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Original Article

Apoptotic activities of the extract from *Moringa oleifera* leaves on human HCT116 colon cancer cells

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ABSTRACT — *Moringa oleifera* Lamk. (*M. oleifera*) is an edible plant and used for traditional medicine formulation. Some bioactive phytochemicals found in *M. oleifera* leaves thus far were identified as quercetin, chlorogenic acid, astragalin, and kaempferol. The flavonoid kaempferol was reported to induce apoptosis in human HCT116 colon cancer cells. Here, we investigated the anti-proliferative activity present in the methanol extract from *M. oleifera* leaves toward human HCT116 colon cancer cells. Fractionation of the methanol extract from *M. oleifera* leaves by gel filtration chromatography on Sephadex LH-20 enabled us to find anti-proliferative and apoptosis-inducing activities. Treatment of HCT116 cells with each pooled fraction (pf1, pf2, or pf3) inhibited the cell proliferation in a dose-dependent manner, and the inhibitory activities contained in pf2 and pf3 were more potent than that in pf1. Compared with kaempferol, pf1, pf2, and pf3 were found to exhibit strong anti-proliferative effects on HCT116 cells. Futhermore, treatment with pf1 induced much larger numbers of cleaved caspase-3-positive cells than that with pf2 or pf3. The apoptosis-inducing activity found in each pooled fraction was higher than that of kaempferol. Cells treated with pf2 displayed the typical characteristics of apoptosis, such as membrane blebbing, nuclear condensation and apoptotic bodies, whereas cells treated with pf1 showed early apoptotic morphologies. In contrast, pf3 barely induced apoptosis despite its strong inhibition of cell proliferation. Taken together, these results suggest that, in addition to kaempferol, *M. oleifera* leaves may contain new substances having anti-proliferative and apotosis-inducing activities on HCT116 cells.

Key words: *Moringa oleifera* leaf, Cytotoxicity, Apoptosis, Cleaved caspase-3, Colon cancer

INTRODUCTION

Cancer is a leading cause of death, with 8.2 million deaths in 2012 (WHO, 2014; Brenner *et al.,* 2014). The phytochemical constituents present in plant-based foods and medical plants are mainly responsible for their anticancer effects (Zhou *et al.,* 2003). Since natural compounds are obtained from diverse sources, many anti-cancer drugs were discovered as a successful story of current drugs, such as ellipticine, paxitaxol, vincristine, and vinblastine (Sultana *et al*., 2014).

Moringa oleifera Lamk. (*M. oleifera*), a short, slender and perennial plant, belongs to the Moringaceae family and is widely cultivated in different locations of Southeast Asia, including India, Sri Lanka, Malaysia, and Philippines, and also in Africa, tropical America, and Mexico (Sharma *et al.,* 2013). *M. oleifera* is an edible plant and used for traditional medicine formulation. Over the past two decades, many studies have examined the nutritional and medicinal properties of *M. oleifera* (Anwar *et al*., 2007; Mbikay, 2012). For example, *M. oleifera* leaves showed anti-bacterial effects (Peixoto *et al*., 2011), anti-

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fungal activities (Chuang *et al.,* 2007), and other medicinal activities. The extracts from roots and leaves of *M. oleifera* showed inhibition of proliferation of epithelial ovarian cancer cells (Bose, 2007) and human epidermal carcinoma KB cells (Sreelatha *et al.,* 2011). Much attention has been paid to phytochemicals that are contained in *M. oleifera* leaves. Some bioactive phytochemicals found in *M. oleifera* leaves were identified as quercetin, chlorogenic acid, astragalin, and kaempferol. Their contents were shown to vary with geography, seasons, plant physiology, and also the methods for collection of leaves and extraction (Iqbal and Bhanger, 2006; Makkar and Becker, 1996; Khalafalla *et al*., 2010; Karthivashan *et al*., 2013).

From our preliminary studies, crude methanol extracts from *M. oleifera* leaves showed anti-proliferative activities against human HCT116 colon cancer cells. In this study, we fractionated the extract from *M. oleifera* leaves by gel filtration chromatography on Sephadex LH-20 and examined the effects of the fractionated extracts on cell viability and apoptosis in HCT116 cells. Furthermore, by comparing with the flavonoid "kaempferol" found in *M. oleifera* leaves, which was reported to induce apoptosis in HCT116 cells (Li *et al*., 2009), we examined whether there was the possibility that new pro-apoptotic substances are present in methanol extracts from *M. oleifera* leaves.

MATERIALS AND METHODS

Chemicals and antibodies

Sephadex LH-20 was purchased from GE Healthcare (Waukesha, WI, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Adriamycin (ADR) and kaempferol were obtained from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (D-MEM) was obtained from Life Technologies (Gaithersburg, MD, USA). Anti-cleaved caspase-3 antibody and Alexa Fluor 647-labeled IgG secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA) and Life Technologies, respectively.

Plant material collection and extraction

Fresh leaves of *M. oleifera* were harvested from January-December 2012-2013 in Nakhon-Pathom province, Thailand. Then, they were washed and dried in a hot air oven at 50-60°C for a few days. The dried leaves were blended by homogenization and submerged in 100% methanol at 50-60°C for 3 days using a Soxhlet Extractor. The resulting extracts were completely dried using an evaporator, and the dried extract was stored at 4°C with protection from light.

Gel filtration on Sephadex LH-20

The dried methanol extract from *M. oleifera* leaves was freshly dissolved in 70% (v/v) aqueous ethanol at 1 mg/mL and filtered through 0.45-µm pore filter membranes (Merck Millipore, Bedford, MA, USA) just before use. The extract from *M. oleifera* leaves was fractionated using a 2.5 × 37.5 cm glass chromatography column (Bio-Rad, Richmond, CA, USA) packed with swollen Sephadex LH-20 in 70% (v/v) ethanol as the mobile phase. Each fraction was collected every 5 mL and determined at UV 260 nm (Robards *et al.*, 1999; Xiao *et al*., 2004; Ma *et al*., 2013) using a SmartSpec™ 3000 spectrophotometer (Bio-Rad). The collected fractions were later grouped into four pooled fractions (pf) on the basis of their spectral readings. The pooled fractions were condensed by evaporation and freeze-drying and stored at -20°C in the dark prior to further analysis.

Cell viability

The effect of the extract from *M. oleifera* leaves on cell viability of human HCT116 colon cancer cells was determined using the MTT assay. In brief, cells suspended in D-MEM containing 10% fetal bovine serum (FBS) were plated 5,000 cells/well in a 96-well plate and incubated overnight at 37°C. The pooled fractions were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted with D-MEM to a final concentration of 0.5% DMSO. The cells were incubated with varying concentrations of the *M. oleifera* pooled fractions for 24 or 48 hr in triplicate cultures, compared with ADR and kaempferol as positive controls. Cells incubated with 0.5% DMSO (vehicle) was used as a negative control. After the incubation period, each well was washed with phosphate-buffed saline (PBS) and replaced with 1 mg/mL MTT solution for 4 hr incubation. The resulting crystals product was dissolved in 100 µL of 100% DMSO and measured at 595 nm using a FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of cell viability was calculated as previously described (Sreelatha *et al*., 2011).

Double staining for cleaved caspase-3 and propidium iodide

Cells were seeded at 1.0×10^4 cells in 96-well plates and cultured in D-MEM containing 10% FBS overnight. Cells treated for 24 hr with the pooled fraction 1-4 (pf1, pf2, pf3, and pf4) were trypsinized, fixed in 4% paraformaldehyde for 30 min, and permeabilized with ice-cold 70% ethanol for 1 hr (Kubota *et al*., 2014). After washing

with PBS containing 0.1% Tween 20, cells were reacted with anti-cleaved caspase-3 antibody for 1 hr at room temperature and then stained with Alexa Fluor 647-conjugated anti-rabbit IgG antibody for 1 hr. Subsequently, cells were treated with 200 µg/mL RNase A and 50 µg/mL propidium iodide (PI) at 37°C for 30 min to stain DNA. A minimum of 3,000 cells per sample was analyzed by flow cytometry using a Guava easyCyte flow cytometer (Merck Millipore) equipped with a 488-nm blue laser and a 640-nm red laser using liner amplification. Data were analyzed using Flowing Software version 2.5.1 (Perttu Terho, Centre for Biotechnology, Turku, Finland).

Immunofluorescence

Confocal images and Nomarski differential-interference-contrast (DIC) were obtained by using a Fluoview FV500 confocal laser scanning microscope with a 40 × 1.00 N.A. dry objective (Olympus, Tokyo, Japan), as described (Aoyama *et al*., 2011; Aoyama *et al*., 2013; Kubota *et al*., 2013). One planar (xy) section slice (1.0-µm thickness) was shown in all experiments. In brief, cells detached by trypsinization were fixed in 4% paraformaldehyde for 30 min, and permeabilized with 70% ethanol for at least 1 hr at -30°C. Cells were subsequently reacted with anti-cleaved caspase-3 antibody for 1 hr, washed with PBS containing 0.1% Tween 20, stained with Alexa Fluor 647-conjugated secondary antibody for 1 hr. For DNA staining, cells were treated with 200 µg/mL RNase A and 50 µg/mL PI for 30 min. After staining, cells were mounted with Prolong Antifade™ reagent (Life Technologies).

Statistical analysis

Data are expressed as means ± S.D. of three independent experiments. Statistical analysis of the experimental data was performed by two-way T-test, and P-values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Fractionation of the methanol extract from *M. oleifera* leaves by Sephadex LH-20 chromatography

To fractionate the components of the extracts from *M. oleifera* leaves on the basis of molecular size, we performed gel filtration chromatography on a Sephadex LH-20 column. Sephadex LH-20, which is characterized by unique chromatographic selectivity due to dual hydrophilic and hydrophobic nature of the gel matrix, is used for fractionation of organic-soluble natural products, such as steroid, fatty acids, and phytochemicals (Amarowicz *et al*., 2003). The elution profile of the extracts from *M. oleifera* leaves was shown using 70% ethanol as an eluent (Fig. 1). Despite the successful fractionation, only limited amounts were yielded from each fraction so that we could not measure the effects of each fraction on cell proliferation. Consequently, we were obliged to combine each fraction into four pooled fractions (pf1-pf4). The amounts yielded in the pooled fraction pf1 were found to contain much larger than those in the subsequent pooled fractions pf2 and pf3, but we were unable to obtain sufficient amounts of the last pooled fraction pf4 for cell proliferation assays. Although small molecules, such as polyphenol, that do not appear to bind

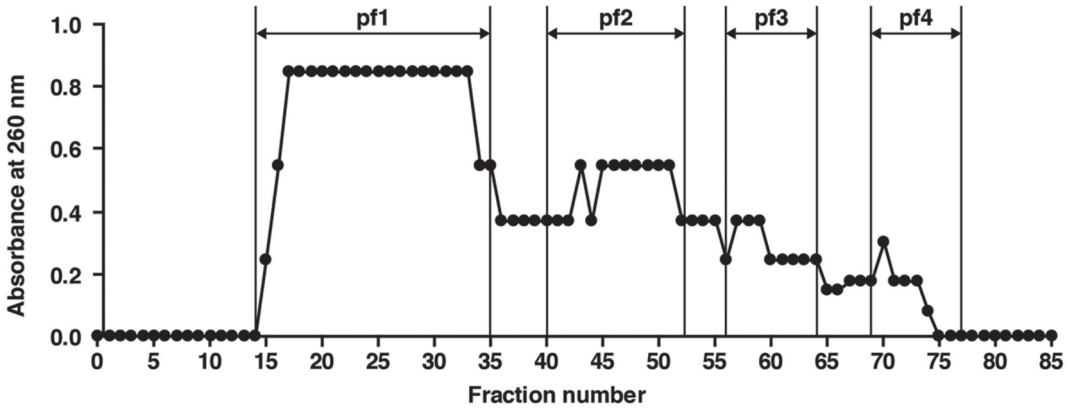

Fig. 1. Gel filtration of the methanol extract from *M. oleifera* leaves on a Sephadex LH-20 column. The elution profile was presented as a chromatogram monitored by UV absorbance at 260 nm. Ethanol:water (70:30) was used as an eluent, and fractions of 5 mL each were collected. Four pooled fractions are indicated as pf1-pf4.

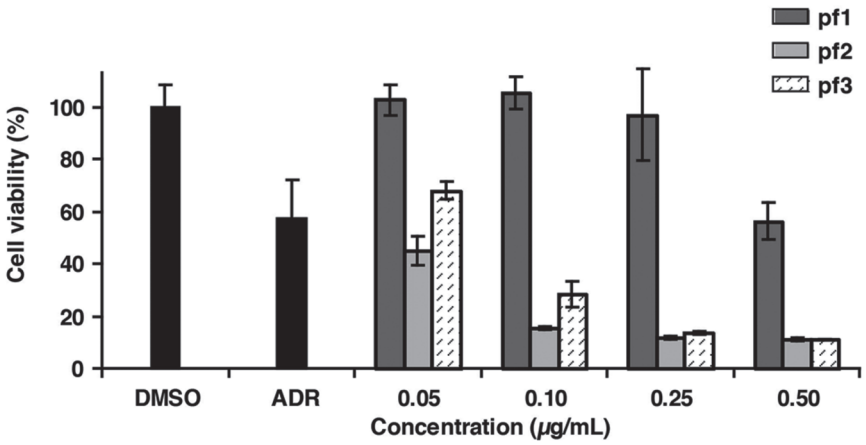

Fig. 2. Effect of pf1, pf2 and pf3 fractions on cell viability. HCT116 cells were treated with DMSO (0.5% v/v), ADR (0.5 μg/mL), or three pooled fractions of *M. oleifera* (pf1, pf2, and pf3) for 24 hr. Viable cell numbers were measured with the MTT assay. Data were calculated for % relative to the negative control and expressed as mean ± S.D. of triplicate samples.

to the gel matrix can be usually eluted before one total column volume of eluent has passed through the column, some components having the ability to interact with the gel matrix to some extent would be retarded and eluted from the column in order of decreasing sizes (Saminathan *et al.*, 2014). Our results indicate that the readings at 260 nm were detected from Fr. 15 to Fr. 74 but almost not in the void volume (Fr. 1-Fr. 14) (Fig.1). Considering that the column bed volume (2.5 × 37.5 cm) was approximately 184 mL (37 fractions per 5 mL each) and the size limit is 4,000-5,000 daltons, these results suggest that some extract components detected at 260 nm may exhibit weakly hydrophilic or hydrophobic interactions with Sephadex LH-20 and their molecular masses are likely to be less than 4,000-5,000 daltons.

Effect of the pooled fractions pf1, pf2, and pf3 on cell viability

The three-pooled fractions pf1, pf2, and pf3 of the methanol extract obtained from *M. oleifera* leaves were tested for cell proliferation using the MTT assay. We treated human HCT116 colon cancer cells with ADR as a positive control and with DMSO alone as a negative solvent control. Treatment with each pooled fraction (pf1, pf2, or pf3) inhibited the proliferation of HCT116 cells in a dose-dependent manner (Fig. 2). The inhibitory activities in pf2 and pf3 toward cell proliferation appeared to be more potent than that in pf1, suggesting that the components present in pf2 and pf3 are more cytotoxic than those in pf1. These results suggest that the different degrees of cell proliferation inhibition induced by each pooled fraction may be due to the variations of the components classified by their molecular size.

Kaempferol, a compound found in *M. oleifera* leaves, was chosen for comparison with each pooled fraction from the *M. oleifera* leaf extract, because kaempferol was reported to induce apoptosis in human HCT116 colon cancer cells (Li *et al*., 2009). Although treatment of HCT116 cells with kaempferol decreased cell viability in a dose-dependent manner, the components in pf1, pf2, and pf3 were found to exhibit strong anti-proliferative effects on HCT116 cells, compared with kaempferol (Fig. 3; see also Fig. 2). The ethanol extract from *M. oleifera* leaves was reported to be rich in glucosinolates, flavonoids, and phenolic acid (Mbikay, 2012), and co-treatment with flavonoids, such as kaempferol and quercetin, showed inhibition of cell proliferation of human HuTu-80 duodenum adenocarcinoma cells, human Caco-2 colon cancer cells, and human PMC42 breast cancer cells (Ackland *et al.,* 2005). In addition, a combination of indole-3-carbinol and genistein showed an augmented effect on cell proliferation inhibition in human HT29 colon cancer cells (Nakamura *et al*., 2009). Taken together, these results suggest that the inhibitory activities for cell proliferation in the pooled fractions pf1-pf3 may be atrributable not to kaempferol alone but rather to new active components or a flavonoid mixture with kaempferol.

Effect of the pooled fractions pf1, pf2, and pf3 on induction of apoptosis

Apoptosis is characterized by caspase activation and chromatin condensation. Procaspases convert into cas-

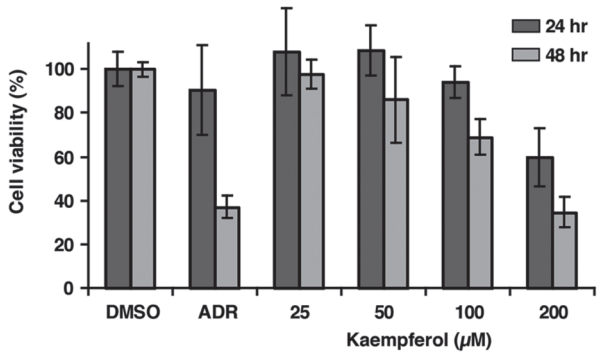

Fig. 3. Effects of kaempferol on cell viability. HCT116 cells were treated with DMSO (0.5% v/v) or ADR (0.5 μg/mL) or kaempferol (25-200 μM) for 24 hr and 48 hr. Viable cell numbers were measured with the MTT assay. Data were calculated for % relative to the negative control and expressed as mean ± S.D. of triplicate samples.

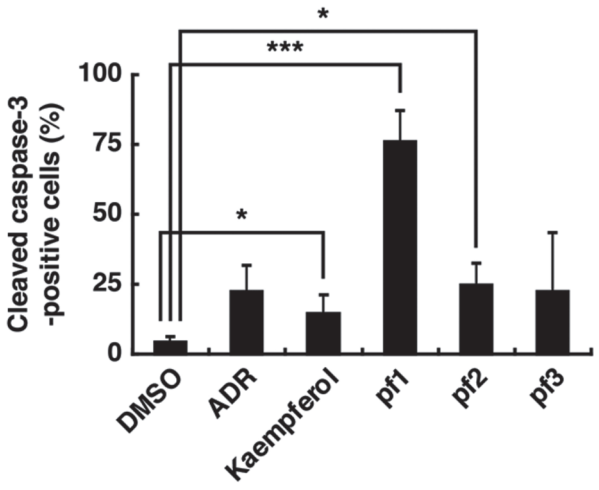

Fig. 4. Effect of pf1, pf2, and pf3 on induction of apoptosis. HCT116 cell were treated with DMSO (0.5% v/v), ADR (1.0 μg/mL), kaempferol (100 μM), or the pooled fraction (0.25 μg/mL) (pf1, pf2, pf3) for 24 hr. Apoptotic cells (% cleaved caspase-3-positive cells) were measured by flow cytometry. Results are averaged from three independent experiments. (*$p < 0.05$, ***$p < 0.005$)

pases, resulting in activation of the protease activities (Alenzi *et al*., 2010). Caspase-3, a crucial component of the apoptotic machinery, is activated during the early stages of apoptosis by proteolytic cleavage (Budihardjo *et al*., 1999). To examine whether inhibition of cell proliferation by the pooled fractions pf1-pf3 was attributable to apoptosis, we treated HCT116 cells with ADR, kaempferol, pf1, pf2, or pf3 for 24 hr, and stained with anti-cleaved caspase-3 antibody and PI for DNA. Treatment with pf1 or pf2 significantly induced cleaved caspase-3-positive cells, and treatment with pf1 in particular induced much larger numbers of cleaved caspase-3-positive cells than that with pf2 or pf3 (Fig. 4). Notably, treatment with kaempferol showed significant but lower level of induction of cleaved caspase-3-positive cells, compared with each pooled fraction (Fig. 4). These results suggest that the apoptosis-inducing activity found in each pooled fraction is higher than that of kaempferol.

The morphological features of apoptosis include cell membrane blebbing, cytoplasm and nuclear condensation, and characteristic apoptotic bodies (Hacker, 2000). Under a confocal microscope, HCT116 cells inducing cleaved caspase-3 were observed upon treatment with pf1 and pf2, and representative cells were displayed (Fig. 5). Cells treated with pf2 displayed the typical characteristics of apoptosis, such as membrane blebbing, nuclear condensation and apoptotic bodies, whereas cells treated with pf1 showed early apoptotic morphologies. In contrast to cells treated with pf1 and pf2, induction of cleaved caspase-3 was hardly seen in cells treated with pf3 (Fig. 5).

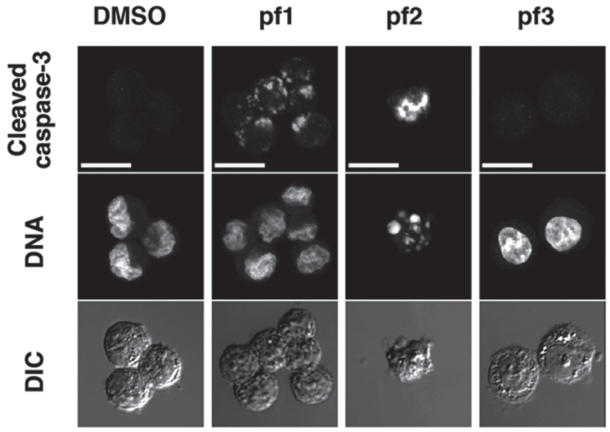

Fig. 5. Photomicrographs of cells treated with pf1, pf2, or pf3. HCT116 cells treated with DMSO (0.5% v/v) or the pooled fraction (0.25 μg/mL) (pf1, pf2, pf3) for 24 hr were doubly stained with anti-cleaved caspase-3 antibody and PI (for DNA). Representative cells are shown as apoptotic cells (cleaved caspase-3-positive), nuclei (DNA), and cell morphology (DIC, Nomarski differential-interference-contrast). Bars, 20 μm.

Note that, upon treatment with pf3, there was a considerable deviation of the number of cleaved caspase-3-positive cells (Fig. 4), suggesting that pf3 barely induces apoptosis despite its strong inhibition of cell proliferation (see also Fig. 2). Taken together, these results suggest that the pooled fractions pf1-pf3 may have different activities on HCT116 cells in terms of induction of anti-proliferation and apoptosis.

Conclusion

In the present study, we fractionated the methanol extract obtained from *M. oleifera* leaves by gel filtration on Sephadex LH-20 and found that the three-pooled fractions contain the different biological activities toward induction of anti-proliferation and apoptosis. We also showed that those activities are more potent than that of kaempferol, a pro-apoptotic flavonoid found in *M. oleifera* leaves. These results encourage us to further identify novel compounds that have the activities toward either or both of pro-apoptosis and anti-proliferation. Identification of the compounds from the *M. oleifera* leaf extract may provide a new clue for the development of new drugs for the treatment of human colon cancer.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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