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Full length research paper

Antiproliferative and cytotoxic properties of honey in human prostate cancer cell line (PC-3): Possible mechanism of cell growth inhibition and apoptosis induction

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Honey has long been used in medicine for different purposes. Only recently, however, its antioxidant property and preventive effects against different diseases, such as cancer, have been highlighted. In this study, we investigated the potential of honey to induce cytotoxic and apoptosis effects in cultured carcinomic human prostate cells (PC-3), a commonly used cell culture system for *in vitro* studies on prostate cancer. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium and treated with different concentrations of honey for three consecutive days. Cell viability was quantitated by MTT assay. Apoptotic cells were determined using Annexin-V-FITC by flow cytometry. Honey could decrease cell viability in malignant cells in a concentration and time-dependent manner. The IC_{50} values against PC-3 were determined at 14.3, 9.2 and 4.3% after 24, 48 and 72 h, respectively. Honey induced apoptosis of PC-3 cells, as determined by flow cytometry histogram of treated cells which inducing apoptotic cell death is involved in honey toxicity. It might be concluded that honey could cause cell death in PC-3 cells, in which apoptosis plays an important role. Honey could also be considered as a promising chemotherapeutic agent in prostate cancer treatment in future.

Key words: Anexin-V, apoptosis, cytotoxicity, honey, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), carcinomic human prostate cells (PC-3), chrysin.

INTRODUCTION

Prostate cancer is one of the leading causes of human male deaths throughout the world. It is a group of cancerous cells (a malignant tumor) that grow mostly from the outer part of the prostate (Jemal et al., 2005). Prostate cancer is clinically diagnosed in men over 50 years of age; expectancy, with aging the incidence of prostate cancer is probably to increase worldwide (Jemal et al., 2005; Marta et al., 2013). There is a promising opportunity for its intervention using cancer chempreventive compounds that can prevent or slow the progression of this downloaded disease (Tsao et al., 2003; Schmitz-Dräger et al., 2012). For a variety of reasons, naturally occurring botanicals and dietary substances are gaining increasing attention as cancer chemopreventive agents (Kumar et al., 2013). Important for prostate cancer chemproprevention is the fact that in

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recent years, the use of dietary and natural substances and botanical products are showing sustained increase by patients. In prostate cancer, a fine balance between cell proliferation and apoptotic death is lost which contributes to increase in cellular mass and tumor progression (Bilbro et al., 2013). In this regard, for prostate cancer chemoprevention at the present time, there is considerable emphasis in identifying novel natural substance that selectively induces apoptosis and growth arrest of prostate cancer cells without producing cytotoxic effects on normal cells.

Developments of new drugs with better efficacy are gaining momentum. The search for food as medicine is constantly evolving and people exploit various antioxidant rich foods for this purpose. Antioxidant rich foods have several preventive effects against different disease such as cancer. Honey has been used as a traditional food source since ancient times. Honey has a long tradition of use in folk medicine for various purposes and has been referred to extensively in the medical literature of Egypt and Greece (Lusby et al., 2002; Gomez-Caravaca et al., 2006). In general, honey is also rich with antioxidants and honey is thought to exhibit a broad spectrum of biological activities and therapeutic properties including antibacterial, antifungal, cytostatic (Estevinho et al., 2008; Molan, 2006), wound healing properties (French et al., 2005) and anti-inflammatory activity (Aljadi and Kammaruddin, 2004). There are also many reports in the medical literature of honey being effective as a dressing for wounds (Ghedolf and Engeseth, 2002; Vijaya and Nishteswar, 2012), burns (Aderounmu et al., 2013) and ulcer (Jull et al., 2008). Honey has been used for the treatment of Fournier's gangrene, abdominal wound disruption, gastric ulcers (Ali et al., 1997), gastroenteritis and burns, and for the storage of skin grafts (Subrahmanyan, 1993). Moreover, honey is harmless and in fact enables faster healing of the wounds by forming new tissues. An important property of honey is its antioxidant capacity; this is mainly due to the presence of flavonoids and phenolic acids, although the exact action mechanism is unknown. Among the mechanisms proposed are free radical sequestration, hydrogen donation, or a combination of these acting as substrates for radicals such as superoxide and hydroxyl. The antioxidant activity of honey is linked to the observed anticancer and antiatherosclerosis effects of honey (Beretta et al., 2007; Gribel and Pashinskii, 1990a). Honey is then substance made when the nectar and sweet deposits from plants are gathered, modified and stored in the honeycomb by honeybees. The major components of honey are fructose and glucose and also consist of carbohydrates, proteins, amino acids, vitamins, water, minerals and enzymes.

Apoptosis, an important process in cell development and maintenance of tissue homeostasis, plays an essential role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells (Schuchmann and Galle, 2004; Yin et al., 2012). Apoptosis is characterized by particular morphological changes, including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Gyulkhandanyan et al., 2012). The relationship between apoptosis and cancer has been a recent focus. Apoptosis provides a number of useful clues when generating effective therapies and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Peng et al., 2014). Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective (Chen et al., 2014; Sharp, 2009).

It has been shown that honey could induce apoptosis in T24, RT4, 253J and MBT-2 bladder cancer cell lines (Chinthalapally et al., 1993). They showed significant inhibition of the proliferation of T24 and MBT-2 cell lines by 1 to 25% honey and of RT4, 253J cell lines by 6 to 25% honey. Honey contained many biologically active compounds including caffeic acid, caffeic acid phenethyl ester 16 and flavonoid glycones, these compounds have been proved to have an inhibitory effect on tumor cell proliferation and transformation by the down regulation of many cellular enzymatic pathways including protein tyrosine kinase, cyclooxygenase and ornithine decarboxylase pathways (Gribel and Pashiniski, 1990b). Research indicates that honey possessed moderate antitumor and pronounced antimetastatic effects in five different strains of rat and mouse tumors (Ferreres et al., 1994; Martos et al., 1997). Since honey is one of the common foods for humans and also because of the properties and potential medical uses that was mentioned earlier, it prompted the investigatation of a potential candidate for prostate cancer treatment and also how honey affects cell growth and induction of apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amerso (USA). Roswell Park Memorial Institute (RPMI) 1640 was purchased from Gibco BRL (Grand Island, NY, USA). Annexin V/FITC (fluorescein isothiocyanate) was obtained from Invitrogene Corporation (USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH, Austria. All other chemicals were of the highest quality commercially available.

Cell culture

The human prostate cancer cells, PC-3, were obtained from Pasteur Institute of Iran, cultured in RPMI medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml streptomycin. PC-3 cells were cultured in CO_2 incubator MCO-17AI (Sanyo Electric Co., Ltd, Japan) at 37°C, in humidified atmosphere

enriched by 5% CO_2 and sub-cultured every 3 to 4 days.

Cell viability assay

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Briefly, PC-3 cells were plated at a density of (1 ×10^3 cells/ml) in 96-well plates and allowed to attach for 24 h to keep the log phase growth at the time of drug treatment. After treatment with different concentrations honey (0, 5, 10, 15 and 20%) for 72 h, 10 µl MTT was added into each well. After 4 h incubation at 37°C, the solution was removed, and the produced formazan was solubilized in 100 µl dimethyl sulfoxide (DMSO). Absorbance was measured at 550 nm using an automated microplate reader (Bio-Rad 550). Cell viability was expressed as a percentage of the control culture value. The cytotoxic effects of honey extract on cell line (PC-3) was expressed as IC_{50} value (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells). All experiments were carried out in triplicate.

Assessment of apoptosis by Annexin V-FITC

Apoptotic cell death of honey was measured using flouresin isothiocynate (FITC)-conjugated Annexin V/PI assay kit by flow cytometry (Deng et al., 2009), briefly 5 ×10^5 cells were washed with ice-cold PBS, re-suspended in 100 µl binding buffer, and stained with 5 µl of FITC-conjugated Annexin V (10 mg/ml) and 10 µl of PI (50 mg/ml). The cells were incubated for 15 min at room temperature in the dark, then 400 µl of binding buffer was added, and analyzed by a FACScan flow cytometry (Becton-Dickinson, USA). For analysis, PC-3 cell lines were gated separately according to their granularity and size on forward scatter (FSC) versus side scatter (SSC) plot. Early apoptosis and late apoptosis were evaluated on fluorescence 2 (FL2 for propidium iodide) versus fluorescence 1 (FL1 for Annexin) plots. The percentage of cells stained with annexin V only was evaluated as early apoptosis; the percentage of cells stained with both annexin V and propidium iodide was evaluated as late apoptosis or necrotic stage.

Statistical analysis

All results were expressed as mean ± standard error of mean (SEM). The significance of difference was evaluated with ANOVA and Bonfrroni's test. A probability level of P<0.05 was considered statistically significant.

RESULTS

Effects of honey on cell viability

PC-3 cancerous cells were incubated with various concentrations of honey for 24, 48 and 72 h. The impact of honey on cell viability was quantitated by MTT assay. Exposure of PC-3 cells with honey showed after 24 h significantly high growth inhibitory effects on prostate cancer cell line in a concentration and time dependent manner (p<0.001). Although, there was no significant result at low concentration of honey (2.5%) (data not shown); however, there were significant decrease in viability at concentration of 5, 10, 15 and 20% (P<0.05, p<0.001 and P<0.001, respectively) versus control after 24 h (Figure 1). On the other hand, treatment of PC-3 cell line for 4 and 72 h at different dose of honey (2.5, 5, 10 and 20%) resulted in marked reduction of number of viable cells (p<0.001) (Figure 1). The dose inducing 50% cell growth inhibition (IC_{50}) against malignant cell (PC-3) was determined at 14.3 ± 0.8, 9.2 ± 0.5 and 4.3 ± 0.3% at 24, 48 and 72 h, respectively (Table1).

Table 1. Doses inducing 50% cell growth inhibition (IC_{50}) of honey extract against prostate cancer cell line (PC-3).

IC_{50}	24 h	48 h	72 h
PC-3 (%)	14±1.0	10±0.9	4±0.05

Cells were treated with different concentration of honey for 24 and 48 h. Viability was quantitated by MTT assay.

Quantification studies for apoptosis by honey

To study the roles of honey in apoptosis, honey was used to setup apoptosis system on PC-3 cell line. PC-3 cells were treated with concentrations of 5 and 20% of honey for 24 h. After treatment, the cells were harvested and apoptosis was examined by flow cytometry (Figure 2). Quantitative analysis using Annexin V/PI assay further showed that the proportion of early stage apoptotic cells (Annexin V+/PI-) increased significantly from 19.12 to 35.61%, while proportion of late stage apoptotic cell (Annexin V+/PI+) increased significantly from 27.89 to 41.93% when the cells were treated with the concentrations of 5 and 20% honey, respectively (Figure 3). Apoptosis induced from 5 and 20% of honey was statistically higher than control and the percentage of the early and late apoptotic cells significantly increased by increasing honey concentration (p<0.001). Although, at concentration of 2.5%, there was no significant difference between percentage of early and late apoptotic cells (data not shown), however, the percentage of the early and late apoptotic cells significantly increased by increasing honey concentration (p<0.001), and also the number of the late apoptotic cells versus early apoptotic cells at concentration of 5 and 20% honey treated cells were statically significant (p<0.01, p<0.001) (Figure 3).

DISCUSSION

The most effective anticancer drugs currently used for treating different types of cancer have serious side effects. Current research has mainly focused on finding

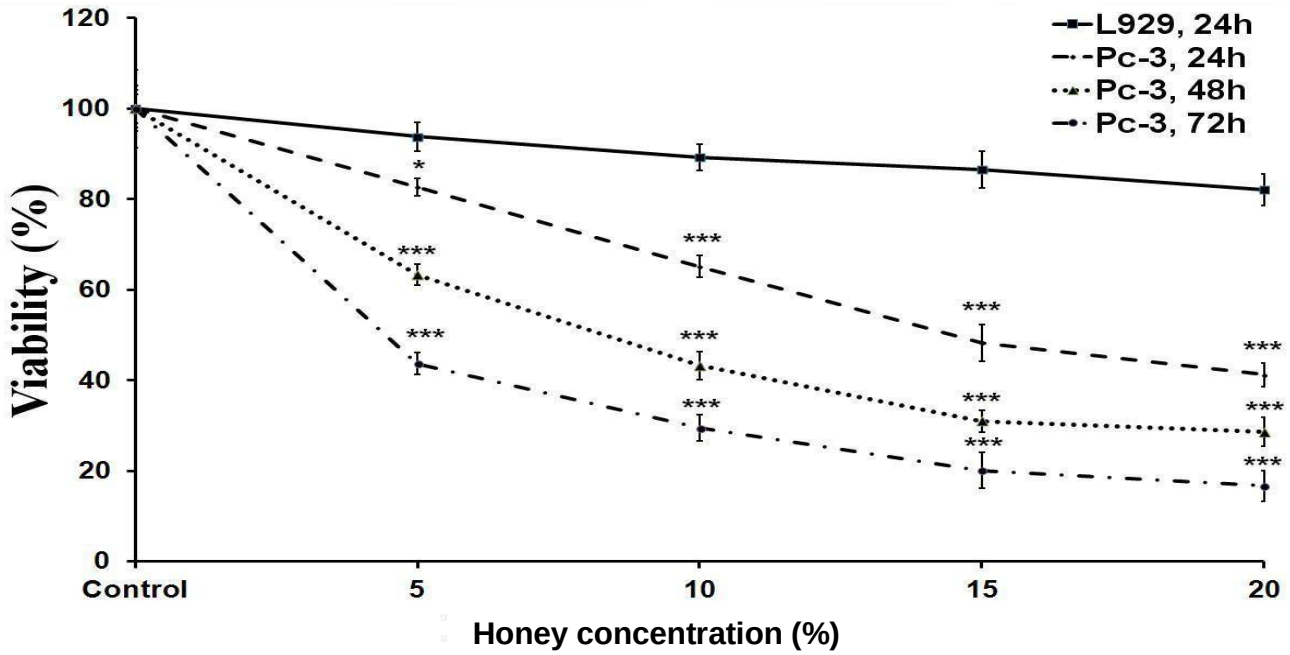

Figure 1. Effect of honey on cell viability of PC-3 cells. Cells were treated with different concentration of honey for 24, 48 and 72 h. Viability was quantitated by MTT assay. Results are mean ± SEM. The asterisks are indicator of statistically difference obtained separately at different time points compared to their controls shown in figure as *P<0.05; ***P<0.001.

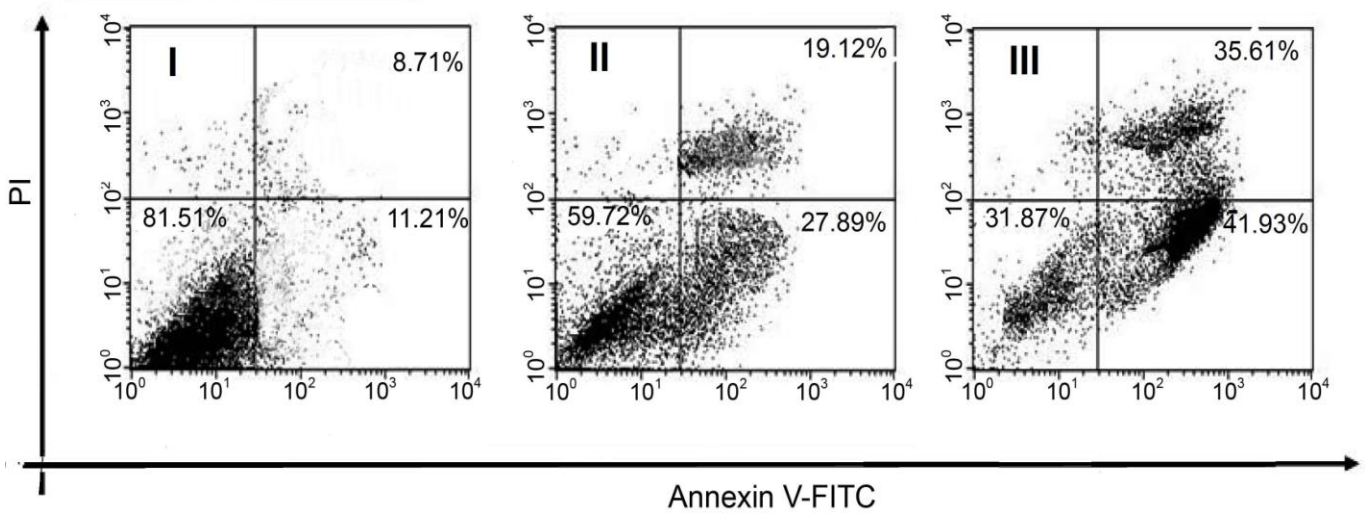

Figure 2. Assessment of apoptosis by Annexin V/PI on human prostate cancer cells (PC-3). The cells were treated with 5 and 20% (symbol II, III, resspectively) or media only (control symbol I), and apoptosis was examined by flow cytometry after Annexin V-PI double staining. Necrotic cells lose membrane integrity, permitting PI entry. Viable cells exhibit Annexin V (-)/PI (-); early apoptotic cells exhibit Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibit Annexin V (+)/PI (+).

ways of reducing these severe side effects (Alizadehnohi et al., 2012). The therapeutic activity of most anticancer drugs in clinical use is limited by their general toxicity to proliferating cells, including some normal cells. Although, chemists continue to develop novel cytotoxic agents with unique mechanisms of action, many of these compounds still lack tumor selectivity and have not been therapeutically useful (Rao et al., 2005; Chari, 2008).

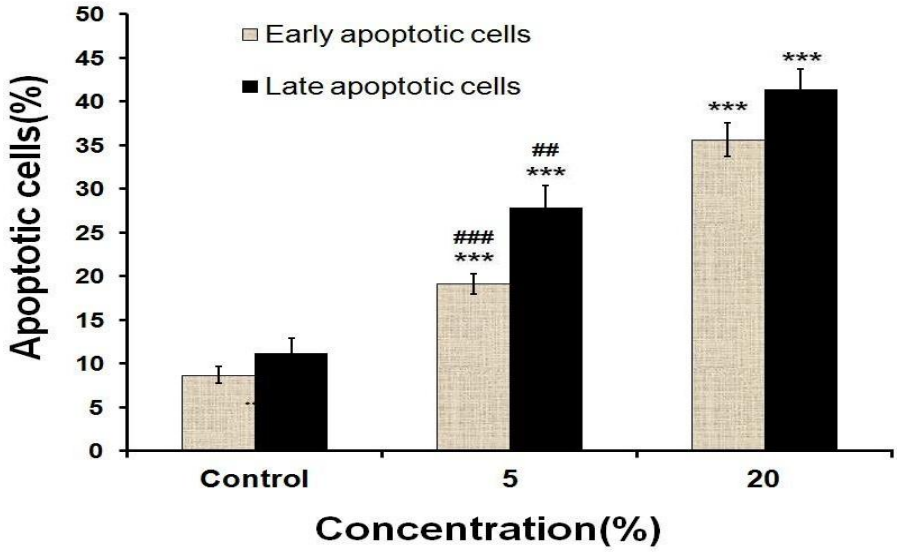

Figure 3. Assessment of apoptosis by Annexin-V/PI on renal cell carcinoma (ACHN). Percentage of cell death based on the assessment of apoptosis by Annexin-V/PI. ***P<0.001 and $^{##}$P<0.01, $^{###}$P<0.001 compared with the control and the other dose, respectively.

New targets for cancer therapy focus on interfering with specific targeted molecules needed for carcinogenesis and tumor growth in order to overcome the problems of traditional therapies (Goldman, 2003). Natural products are perceived as pure, and without side effects medication products (Montbriand, 2004). The purpose of the present study was to determine whether honey, through induction of apoptosis, possess an inhibitory effect on prostate cancer cells. It was demonstrated that honey can induce apoptosis in PC-3 cells. Analysis of cytotoxicity by MTT assay confirmed the cytotoxic effects of honey in a time- and dose-dependent manner.

A wide variety of natural food and products have been recognized to induce apoptosis in various tumor cells. It is thus considered important to screen apoptotic inducers and good candidate for development of anti cancer drug and also offering an opportunity to study the molecular mechanism of tumor genesis (Amit et al., 2009). Many patients with cancer or other chronic conditions use alternative therapies, often herbal or natural products (Montbriand, 1995; Montbriand, 2000). Honey, which is one of the most complex mixtures of carbohydrates produced in nature, has a long history as a medicinal substance. It is a known natural product with several biological activities. Some bioactive compounds have been found in honey such as chrysin which have been used to prevent cancer (Omene et al., 2012; Samarghandian et al., 2011).

For the protection of human health, considerable attention is currently focused on the consumption of functional foods. In particular, the role of dietary antioxidants capable of scavenging the oxidants and free radicals responsible for initiating various diseases has been intensively discussed (Samarghandian et al., 2013a, b). Systematic investigations of the antioxidant properties of various foods, beverages, spices and herbs have been performed (Butkovic et al., 2004; Samini et al., 2013) and the number of papers addressing the health-protective and antioxidant characteristics of honey is increasing. Honey has for a long time been used as a natural source of sugars, as well as an important ingredient in traditional medicine, having antimicrobial and antiinflammatory properties (Ku et al., 2007). Honey contains a variety of compounds including cafeic acid, benzoic acid and esters, substitute's phenolic acids and esters, flavonoid glycones and beeswax (Greenaway et al., 1987; Heby, 1981). Some of the observed biological activities of honey may be traced to its chemical constituents (Spector and Moore, 1988; Honn et al., 1989). Cafeic acid (3, 4-dihydroxycinnamic acid) ester derivates, which are present in honey at levels of 20 to 25%, are thought to exhibit a broad spectrum of activities that possibly include tumor inhibition. Several cellular components that have been associated with cell proliferation, such as polyamines and polyamine synthetic enzyme activities including ornithine decarboxylase, are present at high levels in proliferating normal and neoplastic cells. In addition many kinases, such as tyrosine protein kinase (TPK), mediate proliferative as well as metabolic signals in the cells. Eicosanoids, the metabolits of arachidonic acid through the lipoxygenase and cyclooxygenase pathways, exerts a

variety of biological activities. The health-protective and therapeutic impacts of honeys were formerly attributed to the presence of various antioxidant components, such as flavonoids, phenolic acids, organic acids, enzymes and vitamins (Wang et al., 2004; Samarghandian et al., 2011).

The mechanism of the antitumor effect shown in this study is unclear, but it may be related to the inhibitory effect of caffeic acid esters and flavonoid glycones on TPK, lipoxygenase, and cyclooxygenase pathways metabolites. Honey also contains various simple polyphenols which were found to exert significant antiproliferative potential against various cancer cells *in vitro* (Samarghandian et al., 2011). The apoptosis inducing potential of the honey could be attributed to its phenolic constituents (Samarghandian et al., 2010). Various signaling pathways, including stimulation of tumour necrosis factor-alpha (TNF-alpha) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation, mediate the beneficial effects exerted by honey and its major components such as chrysin and other flavonoids (Woo et al., 2004).

Anti-tumor and anti-cancer effects of honey have been shown in few studies. In this study, the cytotoxic and proapoptotic effects of honey in PC-3 cell lines were investigated. To the author's knowledge, this is the first report on honey-induced apoptosis in human prostate cancer cells. Our data confirmed that honey has cytotoxic activity against carcinomic human prostate cancer cell line, which is consistent with previous studies indicating that honey and its ingredients possess antitumor and anticarcinogenic activities (Samarghandian et al., 2011). Different studies have shown the anti proliferative activity of honey on human breast cancer cell line (MCF7) (Yaacob et al., 2013). The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug, which discriminates between anticancer drugs and toxic compounds (Samarghandian et al., 2010). The MTT assay is thought to be produced by the mitochondrial enzyme succinate dehydrogenase and can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. A multiwell spectrophotometer assay can be semiautomated to process a large number of samples and provide a rapid object measurement of cell number (Samarghandian et al., 2011). Our study showed that honey exerted a significant proliferation inhibitory activity against PC-3 cells in a dose-dependent manner (Figure 1). Much effort has been directed towards the effect of saffron on apoptosis and understanding their mechanisms of action. The apoptosis evoked by honey was confirmed by the Annexin V-FITC (Figure 2). In the present study, honey-induced apoptosis was involved in cell death. Apoptosis is characterized by distinct morphological features including chromatic condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation. As shown in Figures 2 and 3, honey at 5 and 20% induced significant cell toxicity in the PC-3 cells in dose-dependent manner. Apoptosis only partially contributed in this toxicity, it might be suggested that non-apoptotic cell death to be also involved in honey-induced toxicity in these cells.

Overall, this study showed that honey may contain bioactive compounds that inhibit the proliferation of human prostate cell lines (PC-3) with the involvements of apoptosis or programmed cell death. Further studies are needed to fully recognize the mechanism involved in cell death, honey could be considered as promising chemotherapeutic agent in lung cancer treatment.

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