Comparative cellular and molecular analysis of cytotoxicity and apoptosis induction by doxorubicin and *Baneh* in human breast cancer T47D cells

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Abstract It is now widely accepted that dietary phytochemicals inhibit cancer progression and enhance the effects of conventional chemotherapy. In this report, we comparatively studied the cellular and molecular aspects of apoptosis induction by the methanolic extract of *Baneh* fruit skin in comparison to Doxorubicin (Dox), a well-known anticancer drug, in human breast cancer T47D cells. The MTT assay was used to determine the antiproliferative effects. The flow cytometric and microscopic analyses were done to evaluate the apoptosis induction. Furthermore, western blot analyses have been done to study the role of key molecular players of apoptosis including caspase 3 and PARP. The *Baneh* extract showed strong antiproliferative activity

against T47D cells in a dose- and time-dependent manner that was comparable to and even stronger than Dox in certain concentrations. Analysis of *Baneh*-treated cells by flow cytometry and fluorescence microscopy indicated strong apoptosis induction and nuclear morphological alterations similar to or greater than Dox. Finally, molecular analysis of apoptosis by western blotting proved activation of caspase 3 followed by poly ADP ribose polymerase (PARP) cleavage more efficiently in *Baneh* than in Dox treated cancer cells. These findings indicate that *Baneh* extract contains phytochemicals which act as inhibitor of cell proliferation and inducer of apoptosis in human breast cancer T47D cells that makes it a potentially good candidate for new anticancer drug development.

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Abbreviations

AnnV	Annexin-V-FITC
<i>Baneh</i>	<i>Pistacia atlantica sub kurdica</i>
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
Dox	Doxorubicin
IC ₅₀	Half maximal inhibitory concentration
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NF- κ B	Nuclear factor κ B

PARP Poly ADP ribose polymerase
 PI Propidium Iodide

Introduction

Cell populations are tightly under the control of the rates of proliferation, differentiation and death. Any defect in each of these processes may lead to uncontrolled cell growth or uncontrolled cell death. Carcinogenesis as a multi-step process is driven by close interactions between oncogene activation, tumor suppressor inactivation and the cell death machinery. There are six essential alterations in cell physiology causing malignant growth including: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Zhivotovsky and Orrenius 2006). Applied research is focusing in two fields in order to regulate cancer growth: control of cell cycle and induction of cell death.

The ultimate goal of cancer chemotherapy is induction of cell death in tumor cells. There are two main mechanisms in multicellular organisms for elimination of cells including necrosis and apoptosis. Necrosis associates with rupture of the plasma membrane and is an inflammatory process (Satyanarayana and Kaldis 2009).

Apoptosis, a highly evolutionary conserved, ATP-dependent and tightly regulated process plays an essential role in cellular homeostasis and development. The usual cancer therapy approaches such as chemotherapy, irradiation, immunotherapy or suicide gene therapy are noticeably triggering apoptosis in cancer cells (Fulda and Debatin 2004). Uncontrolled apoptosis leads to pathological conditions such as developmental defects, autoimmune disease, neurodegeneration or cancer. Generally, reduced apoptosis is associated with tumorigenesis (Fadeel and Orrenius 2005). Upregulation or over-activation of antiapoptotic proteins and down regulation or mutation of pro-apoptotic proteins leads to resistance of tumor cells to therapy (Jin and El-Deiry 2005). There are two apoptotic pathways, one activated through membrane death receptors (extrinsic) and the other one activated by mitochondria membrane potential alterations (intrinsic): both results in initiating caspase activation, 8 or 9 respectively (Fulda and

Debatin 2006). Both apoptotic signaling pathways lead to the activation of the effective caspases, making these proteases the final effectors of apoptotic cell death. Caspases lead to cleavage of a number of different substrates in the cytoplasm or nucleus, leading to the morphological features of apoptotic cell death including cell shrinkage, nuclear DNA fragmentation and membrane blebbing. Apoptosis is strenuously controlled in multiple intracellular nodes; each of them is induced by pro-and anti-apoptotic proteins (Fulda and Debatin 2004, 2006).

Breast cancer has been known as the most common cancers in women. Epidemiological studies exhibit that alteration in lifestyle and diet results in the increasing rate of breast cancer incidence in countries with a low incidence rate. Each year over 1.15 million women are diagnosed with breast cancer and almost half of them die (Montazeri et al. 2008; Yip 2009). Therefore, an urgent need is felt for developing alternative therapeutic erasures against this deadly disease. Despite the advances in medical research and technology, the complete cure for cancer remains elusive. Current cancer therapies (radical surgery, chemotherapy and radiotherapy) lead to undesired physical and psychological distress of the patients. Thus, incessant global efforts are necessary in research for new anticancer compounds, which have high therapeutic efficacy and less side-effects compared to the existing anti-cancer drugs used in the clinic.

The *Psitacia atlantica sub kurdica* (*Anacardiaceae*), with the local name of *Baneh* grows in Iran which is used in the production of chewing gum and traditionally, in the treatment of peptic ulcer and various diseases. Clear indications of the antiproliferative effect and death inducing characteristic on several tumors were presented for other plants from the same genus of *Baneh*. Indeed, gum mastic of *P. lentiscus* showed strong inhibitory effect on expression and function of androgen receptor in LNCaP prostate cancer cells (He et al. 2006). Moreover, chios mastic of *P. lentiscus* L. var *chia* has antiproliferative and death inducing effects in human colon carcinoma (Balan et al. 2007). Gum mastic of *P. lentiscus* inhibited cell growth and blocked cell cycle in the G1 phase and decreased expression of cyclin D1, p-Akt protein level and I κ B α protein level and suppressed NF- κ B activity in androgen-independent prostate cancer cells (He et al. 2007a). Moreover, it was showed that gum mastic of *P. lentiscus* leads to

upregulation of both mRNA and protein levels of maspin (tumor suppressor) in prostate cancer cells (He et al. 2007b).

Despite numerous reports regarding the chemopreventive effect of mastic gum of *P. lentiscus*, no reports are available about *P. atlantica sub kurdica* on tumors. In order to evaluate the antitumor activity of *P. atlantica* extract on T47D cells, we studied cell death induction in cells treated with *Baneh* in comparison to Dox, one of the most clinically useful chemotherapy drugs.

Materials and methods

Materials

RPMI 1640 and FBS were purchased from Biosera (East Sussex, UK). Pen-strep and Trypsin–EDTA were obtained from Gibco (Paisley, UK). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was purchased from Merck (Darmstadt, Germany). Dox (Ebedoxo) was purchased from Ebewe (Unterach, Austria). Dimethyl sulfoxide (DMSO) and methanol were obtained from Merck. The cell culture dishes were obtained from Greiner Bio-one (Frickenhausen, Germany).

Extraction of plant materials

The fruits of *Pistacia atlantica sub kurdica* (*Baneh*) were collected from trees in Kurdistan province of IRAN and authenticated by Dr Amin, Faculty of Pharmacy, Tehran University of Medical Sciences (6673-TEH). The skin of the fruits were removed, dried in darkness, powdered and stored at 4 °C. This powder was extracted via percolation method by Ethanol: H₂O (70:30) for 7 days and was dried in air. Total extracts were used for fractionation via liquid–liquid method by methanol that after drying were used for in vitro tests. Then the dried extract of *Baneh* was dissolved in methanol (0.5 g in 1 ml), diluted in PBS (1:10) and filter sterilized. This stock solution of extract was freshly diluted with RPMI 1640 complete culture medium before each experiment.

Cell culture

The human breast cancer cell line T47D (ATCC, HTB-133) was cultured in RPMI 1640 medium

supplemented with 10% heat-inactivated fetal bovine serum, 100 µg.ml^{−1} streptomycin and 100 U. ml^{−1} penicillin. The cell cultures were grown in a humidified 5% CO₂ at 37 °C.

Cytotoxicity assay

The colorimetric MTT assay was used to determine cytotoxicity of *Baneh* extract on T47D cells (Azizi et al. 2010). Briefly, 5000 cells/well were seeded in 96-well plates. Cells were exposed to different concentrations of *Baneh* extract (0–2 mg/ml) or IC₅₀ of Dox at 250 nM concentration previously determined and reported by our group (Kaabinejadian et al. 2008) for 24–72 h. Cells were then incubated with MTT solution (4 mg/ml in PBS) for 3 h at 37 °C in dark. Results of the MTT assays were obtained using a microplate reader (TECAN SUNRISE, Grödig, Austria) at 540 nm. The OD₅₄₀ of control cells was considered as 100% and cell proliferation was determined as a percentage to control RPMI. The IC₅₀ value was defined as the concentration that caused a 50% inhibition of cell growth compare to control RPMI. Each experiment was performed 3 times in triplicate format and the results were presented as mean ± SE.

Colonogenic survival assay

For colonogenic assay, 1 × 10³ single cells were seeded in 10-cm² plates, 24 h later treated with *Baneh* (1 mg/ml) or Dox (250 nM) for 3 h, medium was exchanged and recovered for additional 10 days. Then, colonies were stained with Giemsa and observed along with taking pictures.

DNA fragmentation

DNA fragmentation was assessed in order to determine the percent of apoptotic cells as previously described (Fulda et al. 1997). Briefly, T47D cells were seeded with the density of 75,000 cell/well in 24-well plates and treated with *Baneh* extract (1 mg/ml) or Dox (250 nM). Cells were trypsinized and stained with propidium iodide (PI) at each time point. FACS analysis of DNA fragmentation of PI stained nuclei was performed by FACScan (BD Biosciences, USA). Each experiment was repeated 3 times and the results were shown as mean ± SE.

Apoptosis assay

In order to study the different stages of apoptosis, Annexin-V-FITC (AnnV) and PI stained cells were evaluated by Flow Cytometer (Partec, Münster, Germany). Briefly, cells in exponential phase of growth were plated in six-well plates with 3×10^5 cells/well density and incubated with *Baneh* extract (1 mg/ml) and Dox (100 nM). Cells were collected by trypsinisation after 48 h. Stained cells were assessed for apoptosis using Flow Cytometer with flowmax software (Abdollahmohammadi et al. 2009). Results were expressed as mean $\pm$ SE of three independent experiments.

Morphological evaluation of the apoptotic cells

Cells (35,000 cell/well) were seeded in 8-well chamber slides, allowed to settle for 24 h, treated with *Baneh* extract for 24 and 48 h. Methanol-fixed cells were incubated with 0.02% DAPI in Methanol to stain nuclear DNA for 10 min in darkness and 37 °C. The cells were washed with cold PBS two times and observed along with taking pictures using an Olympus IX70 fluorescent microscope.

Western blotting

The T47D cells (5×10^6 cell) were seeded in 10-cm² plates and treated with *Baneh* extract and Dox for indicated time points as described previously. Briefly, cells were lysed for 15 min on ice in lysis buffer [Tris HCl 30 mM, NaCl 150 mM, Triton X100 1% and Glycerol 10%, supplemented with a proteinase inhibitor] followed by high-speed centrifugation. Protein concentration was determined using bicinchoninic acid (Pierce, Rockford, IL). Fifty micrograms of lysate were electrophoresed on a 12% SDS–PAGE and electroblotted onto Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Munich, Germany). Membranes were blocked for 1 h in PBS supplemented with 5% milk powder and 0.1% Tween 20. Western blot analysis was done by using the following antibodies: rabbit polyclonal anti-caspase-3 (Cell Signaling Technology, Beverly/MA, USA); rabbit polyclonal anti-poly ADP ribose polymerase (PARP) (Roche, Mannheim, Germany) and goat-anti-mouse IgG, goat-anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz/CA, USA), conjugated to horseradish peroxidase. Enhanced

chemiluminescence was purchased from Amersham Bioscience (Munich, Germany). Mouse anti- β -actin monoclonal antibody (Sigma) was used as loading control. All western blots are shown representative of at least three independent experiments.

Statistical analysis

All data were shown as mean $\pm$ SE of independent experiments. Data were statistically compared using one-way ANOVA with Tukey post hoc and $p < 0.05$ were considered statistically significant.

Results

Effects of *Baneh* extract on cell viability

The effects of *Baneh* fruit skin extract on the viability of breast cancer T47D cells was assessed by MTT assay at 24–72 h time points at the doses shown in Fig. 1. The *Baneh* extract showed significant growth inhibitory effect in a dose- and time-dependent manner. The IC₅₀ for *Baneh* on T47D cells was 1 mg/ml after 48 h of exposure. Moreover, we used Dox treatment as a positive control with the IC₅₀ concentration of 250 nM after 48 h. The IC₅₀ concentrations were then used to further study the mechanism of action of *Baneh* extract in comparison to Dox. Furthermore, *Baneh* extract and Dox suppressed colony formation, indicating that they also could affect long-term survival (Fig. 1b).

Effects of *Baneh* extract on apoptosis induction

Apoptotic type of cell death was determined by analysis of DNA fragmentation, translocation of phosphatidylserine to the outer membrane leaflet and typical morphologic features. Apoptosis is characterized by changing in DNA integrity and nuclear morphology. For this reason, we performed DNA fragmentation analysis by Flow cytometry as described in methods. *Baneh* induced strong DNA fragmentation after 48 h whereas Dox-treated T47D cells showed strong DNA fragmentation after 72 h (Fig. 2a, b).

The cells distribution profile in quadrants is indicative for the percentage of alive (AnnV[−]/PI[−]), early apoptotic (AnnV⁺/PI[−]) and late apoptotic (AnnV⁺/PI⁺) cells (Fig. 3a). The percentage of viable, early

Fig. 1 Cytotoxicity of *Baneh* extract and Dox. **a** The T47D cells were seeded in 96-well plates (5000 cells/well) in RPMI 1640 culture medium. After 48 h of seeding, cells were exposed to different concentrations of *Baneh* extract (B; 0, 0.5, 0.75, 1, 1.25, 1.5 and 2 mg/ml) and Dox (250 nM) as a positive control. After 24, 48 and 72 h of exposure, the cytotoxicity was determined using colorimetric MTT assay. Data are shown as

mean $\pm$ SE of three independent experiments in triplicate format. * represents the mean difference with $p < 0.001$ in comparison to RPMI. **b** Cells were treated with *Baneh* extract (B; 1 mg/ml), Dox (D; 250 nM) and RPMI (CTRL) then colonogenic assay was performed by giemsa staining. A representative experiment of three independent experiments shown

apoptotic and late apoptotic cells are shown in Fig. 3b. Moreover, primary morphological changes in the nucleus were clearly shown in Fig. 4, including condensation in peripheral zone of the nucleus and DNA fragmentation at 24 h. With increasing the exposure time, more of the cell population was going to die and shrinkage of nucleus was observed.

Induction of caspase activation

In order to further confirm the apoptosis induction at molecular level, western blot analysis of caspase 3 and its main substrate PARP were performed. In *Baneh* treated cells cleavage of caspase 3 to p17/p12 was observed after 24 h (Fig. 5). Following activation of caspase 3, PARP cleavage to p89 was also detected in *Baneh* treated cells after 24 h. In contrast

to *Baneh*, the caspase 3 activation and PARP cleavage were observed only after 48 h upon Dox treatment (Fig. 5).

Discussion

The balance between cell cycle arrest and cell death is necessary to maintain genomic integrity in proliferating cells. Defects in this balance are thought to contribute to the development of cancer and other pathological conditions (Clarke and Allan 2009). Chemo-toxic effects of natural compounds, mediated via apoptotic pathways, have been well established. The compounds with proapoptotic effects could prevent cancer incidence by enhancing elimination of initiated precancerous cells (Khan et al. 2008).

Fig. 2 Effects of *Banah* extract and Dox on DNA fragmentation. The T47D cells were treated with IC₅₀ of *Banah* extract (B; 1 mg/ml), Dox (D; 250 nM) and RPMI (CTRL) for 12, 24, 48 and 72 h. Then DNA-fragmentation of PI-stained nuclei was determined by FACS analysis.

a Representative data of flow cytogram of 3 independent experiments. **b** Data are expressed as mean $\pm$ SE of 3 independent experiments. * $p < 0.001$ and ** $p < 0.0001$ in comparison to control RPMI (CTRL). (M1 is a parameter of the flowJo software for analyzing Sub G1 data to determine the percentage of apoptotic cells with range of 10^1 – 10^3)

Epidemiological studies show that consumption of phytochemicals from whole grains, vegetables and fruits reduce the risk of human cancers such as breast cancer (Er and Radhakrishnan 2007).

Dox is a commonly used drug in clinics against breast cancer. Inhibiting Topoisomerase II, Dox mediates DNA damage, leading to cell cycle arrest at G1 and G2 and programmed cell death (Minotti et al.

Fig. 3 Effects of *Baneh* extract and Dox on apoptosis induction. The T47D cells were treated with *Baneh* extract (B; 1 mg/ml), Dox (D; 250 nM) and RPMI (CTRL) for 48 h. Then apoptosis induction was analyzed by flow cytometry after Annexin V-FITC/PI co-staining (AnnV/PI). **a** Representative dot plots of T47D cells showing the viable cells (AnnV⁻/PI⁻),

early apoptotic (AnnV⁺/PI⁻), late apoptotic or already dead (AnnV⁺/PI⁺), and Necrotic cells (AnnV⁻/PI⁺). **b** The results are shown as percentage of cells that are alive, early apoptotic and late apoptotic. Data are expressed as means $\pm$ SE of 3 independent experiments. * $p < 0.05$ in comparison to control RPMI (CTRL)

2004; Solmon and Gabizon 2008). Unfortunately, the use of this anthracyclin is frequently accompanied by dose-dependent cardiotoxicity (Peng et al. 2005).

Several studies showed that the health benefits of natural mixture of phytochemicals due to nutrients additive and/or synergistic interactions are more effective than of single constituents (Loutrari et al. 2006). Methanolic extracts were traditionally used for anticancer screening because of the observation that polar compounds contained anticancer properties (Tan et al. 2005).

In this study, data clearly showed that *Baneh* fruit skin extract has an inhibitory effect on cell

proliferation in breast cancer cells that is comparable with the effect observed with Dox. The reduction of cell viability showed a time- and dose-dependent pattern. Furthermore, we evaluated the cytotoxic effect of *Baneh* extracts on the immortal NIH 3T3 cell line which has normal-like properties. The extract showed slight cytotoxic effect on NIH 3T3 cells which was significantly lower than cytotoxicity on human breast cancer T47D cells (data not shown).

Reduction in metabolic activity of the cells is due to the reduction in number of cells due to cell cycle block and/or cell death. Recently, it was demonstrated that many plant extracts have the ability of

Fig. 4 Effects of *Baneh* extract on nuclear fragmentation. T47D cells were seeded and exposed to *Baneh* extract (B; 1 mg/ml), Dox (D; 250 nM) and RPMI (CTRL) for 24 and 48 h. Cells were then stained with DAPI and analyzed using invert fluorescence microscope. The white arrows indicate apoptotic cells. Data shown are representative for two independent experiments

Fig. 5 Effects of *Baneh* extract on activation of caspase 3 and PARP cleavage. T47D cells were treated with *Baneh* extract (B; 1 mg/ml), Dox (D; 250 nM) and RPMI (CTRL) for 12–72 h. Then 50 µg of total cell protein lysate were subjected to western blot analysis. Data on *Baneh*-induced apoptosis via caspase 3 activation and PARP cleavage is shown as representative of 3 independent experiments. The expression of β -Actin protein was used as internal control

triggering the apoptotic pathway. In particular, the *Anacardiaceae* family includes many medicinal species with a number of biologically active substances. These compounds exhibit antibacterial, fungicidal and cytotoxic properties. Moreover, cytotoxic activity of the methanol extract of *Lithraea molleoides* (Anacardiaceae) has been reported on HepG2 cells. *Semecarpus anacardium* nut oil showed growth inhibition in leukemia cell lines and its nut had cytotoxic effect on breast cancer cell lines (Chakraborty et al. 2004). Furthermore, an antiproliferating effect of gum mastic (GM) of *P. lentiscus var chia* on prostate and colon cancer cell lines was established (He et al. 2006). Indeed, hexane extract of MG (H-MG) was capable to significantly suppress growth of HCT116 tumor xenografted in immunodeficient SCID mice (Dimas et al. 2009).

Several features of apoptosis, such as distinct chromatin condensation, DNA fragmentation, translocation of membrane phospholipids and nuclear condensation, took place in *Baneh*-treated cells with an earlier kinetics than in Dox-treated cells. For example, both *Baneh* and Dox lead to the activation of caspases 3 followed by the cleavage of PARP, but *Baneh* caused a time dependent increase of this feature, whereas Dox induced only after 48 h of treatment.

Activation of caspases is a final step in most anticancer therapies. Caspase 3 as an executioner caspase is activated by upstream caspases (caspase 8, 9) and is the main downstream effector caspase. Caspase 3 cleaves the majority of the cellular substrates in apoptotic cells which are the cause of morphological changes associated with apoptosis (Kumar 2007; Fulda and Debatin 2004). There are more than 100 substrates, which are cleaved by caspases including: mediators and regulators of apoptosis, structural proteins, cell cycle-related proteins and cellular DNA repairs. DNA-damaging agents such as alkylating agents and camptothecins are the most commonly used and effective chemotherapeutic drugs for cancer treatment (Jin and El-Deiry 2005); (Ricci and Zong 2006; Drew and Plummer 2009).

PARP is a nuclear protein acting as a “molecular nick sensor” that catalysis synthesis of poly ADP-ribose in response to DNA strand breaks (Timmer and Salvesen 2007). Cleavage of PARP is an indicator of caspase 3/7 activation and apoptosis. Its cleavage during apoptosis inhibits the DNA repair

machinery of the cells (Lamkanfi et al. 2007). It is known that both DNA repair and apoptosis are energy consuming processes and therefore, caspases save cellular energy for ATP-dependent apoptosis via PARP cleavage (Jin and El-Deiry 2005).

P53 as a tumor suppressor gene is mutated and nonfunctional in T47D cells (Vayssade et al. 2005). Based on the results which showed caspase 3 activation by *Baneh* extract in T47D cells, it can be postulated that activation of caspase 8 (extrinsic pathway of apoptosis) is involved in caspase 3 activation. It is established that apoptosis via membrane death receptors is independent of p53 which is deleted or inactivated in more than half of human tumors (Fulda and Debatin 2006).

There are some reports on apoptosis induction by extracts of *Anacardiaceae* plants family such as alkyl resorcinol of *L. molleoides* leaves induced p53-independent apoptosis in hepatocarcinoma cell lines and *S. anacardium* nut oil caused caspase activation in leukemia cells. In addition, the nut extract of *S. anacardium* exhibited caspase activation, PARP cleavage and internucleosomal DNA fragmentation in tumor cells (Chakraborty et al. 2004; Mathivadhani et al. 2007). Furthermore, H-MG induced activation of caspase 3, 8, 9 and PARP degradation in HCT116 cells (Balan et al. 2007; Barbini et al. 2006).

We have reported that *Baneh* extract, a rich source of valuable phytochemicals, possesses considerable amounts of polyphenolic compounds, flavonoids and anthocyanins. Additionally, it exerts noticeable antioxidant and radical scavenging activities (submitted manuscript). The anticancer activity of *Baneh* extract can be attributed to the presence of flavonoids, anthocyanins and other phenolic compounds. The promising chemopreventive and/or anticancer efficacy of many phytochemicals, such as bioflavonoids, proanthocyanidins and phytoestrogens have been established in various cell cultures and animal models (Dinicola et al. 2010). Polyphenols are able to affect cancer cell growth via apoptosis induction and cell cycle arrest in many cell lines (Dai and Mumper 2010). Flavonoids could activate apoptotic transcription factors (Mathivadhani et al. 2007).

Taken together, our results suggest antitumor activity of fruit skin extract of *P. atlantica sub kurdica* (*Baneh*) and induction of apoptosis in breast cancer cells which is comparable to or even stronger than Dox in certain molecular events. Further

experiments are needed to more elaborate on other molecular aspects of antitumor properties of *Baneh* in breast and other cancers.

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