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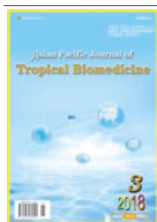

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Synsepalum dulcificum extracts exhibit cytotoxic activity on human colorectal cancer cells and upregulate *c–fos* and *c–jun* early apoptotic gene expression

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ABSTRACT

Objective: To explore cytotoxicity of *Synsepalum dulcificum* (*S. dulcificum*) Daniell (Sapotaceae) on human colon cancer (HCT-116 and HT-29), human monocytic leukemia (THP-1) and normal (HDFn) cell lines, and its effect on the expression of early apoptotic genes, *c–fos* and *c–jun*. **Methods:** Leaf, stem and berry of *S. dulcificum* were separately extracted by using 2 solvents, 10% ethanol (EtOH) and 80% methanol (MeOH). PrestoBlue® cell viability assay and qRT-PCR assay were conducted to examine the above objectives respectively. **Results:** Stem MeOH, stem EtOH, and berry EtOH extracts of *S. dulcificum* were cytotoxic to HCT-116 and HT-29 human colon cancer cells. For HCT-116, IC_{50} values of these 3 extracts were not significantly different ($P>0.05$) from that of the positive control bleomycin (IC_{50} of 33.57 μg/mL), while for HT-29, IC_{50} values of these 3 extracts were significantly lower ($P<0.05$) than that of bleomycin (IC_{50} of 25.24 μg/mL). None of the extracts were cytotoxic to the THP-1 monocytic leukemia cells and HDFn normal human dermal fibroblasts. For both HCT-116 and HT-29, these extracts significantly up-regulated ($P<0.05$) the expression of *c–fos* and *c–jun* compared to the untreated negative control. **Conclusions:** The results of this study suggest that cytotoxicity of stem MeOH, stem EtOH, and berry EtOH extracts of *S. dulcificum* on HCT-116 and HT-29 colon cancer cells is due to the induced apoptosis which is caused by the up-regulation of the expression of early apoptotic genes, *c–fos* and *c–jun*.

1. Introduction

Cancer is one of the major causes of death in both economically developed and developing countries[1,2]. In 2012, about 14.1 million people were diagnosed with cancer and 8.2 million deaths due to cancer were reported[2]. It has been estimated that about 1.68 million people will be diagnosed with cancer and 595 690 deaths will occur due to cancer in 2016 in United States alone[3]. Among all types of cancer, colorectal cancer is the third leading cause of death in women and fourth in men[2]. Cancer originates from the uncontrolled cell proliferation and suppression of apoptosis[4,5], an autonomous, genetically programmed cell death necessary for animal development and homeostasis of cell population[6–8]. Due to

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the enormous side effects which are brought together by the modern cancer therapies such as chemotherapy and radiotherapy, minimal side effect-causing methods, such as apoptosis induction through plant-derived anticancer agents, is being favored[9].

Synsepalum dulcificum (*S. dulcificum*) Daniell (Sapotaceae), which is popularly known as miracle fruit, is an ever-green shrub native in tropical west and west Central Africa. The miracle fruit is well known for its distinctive property of altering the tongue's reception of sour taste into sweet taste. This taste alteration is due to a protein called miraculin found in the berries[10–12]. Further studies on *S. dulcificum* revealed that the shrub not only possesses the ability to alter taste, but also possesses antioxidant, antibacterial, as well as anticancer activities[13–16]. Du *et al*. reported that the flesh and seed extracts exhibited antioxidant property, wherein the flesh extract exhibited significantly more or similar potencies as antioxidant standards[13]. Other groups also reported that the leaf[14] and stem[15] extracts showed antioxidant activity. Furthermore, Lu *et al*. reported that the leaf essential oil showed antibacterial activity against common experimental bacteria such as *Bacillus subtilis* and *Escherichia coli*[16]. Several groups showed that various part extracts of *S. dulcificum* exhibited anticancer activities on A375.S2 human melanoma cells[15], HepG2 human liver cancer cells[14], and K562 human myelogenous leukemia cells[16] in a dose-dependent manner.

Although the chemical compositions of all the parts of *S. dulcificum* are identified[13–19], and various effects of certain parts of the plant have been studied[13–16], the bioactivities, especially cytotoxicity of the parts of *S. dulcificum* and the molecular mechanism on how the extracts exhibit cytotoxicity on different cancer cell lines need further studies. Thus, this study aimed to determine the cytotoxicity of *S. dulcificum* extracts on HCT-116 and HT-29 human colorectal cancer cells, THP-1 human monocytic leukemia cells, and HDFn normal human fibroblasts, and to determine the effect of plant extracts on the expression of the early apoptotic genes, *c–fos* and *c–jun*.

2. Materials and methods

2.1. Plant material

S. dulcificum shrubs were obtained from the Agri-Aqua Network International, Inc. (AANI), Quezon City, Philippines. The identity of the shrub was authenticated by Dr. Emelina Mandia of the Biology Department, College of Science, De La Salle University, Manila.

2.2. Cell cultures

HCT-116 and HT-29 human colorectal cancer cells, and THP-1 human monocytic leukemia cells were previously procured from the American Type Culture Collection (USA), while HDFn human neonatal dermal fibroblasts were previously procured from Invitrogen (USA). The cell cultures were kindly provided by the Molecular Science Unit Laboratory of the Center for Natural Science and Environmental Research of De La Salle University, Manila. HCT-116, HT-29, and HDFn were cultured to 90% confluency in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (Invitrogen, USA) and 1 × anti-mycotic antibiotic (Invitrogen, USA) in 50 mL T-flasks (Falcon, USA) at 37 ℃ in 95% humidity and 5% CO_2 atmosphere. THP-1 was cultured to 90% confluence in Roswell Park Memorial Institute medium (Gibco, USA) with the same supplements and conditions as above. All cells were harvested by using 0.05% trypsin-EDTA (Gibco, USA) in phosphate buffered saline at pH 7.4 (Gibco, USA). All cells were harvested at their log phase.

2.3. Preparation of *S. dulcificum* extracts

The plant extracts were prepared following the methods of Du *et al*.[13] with slight modifications. The leaves, stems, and berries of *S. dulcificum* were harvested and cleaned first with tap water and rinsed twice in sterile distilled water. Thereafter, cleaned plant materials were dried for 1 wk at room temperature. The extraction procedure was as follows: Ten grams of each powdered sample were separately mixed with 100 mL of freshly prepared 80% MeOH and 10% EtOH solvent for 24 h with stirring using a magnetic stirrer. The mixtures were then centrifuged at 4 500 rpm for 10 min, and the solvent layers were collected in individual flasks. Another 100 mL of freshly prepared solvents were separately added to the residues, and the same procedure was followed to extract once more. The two collected solvent layers were combined accordingly and evaporated through rotary evaporator (IKA® RV10, USA). The evaporated solutions were then lyophilized at -40 ℃ to get the dry extracts (LABIONIO Freeze dry system/freezone®4.5, USA). The dried extracts were diluted with 0.2% DMSO in phosphate buffered saline to the concentration of 1 g/mL.

2.4. Cell viability assay

All cell lines were subjected to viable cell counts by using Trypan blue exclusion method and adjusted to the cell density of 1.0×10^5 viable cells/mL. One hundred microliters of each cell line were independently dispensed into sterile 96-well plates (Falcon, USA) resulting in 1.0×10^4 cells/well, and incubated for 24 h to allow the cells to attach and form monolayers on the bottom of the wells. The test extracts were freshly prepared by diluting the crude extracts to a concentration of 200 μg/mL using 0.2% DMSO in complete DMEM as vehicle solvent and filter-sterilized by using 0.45 μm membrane filter (Millipore, USA). The extracts were applied to the respective wells following 2-fold serial dilution. For positive control, bleomycin at a concentration of 200 μg/mL was added into the respective wells, and 2-fold serial dilution was likewise conducted. For vehicle control, 2 rows of wells with cells were grown in complete DMEM

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with 0.2% DMSO. For negative control, another 2 rows of wells with cells were grown in complete DMEM and left untreated. Thereafter, the plates were incubated for a week and the applied plant extracts were removed from the well. The wells were washed with phosphate buffered saline to remove the pigments of the extracts, and 100 µL of fresh complete DMEM were added. Each well was added with 10 µL of PrestoBlue® resazurin reagent (Invitrogen, USA), and re-incubated as before for 4 h. Absorbance measurement was accomplished at 570 nm in a microplate reader (Biotek EL × 800, BioTek Instruments, USA). Percent cell death from each treatment concentration was derived using the following formula[20]:

% cell death=100-[(Absorbance of sample/Absorbance of negative control) × 100]

Cytotoxicity graphs were constructed by plotting the % cell death against plant extract treatment concentration (µg/mL). Cytotoxicity index affecting 50% of the cells (IC_{50}) were derived from the cytotoxicity plots via linear regression.

2.5. qRT–PCR assay

The qRT-PCR assay procedure was adapted from the study of Shyu *et al*. with some modifications[21]. HCT-116 and HT-29, to which *S. dulcificum* extracts showed IC_{50} activities, were further subjected to qRT-PCR to determine the effect on the expression of the early apoptotic-response genes, *c–fos* and *c–jun*. One hundred microliters of each cell line with 1.0×10^5 viable cells/mL were separately placed into 96-well plates (1.0×10^4 cells/well), and incubated for 24 h to allow cell attachment and monolayer formation. Thereafter, each cancer cell line was exposed to 0.2% DMSO (vehicle control), bleomycin (positive control), and corresponding extracts of *S. dulcificum* which were cytotoxic to the cell lines using their IC_{50} concentrations. Treatment exposure was done for 30 min. Untreated cancer cell lines were used as negative controls.

Total RNA was then extracted from the cells by using TriZol® reagent (Invitrogen, USA). A final volume of 10 µL reaction was prepared containing 1 × KAPA SYBR FAST One-Step (KAPA Biosystems, USA), 0.3 µM of each forward and reverse primers, and 1 µL of extracted RNA template. Separate reactions were performed for *c–fos* and *c–jun* respectively, using the following primers[22,23]: *c–fos* forward (5′-AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT-3′) and *c–fos* reverse (5′-AGACGAAGGAAGACGTGTAAGCAGTGCAGCT-3′) with the expected product size of 612 bp; *c–jun* forward (5′-GCATGAGGAACCGCATTGCCGCCTCCAAGT-3′) and *c–jun* reverse (5′-GCGACCAAGTCCTTCCCACRCGTGCACACT-3′) with the expected product size of 409 bp. Simultaneously, amplified human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA at standard known concentrations of 10^9, 10^8, 10^7, 10^6 and 10^5 were used as standards. All qRT-PCR reactions were conducted by using the Rotor Gene thermocycler (Rotor Gene 3000 and Rotor Gene Q5-Plex HRM) which was programmed to run initial cDNA synthesis at 50 ℃ for 3 min, followed by 45 cycles of cDNA amplification (denaturation at 95 ℃ for 20 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 35 s). Melting analysis was set at 1 min increment from 72 ℃ to 95 ℃ to check and confirm PCR product specificity. Critical cycle thresholds (Ct) were automatically determined by using the built-in Rotor Gene software (RGQ v. 2.3.1.49.).

2.6. Statistical analysis

Student's *t*-test was performed to determine the statistical differences between the test reactions and controls. Data were presented as mean±SEM and probability value less than 0.05 ($P<0.05$) was considered significant.

3. Results

3.1. Cytotoxicity assay

Stem, leaf, and berry extracts of *S. dulcificum* were tested for their cytotoxicity on 4 cell lines, namely: HCT-116, HT-29, THP-1, and HDFn. Figure 1 shows the cytotoxicity graphs of the respective cell lines. When the extract concentration that was cytotoxic to at least 50% of the cells (IC_{50}) was higher than 100 µg/mL, the extract was considered non-cytotoxic[24,25]. The results showed that the extracts exhibited cytotoxicity in a concentration-dependent manner.

Stem methanol extract (Stem MeOH), stem ethanol extract (Stem EtOH), and berry ethanol extract (Berry EtOH) exhibited cytotoxicity on HCT-116 and HT-29, while leaf methanol extract (Leaf MeOH), berry methanol extract (Berry MeOH), and leaf ethanol extract (Leaf EtOH) were non-cytotoxic. None of the extracts were cytotoxic to THP-1 leukemia cell line and the normal human dermal fibroblast HDFn. Similarly, vehicle control (0.2% DMSO in complete DMEM) showed non-cytotoxicity to all tested cell lines (data were not shown). Positive control bleomycin was cytotoxic to all tested cell lines. Plant extracts that showed cytotoxicity were then calculated for their respective IC_{50} values by plotting respective linear regressions.

For HCT-116, IC_{50} values of stem MeOH, stem EtOH, berry EtOH, and bleomycin were 49.45 µg/mL, 44.19 µg/mL, 54.37 µg/mL, and 33.58 µg/mL, respectively. Statistical analysis showed that IC_{50} values of stem MeOH, stem EtOH, berry EtOH were not significantly different from that of bleomycin ($P>0.05$), indicating similar potencies of the plant extracts compared to bleomycin. Conversely for HT-29, IC_{50} values of stem MeOH, stem EtOH, berry EtOH, and bleomycin were 63.97 µg/mL, 54.46 µg/mL, 48.11 µg/mL, and 25.12 µg/mL, respectively. Statistical analysis showed that IC_{50} values of stem MeOH, stem EtOH, berry EtOH were significantly higher than that of bleomycin ($P<0.05$), indicating that bleomycin was significantly more cytotoxic than the plant extracts.

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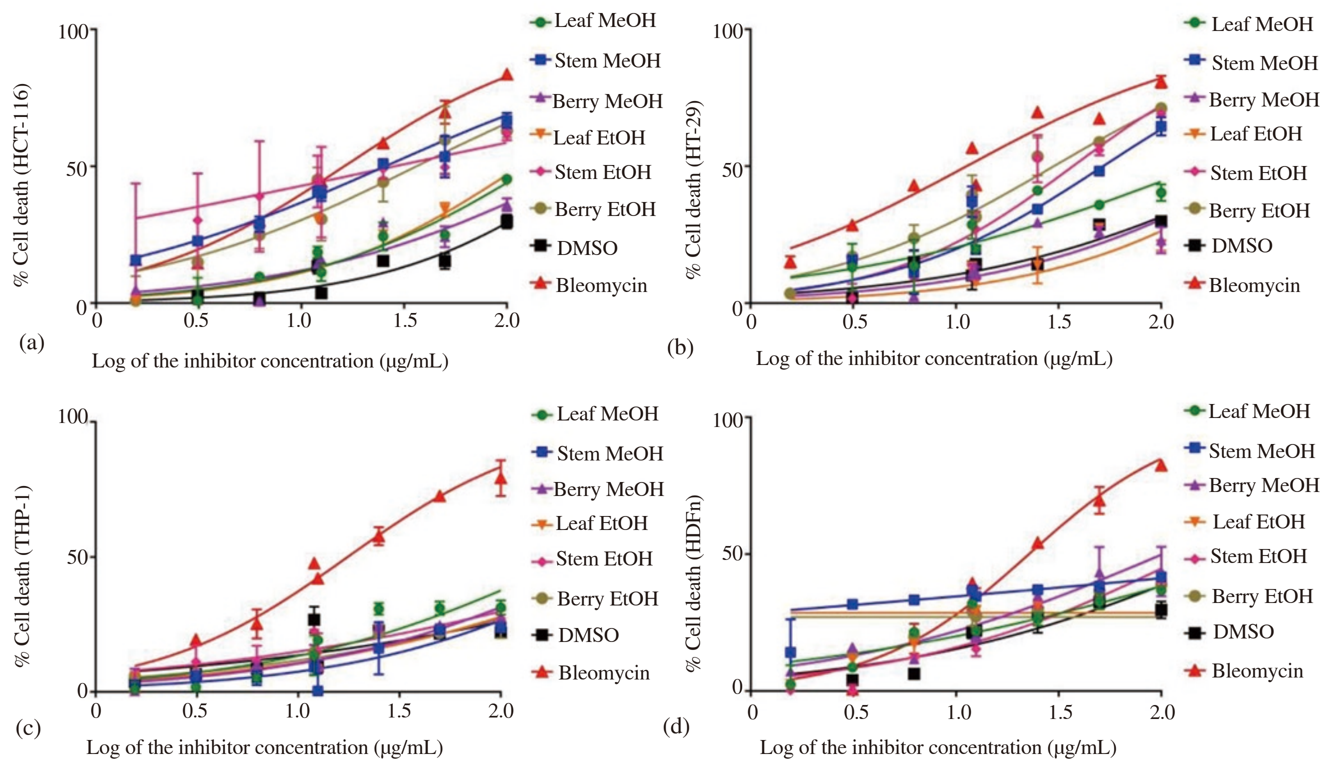

Figure 1. Cytotoxicity graphs for (a) HCT 116, (b) HT 29, (c) THP 1 and (d) HDFn after a week exposure to respective extracts of *S. dulcificum* and bleomycin.

3.2. qRT–PCR assay

qRT-PCR assay was performed to quantify the expressed *c–fos* and *c–jun* transcripts in HCT-116 and HT-29 colorectal cancer cells treated with the plant extracts using their respective IC_{50} concentrations which was derived from the cytotoxicity graphs. Melting analysis confirmed that the fluorescent signals of the amplicons in the extracts and bleomycin treated samples were *c–fos* and *c–jun*, and were not secondary to primer dimers. Figure 2 shows the quantified *c–fos* and *c–jun* transcripts after the exposure of HCT-116 and HT-29 to stem MeOH, stem EtOH, berry EtOH, bleomycin, and 0.2% DMSO. The results showed that both *c–fos* and *c–jun* transcripts expression were significantly up-regulated in both the colorectal cancer cells treated with the three plant extracts and bleomycin compared to the respective untreated cells ($P<0.05$). Expectedly, no significant up-regulation of transcript expression was observed in both colorectal cancer cell lines treated with 0.2% DMSO vehicle control compared to that of respective untreated controls ($P>0.05$).

4. Discussion

The present study showed that stem and berry extracts of *S. dulcificum* possess anticancer activity against HCT116 and HT29 human colorectal cancer cells, but not on THP-1 leukemia cells and HDFn normal human dermal fibroblasts. It has been known that the *S. dulcificum* extracts are rich in various amides and monoterpene-sesquiterpene derivatives, which possess antibacterial and anticancer activities[16,26]. Furthermore, it has been reported that plant-derived phenolics and flavonoids possess anticancer activities on various tumors[27]. *S. dulcificum* berry and stem were found to have phenolic compounds such as gallic acid, ferulic acid[13], syringic acid, and vanillic acid[15]. Moreover, *S. dulcificum* berry was found to have flavonoids such as quercetin, myricetin and kaempferol[13], which are known to bring low incidence of colon cancer[27]. However, for unknown reason, berry MeOH failed to induce apoptosis in tested cell lines. This may be due to the different capability of solvents to extract the potent components.

Although several groups have reported that the extracts of *S. dulcificum* exhibit anticancer activities against various cancer cell lines[14–16], the molecular mechanism by which the extracts exhibited these effects was not elucidated. Here we showed that stem and berry extracts of *S. dulcificum* induced apoptosis through upregulation of early apoptotic genes *c–fos* and *c–jun*. Several groups have reported that up-regulation of *c–fos* and *c–jun* mRNA expression is one of the markers of apoptosis[28–31]. The proto-oncogenes *c–fos* and *c–jun* are immediate-early genes that encode for proteins which form a transcription factor called activator protein-1 (AP-1)[32,33], which can be in heterodimer form of *c–fos* and *c–jun* products or homodimer form of *c–jun* products[32]. AP-1 is linked to various cell functions such as cell proliferation, differentiation and apoptosis[34]. It has

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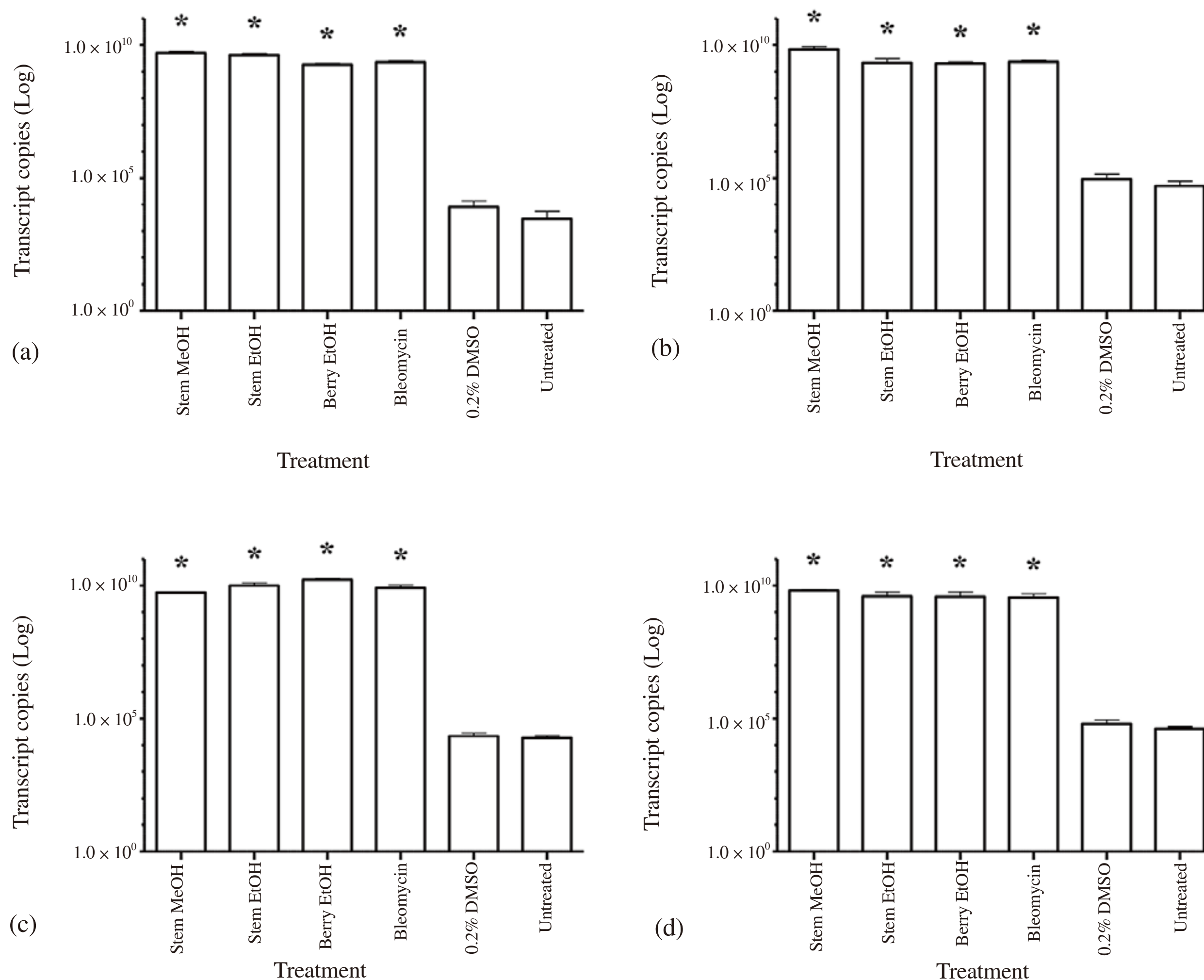

Figure 2. Quantified *c–fos* transcript copies on (a) HCT-116 and (c) HT-29 and the quantified *c–jun* on (b) HCT-116 and (d) HT-29. Significant difference (P<0.05) between the extracts and the untreated control is indicated with an asterisk (*).

been reported that AP-1 is a crucial element that is needed in the induction of transforming growth factor- β 1, a homodimer protein that induces apoptosis in cancer cells[35]. On the other hand, Shyu *et al*. assumed that upregulation of *c–fos* and *c–jun* transcripts and following apoptosis may be due to the subsequent AP-1 formation and its involvement in mitogen-activated protein kinase pathway or sensitization of the cancer cells to the tumor necrosis factor-related apoptosis-inducing ligand pathway[21]. However, the present study is limited to viewing the upregulation of *c–fos* and *c–jun*. Thus, further research on the cellular mechanisms by which the extracts mediate apoptosis needs to be elucidated.

Cytotoxic activity of *S. dulcificum* extracts were examined in the present study by using the PrestoBlue® resazurin assay. Stem MeOH, stem EtOH and berry EtOH extracts of *S. dulcificum* were found to be cytotoxic to HCT-116 and HT-29 colorectal cancer cells. The IC_{50} values of the three plant extracts showed that these were of comparable potencies with bleomycin for HCT-116 cells, but were less potent compared to bleomycin for HT-29 cells. None of the extracts were cytotoxic to THP-1 monocytic leukemia cells and HDFn normal human dermal fibroblasts. The study also revealed that the cytotoxicity of *S. dulcificum* on HCT-116 and HT-29 was due to the up-regulation of *c–fos* and *c–jun* transcript expression, suggesting an early apoptosis mechanism.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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