Apoptosis-inducing effects of jujube (Zǎo) seed extracts on human Jurkat leukemia T cells

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Abstract

Background: Jujube (Zǎo) seeds exhibited anticancer effects and used in Chinese medicine for many years. This study aims to investigate the apoptosis-inducing effects of seed extracts from eight different cultivated species (Apple, Bombay, Jumbo, Kaew, Nomsod, Riantong, Samros, and Taiwan) on human Jurkat leukemia T cells.

Methods: We evaluated the effects of seed extracts from eight jujube cultivated species on human Jurkat leukemia T cells. The crude seed extracts were prepared sequentially by using water, 95% ethanol, dichloromethane, ethyl acetate, chloroform or hexane. The antiproliferative effects of the jujube seed extracts relative to that of melphalan were evaluated by neutral red assays. Apoptotic cell death induced by the ethanolic extracts at 1 × IC₅₀ and 2 × IC₅₀ concentrations was demonstrated by DAPI staining, gel electrophoresis, flow cytometry with Annexin V/propidium iodide staining, and caspase-3, -8, and -9 enzyme activities.

Results: Ethanolic extracts of ‘Taiwan’, Jumbo, Nomsod, Riantong, Samros, and Bombay, significantly inhibited the proliferation of Jurkat cells compared with untreated cells (all $P < 0.001$), while the extracts of ‘Kaew’ and ‘Apple’ were inactive. The six active extracts preferentially induced apoptotic cell death in a concentration-dependent manner with DNA fragmentation ($2 \times IC_{50}$). Increased caspase-3 activity was detected after treatment with the six extracts. The ‘Taiwan’, Nomsod, Jumbo, and Riantong extracts ($2 \times IC_{50}$) induced both the extrinsic and intrinsic apoptosis pathways by increasing caspase-8 and caspase-9 activity, respectively. Alkaloids (Dragendorff’s method) and reducing sugars (Fehling’s test) were mainly identified in the apoptosis-inducing extracts.

Conclusions: The tested of six active extracts (‘Taiwan’, ‘Jumbo’, ‘Nomsod’, ‘Riantong’, ‘Samros’ and ‘Bombay’) contained alkaloids or reducing sugars, and induced caspase-dependent apoptosis in human Jurkat leukemia T cells.

Keywords: Jujube, Apoptosis, Jurkat leukemia T cells, Cancer

Background

Complementary and alternative medicines have been evaluated in addition to chemotherapy, radiation, and surgery in treating cancer. The development of an effective strategy for cancer drug discovery has been outlined [1]. Apoptosis is a major target for chemoprevention and chemotherapy [2].

Jujube (Zǎo) is a functional food with nutritional value that is consumed in Asia, the Mediterranean and the United States [3–6]. The two extant species include Indian jujube (Ziziphus mauritiana Lam.) and Chinese jujube (Ziziphus jujuba Mill.) [4], and were reported to exhibit sedative, anodyne, pectoral, stomachic, styptic, and tonic effects [7], as well as anticancer, antianxiolytic, antifungal, and antispastic effects [8].

Different parts of Z. mauritiana and Z. jujuba exhibit various medicinal effects. Z. jujuba has long been used as a crude drug in Chinese medicine [5]. The seeds of Z. jujuba enhanced cell membrane permeability for drugs [9] and exhibited sedative effects [10]. Linoleic and stearic acids from seed extracts of Z. jujuba significantly inhibited cyclooxygenase (COX)-1 and COX-2 activity.
4,6-diamidino-2-phenylindole (DAPI), copper (II) sulfoxide, and dimethylsulfoxide (DMSO) (molecular grade) were from Sigma-Aldrich Chemie GmbH (Saint-Quentin Fallavier, France). Trypan blue was from Fluka Chemika (Buchs, Switzerland) and potassium sodium tartrate was from Loba Chemie (Mumbai, India). Agarose molecular grade was purchased from Bio-Rad (Hercules, CA, USA). A 100 bp DNA ladder marker was purchased from Invitrogen (Carlsbad, CA, USA). Caspase-Glo® 3/7, 8 and 9 were purchased from Promega (Madison, WI, USA).

Preparation of crude extracts
Eight jujube cultivated species (‘Apple’, ‘Bombay’, ‘Jumbo’, ‘Kaew’, ‘Nomsod’, ‘Rianthong’, ‘Samros’ and ‘Taiwan’) were commercially cultivated and authenticated based on fruit and seed morphological characters by Assistant Professor Thaweesak Thitimatharoch, Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University. The jujubes were collected from the local market in Amphoe Muang, Khon Kaen, Thailand, in 2012. The picture vouchers of the specimens (NW-55-2012–NW-62-2012) were deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen province, Thailand. The seed characteristics of each jujube cultivated species (five samples for each) are presented in Fig. 1. The seed was separated and dried at ≤55 °C. The seeds were macerated in deionized water (1 g:3 mL) overnight. The water crude extract was collected after being freeze-dried. The remaining residue from water extraction was dried at 55 °C and further macerated with 95 % ethanol, dichloromethane, ethyl acetate, chloroform or hexane, respectively. Each round of solvent extraction lasted 72 h, using different dry residue to solvent ratios (1 g:3 mL). Solvent was removed by a rotary evaporator (IKA®, Staufen, Germany), resulting in a dry residue. Six types of crude extract were obtained for each cultivars. The crude extracts were stored at −20 °C. The concentrations of 10, 25, 50, 100, 250 and 500 µg/mL were used in the antiproliferation study.

Cell culture
The human Jurkat leukemia T cells were cultured in RPMI 1640 media while the normal African green monkey kidney epithelial cell line (Vero) was cultured in DMEM. Both cell lines were provided by Associate Professor Sahapat Barusrux. Both media were maintained with 10 % fetal bovine serum and 1 % penicillin–streptomycin. Cells were incubated at 37 °C and 5 % CO₂.
| Cultivated species | Seeds characteristics (n=5) |
|--------------------|---------------------------|
| 'Samro'            | 0.86 ±0.18 cm wide × 1.58 ±0.19 cm long, round shape, light brown |
| 'Bombay'           | 0.66 ±0.11 cm wide × 2.4 ±0.12 cm long, ovate, tapered at both ends, pale yellow |
| 'Apple'            | 1.1 ±0.11 cm wide × 1.5 ±0.18 cm long, round shape, rough surface with tiny spines, off-white |
| 'Taiwan'           | 1.04 ±0.11 cm wide × 2.94 ±0.19 cm long, ovate, pale yellow |
| 'Nomsod'           | 1.12 ±0.21 cm wide × 2.6 ±0.21 cm long, ovate, light brown |
| 'Jumbo'            | 1.1 ±0.15 cm wide × 2.52 ±0.16 cm long, ovate, brown |
| 'Kaew'             | 0.54 ±0.11 cm wide × 1.16 ±0.21 cm long, ovate, light brown |
| 'Rianthong'        | 0.6 ±0.12 cm wide × 1.7 ±0.16 cm long, ovate, brown |

Fig. 1 Characteristics of jujube seeds
**Antiproliferation assay**
The antiproliferation test was determined based on modified neutral red assay [30]. A Jurkat cell density of $5 \times 10^5$ cells/mL and Vero $3 \times 10^5$ cells/mL were seeded in 96-well plates and maintained at 37 °C for 24 h in humidified 5 % CO₂. The extracts dissolved in DMSO were pipetted into each well to generate various final concentrations ranging between 10 and 500 µg/mL. The final concentration of DMSO did not exceed 1 % v/v to minimize the cytotoxic effect of DMSO [30]. After treatment with the extracts for 24 h, cells were re-suspended. Neutral red dyes (50 µg/mL) were added to each well. Cells were incubated for another 2 h, then washed with 1 × PBS (pH 7.4) and lysed with 0.33 % HCl in isopropanol. The absorbance of viable cells and dye solution was measured at 537 nm (650 nm reference wavelength) by a microplate reader (TECAN, Grödig, Austria). The untreated cells comprised the control group and cells treated with alkylating drug melphalan are the positive control. A plot of %cell viability and various extract concentrations was used to calculate the IC₅₀—which represents the concentration possessing 50 % antiproliferation. The selectivity index (SI) was calculated from the IC₅₀ of the extracts in normal Vero cells over the Jurkat cancer cells to indicate the cytotoxic selectivity (i.e., safety) of the crude extracts [31].

**Detection of apoptosis**

**Nuclear morphological alteration**

Morphological changes in cell nuclei undergoing apoptosis was determined by DAPI staining [32]. The stained nuclei were then detected by a fluorescent microscope (Nikon Eclipse 80i, Kanagawa, Japan). Cells were seeded in 24-well plates at a density of $3 \times 10^5$ cells/mL per well and incubated for 24 h. Cells were treated with 500 µg/mL extracts and incubated for another 24 h. Then cells were washed with PBS and collected by centrifugation (Daihan Scientific, Seoul, Korea) at 1677g for 5 min. Cells were fixed in ice-cold methanol for 15 min. After the methanol was removed, the cells were incubated for 30 min, at room temperature, in the dark, with 0.3 µg/mL DAPI. The mixture of PBS to glycerin (at a 1:1 ratio) was added to achieve a 20-µL volume. Cells were wet-mounted on a glass slide and observed under a fluorescent microscope by fluorescence filters with an excitation band of 358 nm and an emission of 461 nm. The images of stained nuclei were captured by the NIS-Element AR 3.2 imaging software (Nikon Instruments Inc, NY, USA).

**Mode of cell death**

Flow cytometry was used to determine various types of cell death including early and late stage apoptosis as well as necrosis. Annexin V-FITC and propidium iodide (Annexin V-FITC apoptosis detection kit, eBiosciences, Inc., San Diego, CA, USA) [33]. Cells were seeded in 24-well plates at a density of $5 \times 10^5$ cells/mL per well and incubated for 24 h. Cells were treated with the crude extracts at a concentration of $1 \times \text{IC}_{50}$ and $2 \times \text{IC}_{50}$ obtained from the antiproliferation study at 12 and 24 h. Afterward, cells were harvested by centrifugation (Wisds’ Laboratory Instruments, Korea) at 1677g for 5 min then the supernatant was removed. The cells were washed with 200 µL of 1x binding buffer. After removal of the supernatant, 95 µL of binding buffer and 5 µL Annexin V-FITC were added and the mixture incubated in the dark for 15 min at room temperature. Then 95 µL of binding buffer and 5 µL of propidium iodide (final concentration of 2 µg/mL per cell sample) were pipetted into an Eppendorf tube and the mixture incubated for 15 min in the dark at the room temperature. Cells were re-suspended in 200 µL of binding buffer. The stained cells were analyzed immediately by flow cytometry (BD FACSCanto II, Franklin Lakes, NJ, USA) by FACSDiva software version 6.1.3 (BD Biosciences, San Jose, CA, USA).

**Caspases activity**

The activity of caspase-3/7, -8 and -9 were evaluated to confirm whether apoptosis was induced by the jujube seed extract(s) and to determine the apoptosis induction pathway. Jurkat cells ($1.3 \times 10^4$ cells/well) were seeded into 96-well white plates (Costar™, Corning, NY, USA). Cells were incubated at 37 °C for 24 h and extracts added to each well for a final concentration of $2 \times \text{IC}_{50}$. Treated cells were incubated at various intervals. Supernatant (50 µL) was pipetted out of each well and 50 µL of caspase reagent mixture added and incubated in the dark for 40 min [34]. The relative luminescence units (RLU) were measured at 562 nm by a Multifunction Microplate Reader (Varioskan™ Flash Multimode Reader, Thermo Scientific, USA) equipped with SkanIt Software 2.4.3 DDE’s program (Thermo Scientific, Waltham, MA USA).

**DNA fragmentation**

Late stage apoptotic death mode was confirmed by a DNA fragment assay. Cells ($2 \times 10^6$ cells/mL density) were seeded in 24-well plates and incubated for 24 h. Cells were treated with the jujube seed extracts as described above in the cell death mode assay. Cells were harvested and washed with PBS. After PBS was removed, 300 µL of lysis buffer (FlexiGenen DNA kit; Qiagen, Germany) was added to each well and mixed thoroughly. After that 150 µL of denaturation buffer and 20 µL of Protease K (10 mg/mL) were added to the reaction mixture. Cells were incubated at 65 °C for 15 min and 600 µL of absolute isopropanol added and thoroughly mixed until the DNA became visible. DNA was collected...
after centrifugation (Daihan Scientific, Seoul, Korea) at 10,000 g for 5 min. The supernatant was discarded and the pellet washed with 70 % ethanol. After the liquid was removed by inverting the Eppendorf tube onto a clean piece of paper, 15 µL of hydration buffer was added to dissolve the DNA for 15 min at 65 °C. The resulting 300 ng/µL of DNA (DNA counting by GE healthcare Life Sciences, United Kingdom) was analyzed by electrophoresis on 1.8 % agarose gels containing 0.75 % ethidium bromide. The DNA was mixed with 6X loading dye and the gel was electrophoresed in 0.5xTBE buffer at 90 V for 1 min and 50 V for 1 h. DNA fragmentation was visualized by a UV transilluminator (Vilberlourmat, Germany) and the image was captured.

Alkylating reaction in vitro
An alkylation reaction of the jujube seed extract was performed to understand the interaction of the extract with DNA resulting DNA damage. The alkylation assay was conducted following the method modified by Machana et al. [30]. NBP (final concentration 20 mM) was prepared in acetonitrile. The buffer solution (pH 4) comprised 0.5 µM of citric acid, 1 µM of boric acid, 4.9 µM of sodium chloride, and 1 M sodium hydroxide adjusted to a volume of 10 mL with deionized water. The mixture of 180 µL NBP and 420 µL buffer solution was prepared and heated to 70 °C for 30 min. The extracts at 2 × IC$_{50}$ concentrations dissolved in acetonitrile were pipetted into the NBP mixture. Then, 120 µL of solution mixture was pipetted and added into a 160 µL ethanol–acetonitrile mixture at a ratio of 1:3. The wavelength of NBP adduct was measured at 580 nm at different time points (5, 15, 30, 45, 60 and 75 min).

Phytochemicals screening
Phytochemical screening was performed to identify the presence of the main phytoconstituents in 0.025 g of jujube seed extract of each cultivar. Identification of alkaloids, saponins and carbohydrates were tested as per Anandanayaki [35], while identification of flavonoids was conducted as per Bello et al. [36].

Alkaloids identification
Jujube seed extracts were dissolved in 500 µL of methanol and centrifuged (Daihan Scientific, Seoul, Korea) at 268.3 g for 10 min. HCl (1 %, 1 mL) and 1 drop of Dragendorff’s reagent (potassium bismuth iodide) was added to the supernatant. An orange or reddish-brown precipitate indicated a positive test.

Flavonoids identification
Methanol (2.5 mL) and 0.5 mL of 10 % NaOH were added to the extracts. A few drop of 1 % HCl was added to 0.5 ml of supernatant. The disappearance of the yellow color indicated the presence of flavonoids.

Saponins identification
The extracts were diluted with deionized water to a final volume of 1.5 mL and shaken for 30 min. Permanent foaming indicated the presence of saponins.

Reducing sugar identification
Fehling’s solution A was prepared by dissolving 3.5 g of copper II sulfate in deionized water and adjusted to 50 mL. Fehling’s solution B was prepared by dissolving 17.5 g potassium sodium tartrate with 5 g sodium hydroxide in 50 mL of deionized water. The extracts were diluted in 1.5 mL of deionized water, heated with Fehling’s A and B solution (mixture 1:1) for 10 min. An orange-red precipitate indicated a positive result and the presence of reducing sugar.

Statistical analysis
The results from multiple independent experiments were expressed as mean ± standard deviation. One-way ANOVA was used to test for significant differences between means. P < 0.05 was considered statistically significant. The variation between groups was tested by the Tukey range test with IBM SPSS software version 19.0 (SPSS Inc., USA). The concentration- and time-dependence shown in the graphs was visually determined.

Results and discussion
Characteristics of seeds
The eight jujube cultivars had diverse seed characteristics (Fig. 1). Generally, the cultivars had different shapes, sizes, and gnarled seed surfaces, with the average width and length being 0.5 cm and 1.6 cm, respectively. The ‘Nomsod’ and ‘Taiwan’ cultivars had the largest seed sizes, while the ‘Kaew’ cultivar had the smallest.

Antiproliferative effects
The antiproliferative effects of seed extracts from the eight jujube cultivars were evaluated on the leukemic Jurkat cell line, in comparison with the non-cancer Vero cell line, by neutral red assays (Table 1). The cationic neutral red dye can penetrate through cell membranes and lysosomal membranes to attach to anionic charges in the lysosomal matrix of living cells [37]. The antiproliferative effects were represented as IC$_{50}$ values calculated from linear plots between the concentrations and the %cell viability. Extracts with the maximum concentration (500 µg/mL) that inhibited cell viability by <50 % were classified as inactive extracts [30]. Seeds extracted using water, dichloromethane, ethyl acetate, or hexane inhibited Jurkat cell viability by <50 %
Table 1 Antiproliferative activity of different jujube seed extracts on non-cancer Vero cell line and T cell leukemic Jurkat cell line evaluated by the NR assay for 24 h incubation

| Sample      | IC_{50} (µg/mL) | Selectivity index |
|-------------|-----------------|-------------------|
| Vero cell line |                 |                   |
| Water       |                 |                   |
| Ethanol     |                 |                   |
| Dichloro-methane |           |                   |
| Ethyl acetate |               |                   |
| Chloroform  |                 |                   |
| Hexane      |                 |                   |
| Jurkat cell line |            |                   |
| Water       |                 |                   |
| Ethanol     |                 |                   |
| Dichloro-methane |           |                   |
| Ethyl acetate |               |                   |
| Chloroform  |                 |                   |
| Hexane      |                 |                   |

| Sample      | IC_{50} (µg/mL) | Selectivity index |
|-------------|-----------------|-------------------|
| 'Samros'    | Inactive        |                   |
| 'Bombay'    | Inactive        |                   |
| 'Apple'     | Inactive        |                   |
| 'Taiwan'    | Inactive        |                   |
| 'Nomsod'    | Inactive        |                   |
| 'Jumbo'     | Inactive        |                   |
| 'Kaew'      | Inactive        |                   |
| 'Rianthong' | Inactive        |                   |
| Melphalan   | 75.0 ± 2.3      |                   |

Data presented as IC_{50} values and selectivity index in Jurkat cell line (n = 3)

Means in the same column with different superscript letter(s) are significantly different, P < 0.05 (one-way ANOVA). Inactive means at the extract caused <50 % cytotoxicity even using the maximum concentration at 500 µg/mL.

nd not determined due to their low yield
even when the maximum concentration of 500 µg/mL was used. Therefore, these solvent extracts were defined as inactive against both Jurkat and Vero cell lines. The ethanolic extract of 'Taiwan' (232.4 ± 7.8 µg/mL) exhibited the highest antiproliferative effect, followed by the ethanolic extracts of 'Jumbo' (312.2 ± 18.3 µg/mL), 'Nomsod' (333.4 ± 2.8 µg/mL), 'Rianthong' (401.6 ± 9.9 µg/mL), 'Samros' (417.7 ± 10.4 µg/mL), and 'Bombay' (487.9 ± 17.5 µg/mL). These six ethanolic extracts showed significant antiproliferative effects compared with the untreated cells (all P < 0.001). The antiproliferative effects of individual pairs of 'Jumbo' vs. 'Nomsod' (P = 0.322) and 'Samros' vs. 'Rianthong' (P = 0.841) did not differ significantly. The chemotherapeutic drug melphalan, used as the positive control, showed nonselective antiproliferative effects on Jurkat cells over Vero cells with IC50 values of 119.1 ± 10.4 µg/mL vs. 75.0 ± 2.3 µg/mL, respectively. Although the IC50 values of the ethanolic extracts were higher than those of melphalan (i.e., lesser antiproliferative effects) (all P < 0.001), the selectivity index of the ethanolic extracts toward Jurkat cells was higher than that of melphalan.

The antiproliferative effects of the ethanolic extracts of the jujube seeds on normal cells were in agreement with previous studies [23, 24, 38]. Ethanolic extracts of Z. mauritiana seeds showed antiproliferative effects on HL-60, HeLa, and Molt-4 cell lines without affecting the normal HGF cell line [24], and exerted no cytotoxicity on normal rat liver cells [38]. A chloroform extract of Z. jujuba fruit pulp showed antiproliferative effects on MCF-7 and SKBR3 cells without toxicity on normal cells [23]. The present study documented the nonselective cytotoxicity of chloroform seed extracts on Jurkat cells. Owing to the low yields of some chloroform extracts, their cytotoxicity was not determined. Six of the eight ethanolic extracts (i.e., ‘Samros,’ ‘Bombay,’ ‘Taiwan,’ ‘Nomsod,’ ‘Jumbo,’ and ‘Rianthong’) were selected to determine the mode of cell death.

Apoptosis morphological changes

As shown in Fig. 2a, the normal nuclei of untreated cells had an intact round morphology, whereas the apoptotic nuclei of cells treated with 500 µg/mL melphalan showed chromatin condensation with heterogeneous staining (Fig. 2b, c) and apoptotic body formation (Fig. 2d). The nuclear morphologies of Jurkat cells treated with the ethanolic seed extracts (500 µg/mL) for 24 h were shown in Fig. 2e. The six ethanolic jujube seed extracts induced apoptosis with nuclear morphological changes reminiscent of apoptotic characteristics (i.e., cell shrinkage, chromatin condensation, membrane blebbing, and apoptotic body formation).

Mode of cell death

The stages of apoptosis can be differentiated from necrotic cell death by flow cytometry using Annexin V-FITC/propidium iodide (PI) staining of cells. Apoptotic lymphocytes are identified by their externalized phosphatidylserine (PS) bound with Annexin V-FITC (green), while necrotic cells are identified by their DNA intercalation of PI (red) [39]. During the early stages of apoptosis, the PS comprising the cell membrane flip to the outside and bind with Annexin V-FITC, showing a cell dot plot in quadrant 4. Damaged or injured cells lose their membrane integrity, including nuclear membrane integrity, such that PI can pass through the nuclear membrane and appear in quadrant 1 (necrotic cells). In the late stages of apoptosis, cells are double-stained and appear in quadrant 2. Normal cells are not stained and appear in quadrant 3.

The ethanolic jujube seed extracts induced cell death in a concentration- and time-dependent manner through diverse modes of cell death (Fig. 3). In general, the extracts induced relatively high late-stage apoptosis and low necrosis compared with melphalan, which induced more early-stage apoptosis. After incubation for 12 h, the 2 × IC50 concentrations of ‘Bombay’ (99.2 ± 0.4 %), ‘Samros’ (95.1 ± 1.8 %), and ‘Nomsod’ (90.2 ± 2.7 %) were the first three extracts to induce high total apoptosis (>90 %) in Jurkat cells. The total apoptosis inductions for ‘Bombay’ vs. ‘Samros’ (P = 0.988), ‘Bombay’ vs. ‘Nomsod’ (P = 0.928), and ‘Samros’ vs. ‘Nomsod’ (P = 0.050) did not differ significantly. Under the same conditions (12 h, 2 × IC50), only ‘Samros’ (29.9 ± 2.6 %) (P = 0.004) and ‘Bombay’ (20.9 ± 1.4 %) (P < 0.001) significantly induced higher %early-stage apoptosis than melphalan (10.7 ± 0.4 %). By contrast, the 2 × IC50 concentrations of ‘Nomsod’ (79.2 ± 3.0 %) (P < 0.001), ‘Bombay’ (78.3 ± 1.6 %) (P < 0.001), and ‘Samros’ (65.2 ± 1.2 %) (P < 0.001) caused more significant late-stage apoptosis than melphalan (3.5 ± 0.3 %) and the other extracts.

At 24 h, the 2 × IC50 concentration of ‘Bombay’ showed the highest %total apoptosis (97.3 ± 2.9 %) followed by those of ‘Jumbo’ (93.7 ± 2.4 %), ‘Nomsod’ (86.0 ± 1.9 %), ‘Samros’ (80.1 ± 3.2 %), ‘Rianthong’ (53.9 ± 2.6 %), melphalan (51.6 ± 1.1 %), and ‘Taiwan’ (45.8 ± 3.0 %). Only the 2 × IC50 concentration of ‘Bombay’ (46.1 ± 2.5 %) (P < 0.001) was found to induce early-stage apoptosis significantly more than melphalan (35.0 ± 2.2 %). Furthermore, the first three jujube seed extracts that induced high %late-stage apoptosis were ‘Jumbo’ (80.8 ± 0.8 %), ‘Nomsod’ (75.8 ± 2.3 %), and ‘Bombay’ (51.2 ± 3.7 %). The %late-stage apoptosis for ‘Jumbo’ vs. ‘Nomsod’ did not differ significantly (P = 0.599, one-way ANOVA), while the %late-stage apoptosis of ‘Jumbo’ and ‘Nomsod’
'Bombay' did differ significantly ($P < 0.001$ and $P < 0.0001$, one-way ANOVA).

The six ethanolic seed extracts induced apoptosis in a concentration-dependent manner. The ranks of the %total apoptosis-inducing effects at the $2 \times IC_{50}$ concentrations after 24 h of incubation were in the descending order: 'Bombay', 'Jumbo', 'Nomsod', 'Samros', 'Rianthong', melphalan, and 'Taiwan'. Apoptosis in the HL-60 cell line was induced by the ethanolic jujube seed extracts in a concentration-dependent manner, and our findings were in agreement with a previous report [24].

The extracts needed to cause less necrotic cell death to achieve effectiveness of their anticancer action. After incubation for 12 h, the $2 \times IC_{50}$ concentrations of 'Jumbo' (13.0 ± 1.5 %), 'Taiwan' (12.6 ± 4.9 %), and 'Nomsod' (6.8 ± 2.4 %) induced relatively high necrotic cell death compared with the other extracts and melphalan (0.5 ± 0.3 %). At 24 h, the $2 \times IC_{50}$ concentrations of the extracts increased necrotic cell death in the descending order: 'Taiwan' (19.8 ± 2.0 %), 'Nomsod' (7.6 ± 0.8 %), 'Samros' (4.2 ± 0.5 %), and 'Jumbo' (2.3 ± 0.8 %).

**Caspase activity**

Apoptosis can be triggered through both extrinsic and intrinsic pathways, involving caspase cascades that act through initiator caspases and executioner caspases [40, 41]. The initiators are caspase-8 for the extrinsic pathway and caspase-9 for the intrinsic pathway. When the initiator caspases are activated by apoptotic stimuli, the
function of the executioner caspase-3 is triggered, which culminates in DNA fragmentation and apoptotic body formation [2, 39, 41]. The present study assessed the activity of both initiator caspases, caspase-8 and caspase-9, to distinguish the extrinsic pathway from the intrinsic apoptotic pathway. Executioner caspase-3 activity was also determined.

Increased caspase-3 activity was detected at 30 min in Jurkat cells (Fig. 4), suggesting that apoptosis induction by the jujube seed extracts was mediated through the caspase-mediated pathways. 'Taiwan', 'Nomsod', 'Jumbo', 'Rianthong', and melphalan all significantly increased caspase-8 and caspase-9 activity compared with untreated cells at different time points (all \( P < 0.001 \)). The increases in caspase-8 activity for 'Taiwan' vs. melphalan differed significantly (\( P = 0.006 \)). However, there were no significant differences in the increases in caspase-8 activity for 'Rianthong' vs. melphalan (\( P = 0.344 \)), 'Jumbo' vs. melphalan (\( P = 0.402 \)), 'Taiwan' vs. 'Rianthong' (\( P = 0.064 \)), 'Taiwan' vs. 'Jumbo' (\( P = 0.053 \)), and 'Rianthong' vs. 'Jumbo' (\( P = 0.999 \)). The increases in caspase-9 activity for 'Nomsod' vs. 'Taiwan' (\( P = 0.018 \)), 'Nomsod' vs. melphalan (\( P = 0.009 \)), 'Taiwan' vs. 'Jumbo' (\( P = 0.019 \)), 'Taiwan' vs. 'Rianthong' (\( P = 0.003 \)), 'Taiwan' vs. melphalan (\( P < 0.001 \)), and 'Jumbo' vs. melphalan (\( P = 0.009 \)) all differed significantly. In contrast, the increases in caspase-9 activity for 'Nomsod' vs. 'Taiwan' (\( P = 0.018 \)), 'Nomsod' vs. 'Jumbo' (\( P = 1.000 \)), 'Nomsod' vs. 'Rianthong' (\( P = 0.773 \)), 'Nomsod' vs. melphalan (\( P = 0.009 \)), and 'Jumbo' vs. 'Rianthong' (\( P = 0.771 \)) did not differ significantly.

'Samros' and 'Bombay' did not increase the caspase-8 and -9 activities, although they did activate caspase-3 activity. While activation of caspase-9 was not evident, the 'Samros' and 'Bombay' extracts were postulated to cleave the caspase-8 substrate earlier than 15 min because of the high activity at 15 min and lower activities at later time points. Activation of caspase-9 and -3 in a concentration-dependent manner in melanoma cells was previously reported for polysaccharides extracted from the fruit part of Z. jujuba [42]. The ethanolic extracts of 'Taiwan', 'Jumbo', 'Nomsod', 'Rianthong', 'Samros', and 'Bombay' induced apoptosis via a caspase-mediated pathway in a similar manner to melphalan, but to different extents. The ethanolic extracts of 'Taiwan', 'Nomsod', 'Jumbo', and 'Rianthong' and melphalan evidently induced apoptosis via the intrinsic and extrinsic pathways.
DNA fragmentation

DNA fragmentation occurs through the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of 180 bp and multiples thereof. Similar to the case for melphalan, all six ethanolic jujube seed extracts at 2×IC₅₀ concentrations clearly exhibited the characteristic DNA ladder formation (Fig. 5).

Alkylation effects of the selected crude extracts compared with melphalan

An alkylation reaction of the jujube seed extracts was performed to understand the interactions of the extracts with DNA resulting in DNA damage, by testing their alkylating activity. The nitrobenzylpyridine (NBP) assay was used as a model for nucleophilic DNA bases [43–45]. This reaction depends on high temperature and low acidic media to catalyze NBP, which is converted into a blue/violet chromophore product. This specific reaction is positively detected in the presence of electrophilic functional groups or alkylating agents. Only the alkylating drug melphalan led to NBP product formation, suggesting that the DNA damage induced by the jujube seed extracts did not arise from an alkylation reaction. The findings also implied that no electrophilic functional groups were present in the constituents of the jujube seed extracts.

Phytochemical constituents of the extracts

The phytochemical identification of the ethanolic jujube seed extracts showed that alkaloids predominated in all jujube cultivated species followed by reducing sugars (Table 2). Neither saponins nor flavonoids were found in the cultivars tested. The ‘Apple’, ‘Taiwan’, and ‘Nomsod’ extracts had relatively high amounts of alkaloids, followed by the ‘Samros’, ‘Bombay’, ‘Jumbo’, ‘Kaew’, and ‘Rianthong’ extracts. The ‘Nomsod’ and ‘Rianthong’ extracts had relatively high amounts of reducing sugars.

Ethanolic extracts of *Z. mauritiana* seeds contained alkaloids, terpenes, flavonoids, saponins, and tannins [46]. The alkaloids exhibited anticancer activity [47]. Alkaloids from *Z. jujuba* seeds (i.e., sanjoine K, zizyphusine, and amphibine) processed a sedative action [48, 49]. The fruit pulp of *Z. jujuba* induced apoptosis in various cancer cells, including breast cancer, liver cancer, and leukemia cells [7, 23]. The bioactive compounds in jujube with the anticancer effects (through induction of apoptotic cell death) were triterpene and betulinic acid [23, 24, 46, 50, 51].

The ethanolic extracts of jujube seeds from different cultivars exhibited diverse inhibition of leukemic Jurkat cell viability. The chloroform extracts and melphalan were toxic toward both Jurkat and Vero cells. The ethanolic extracts selectively caused death in Jurkat cells, but not in normal Vero cells. The ‘Taiwan’ extract exhibited the highest cytotoxicity followed by the ‘Jumbo’, ‘Nomsod’, ‘Rianthong’, ‘Samros’, and ‘Bombay’ extracts. The modes of cell death induced by these six jujube extracts were distinguished and confirmed by different methods. The Jurkat cells treated with these six ethanolic extracts exhibited various stages of apoptotic nuclear morphological alterations similar to those found in Jurkat cells treated with melphalan. The analyses for mode of cell death at 12 h, detected by flow cytometry, revealed that the ‘Bombay’ extract at 2×IC₅₀ induced significantly more apoptosis than the other extracts and melphalan (99.2 ± 0.4%; *P* < 0.001). At 24 h, the ‘Bombay’, ‘Jumbo’, and ‘Nomsod’ extracts induced apoptotic cell death at
97.3 ± 2.9 %, 93.7 ± 2.4 %, and 86.0 ± 1.9 %, respectively. Although ‘Taiwan’ and ‘Jumbo’ were the first two cultivars possessing high cytotoxicity, they also caused higher degrees of undesirable necrotic cell death. DNA damage was detected in the Jurkat cells treated with the ‘Taiwan’, ‘Jumbo’, ‘Nomsod’, ‘Rianthong’, ‘Samros’, and ‘Bombay’ extracts. This DNA damage did not occur via an alkylation reaction between the extracts and the nucleophilic DNA model, as confirmed by the NBP assays. The Jurkat cellular changes, reflecting apoptosis induced by the seed extracts of the six jujube cultivars, were mediated by caspases. Increased caspase-3 activity was detected after all six extract treatments. The ‘Taiwan’, ‘Nomsod’, ‘Jumbo’, and ‘Rianthong’ extracts induced apoptosis through both extrinsic and intrinsic apoptosis pathways, based on increases in caspase-8 and -9 activities, respectively. The ‘Samros’ and ‘Bombay’ extracts seemed to augment the initiator caspase-8 activity at an earlier time point, but did not induce the intrinsic apoptosis pathway.

Conclusions

The six active extracts (‘Taiwan’, ‘Jumbo’, ‘Nomsod’, ‘Rianthong’, ‘Samros’ and ‘Bombay’) contained alkaloids and reducing sugars, and induced caspase-dependent apoptosis in human Jurkat leukemia T cells.

Abbreviations

APP: ‘Apple’ cultivar; BOM: ‘Bombay’ cultivar; JUM: ‘Jumbo’ cultivar; KAE: ‘Kaew’ cultivar; Mel: chemotherapeutic drug melphalan; NOM: ‘Nomsod’ cultivar; RIA: ‘Rianthong’ cultivar; SAM: ‘Samros’ cultivar; TAI: ‘Taiwan’ cultivar.

Authors’ contributions

NW designed the study. NT performed the experiment, collected, and analyzed the data. NW and NT wrote the manuscript. NW, SB and SS revised the manuscript. All authors read and approved the final manuscript.

Table 2 Phytochemicals screening of the ethanolic seed extracts of eight jujube cultivars

| Phyto-chemicals     | Presence of phyto-chemical | 'Samros' | 'Bombay' | 'Apple' | 'Taiwan' | 'Nomsod' | 'Jumbo' | 'Kaew' | 'Rianthong' |
|---------------------|-----------------------------|----------|----------|---------|----------|----------|---------|--------|-------------|
| Alkaloids           | Orange or reddish brown precipitate | ++       | ++       | +++     | +++      | +++      | ++      | ++     | +           |
| Flavonoids          | Colorless                   | −        | −        | −       | −        | −        | −       | −      | −           |
| Saponins            | Permanent foam              | −        | −        | −       | −        | −        | −       | −      | −           |
| Reducing sugar      | Orange red precipitate      | −        | −        | −       | −        | −        | −       | −      | +           |

- −, absence of phytochemicals; +, presence of phytochemicals; +++, indicates the highest level; +, indicates the lowest level of phytochemical in positive results.

Fig. 5 DNA laddering based on gel electrophoresis after the cells were treated with 2 × IC50 of ethanolic jujube seed extracts and melphalan for 24 h.
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Competing interests
The authors declare that they have no competing interests.

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