RESEARCH ARTICLE

Pectic-Oligoshaccharides from Apples Induce Apoptosis and Cell Cycle Arrest in MDA-MB-231 Cells, a Model of Human Breast Cancer

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Abstract

Background: The effects of plant products on cancer cells has become a field of major importance. Many substancesmay induce apoptosis in anti-cancer treatment. Pectins, a family of complex polysaccharides, and their degradation products may for exasmple exert apoptotic effects in cancer cells. Apples and citrus fruits are the main sources of pectin which can be applied for anti-cancer research. The present study concerned an intact form of pectic-oligoshaccharide named pectic acid (poly galactronic acid). Materials and Methods: Inhibition of cell proliferation assays (MTT), light microscopy, fluorescence microscopy (acridin orange/ethidium bromide), DNA fragmentation tests, cell cycle analysis, annexin PI and Western blotting methods were applied to evaluate apoptosis. Results: The results indicated that pectic acid inhibited cell growth and reduced cell attachment after 24h incubation. This did not appear to be due to necrosis, since morphological features of apoptosis were detected with AO/EB staining and cell cycling was blocked in the sub-G1 phase. Annexin/PI and DNA fragmentation findings indicated that apoptosis frequency increased after 24h incubation with pectic acid. In addition, the data showed pectic acid induced caspase-dependent apoptosis. Conclusions: These data indicate that apple pectic acid without any modification could trigger apoptosis in MDA-MB-231 human breast cancer cells and has potential to improve cancer treatment as a natural product.

Keywords: Apple - appotosis - breast cancer - cell cycle - MDA-MB-231 - pectic acid

Asian Pac J Cancer Prev, **16 (13)**, 5265-5271

Introduction

Breast cancer is one of the most common malignancies and an important reason of death among women (Kociecka et al., 2010). It is observed that common chemotherapies used to treat breast cancer induce apoptosis to control tumorgenisity (Allan et al., 1992). Numerous studies suggest that products from plant origin may contain cytotoxic effect on cancer cells. These products as therapeutic agents should be able to induce apoptosis and arrest cell cycle as a result of cancer attenuation. Apple has been known as a healthy fruit with high amounts of antioxidant. The advantages and therapeutic effects of apple on human health has been known for long times. It has been demonstrated that apple component such as flavanoids may play role as anti-cancer agents (Xiangjiu et al., 2008). Furthermore, it was reported that apple polysaccharides play role in inhibition of cancer growth progression (Qian et al., 2013). Pectin, abundant in apple, is a well-known polysaccharide. Commonly, pectins are extracted from citrus and apple for various applications (Kurita et al., 2008). Structurally, pectins contain α-D-galacturonic acid residue with gelling ability (Schols et al., 1992). Studies showed administration of citrus pectin in its heat or pH modified form could control and treating cancer metastasis (Platt et al., 1992). It is important that the pectin anti-metastatic effects have been done without any cell toxicity (Inohara et al., 1994). Plus, the anti-cancer effects of modified citrus pectin was observed in both *in vitro* and *in vivo* models of prostate, colon, myeloma and melanoma cancers (Qian et al., 2013).

In the case of breast cancer, it was indicated that fruits and vegetables reduced breast cancer risk (Malin et al., 2003). Other studies illustrated that whole-apple extract included protective effects in animal model of mammary cancer (Liu et al., 2005). Based on the studies, apple derivates play role in biological activities especially cancer protection and the anti-cancer effects of oligosaccharides in different tumors like prostate (Jackson et al., 2007) and colon cancer (Li et al., 2010). In the present study

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pectic acid, a type of pectic-oligoshaccharide (α-D-galacturonic acid) from apple is selected to investigate its anti-apoptotic properties in human breast cancer model, MDA-MD-231 cells. Biological roles of apple derivates and oligosaccharides in cancer protection were studied (Boyer et al., 2004), yet pectic acid as an anti-cancer agent was not characterized. The data presented here, demonstrated that in contrast with citrus pectin, which induced apoptosis weakly, pectic acid, itself includes apoptotic activity in cancer cells.

Materials and Methods

Cell culture

Human breast cancer cells, MDA-MB-231, and HUVEC (Human umbilical vein endothelial cell) were obtained from National Cell bank of Iran (NCBI). MDA-MB-231 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) contained penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO_2 humidified air. The same culture condition was used for HUVEC cells in Ham'F12/DMEM media. The medium was changed two times a week. The cells were detached with 0.25% (w/v) trypsin-EDTA (Sigma). Cell number and viability were determined using trypan blue (Sigma) exclusive dye and haemocytometer.

Cell Viability Assay (MTT assay)

The effect of pectic acid (α-D-galacturonic acid) (Fluka) on cell proliferation was examined by MTT assay. Approximately 2× 10^4 cells were seeded into 96-well plates and incubated for 24 h. Then different concentrations of pectic acid dissolved in the media were added and cells were incubated for 24h and 48h. RPMI 1640 alone was used as control. At the end of the time, 10 µL of MTT stock solution (Cell Proliferation Kit, Roche) was added to each well and incubated for 3 h at 37°C. Subsequently, MTT-formazan product in viable cells was dissolved by adding 100 µL of solubilization solution. The plates were incubated overnight at 37°C and the absorbance was measured at 550nm with Elisa reader (BioTec).

Fluorescence microscopic analysis of cell death

Apoptotic morphological changes were determined with Acridine orange (AO)-ethidium bromide (EB) (Merck) staining and fluorescence microscopy (Ziess). Both viable and dead cells have been stained with AO. AO emits green fluorescence by binding into DNA in viable cells. On the other hand, AO binds into RNA or single stranded DNA and emits red fluorescence in dead cells. The cells with ruptured membrane (late apoptotic or necrotic cells) stain with EB and their DNA emit red fluorescence. 1×10^6 cells were treated with different concentrations of AP for 24. After incubation, cells were detached and washed three times with phosphate buffer saline (PBS). The cell suspension then mixed with AO/EB solution in a final concentration of 100μg/ml and the cells were analyzed under fluorescence microscope and photographed.

DNA laddering assay

2×10^6 cells were treated with different concentrations of AP for 24 and 48h. After incubation, cell pellets were lysed with lysis buffer (TE- Triton X-100, proteinase K). DNA was precipitated with isopropanol on ice- cold and finally washed with ethanol and dissolve in TE buffer. DNA samples (2-4 μg) were electrophoresed on 1.5% agarose gel and stained with DNA safe red and visualized with UV gel documentation (uvdoc).

Cell cycle analysis by Flow cytometry

MDA-MD- 231 cells were cultured in 6-well plates and treated with pectic acid for 24h. After treatment, both floating and the attached cells were collected and fixed in 70% ethanol at 4°C with vortexing. After 2 h, the cells were washed with cold PBS and stained with propidium iodide (20 µg/ml) in the presence of RNase A (100 µg/ml) for 30 min at 37°C. DNA content was analysed with a flow cytometer using WinMDI software.

Annexin V apoptosis detection

Cells were treated with different concentration of pectic acid for 24h .After treatment, cells were stained by annexin-V- FLUOS staining kit (Roche). Next, they were washed with PBS and resuspended in 100 μl of annexin-V-FLUOS labeling solution (HEPES buffer, annexin-V-FLUOS labeling reagent and propidium iodide). Cells were incubated 10- 15 min at room temperature (RT) and then read by a flow cytometer.

Immunoblotting

Following pectic acid treatments, the cells lysed in lysis buffer (Tris- HCl 62.5 mM, pH=6.5, Sodium dodecyl sulfate 200 mg, Glycerol 10%, (phenylmethanesulfonylfluoride 1 mM). Protein concentration was assessed using Bradford's method (Bradford 1974). The lysates were separated on 12% SDS-PAGE gel and transformed to nitrocellulose membrane. Finally, the membranes were probed with caspase 3, and detected by chemiluminescence using enhanced electrochemiluminescence (ECL) reagents (Amersham Bioscience). Subsequently autoradiography was done and the bands were quantified with Image J software.

Anchorage-independent growth assay

In order to determine cells ability to grow in an anchorage-independent manner, MDA-MB-231 cells were plated in six- well dishes coated with 0.5% agarose (Merck) dissolved in culture medium containing 10% FBS. The plates then covered with medium containing 0.2% agar and replaced every 3 days for 2 weeks. Cells colonies were counted using phase-contrast photomicrography. Colony formation efficiency was calculated as the ratio of the number of colonies to total seeded cells.

Statistical analysis

The values are reported as mean ± S.E.M. The experiments were repeated at least four times. The results were analyzed using one-way ANOVA followed by Tukey's post hoc. $P \leq 0.05$ were considered statistically significant.

Results

Effect of pectic acid on cell viability

In the primary step, the cell viability assay was done in order to determine the most effective time and concentration of pectic acid. To reach this goal, MDA-MB-231 cells and HUVEC cells were treated with different concentration of pectic acid (0, 0.01, 0.1, 0.5, 1, 2.5, 5 mg/ml) for 24 and 48h. HUVEC cells were tested as a control for the effect of pectic acid on non-cancerous cells. Figure 1 indicates that Pectic acid inhibited cell growth after 24h significantly and in 48h the highest rate of tumor cell was achieved. As well as this, incubation with pectic acid reduced the number of attached cells (Figure 2). As the IC50 of pectic acid did not mainly differ in 24h and 48h, next procedures were performed for 24h pectic acid treatments. In this condition, pectic acid did not exert strong effect on HUVEC cells (Figure 3). Thus, it seems pectic acid had no strong cytotoxic effect on non-cancerous cells, while it caused cell growth inhibition in cancer cells.

Morphological assessment of apoptosis with AO/EB double staining

Under fluorescence light, intact DNA of viable cells is bound to AO and emits green fluorescence. Fragmented DNA in early apoptotic cells are seen as several green nuclei. EB stains DNA in unhealthy cells and emits orange/red fluorescence. Necrotic and late apoptotic cells are distinguished as orange/red nuclei with condensed or fragmented chromatin. Figure 4 shows results from AO/EB staining of MDA-MB-231 cells treated with pectic acid. It can be distinguished that the number of viable cells reduced by increasing concentration of pectic acid. As well as apoptotic features such as fragmented chromatin and membrane blebbing were also determined. Since that the necrotic cells did not increase, it can be claimed that pectic acid caused cell death through apoptosis. Quantitative analysis of AO/EB double staining is summarized in Table 1.

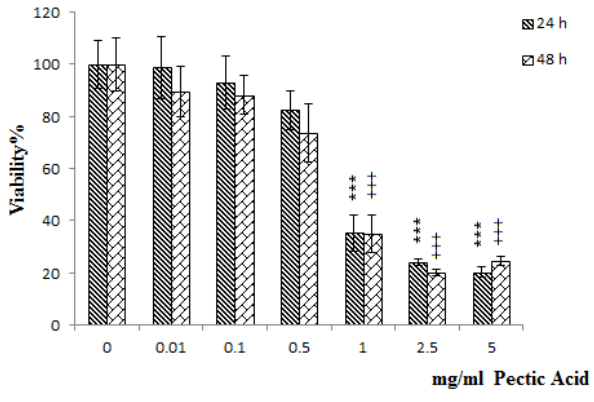

Figure 1. Pectic Acid Inhibited Cell Growth of MDA-MD-231 Breast Cancer Cell Line. The cells were treated with pectic acid for 24 and 48 h. Columns are the mean values percent of viability and differences between groups were determined using one-way ANOVA. n=12, +++ and ***, significant differences (P<0.001)

DNA laddering assay

One of the hallmarks of apoptosis is DNA fragmentation in which chromosomal DNA is fragmented into 180-200 bp fragments in many different cell types (Cohen et al., 1992). In the next step, DNA fragmentation was evaluated in pectic acid treated MDA- MD 231cells. As shown in Figure5 more DNA fragmentation was detected in presence of higher pectic acid concentration (2.5 and 5 mg/ml) and no fragmentation was seen in untreated cells. DNA fragmentation is an early event in apoptosis whereas MDA- MD 231cells treated with pectic acid started to undergo apoptosis up to 24 h.

Cell cycle analysis by Flow cytometry

To investigate how pectic acid affects cell growth, cell-cycle analysis was determined by flow cytometry. During apoptosis, low-weight fragmented DNA leaked

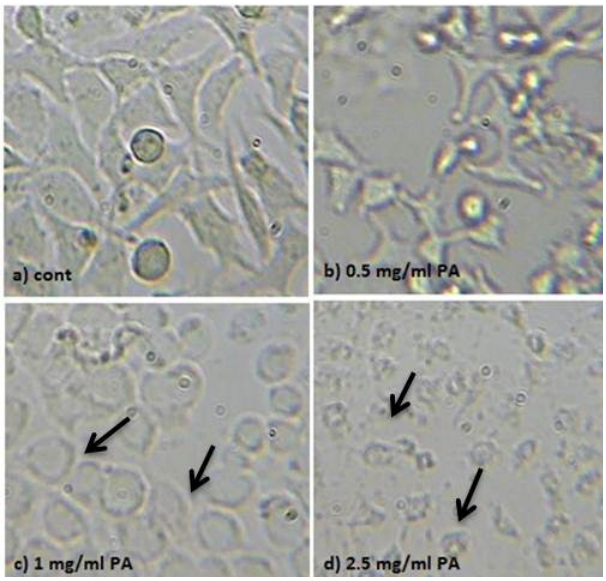

Figure 2. Microscopic Observation of Morphological Changes in MDA-MD-231 Cells After Pectic Acid Treatment for 24h. Cell attachment decreases with increasing concentration of pectic acid. The arrows show round cells with decreasing attachment to the surface (100X)

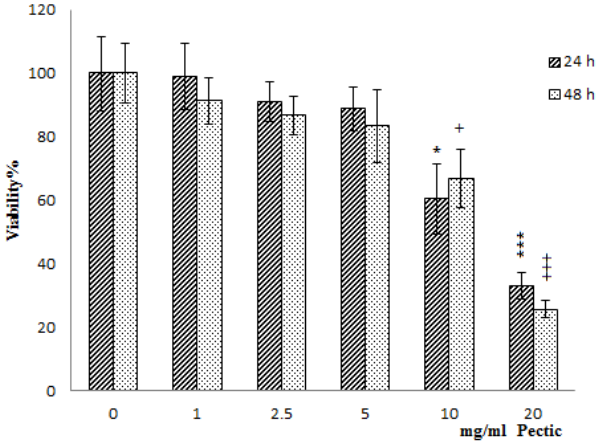

Figure 3. Pectic Acid Effects on Cell Growth Inhibition on HUVEC Cells. Pectic acid could not able to inhibit HUVEC cell growth. Columns are the mean values percent of viability and differences between groups were determined using one-way ANOVA. n=12, ***, significant differences (P < 0.001)

from cells, suggesting that apoptotic cells usually include a distinct sub-G1 peak (Xuan Te al,. 2005). As shown in table 1 a higher proportion of MDA-MD 231 cells treated with 1 and 2.5 mg/ml of pectic acid showed high peak in G0/G1 phase (66.74%±7.2) in comparison with untreated cells (2%± 0.3). The amount of sub-G1 region was not significantly increased in lower concentrations of pectic acid treatments (Figure 6) indicating that pectic acid induced a very strong sub-G1 arrest representing apoptotic cells.

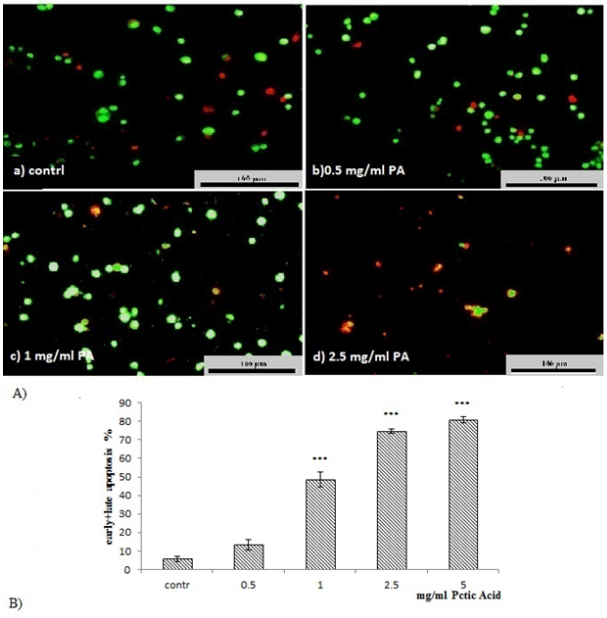

Figure 4. Morphological Study of MDA-MB-231 Cells by AO/ EB Staining Treated with Pectic Acid for 24h. The number of stained cells at least counted in 10 fields and each test was done three times. Cells blebbing were shown with arrows

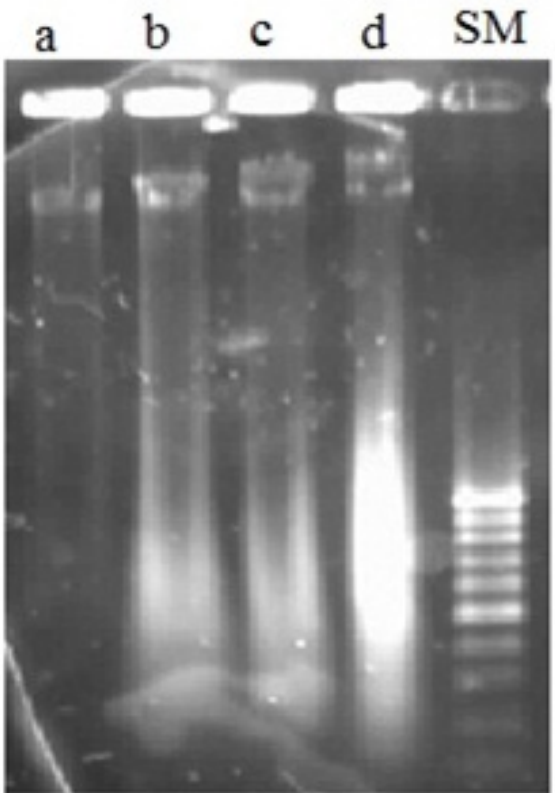

Figure 5. DNA Laddering Assay of DNA Extracted from MDA-MB-231 Cells Treated with Pectic Acid for 24h. Control cells (0 mg/ml pectic aid) were innate while fragmentation occurs in the presence of pectic acid. (a) 0 mg/ml; (b) 0.5 mg/ml; (c) 1 mg/ml; (d) 2.5 mg/ml (SM) DNA size marker

Annexin V staining apoptosis detection

The loss of plasma membrane integrity is the latest stage of cell death (Neves and Brindle, 2014). Annexin V/PI staining was performed to quantify last stages of apoptosis. In FACS analysis, MDA-MB 231 cells treated with pectic acid, a dose dependent apoptotic death was observed in 24h. The data (Figure 7) showed less than 5% of cells in untreated group were in late apoptosis region (3.78%), while the percentage of apoptosis (early and late) increased mainly in presence of pectic acid. In the higher concentrations of pectic acid (1 and 2.5 mg/ml) the percentage of late apoptosis increased according to dose increasing. The PI analysis showed no significant number of cells with either annexin V (+) and PI (+) suggesting that the cell death occured with pectic acid was apotosis not necrosis (Table 1).

Immunoblotting

Cell death caused upregulation of the pro apoptotic proteins such as Bax, which lead to caspase activation. Caspases a family of cysteinedependent enzymes are key effectors in apoptosis pathway. Caspase-3 is an important member of this family, which is accountable for PARP-1 cleavage (Kaufmann et al., 1993). Pectic acid mediated apoptosis via caspase-3 activation. As shown in Figure 8 increasing in pectic acid concentration caused over expression of caspase-3. It seems that pectic acid was capable to upregulate pro apoptotic proteins and induced caspase-3 dependent apoptosis in MDA-MD-231 breast cancer cells after a period of 24h.

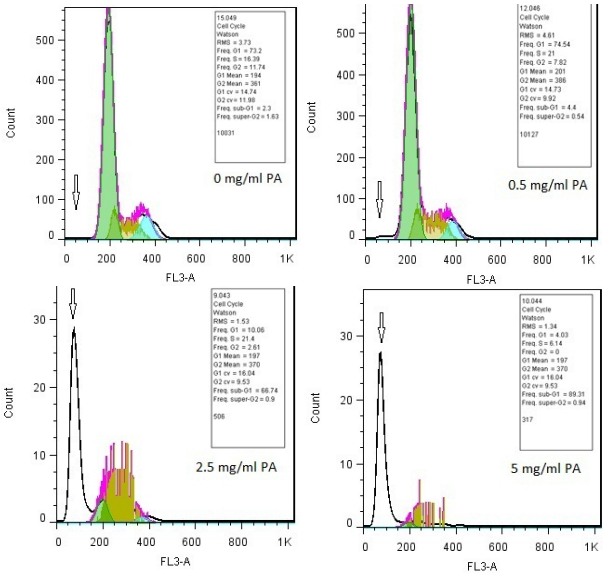

Figure 6. Flowcytometric Analysis of Cell Cycle in MDA-MB-231 Cells Treated with Pectic Acid for 24 h. The Sub-G1 region was shown with arrows. Figures are indicated one of three experiments

Table 1. Effects of Pectic Acid on Various Apoptotic Parameters in MDA-MB-231 Cells

PA (mg/ml)	AO/EB double staining	cell cycle analysis	Annexin V analysis		
	apoptosis % (Late+ Early)	subG1%	Early apop. %	Late apop.%	Necrosis%
control (0)	5.71±1.37	2±0.3	1.9	1.14	2.7
0.5	13.38±2.73 (p<0.05)	5.4±1.08 (p>0.05)	11.64	5.5	2.7
1	48.62±3.84 (p<0.001)	12.46±1.35 (p<0.001)	18.25	5.2	2.9
2.5	74.58±1.044 (p<0.001)	59.14±7.28 (p<0.001)	28.15	10.75	2.58
5	80.85±1.52 (p<0.001)	82.05±7.28 (p<0.001)	16.63	43.63	8.1

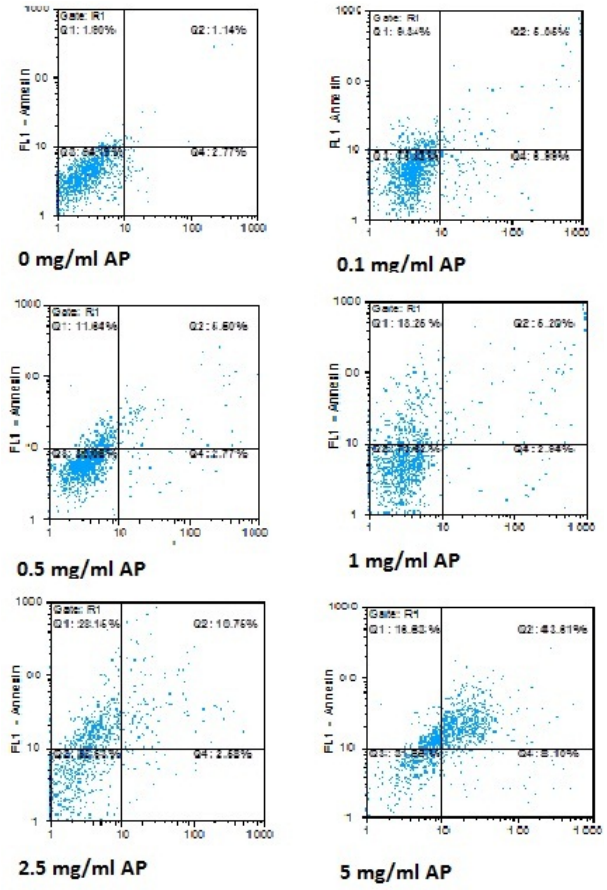

Figure 7. Annexin V Apoptosis Detection of MDA-MB-231 Cells in Presence of Pectic Acid After 24h. Q1 showed early apoptotic cells, Q2 showed cells in the end stage of apoptosis (late apoptosis). Q3 showed viable cells, Q4 showed necrotic cells

Anchorage-independent growth assay

Lastly, the effect of pectic acid on clonogenic properties in semisolid medium was determined. The data (Figure 9) indicated that in the presence of pectic acid colony-forming efficiency was significantly reduced. It appeared pectic acid caused reduction in both colony formation and the size of colonies. The cells with no pectic acid formed the highest number and largest of colonies, while the cells treated with 1 mg/ml pectic acid had the fewest number of small colonies. In the presence of 2.5 mg/ml pectic acid, no colonies formed which was due to cell death. The reduction of anchorage-independent growth owing to pectic acid could be suggested that cells undergo transformation and loss tumorigenic properties.

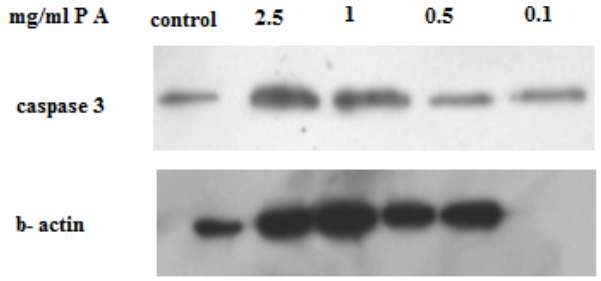

Figure 8. The Effect Pectic Acid on Caspase 3 Level in MDA-MB-231 Cells. The cells were treated with pectic acid at the indicated dose. An amount of 25 mg proteins was loaded for western blot assay (n= 3)

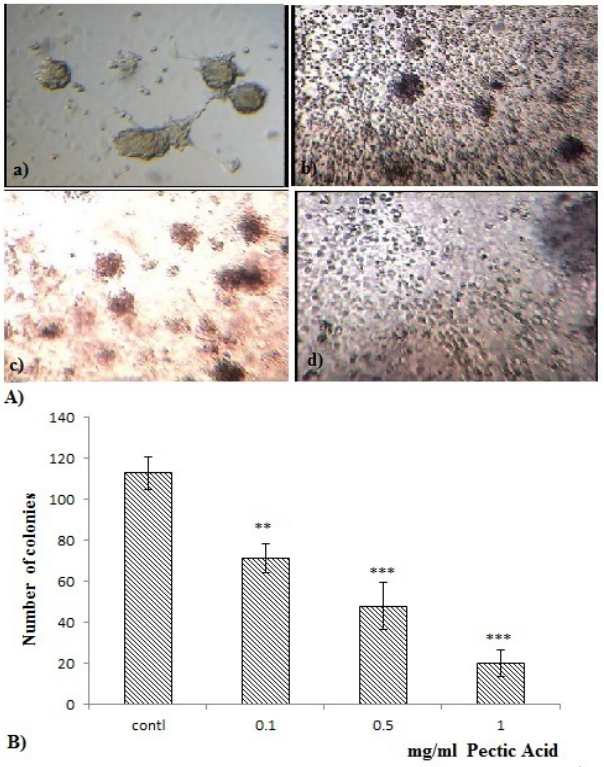

Figure 9. The Colony-Formation Assay of MDA-MD 231 Cells in the Presence of Pectic Acid. A) Colony images are (a) 0 mg/ml, (b) 0.1 mg/ml, (c) 0.5 mg/ml, (d) 1 mg/ml pectic acid. B) Data analysis of colony numbers. Each row demonstrates mean ± SEM. n=5, ** and ***, significant differences (P < 0.01 and P < 0.001)

Discussion

It has been reported that regular consumption of natural substances promoted cancer therapy without increasing

vulnerability to immunologic side effects of chemical drugs (Li et al., 2012). The efficacy of these substances as an anticancer treatment may be depending on apoptosis induction in cancer cells or biodegradable transporters for drug delivery systems to rich more effective anti-cancer compounds (Ghasemali et al., 2013). Pectin, a complex carbohydrate is abundantly present in fruits such as apple, lime and citrus. As a natural substance, it plays role in cancer treatment. Newly, the preventive effect of pectins on tumor or metastatic cells has been unveiled. Even more, pectins are able to reduce primary tumor growth (Nangia-Makker et al., 2002). Thus, inevitable role of pectin to induce apoptosis in cancer cells is undeniable. In the present study, anticancer effects of pectic acid from apple on cell proliferation inhibition and induction of apoptosis in cellular model of breast cancer were investigated. Studies illustrated that apple juice decreased the rate of DNA damage and hyper proliferation in animal model of colon cancer. These cancer-protective effects were due to procyanidins and pectin abundance in apple (Barth et al., 2005).

This study indicated that pectic acid in its intact form caused growth inhibition correlated with cell death and apoptosis induction in breast cancer cells. The results of cell growth inhibition showed that pectic acid contains cytotoxic effects on cancer cells in comparison with noncancerous cells. Interestingly, it has been demonstrated that apple pectin (oligogalactan) includes protective effects on colon carcinogenesis mouse (Chang et al., 2005). Cell blebbing and DNA fragmentation observed in dual staining of AO/EB illustrated that pectic acid could induce apoptosis morphological. The increasing of sub-G1 area in cell cycle analysis intensified that pectic acid induced cell death via apoptosis. In addition, in higher concentrations of pectic acid G2/M arrest was occurred which may be related to the time of DNA repairing (Ho et al., 2009). It should be noted that in cell viability assay the whole of sub-G1 and G2/M arrested cells were measured as death cells. These data confirm the outcomes of previous studies on the effects of citrus pectin on sub-G1 arrest in LNCap, DU-145 and PC3 prostate cancer cell lines (Tehranian et al., 2012; Yan et al., 2010). Finally, employing annexin V/PI staining confirmed that pectic acid induced cell death following early and late apoptosis. It showed that pectic acid caused apoptosis rather than an accidental uncontrolled cell death, necrosis. Likewise, pectic acid triggered apoptosis by interfering with proteins take part in apoptotic pathways, such as caspase-3 and Bax. Other studies unveiled the apoptotic effects of pectin on colorectal, prostate, and colon cancer cells (Zhanga et al., 2013; Avivi-Green te al,. 2000; Bergman et al., 2010). Similarly, pectin affected cancer progression via induction of apoptosis and upregulation of caspase-3 in colon and prostate cancer (Avivi-Green te al,. 2000; Jacson et al., 2007). It was shown that Pectins are able to increase caspase-3 activity and apoptosis in human SKOV-3 ovarian cancer cells (Hossein et al., 2013). Pectins used in these studies were modified and fragmented such as pectasol, a PH-/temperature-modified form of citrus pectin. Similarly Qian Li demonstrated that apple oligosaccharide acts as a chemoprevention agent or anti-tumor agent in colon cancer (Li et al., 2013), while the present data showed that pectin extracted from apple in the form of pectic acid (oligogalactan) is a potential anti-cancer agent. According to Liu et al, whole-apple extracts contained protective effects in animal model of mammary cancer (Liu et al., 2005). Hence, these observations suggest that entire apple pectic acid can be used as anti-cancer drug to improve traditional therapy used for breast cancer in order to reduce tumorgenicity. Due to the fact that heat modified form of pectic acid prepared according to Debra Mohnen methods, (Avivi-Green et al., 2000) did not intensify pectic acid effects in MDA-MD 231 breast cancer cells (data did not show). Thus, intact pectic acid might improve cancer therapy quality without worrying about long-term administration and side effects, as pectic acid originates from fruit.

Of these data, the former is the mechanisms of pectic acid in cancer cells. It was reported that MCP (modified citrus pectin) is bound to glycan-binding proteins, Galectin-3 and acted (Inohara et al., 1994; Nangia-Makker et al., 2000). The possible mechanisms of pectin via Galectin-3 function were studied in colorectal and prostate cancers (Li et al., 2010; Wang et al., 2010) . Moreover, it was reported that Galectin is expressed in human breast cancer cell, MDA-MB-231(Honjo et al., 2001), even though the possible mechanisms of pectic acid in apoptosis induction in MDA-MD-231 cancer breast cells need more investigations.

Acknowledgements

This study was supported in part by the grant from University College of Sciences, University of Tehran, Tehran, Iran and Endocrinology and Metabolic Research Institute of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. The authors are thankful to Mr. Omid Lotfi and Azar Berahmeh for their useful advises.

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