

Antitumor Effect of Honey and Squirting Cucumber Fruit Juice Mixture on Glioblastoma Cells *in Vitro*

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ABSTRACT

The study aimed to investigate the antitumor effects of *Ecbalium elaterium* (squirting cucumber) (Cucurbitaceae) fruit juice and some fruit extracts as well as bee honey separately and in mixture. The fruit juice of *E. elaterium*, which is found to be rich in cucurbitacins, was selected for further evaluation of antitumor activity on the bases of a phytochemical study by thin layer chromatographic technique (TLC). The antitumor effects of the fruit juice and bee honey (separately and in mixture) were assessed for human brain tumor cell line (U₂₅₁) using trypan blue exclusion assay for evaluating cytotoxicity, and H³-thymidine assay for estimating DNA synthesis. ELISA technique for measuring cell cycle regulation and the evaluation of the effects on metastasis and angiogenesis were also achieved. Results suggested that honey did not inhibit tumor cell growth through cyclin D₁, P₂₁ regulation or cell number reduction but through a significant inhibition of DNA synthesis. Results showed that *E. elaterium* fruit juice significantly down regulated P₂₁, up-regulated cyclin D₁, decreased cell number and inhibited DNA synthesis. Mixing of honey with *E. elaterium* fruit juice decreased the cytotoxicity induced by the latter and produced intermediate inhibition of DNA synthesis and down regulation of P₂₁. All treatments inhibited MMP₂ and MMP₉. Thus, honey was able to reduce the cytotoxic effect of *E. elaterium* juice on glioblastoma cells enhancing its ability of inhibiting DNA synthesis and they acting together as antiangiogenic and antimetastatic agents.

Keywords: angiogenesis, cucurbitacins, cyclin D1, MMP, P21

INTRODUCTION

Natural products form the basis of many of the drugs currently in commercial use or under development. More than 3000 species of herbs used in cancer treatment since ancient times are known to biomedicine (Montbriand 1999; Cragg *et al.* 2006). *Ecbalium elaterium* fruit juice (a Mediterranean plant belonging to Cucurbitaceae) possesses marked antitumor activity and tumor necrotizing capacity (Seger *et al.* 2005). It has high content of cucurbitacins, the bitter principles that cause the high toxicity of this plant especially of the fruit and they are well known biologically active compounds (Lavie and Glotter 1971). The high toxicity of the fruit juice of *E. elaterium* in spite of its medicinal properties limits further researches as antitumor drug. The alleged uses of the fruit juice are as a potent cathartic, analgesic, and antiinflammatory agent. Cucurbitacin B, a triterpene derivative is the active antiinflammatory principal (Raikhlin-Eisenkraft and Bentur 2000).

Honey, as a natural product, is known with its several biological activities (Molan and Allen 1996; Sesta *et al.* 2006). It is considered one of the most complex mixtures of carbohydrates produced in nature (Swallow 1990). Studies that report the use of honey as an antitumor are few. Crude honey is known to enhance proliferation of both normal and malignant cells (Suguna *et al.* 1992; Abuharfeil 1999; Al-Jady *et al.* 2000; Tonks 2001) because it provides substrates for glycolysis, which is the major mechanism for energy production for cell proliferation (Spoden *et al.* 2009).

On the other hand, it was reported that honey is a promising antitumor agent with pronounced antimetastatic and antiangiogenic effects in different model systems (Gribel and Pashinskii 1990; Dunford *et al.* 2000; Egeblad and Werb 2002; Nada and Ivan 2004; Orsolice *et al.* 2005). It

was found that honey decreases the total extracellular protease and extracellular gelatinolytic activities of HepG2 cells which have been implicated in the induction of the angiogenic switch (Amer *et al.* 2008). Most of the biological activities of honey were attributed mainly to its high content of vitamins such as vitamin C which is known by its powerful medicinal effects including its antitumor effect (Kong *et al.* 2009). The biological activity of honey may also be attributed to the presence of an unknown substance(s) called inhibine(s) (Molan and Allen 1996; Molan and Brett 1998).

Therapy directed against oncogenes or their products would be more specific to cancer and less harmful to normal tissues of the body. New targets for therapy no longer focus on the traditional foundations of cytotoxic therapy, but focus instead on the processes of invasion, metastasis, cell cycle control, differentiation. Proteolytic degradation of the basement membrane and the extracellular matrix is a central aspect of neovascularization (angiogenesis) and malignant growth (Scorilas *et al.* 2001). Matrix metalloproteinases (MMPs) are likely to be play a key role as relevant mediators of the extracellular matrix (ECM) degradation and hence neovascularization (Yu *et al.* 1998; Belleguic *et al.* 2002). An inhibitor of metastasis or angiogenesis might prevent new lesions from appearing and/or preexisting lesions from growing (Almholt 2008). Therefore, MMPs are considered as drug targets (Sang 1998; Lubbe *et al.* 2006). In addition, the key genes in controlling cell cycle provide obvious target for new generation of anticancer drugs. P₂₁ protein is an important regulator at the G₁ checkpoint in the cell cycle (Li *et al.* 2004) and Cyclin D₁ is a key regulator of G₁ to S phase progression of the cell cycle (Zhao *et al.* 2001).

MATERIALS AND METHODS

Plant materials

Ecbalium elaterium fruits were collected from Libya and transferred to Egypt on dry ice. The juice of the fruits was prepared by direct squeezing then extracted by 70% ethanol. The *E. elaterium* fruits were also extracted with 70% methanol and then a successive extraction with petroleum ether, ether, ethyl acetate, chloroform, and chloroform/methanol respectively. A dark brown residue was remained. All extracts were distilled off under reduced pressure at 50°C. Cucurbitacins were detected using thin layer chromatographic technique and vanillin-phosphoric acid spray reagent.

Tests of honey adulteration

Three commercial honey samples were collected from Egyptian markets and then tested for adulteration and improper condition of storage (Amer *et al.* 2008). Honey (I) called El Semny brought from Cairo markets, honey (II) was brought from El Sharkia Governorate and honey (III) was brought from El Fayoum Governorate. Honey samples were subjected to tests of adulteration [diastase activity, 5hydroxymethylfurfural (HMF) content and detection of commercial glucose]. Unadulterated honey II was selected for further bioassays. Honey samples were brought as one lot (1 Kg) to avoid any consistency in the results.

Biological studies

1. Tissue culture

Tumor brain glioblastoma cell line (U₂₅₁), established by Tsuboi *et al.* (1996), was supplied by the National Cancer Institute, Department of Cancer Biology, Cairo University. Cells were propagated and maintained in RPMI-1640 medium with L-glutamine (Sigma) supplemented with 10% fetal calf serum (Sigma) for growth and 2% for maintenance medium, 1% of 4% sodium bicarbonate (Merck) and 1% antibiotic mixture (Sigma). Exponentially growing cells were used in all experiments.

2. Cytotoxicity

Cells were treated with *Ecbalium elaterium* fruit juice, ethanol extract, and ether extract of the fruits at fixed concentration of 2 µg/5 × 10⁵ cells and then left for 72 hrs. Viable cells were counted using trypan blue exclusion assay (Sugibayashi *et al.* 2002). Based on the results of the cytotoxicity, the fruit juice was selected for further experiments because of their low cytotoxicity. LC₅₀ of *E. elaterium* fruit juice on glioblastoma U₂₅₁ cells propagated *in vitro* was estimated. Cells were also treated with different dilutions of honey (10, 100, 1000 µg/2 × 10⁶ cells). The viable cell number was also determined.

3. Bioassays

Cells were treated with honey (1000 µg/2 × 10⁶ cells), fruit juice (30 µg/2 × 10⁶ cells) and honey-fruit juice mixture (1000: 30 µg/2 × 10⁶ cells) then incubated for different time intervals. The selected concentration of the fruit juice was below the LC₅₀ range in order to avoid its high toxicity. Cells were treated for three days then harvested and then subjected for further bioassays.

4. Determination of DNA synthesis

DNA synthesis was measured according to the method described by Yamao *et al.* 1993. In brief, a 6 well plate, cells were seeded in a concentration of 500 × 10³ cells/well and treated with 1000 µg/2 × 10⁶ of honey, 30 µg/2 × 10⁶ of juice or 1000: 30 µg/2 × 10⁶ honey and fruit juice mixture for three days. Wells were pulsed with 0.5 µCi H³-thymidine /well and left over night in the incubator at 37°C. Cells were trypsinized, harvested and dried then counted in a liquid scintillation counter using a scintillation cocktail (5 g 2, 5, diphenyloxazole pop + 1 liter toluene + 500 ml of 10% triton X-100).

5. Enzyme-linked immunosorbent assay (ELISA)

The method was carried out according to Hahn-Dantona *et al.* (2001) to evaluate the effect of honey, fruit juice and honey fruit juice mixture on protein levels of P₂₁, cyclin D₁ and matrix metalloproteinases (MMP₂ and MMP₉). A microtiter plate (96- well) was coated with 100 µl of the lysate of cells which were treated with different treatments at 4°C overnight. The plate was washed three times with PBS-NP40 and blocked with 1% BSA buffer containing 5 mM CaCl₂ for 1 hr at room temperature. The plate was washed again with PBS-NP40 three times to remove the unbound proteins. Subsequently, the anti-mouse anti-human IgG monoclonal antibodies of P₂₁, D₁, MMP₂ or MMP₉ (1 µg/ml) was added, respectively to each cell lysate for 2 hrs. To develop the color of the reaction 10 µl of the peroxidase substrate (1 mg/ml *p*-nitrophenyl phosphate substrate in 10 mM diethanolamine) were added. The reaction was measured as absorbance values at 405 nm using a microplate reader.

Statistical analysis

Results were analyzed using SPSS computer program version 1.1 to evaluate all the above determinations. The analysis included the means ± standard deviation and the significance levels of $P \leq 0.05$ or 0.001. Two tailed Spearman's Rho test was used to analyze the effect of different concentrations of the fruit juice on the cell viability. One way ANOVA *post-hoc* test was used for the analysis of the cytotoxicity of honey, juice and the mixture on the cells at different interval. MANOVA test was used to analyze the effect of the different treatments on H³-thymidine uptake, cell cycle (P₂₁ and cyclin D₁) and angiogenesis and metastasis processes (MMP₂ and MMP₉).

RESULTS

Phytochemical studies revealed the presence of cucurbitacins at different proportions and types in all *E. elaterium* fruit juice fractions tested. The extracts of petroleum ether, ethyl acetate, chloroform, chloroform-methanol and residual portion were excluded from the study because of their relatively low content of cucurbitacins. Fruit juice, ethanol and ether extracts were considered suitable for further biological studies according to thin layer chromatography (TLC).

The adulteration tests showed that honey II was considered unadulterated and used for further bioassays due to its neglected amounts of HMF, 3.36 × 10⁻⁶ mg/kg, negative commercial glucose test and a diastase activity around the required limits (7.32 units/kg) whereas the required limits is 8 units/g (Amer *et al.* 2008).

Cytotoxicity

Fig. 1 represents the growth curve of brain tumor cell line (U₂₅₁) propagated as a monolayer in RPMI-1640 for six days. The doubling time of the tumor cells as elicited from the growth curve of U₂₅₁ equals to 72 hrs. Cell growth of U₂₅₁ cells was inhibited significantly in a concentration-dependent manner when treated with the *E. elatrium* fruit juice and the LC₅₀ range equal to 40 to 50 µg/2 × 10⁶ cells (**Fig. 2**). While treatments with ethanol or ether extracts did not affect U₂₅₁ cell growth. A concentration of *E. elatrium* fruit juice lower than LC₅₀ values was selected for further tests. Ethanol and ether extracts were excluded for further biological tests because of their nonsignificant effects (data not shown). Honey, at different concentrations (10, 100, 1000 µg/2 × 10⁶ cells), exhibited non significant effects on the viability of U₂₅₁ cells (**Fig. 3**). Treatment with honey at different time intervals (for six days) revealed that cell growth of U₂₅₁ cells was not affected. On the other hand, treatment with *E. elatrium* fruit juice and honey-juice mixture for six days inhibited cell growth of U₂₅₁ significantly (**Fig. 4**).

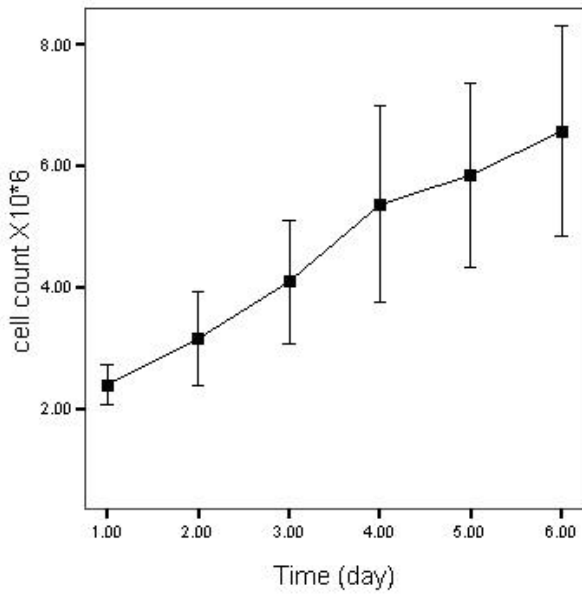


Fig. 1 Growth curve of glioblastoma cells (U₂₅₁) propagated in RPMI-1640 medium with L-glutamine *in vitro*.

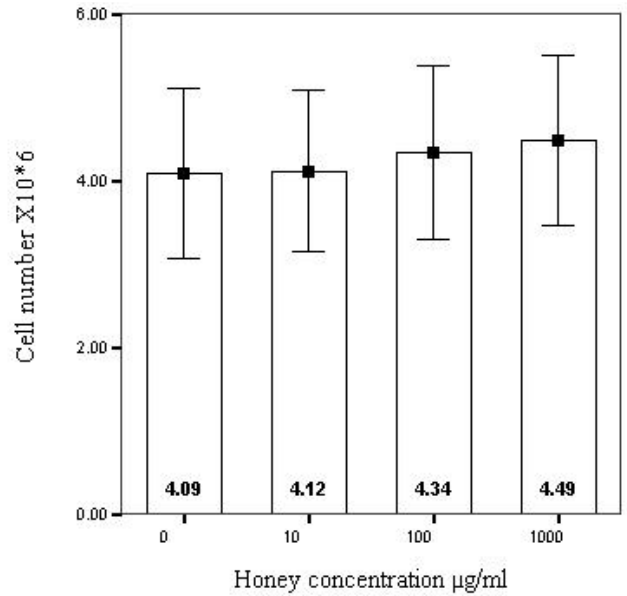


Fig. 3 Effect of different concentrations of honey on glioblastoma cell growth.

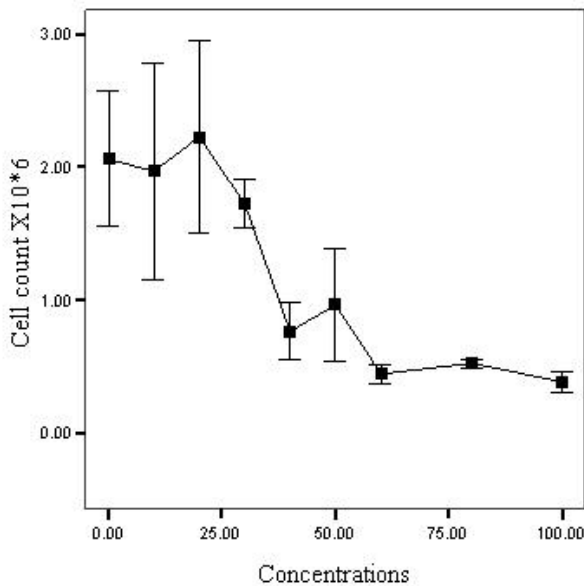


Fig. 2 Effect of different concentrations of *E. elaterium* fruit juice on proliferation of glioblastoma cells *in vitro*.

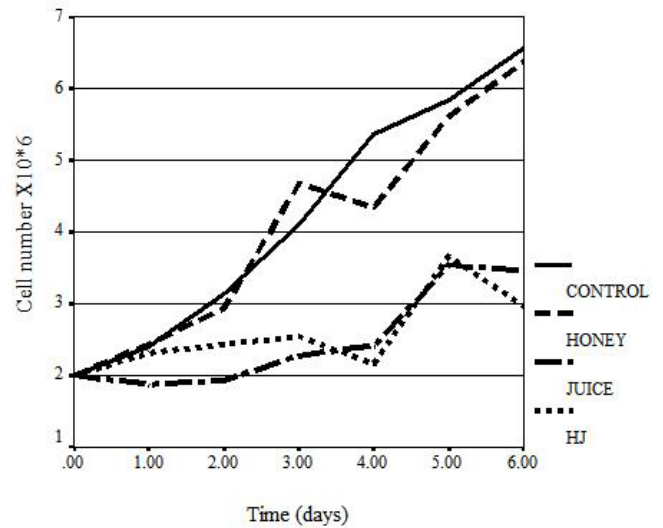


Fig. 4 Effect of honey, *E. elaterium* fruit juice and honey-juice mixture on cell growth at different time intervals on glioblastoma cells *in vitro*.

DNA synthesis

There was no significant difference between various treatments. Fig. 5 illustrates the inhibition of H³-thymidine uptake in glioblastoma cells (U₂₅₁) after treatments with honey, fruit juice and honey-juice mixture.

Cell cycle regulation

The treatment of glioblastoma cells with honey, *E. elaterium* fruit juice and honey-juice mixture upregulated cyclin D₁ protein level with no significant difference between the treatments of juice and honey-juice mixture (Fig. 6). On the other hand, the same treatments decreased P₂₁ protein level significantly.

Regulation of angiogenesis

The gene expression of protein levels of matrix metalloproteinases (MMP₂, MMP₉) of glioblastoma cells were down regulated after the treatment with honey, fruit juice and honey-juice mixture. The down regulation in case of honey

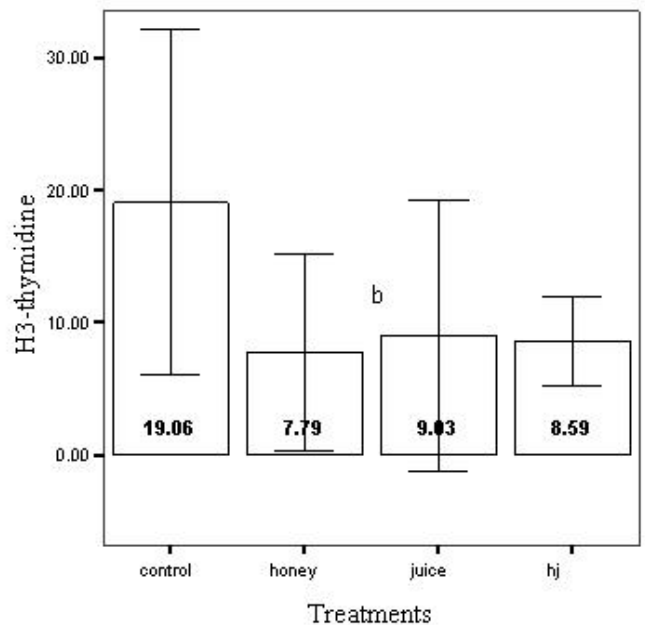


Fig. 5 Effect of honey, *E. elaterium* fruit juice and honey-juice mixture on DNA synthesis (H³ thymidine uptake) of glioblastoma cells *in vitro*.

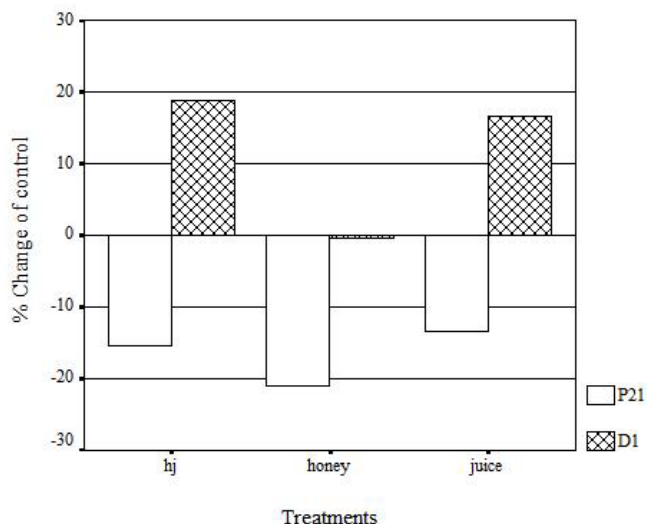


Fig. 6 Effect of honey, *E. elaterium* fruit juice and honey-juice mixture on gene expression level of P₂₁ and cyclin D₁ of glioblastoma cells *in vitro*.

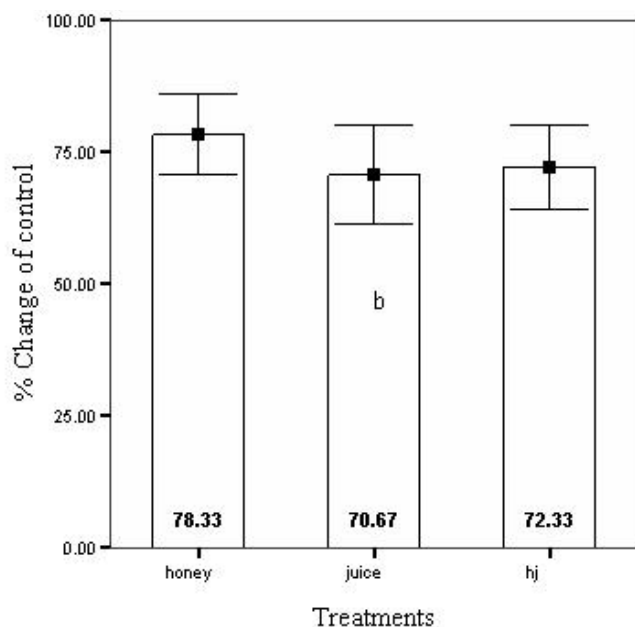


Fig. 7 Effect of honey, *E. elaterium* fruit juice and honey-juice mixture on MMP2 gene expression level of glioblastoma cells *in vitro*.

was higher than that caused by other treatments and was significantly different to that in case of *E. elaterium* fruit juice treatment (Figs. 7, 8).

DISCUSSION

Nature is an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of species of plants and animal products. *E. elaterium* has a high content of cucurbitacins which are well known biologically active compounds. Cucurbitacins, the bitter principles, cause the high toxicity of this plant, especially the fruit (Raikhlin-Eisenkraft and Bentur 2000).

Another natural product of animal and plant origins with several biological activities is honey which known with its safe biological activities (Molan and Brett 1998). Reports in the literature on honey as antitumor substance are few (Valavichyus *et al.* 1975; Keast-Butler 1980; Rady 1996; Dunford *et al.* 2000; Egeblad and Werb 2002).

Antitumor therapy has expanded beyond previous concepts of cytotoxicity and includes agents that induce differentiation through non-cytotoxic pathways (Braverman

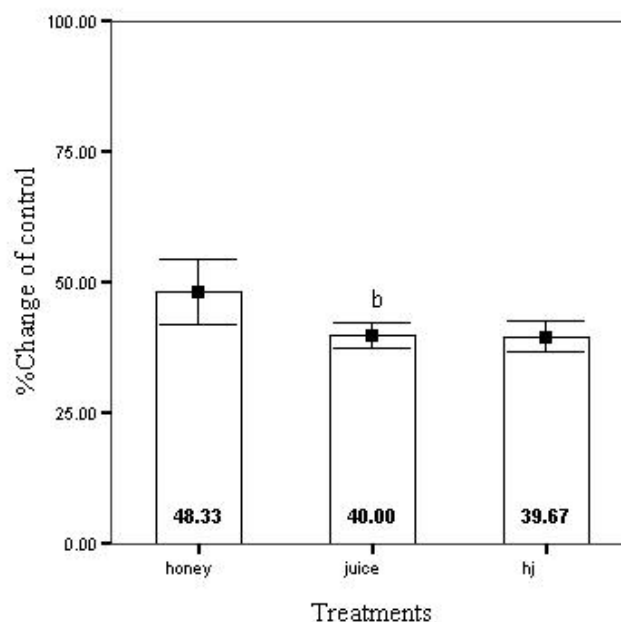


Fig. 8 Effect of honey, *E. elaterium* fruit juice and honey-juice mixture on MMP9 gene expression level of glioblastoma cells *in vitro*.

1993). Angiogenesis, the process of new blood vessel formation, is considered as a central pathogenic step in the process of tumor growth, invasion and metastasis (Amer *et al.* 2008). Antiangiogenic agents have been recently recognized to be potentially useful in the treatment of malignant processes (Marshall and Hawkins 1995; Gately *et al.* 1997).

Our results revealed that honey had no cytotoxic effect on the human tumor brain cell line (U₂₅₁) propagated *in vitro* at different concentrations and different time intervals. These results are concomitant with the fact proved by many authors that honey causes no tissue or cell damage and is not harmful to tissues or cells (Phuapradit and Saropala 1992; Rady 1996; Subrahmanyam 1996; Molan and Brett 1998; Amer *et al.* 2008). However, honey inhibited DNA synthesis (H³-thymidine uptake) of U₂₅₁ after the treatment for three days. These results proved that honey is not a mitogenic agent and the high proliferation rate induced by honey can be attributed to a direct nutritional effect on the cells (Schmidt *et al.* 1992).

Cyclin D₁ protein level remains unchanged after the treatment with honey like other antitumor antibiotics (Galoustian 2002; Bogner *et al.* 2003). P₂₁ is over-expressed in brain metastasis on the contrary with other tumors (Zhang 1995). The down regulation of P₂₁ protein level of glioblastoma U₂₅₁ cells after treatment with honey may be specific for this type of tumor. Effect of honey on P₂₁ protein level of other tumor cell lines will be evaluated in further studies. Honey also down regulated the protein level of matrix metalloproteinases (MMP₂ and MMP₉) of U₂₅₁ cells significantly. These results suggested that honey may inhibit the angiogenesis and metastasis of brain tumors. These findings in agreement with the results reported by Molan and Brett (1998), who found that treatment with honey, increased the synthesis and cross-linking of collagen (the main component of the extracellular matrix). In addition, honey was implicated in the induction of the angiogenic switch in different other model systems (Egeblad and Werb 2002). These results may attributed to the fact that high sugar concentration is known to increase the synthesis of many matrix components, decreases the expression of MMPs and increases expression of tissue inhibitors of matrix metalloproteinases (McLennan *et al.* 2000).

On the other hand, the treatment of glioblastoma cells (U₂₅₁) with *E. elaterium* fruit juice showed significant cytotoxic effect in a dose dependent manner with LC₅₀ of 50-40 µg/2 × 10⁶ cells. According to D'Atri *et al.* (2000), a low

cytotoxic dose of the fruit juice was selected in order to induce changes in tumor cells without killing it. Our results suggested that *E. elaterium* fruit juice capable of down regulating matrix metalloproteinases when used in low, non-cytotoxic concentrations. Many of the promising antiangiogenic factors are examples for noncytotoxic antitumor agents for example, squalamine (Sills *et al.* 1998).

Combined treatment of glioblastoma cells (U₂₅₁) with honey and *Ecballium elaterium* fruit juice reduced the cytotoxic effect of *E. elaterium* juice on glioblastoma cells and enhanced its ability of inhibiting DNA synthesis and also maintained the antiangiogenic and antimetastatic potential. Further studies are recommended to elucidate the mechanism(s) by which these effects are exerted on the tumor cells *in vitro* and *in vivo*.

CONCLUSION

The results of the current study revealed that the effect of honey, like other antitumor antibiotics, was not exerted through cell cycle control, where it did not change either the cell number or the expression of cyclin D₁ although it down regulated P₂₁. These results, together with the inhibition of DNA synthesis suggest that the treated cells may enter the cell cycle but DNA synthesis was blocked. Therefore, glioblastoma cells after treatment with honey, characterized by low DNA content compared with the control tumor cells.

E. elaterium juice, at the concentration used in the current study (< LC₅₀) caused a reduction in the cell number and inhibited DNA synthesis. Our results suggested that honey modulates the cytotoxic effect of *E. elaterium* fruit juice and at the same time enhanced its inhibitory effect on DNA synthesis when used in a mixture. The mixture also retained the inhibitory effect on MMP₂ and MMP₉ of glioblastoma cells.

Thus, honey was able to reduce the cytotoxic effect of *E. elaterium* juice on glioblastoma cells enhancing its ability to inhibit DNA synthesis and maintains its antiangiogenic and antimetastatic potential. Further studies are recommended to elucidate the mechanism(s) by which these effects are exerted on the tumor cells *in vitro* and *in vivo*.

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