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ORIGINAL ARTICLE

Mango (*Mangifera indica* L.) Peel Extracts Inhibit Proliferation of HeLa Human Cervical Carcinoma Cell *via* Induction of Apoptosis

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Abstract Flesh and peel extracts of five mango varieties were compared for their antioxidant and antiproliferative activities. Fozli peel (FP) extract was found to have the highest antioxidant activity and to significantly inhibit the proliferation of HeLa human cervical carcinoma cells in a dose-dependent manner. Hoechst 33342 staining revealed apoptotic nuclei with condensed chromatin, and cell growth arrest was confirmed by flow cytometry. Apoptotic signaling induced by FP extract was characterized by an increased Bax/Bcl-2 ratio and down-regulation of Bcl-2. Moreover, FP extract treatment triggered the proteolytic activation of caspases-3, -8, and -9 and the degradation of poly ADP-ribose polymerase in HeLa cells. These results indicate that FP is an excellent source of phenolic and/or flavonoid compounds, and may have applications in the treatment of human cervical carcinoma and as an antioxidant in the food and pharmaceutical industries. Gas chromatography-mass spectrometry analysis of FP extract revealed that 2,5-dihydroxyphenol was detected with highest amount and other compounds such as 5-hydroxymethyl-2-furancarboxaldehyde, pentadecanoic acid, (Z,Z)-9,12-octadecadienoic acid, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one were also found in high amounts.

Keywords antioxidant · apoptosis · gas chromatography-mass spectrometry · HeLa human cervical carcinoma cells · mango · phytochemicals

Introduction

High consumption of fruits and vegetables has been associated with a lower incidence of degenerative diseases. Such protective effects are thought to stem partly from the various antioxidant compounds contained in these foods. Specifically, fruit peels are rich in polyphenolic compounds, flavonoids, ascorbic acid, and many other biologically active components that positively influence health (Leontowicz et al., 2003). Mango (*Mangifera indica* L.), a rich source of antioxidants (Kim et al., 2007), is one of the most important tropical fruits worldwide in terms of production and consumer acceptance. Mango is one of the most popular fruits in Bangladesh; about 9.5 tons are produced there per hectare, and several varieties, including Fozli, Khershapat, Langra, Dudsagor, Lakhna, Gopalvog, Mohonvog, and Amrupali, are cultivated. Because mango is a seasonal fruit, approximately 20% is processed, yielding products such as purees, nectar, leather, pickles, and canned slices, which are popular worldwide (Maisuthisakul, 2008). Mango peel, which constitutes about 15–20% of the fruit weight, is a major by-product of these processings and is discarded as waste, making it a source of pollution. However, the peel has been found to be a good source of phytochemicals such as polyphenols, carotenoids, vitamin E, and vitamin C (Ajila et al., 2007a), as well as exhibits antioxidant properties (Ajila et al., 2007b). Using a single variety, we previously showed that mango peel contains more polyphenols and flavonoids than the flesh and

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that it exhibits strong antioxidant activity. In addition, mango peel has been demonstrated to be a potent antiproliferative agent, regardless of ripeness, and that HeLa human cervical carcinoma cells are highly susceptible to treatment with mango peel extracts (Kim et al., 2010). Though some studies have tested the antiproliferative activities of mango extracts, few reports have described the mechanism responsible for these effects.

Apoptosis is an active physiological process resulting in cellular self-destruction. It is characterized by distinct morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies. It is well established that activation of a caspase cascade during apoptosis occurs via the activation of either the mitochondrial (intrinsic) or death receptor (extrinsic) pathway (Green, 2000). Bax, which is pro-apoptotic, and Bcl-2, which is anti-apoptotic, are key regulators of the intrinsic pathway of apoptosis, controlling the point of no return and setting the threshold for engagement of the death machinery (Marzo and Naval, 2008). The mitochondrial pathway generally involves the induction of a mitochondrial permeability transition and subsequent release of cytochrome *c*. Procaspase-9, cytochrome *c*, and oligomerized apoptotic protease-activating factor 1 (Apaf-1) form massive complexes known as apoptosomes, resulting in the activation of the initiator procaspase-9. Activated caspase-9 activates the downstream executioners, procaspases-3 and -7. Active caspase-3 cleaves the 116-kDa poly(ADP-ribose) polymerase (PARP) protein, yielding an 89-kDa fragment, which is a characteristic marker of apoptosis (Anuradha et al., 2000; Herr and Debatin, 2001; Zimmermann et al., 2001). The death receptor pathway involves engagement of death receptors on the cell membrane and recruitment of procaspase-8 and the adaptor protein Fas-associated death domain (FADD), which form a complex known as the death-inducing signaling complex. To initiate apoptosis, procaspase-8 is cleaved to active caspase-8, which directly activates caspase-3. Active caspase-3 in turn cleaves procaspases-3 and -7, yielding active caspases-3 and -7, respectively, which cause cells to undergo apoptosis (Herr and Debatin, 2001; Zimmermann et al., 2001). Because cancer is characterized by cellular proliferation and impediments to apoptosis, the therapeutic targeting of apoptosis has long been regarded as a valid strategy for developing anti-tumor drugs (Hong et al., 2003). In our continuing search for new and safe anticancer agents from natural products, the present study was conducted to elucidate the anticancer ability of mango peel to induce apoptosis in HeLa cells. Furthermore, a compositional analysis of the FP extract of mango was performed using gas chromatography-mass spectrometry (GC-MS) to identify which phytochemicals would be responsible for the anticancer activity. Thus, the aim of the present study was to assess the anti-carcinogenic potency of mango and determine the biochemical mechanisms by which it induces cell death.

Materials and Methods

Materials. The mango (*M. indica* L.) varieties used in this study were collected from a well-nourished garden in Gurudaspur, Natore, Bangladesh, in 2010 and were authenticated by the Department of Botany, University of Rajshahi, where voucher specimens (224/10, 225/10, 321/10, 341/10, and 342/10; collection date 17.08. 2010) were deposited. The fruits were continually observed during their maturation and ripening. Selected fruits of varieties known locally as Fozli, Khershapat, Langra, Dudsagor, and Lakhna were harvested at the mature stage (when they show a moderately soft yellowish color), shipped on the same day, and processed upon receipt.

Chemicals. Acetone and methanol were purchased from Merck KGaA (Germany). Folin-Ciocalteu's phenol reagent; 10% $(Na)_2CO_3$; gallic acid; 5% $NaNO_2$; 10% $AlCl_3$; 1 M NaOH; rutin; 1,1-diphenyl-2-picrylhydrazyl (DPPH); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO); ethanol (EtOH); ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$); H_2O_2; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); quercetin; PI; and RNase A were purchased from Sigma-Aldrich Inc. (USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) were purchased from Amresco Inc. (USA). Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin, streptomycin, and Hoechst 33342 dye were purchased from Invitrogen Life Technologies Inc. (USA). Anti-cleaved PARP, -caspase-9, -caspase-8, -caspase-3, -Bcl-2, -Bax, and -β-actin antibodies were purchased from Cell Signaling Technology Inc. (USA). BCA Protein Assay Kits and polyvinylidene fluoride (PVDF) membranes were purchased from Thermo Scientific (USA) and Millipore Corp. (USA). All other chemicals and reagents used were of analytical grade.

Preparation of peel and flesh extracts from five mango varieties. Fozli, Khershapat, Langra, Dudsagor, and Lakhna mango peels were removed using a sharp knife and peeler, and the edible flesh from the seed kernel was removed by gently scraping with the blunt edge. A spoon was used to separate the flesh from the peel. The samples were sun-dried and then oven-dried at <40°C. The peel and flesh samples were then pulverized, and extracted in 80% acetone, 80% methanol, or hot water by three cycles of periodic sonication, followed by an overnight cold extraction for 3 consecutive days at room temperature. The extracts were filtered, concentrated with a vacuum rotary evaporator under reduced pressure at 40°C, and lyophilized. The extracts were dissolved in DMSO to a concentration of 400 mg/mL and diluted with phosphate-buffered saline (PBS; pH 7.4) or selected solvents to the desired final concentrations.

Estimation of the total polyphenol content. The total polyphenol content was determined as described previously (Ribeiro et al., 2008) with slight modifications. A 1.5 mL aliquot was mixed with 0.5 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 1 mL of

Na_2CO_3 (10% [v/v]) was added to the mixture. The reaction was kept in the dark for 30 min, after which 200 μL of the solution was transferred to a microplate and its absorbance read at 700 nm using a microplate reader (Tecan, Austria). All peel and flesh extracts were analyzed under similar conditions; the results are expressed as gallic acid equivalents (GAE) in mg/g dried sample.

Estimation of the total flavonoid content. The flavonoid content was measured by a colorimetric assay according to the method of Wang et al. (2008) with slight modifications. A total of 6 μL of 5% $NaNO_2$ were mixed with a 120 μL aliquot in a microplate well. The resulting mixture was incubated for 6 min at room temperature, mixed with 12 μL of 10% $AlCl_3$, and incubated for an additional 6 min. Subsequently, 40 μL of 1 N NaOH and distilled water were added to a final volume of 200 μL. The absorbance at 510 nm was read immediately against a blank (PBS). All peel and flesh extracts were analyzed under similar conditions; the flavonoid content is expressed as rutin equivalents (RE) in mg/g dried sample.

Measurement of DPPH radical scavenging activity by ESR spectrometry. DPPH free radical scavenging activity was measured using a previously described method (Nanjo et al., 1996). A 30 μL aliquot of each sample in PBS (or, as a control, 30 μL of PBS) was added to 30 μL of 60 μM DPPH in EtOH. After mixing vigorously for 10 s, the solutions were transferred to a 50-μL Teflon capillary tube and fitted into the cavity of an ESR spectrometer. The spin adduct was measured exactly 2 min later. The measurement conditions were as follows: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 500 mT, modulation width 0.8 mT, sweep width 10 mT, sweep time 30 s, microwave power 5 mW, and temperature 297 K. The radical scavenging activities of the mango peel and flesh extracts were calculated using the following formula: Scavenging rate = $[(A_0 - A_x)/A_0] \times 100\%$, where A_0 and A_x are the ESR signal intensities of the samples in the absence and presence of the extract, respectively.

Measurement of hydroxyl radical scavenging activity by ESR spectrometry. Hydroxyl radicals were generated by the Fenton reaction and reacted rapidly with nitrone spin-trap DMPO. The resulting DMPO-OH adduct was detected using an ESR spectrometer (Li et al., 2004). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) containing 20 μL of 0.3 M DMPO, 20 μL of 10 mM $FeSO_4$, and 20 μL of 10 mM H_2O_2 using a JESFA electron spin resonance spectrometer (JEOL, Japan). The following measurement conditions were used: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 140 mT, modulation width 0.2 mT, sweep width 10 mT, sweep time 30 s, microwave power 1 mW, and temperature 298 K. The radical scavenging activities of the mango extracts were calculated using the formula described above.

Determination of ABTS free radical cation scavenging activity by UV spectrometry. This experiment was carried out using a previous method (Kumaraswamy and Satish, 2008) with slight modifications. The $ABTS^{\bullet+}$ chromophore was produced by the oxidation of 7 mM ABTS in water at a ratio of 1:1 with 2.45 mM potassium persulfate in water (final concentration), following incubation in the dark at room temperature for 16 h. The radical was diluted in PBS (pH 7.4) to give an absorbance of 0.700± 0.005 at 734 nm, as measured using a UV 1800 spectrophotometer (Shimadzu, Japan). Next, 50 μL aliquots of different concentrations of the test sample in PBS (and, as a control, 50 μL of PBS) were added to 950 μL of this diluted solution to give a final volume of 1 mL. The absorbance at 734 nm was measured 2 min after mixing. Gallic acid was used as the reference standard. The antioxidant solution reduced the radical cation to ABTS, which reduced the amount of color. The percent inhibition was calculated using the following equation: % inhibition = $[(A_0 - A_x)/A_0] \times 100\%$, where A_0 and A_x are the absorbances of the control and test samples, respectively.

Cell culture. HeLa cells obtained from the Korean Cell Line Bank (KCLB, Korea) were cultured in DMEM containing 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

Cell viability assay. The effects of the extracts on HeLa cell viability were determined by a colorimetric MTT assay which measures the mitochondrial-dependent reduction of MTT to formazone. HeLa cells were cultured in DMEM until the mid-exponential phase, and then seeded in a 96-well plate at a density of 2×10^4 cells per well in 190 μL of medium. After incubation for 24 h, the cells were exposed to peel or flesh extracts (12.5, 25, 50, 100 or 200 μg/mL) or PBS (control) for 72 h. After treatment, 20 μL of 5 mg/mL MTT was added, and the cells were incubated for 4 h at 37°C. The supernatant was then discarded, and 150 μL of DMSO was added to each well. The mixture was shaken at room temperature for 30 min on a mini shaker to dissolve the formazan crystals, and the spectrophotometric absorbance at 570 nm was measured using a microplate reader (Tecan). Triplicate experiments were performed in a parallel for each concentration. The results are presented as the mean ± SEM.

Hoechst 33342 staining. Hoechst 33342, a fluorescent DNA-binding dye, was used to visualize nuclear changes and apoptotic body formation. Cells were plated in 12-well plates and treated with Fozli extracts (25, 50, 100 or 200 μg/mL). After 24 h, 10 μM Hoechst 33342 was added to the solution in each well, and the plates were incubated for 10 min at 37°C. The stained cells were then observed at 340 nm under an Olympus fluorescence microscope.

Flow cytometric cell cycle analysis. HeLa cells (5×10^5 cells/culture plate) were treated with FP extract (0, 50, 100, or 200 μg/mL) for 48 h then harvested and washed twice with cold PBS, fixed in ice-cold 70% EtOH, and incubated at 4°C. The cells were then centrifuged and washed twice with 2 mM EDTA-PBS. The supernatant was discarded, and the cells were resuspended in 0.5 mL of DNA staining solution (40 μg/mL propidium iodide, 250 μg/mL RNase A, and 2 mM EDTA in PBS) and incubated for 30 min at 37°C. The DNA content (population of cells in each cell

cycle phase) was analyzed by FACSCalibur flow cytometry using Cell Quest (Becton Dickinson Bioscience, USA).

Protein extraction and Western blot analysis. After treatment with FP extract (0, 50, 100, 200 or 400 μg/mL) for 48 h, HeLa cells were collected and washed twice with cold PBS. The cells were then lysed in lysis buffer (40 mM Tris-HCl [pH 7.5], 120 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) using a D100 sonic dismembrator (Fisher Scientific, USA) and kept on ice for 30 min. The cell debris was pelleted by centrifugation at 13,000 rpm at 4°C for 30 min. The supernatants were stored at −70°C prior to use. Protein concentrations were quantified using BCA protein detection assay kits. Western blotting was performed as described previously (Moon et al., 2011) with slight modifications. Briefly, equal amounts of protein (90–100 μg) were separated by 10–15% SDS-PAGE and transferred to a PVDF membrane using glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% [v/v] methanol). After blocking with 5% nonfat skim milk in Tween 20-Tris-buffered saline (T-TBS), the membrane was incubated overnight with primary antibodies, and then for 1 h with secondary antibodies diluted in TBS and 0.1% Tween 20. Human anti-caspase-3, -caspase-8, -caspase-9, -PARP, -Bcl-2 and -Bax antibodies (diluted 1:1,000) and anti-β-actin antibodies (1:10,000) were used as the primary antibodies with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Vector Laboratories, USA) and HRP-conjugated goat anti-mouse IgG (Invitrogen, USA) (both 1:5,000) as secondary antibodies. The membrane was then exposed to X-ray film. Protein bands were detected using the WEST-ZOL® plus Western Blot Detection System (iNtRON, Korea).

Analysis by GC-MS. Chromatographic analysis was carried out using a Shimadzu GC-MS (Model QP-2010, Shimadzu Co.) in electron impact mode. The ionization voltage was 70 eV, and the injector and detector temperatures were 230 and 280°C, respectively. The capillary column used was an Rtx-5MS (30 m length, 0.25 mm internal diameter, and 0.25 μm film thicknesses). The oven temperature began at 80°C (isothermal for 5 min), and was ramped up to 200°C at 5°C/min (isothermal for 5 min) and up to 250°C at 5°C/min (isothermal for 16 min). Helium was used as the carrier gas at a flow rate of 1 mL/min, with an injector volume of 1 μL using a 1:10 split ratio. The methanolic extracts of peel was solubilised with MeOH and filtered through a 0.20-μm syringe filter (Advantec, Japan), and aliquots were injected onto the GC-MS. The mass spectra of each compound were tentatively identified by comparing our data to those in the WILEY7 and NIST libraries. Further identification was completed by comparing the mass spectra to data in publications.

Statistical analysis. All experiments were performed in triplicate. The results were analyzed using SPSS 12.0 for Windows (SPSS Inc., USA). Data were analyzed by one-way ANOVA, followed by unpaired Student's *t*-tests, and are expressed as the mean ± SD. A difference at *: $p<0.01$; **: $p<0.001$ was considered to be statistically significant.

Results and Discussion

Total phenolic contents of the mango peels and flesh extracts. The extraction of phenolic compounds from plant material is influenced by their chemical nature as well as the polarity of the solvent used (Xu and Chang, 2007). Methanol has generally been found to be more efficient for the extraction of lower molecular weight polyphenols, whereas the higher molecular weight flavanols are more easily extracted with aqueous acetone (Dai and Mumper, 2010). Hot water, 80% methanol, and 80% acetone were tested as extractants with total polyphenols from powdered mango peel. Results showed that extraction in 80% acetone yield higher concentrations of phenolic compounds than extraction in either 80% methanol or hot water for all varieties of mango peel tested, in agreement with previous data (Ajila et al., 2007a; Liu et al., 2009). The total phenolic contents of the 80% acetone peel extracts for all mango varieties were about eight to ten times higher than those for the mango flesh extracts. FP had the highest polyphenol content (Table 1), indicating that these varieties are promising sources of polyphenolics. Overall, the total phenolic contents of the mango peels were higher than those of apple peels, as analyzed using Folin-Ciocalteu's reagent and expressed as GAE units (Wolfe and Liu, 2003).

Total flavonoid contents of the mango peels and flesh extracts. The total flavonoid contents in the peel extracts of the five mango varieties were higher than those in the flesh extracts (Table 2). Total flavonoid contents of the peels of the five mango varieties extracted in three different solvents ranged from 2.57±0.05 to 9.06±0.16 mg/g dry matter (dm) (RE). The highest level was found in FP extracted in 80% acetone (9.06±0.16 mg/gm dm [RE]) followed by Langra (8.09±0.12 mg/g), Dudsagor (7.38±0.24 mg/g), Khershapat (5.61±0.09 mg/g), and Lakhna (5.27±0.03 mg/g) peel. Total flavonoid contents in the five mango flesh extracts ranged from 0.28±0.04 to 0.65±0.05 mg/g dm. The data obtained are in agreement with a previous report showing that the total flavonoid contents of citrus fruit peels were much higher than those of the corresponding fleshes (Wang et al., 2008).

Table 1 Total polyphenol contents of five mango varieties extracted in various solvents

	TPC[a] (mg GAE[b]/g dm[c])			
	Peel			Flesh
Sample	80% acetone extract	80% methanol extract	Hot water extract	80% acetone extract
Fozli	42.73±0.49	39.64±1.23	29.07±0.98	4.47±0.06
Khershapat	37.04±0.75	32.65±0.75	23.87±0.75	3.82±0.06
Langra	41.10±0.75	39.80±0.98	20.61±0.75	4.81±0.08
Dudsagor	37.04±0.28	38.01±1.23	26.63±0.49	4.59±0.05
Lakhna	34.76±0.75	29.39±0.75	29.88±0.75	3.80±0.11

[a]TPC = total polyphenol content
[b]GAE = gallic acid equivalents
[c]dm = dry matter
All data are presented as the mean ± SD of three replicates.

Table 2 Total flavonoid contents of five mango varieties extracted in various solvents

Sample	TFC[a] (mg RE[b]/g dm[c])			
	Peel			Flesh
	80% acetone extract	80% methanol extract	Hot water extract	80% acetone extract
Fozli	9.06±1.16	7.14±0.12	4.26±0.12	0.36±0.05
Khershapat	5.61±0.09	4.53±0.09	3.72±0.07	0.30±0.03
Langra	8.09±0.12	6.61±0.22	2.57±0.05	0.65±0.05
Dudsagor	7.38±0.24	6.44±0.12	3.16±0.08	0.35±0.03
Lakhna	5.27±0.03	4.02±0.18	4.15±0.07	0.28±0.04

[a]TFC = total flavonoid content
[b]RE = rutin equivalents
[c]dm = dry matter
All data are presented as the mean ± SD of three replicates.

DPPH radical scavenging activity. The DPPH radical assay is used to evaluate the antioxidant properties of fruits and vegetables (Baydar et al., 2007). The effects of the 80% acetone extracts of five types of mango peel and flesh on DPPH free radical scavenging was tested at concentrations of 6.25, 12.5, and 25 μg/mL (Fig. 1A). Significant DPPH free radical scavenging activity was evident in the peel extracts. Whereas the radical scavenging activity of the peel extracts was markedly higher at a concentration of 12.5 μg/mL than at 6.25 μg/mL, scavenging activity at 25 μg/mL was only slightly higher than that at 12.5 μg/mL. At 12.5 μg/mL, FP exhibited 78.46% scavenging activity, followed by Khershapat peel (72.94%), Langra peel (78.74%), Dudsagor peel (76.54%), and Lakhna peel (71.63%); however, the mango flesh extracts showed lower scavenging activities, 13.37, 15.56, 22.29, 13.33, and 7.56%, respectively, at 25.0 μg/mL. The positive control catechin exhibited 66.26% DPPH radical scavenging activity, which is lower than those of the mango peel extracts at 25 μg/mL. Thus, the peels were found to possess hydrogen-donating abilities, suggesting they have the potential to react with the DPPH radical.

Hydroxyl radical scavenging activity. Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and large-scale biological damage (Huang et al., 2002). The DMPO spin-trap method has been widely used to measure the hydroxyl radical scavenging activity of compounds, with the Fenton reaction as the hydroxyl radical source. In the study of Li et al. (2004), hydroxyl radicals generated in the Fe_2^+/H_2O_2 system were trapped using a DMPO-forming spin adduct and could be detected using an ESR spectrometer. No significant differences were found between the scavenging activities of the peel and flesh extracts at concentrations of 250, 500, and 750 μg/mL (Fig. 1B). The scavenging rates for the peel extracts ranged from 40.82 to 80.85%, whereas those for the flesh extracts ranged from 38.75 to 79.87%. The scavenging activities were higher than that of catechin, which exhibited 57.21% scavenging activity at 2 mg/mL. The data obtained for the mango flesh and peel extracts are in agreement with those

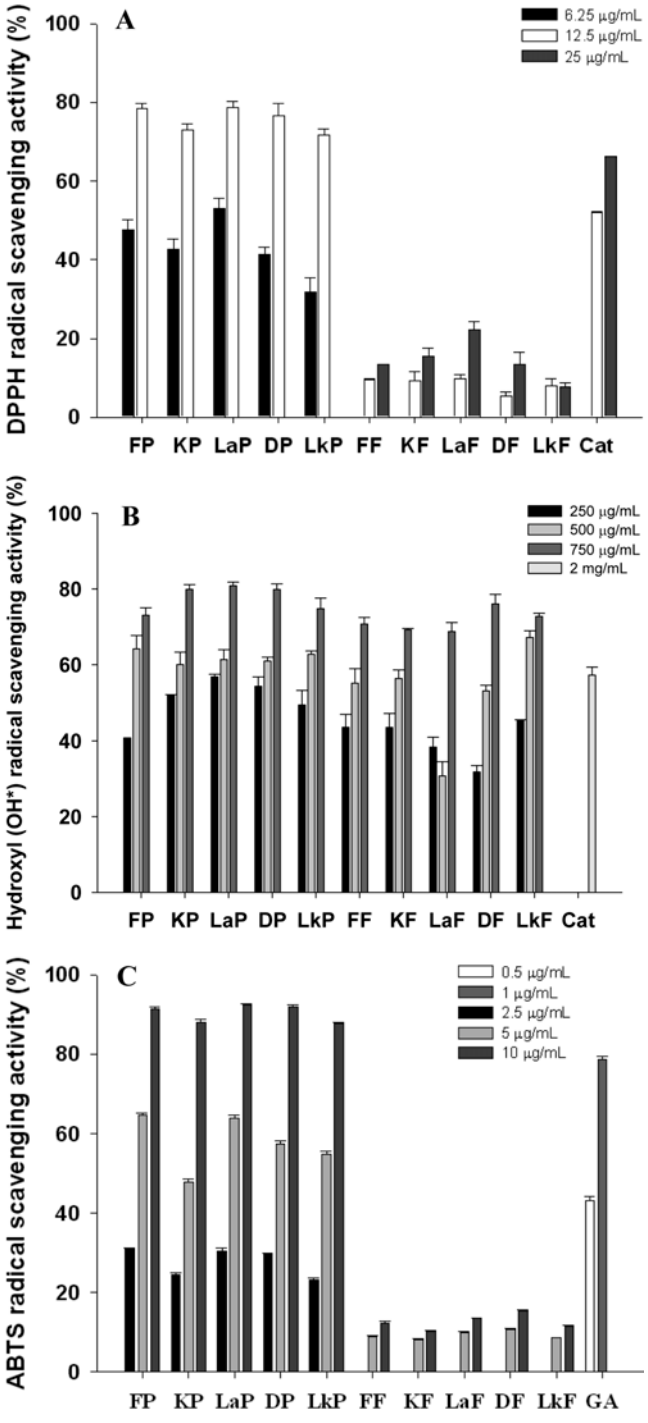

Fig. 1 Effects of acetone extracts of mango peel and flesh on DPPH and OH radicals and ABTS radical cation scavenging. The data show the percent inhibition of the scavenging of (A) DPPH radicals (extracts tested at concentrations of 6.25, 12.5, and 25 μg/mL) and (B) hydroxyl radicals (250, 500, and 750 μg/mL), with an appropriate amount of catechin (Cat) as a positive control in both experiments, and of (C) ABTS radical cations (2.5, 5, and 10 μg/mL), with gallic acid (GA) as the reference standard. All data are expressed as the mean ± SD of three replicates. Key: FP, KP, LaP, DP, and LkP and FF, KF, LaF, DF, and LkF = Fozli, Khershapat, Langra, Dudsagor, and Lakhna peel and flesh, respectively.

reported previously by Kim et al. (2010).

ABTS radical cation scavenging activity. The effects of 80% acetone extracts of Fozli, Khershapat, Langra, Dudsagor, and Lakhna peel and flesh samples on ABTS free radical scavenging were assayed at concentrations of 2.5, 5.0, and 10 μg/mL (Fig. 1C). ABTS is used as a free radical to evaluate the antioxidant activity of extracts. The method is based on the ability of antioxidant molecules, especially phenolic and flavonoid compounds, to quench the long-lived ABTS radical cation ($ABTS^{\bullet+}$). Fozli and Langra peel extracts showed significant ABTS free radical scavenging activity, whereas Khershapat, Dudsagor, and Lakhna peel extracts exhibited moderate scavenging activity. Mango flesh extracts exhibited lower scavenging activities—12.33, 10.19, 13.50, 15.28, and 11.51%, respectively, at 10 μg/mL. The radical scavenging activities of the peel extracts increased in a dose-dependent manner. The positive control (gallic acid, used at concentrations of 0.5 and 1 μg/mL) showed higher activity than the tested extracts.

Cell viability assay. The effects of the mango peel and flesh extracts on the proliferation of HeLa cells were examined in an MTT-based assay. Treatment with mango peel extracts significantly inhibited the growth of HeLa cells, and the number of viable cells decreased in a dose-dependent manner (Fig. 2A). The viability (%) of HeLa cells treated with extracts of the five different varieties of peels at a concentration of 200 μg/mL were 33.33±1.37 (FP), 54.44±2.92 (Khershapat), 46.90±1.36 (Langra), 49.81±4.69 (Dudsagor), and 55.03±2.21% (Lakhna). The growth inhibition of HeLa cells treated with the five mango flesh extracts at 200 μg/mL was lower than that of cells treated with the peel extracts (Fig. 2B).

Induction of apoptosis by FP extract. The most conspicuous changes in the morphology of FP extract treated-cells as observed by light microscopy included cell shrinkage and extensive detachment of the cells from the cell culture substratum (data not shown). These changes are characteristic of apoptotic cell death. Therefore, Hoechst 33342 nuclear staining was performed to determine whether the FP-induced reduction in viability was attributable to apoptosis. Hoechst 33342 staining revealed fragmented and condensed nuclei in cells treated with FP extract or quercetin (Fig. 3A). These results demonstrate that FP extract has the ability to induce apoptosis in HeLa cells. The mechanism of FP-induced apoptosis was also examined by Western blotting. In FP-treated HeLa cells, expression of the anti-apoptotic protein Bcl-2 was inhibited in a concentration-dependent manner, whereas that of Bax was unchanged (Fig. 3B). It has been reported that the ratio of pro-apoptotic and anti-apoptotic Bcl-2 members is a critical determinant of susceptibility to apoptosis. Changes in the Bax/Bcl-2 ratio have been reported to be caused by down-regulation of Bcl-2 and slight down-regulation of Bax (Cha et al., 2004), down-regulation of Bcl-2 and up-regulation of Bax (Bhutia et al., 2009), as well as down-regulation of Bcl-2 with no significant change in the level of Bax (Pavlovic et al., 2007). Our data clearly suggest

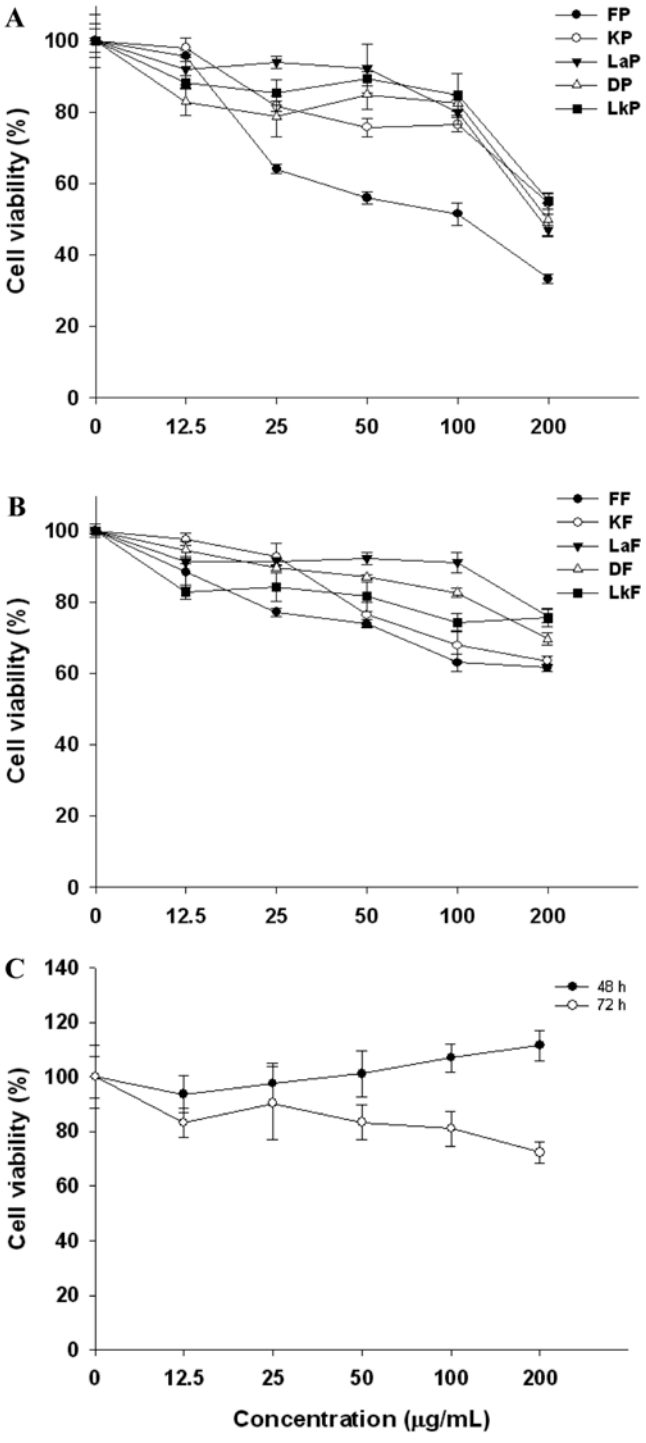

Fig. 2 Cell growth inhibition. Cell viability was assessed on the basis of MTT reduction. All data are expressed as the mean ± SD ($n = 4$). Cells treated with vehicle (DMSO) alone were used as controls. Inhibition of HeLa cell growth by acetone extracts of (A) mango peel and (B) mango flesh. Mango varieties: ●, Fozli; ○, Khershapat; ▼, Langra; △, Dudsagor; and ■, Lakhna. Normal fibroblast cell were treated with the FP for 48 and 72 h (C).

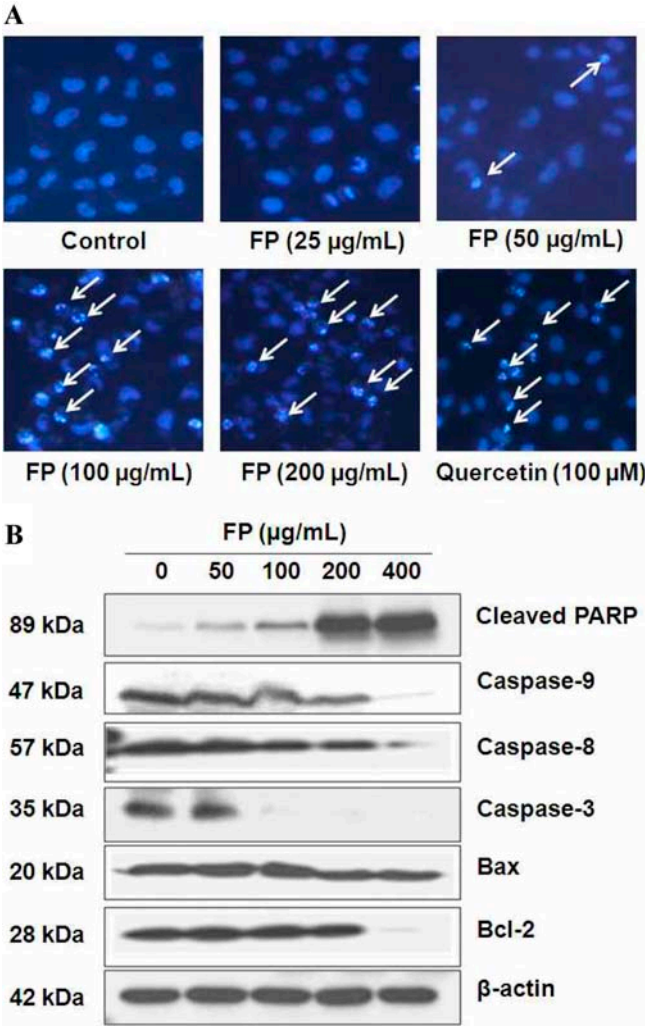

Fig. 3 FP induced apoptosis in HeLa cells. (A) Nuclear Hoechst staining. Cells were treated with the indicated concentration of FP extract, 0.1% DMSO (control) or quercetin (positive control) for 24 h, stained with Hoechst 33342, and photographed with a fluorescence microscope. Arrows indicate the formation of apoptotic bodies. (B) Western blot analysis of apoptosis-related protein expression in FP extract-treated HeLa cells. Cells were lysed after incubation for 48 h with the indicated concentration of FP extract. Cellular proteins were then separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with the indicated primary antibodies. β-Actin was used as an internal control.

that the FP extract-induced apoptosis of HeLa cells resulted from an alteration in the Bax/Bcl-2 ratio. The central executioners of apoptosis, caspases, cleave numerous vital cellular proteins to affect the apoptotic cascade (Straszewski-Chavez et al., 2005). Initiator caspases, including caspases-8 and -9, are activated by two alternative pathways. The first involves cell death receptor-mediated apoptosis and caspase-8. The second is mitochondria-mediated apoptosis involving the release of mitochondrial cytochrome *c* into the cytoplasm, where it induces formation of an apoptosome, leading to the activation of caspase-9 (Stefanis, 2005). Both pathways converge at a final common pathway involving the activation of effector caspases such as caspase-3, which cleaves regulatory and structural molecules, culminating in apoptotic death (Ghobrial et al., 2005). Our results revealed that FP induced the activation of caspases-3, -8, and -9 in a dose-dependent manner. In particular, caspase-3 activation was dominant at concentrations ranging from 100 to 400 μg/mL, which also caused the proteolytic cleavage of PARP, a well-known caspase-3 substrate (Fig. 3B). These results suggest that the apoptotic effects of FP extract on HeLa cells are associated with an increase in the Bax/Bcl-2 ratio and caspase activation via both the intrinsic and extrinsic pathways.

Table 3 Cell cycle analysis of HeLa cells treated with Fozli Mango Peel extract

Phase (%)	Concentration (μg/mL)			
	0	50	100	200
Sub-G1	5.83±3.66	9.28±3.53	16.66±1.80*	52.92±3.32**
G1	58.46±1.77	55.68±3.42	44.89±4.56	30.86±7.83
S	12.89±4.82	13.59±4.23	13.44±6.14	8.31±5.81
G2/M	21.85±2.11	20.65±3.16	23.66±4.45	1.54±0.90

*: p <0.01, **: p <0.001 compared with non-treatment (0 μg/mL)
All data are presented as the mean ± SD of three replicates.

FP extract-induced disruption of the cell cycle. The effect of FP on cell cycle progression was analyzed by flow cytometry. The percentage of cells in sub-G1 phase after FP treatment was measured as the apoptotic index. HeLa cells were treated with FP, and the percentage of cells in each phase of the cell cycle was quantified (Table 3). This treatment increased the percentage of cells in sub-G1 phase from 5.83±3.66 (0 μg/mL) to 52.92±3.32% (200 μg/mL) in a dose-dependent manner. These results indicate that growth inhibition in response to FP is associated with a sub-G1 arrest.

Compositional analysis by GC-MS. GC-MS analysis tentatively identified 22 compounds from FP (Fig. 4). Compound 2,5-dihydroxyphenol was detected with highest amount followed by 5-hydroxymethyl-2-furancarboxaldehyde, pentadecanoic acid, (Z,Z)-9,12-octadecadienoic acid, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (Table 4). The fatty acids pentadecanoic acid, ethyl linoleate, linoleic acid, and oleic acid detected in present study, have previously been reported to be present in mango (Pino et al., 2005). Compound 2,5-dihydroxyphenol have three hydroxy groups bonded to the aromatic ring and similar type of model of substitution could be the important factor associated with a H_2O_2 and DPPH scavenging activities of these phenolic compounds (Sroka and Cisowski, 2003). Moreover, the polyunsaturated fatty acid (Z,Z)-9,12-octadecadienoic acid known as an antioxidant (Ha et al., 1990), increases β-oxidation in the liver and reduces the levels of total body lipid and liver triacylglycerol as well as unsaturated fatty acid biosynthetic capacity (Leaver et al., 2006). Increasing evidence shows that α-tocopherol, a well-known potent natural lipophilic antioxidant,

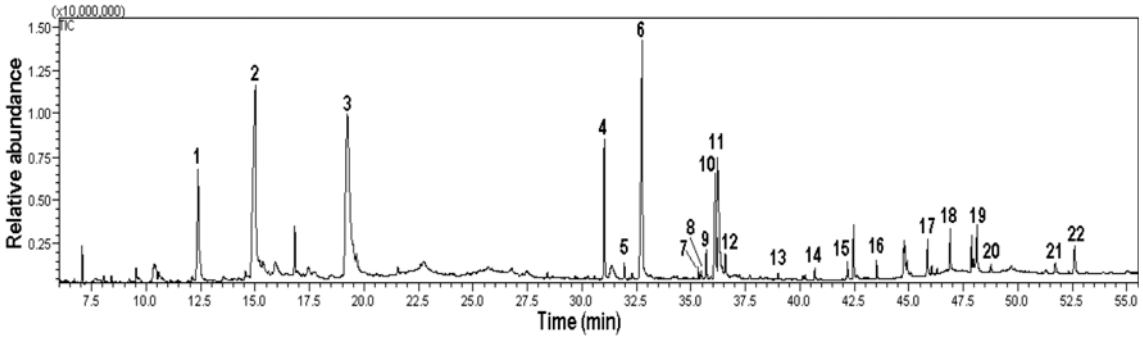

Fig. 4 GC-MS chromatogram of methanolic extract of Fozli peel.

can inhibit proliferation of cancer cells and influence apoptosis (Reddy Avula and Femandes, 2000).

In conclusion, the antioxidant effects and total phenolic and flavonoid contents of extracts of peel and flesh from five mango varieties were evaluated, and their potential antioxidant activities were assessed for the first time. The results of the present study demonstrate that the mango peel extracts have greater DPPH, OH, and ABTS free radical scavenging activities than the mango flesh extracts and that the antioxidant activities of mango peel may be mainly ascribed to its phenolic and, especially, flavonoid contents. In addition, the results clearly demonstrate that mango peel extract strongly inhibited the proliferation of HeLa cells via a mechanism involving the induction of apoptosis through the down-regulation of Bcl-2 and activation of caspases-3, -8, and -9. The results of present study suggest mango peel, rich in plenolics and unsaturated fatty acids, as a candidate in the development of anti-cancer drugs for the treatment of cervical cancer.

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