lymphoblastic leukemia cells (Molt-4) and human cervical cancer cells (HeLa) without showing toxicity against normal human gingival fibroblast cells (HGF) [13].

IV. Conclusion

Here we reveal that ethanolic extracts from seeds of three jujube cultivars selectively induce cytotoxicity in a human T cell leukemic cell line via the apoptosis pathway. The different abilities of the three jujube cultivars to induce apoptosis urges for further studies to define the bioactive components and to understand the therapeutic actions of

these extracts.

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