Effect of Some Honey Bee Extracts on the Proliferation, Proteolytic and Gelatinolytic Activities of the Hepatocellular Carcinoma Hepg2 Cell Line

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Abstract: The cytotoxic, antimetastatic, and antiangiogenic effects of some honey bee extracts were evaluated in order to determine the effective antitumor fractions of bee honey. Four different samples of honey with different qualities were tested in order to examine the effect of honey quality on its antitumor effect. The cytotoxic effect of crude honeys, residues (100 μg / ml assay) and the extracts of one gram of each honey was assessed by the 3-[4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cytotoxicity assay; the antimetastatic and antiangiogenic effects were determined by measuring their effects on the proteolytic and gelatinolytic activities of HepG2 cells using the total extracellular protease and gelatin zymography assays, respectively. Some extracts (ethyl acetate, chloroform/methanol extracts of all honeys, ether extract of H1, H2 and H4 and chloroform extract of H1) exerted an inhibitory effect on cell proliferation, protease and gelatinase activities with different degrees depending on the honey quality. Petroleum ether extract and the residue of H1, H2 and H4 enhanced cell proliferation but inhibited protease and gelatinase activities with different degrees depending on the honey quality. H3, which had the lowest quality, and its extracts showed disappointing effects against HepG2 cells.

Key words: Hepatocellular carcinoma, honey bee extracts, protease, gelatin zymography.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy, and is responsible for more than one million deaths annually worldwide (Yang et al., 2003). HCC is the most frequent epithelial malignancy of the liver, although great improvements have been made in the diagnosis and therapy of HCC, survival is still poor even for those patients with better clinical and pathological features (Giannelli et al., 2002).

Angiogenesis angiogenesis can be clarified by the fact that, one cubic centimeter of tumor contains approximately 10^7 cancer cells, which, in turn, are supported by 10^7-10^8 vascular endothelial cells. Experimental studies have shown that this co-population sheds between 1x10^4 – 2 x10^6 cancer cells per 24 hours into the circulation. While most circulating cancer cells are eliminated by host defense systems, a few survive after lodging in distant organs, remaining dormant until angiogenesis takes place (Singh, 2004). Investigators have long recognized the dynamic nature between cell growth and cellular interaction with the extracellular matrix(ECM) (Gilbert, 2003). The role of ECM in the tumor microenvironment is not limited to being a barrier against tumor invasion; ECM is an integral part of the tumor microenvironment that represents a reservoir of cell binding proteins and growth factors that affect tumor cell behavior (Brown and Giavazzi, 1995; DeClerck et al., 2004). Both tumor angiogenesis and metastasis require extracellular matrix (ECM) degradation (Shian et al., 2003); the degradation of ECM is achieved through extracellular proteolysis by several proteases that facilitate metastasis and angiogenesis, and enhance cell proliferation, invasion and migration (DeClerck et al., 2004; Garcia-Touchard et al., 2005). Many attempts have been done to fight cancer; traditional treatments of cancer are limited by their general toxicity to proliferating cells, including some normal cells (chari, 2008). 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; Silvestri and Rivera, 2005). One of these strategies is the prevention of cancer metastasis and angiogenesis using protease inhibitors which were found to be very effective in their ability to suppress carcinogenesis in many different in vivo and in vitro assay systems (Kennedy, 1994; Wall et al., 2003). Natural products have served to provide a basis for many of the pharmaceutical agents in current use in cancer therapy, and there is a need for a
continued search for novel natural products that may be used as cancer chemopreventive and/or chemotherapeutic agents (Izevbigie, 2003; Pietras and Weinberg, 2005). Honey is a known natural product with several biological activities (Molan and Allen, 1996; Sesta et al., 2006), many papers and articles have been published in scientific and medical journals describing various biological activities of honey such as antibacterial, antifungal, and antiprotozoal effects. Honey also was used in wound healing and treatment of ulcers and inflammations (Obeseiki-Ebor et al., 1983; Emarah, 1985; Haffejee and Moosa, 1985; McInerney, 1990; Molan, 1992; Basson et al., 1994; Ali, 1995; Molan, 1995; Brady et al., 1997; Cooper et al., 1999; Nzeako and Hamdi, 2000; Dunford and Hanano, 2004).

MATERIALS AND METHODS

Four different samples of honey were selected, on the basis of their results of adulteration tests (diastase activity, 5-hydroxymethyl furfural level and commercial glucose content). The honey samples were brought, each as one lot, from different sources. Sider Kashmiry (H₄) and Magarian (H₅) honeys were brought from the kingdom of Saudi Arabia. El-Madina El-Monawara (H₆) honey was brought from the Egyptian market, Clover (H₇) honey was brought from a source that was random and doubtful in the Egyptian market to be tested as a control adulterated honey.

Honey samples were successively fractionated with petroleum ether, ether, ethyl acetate, chloroform and chloroform/methanol (6:4), respectively. Honeys and their extracts (as well as the residues) were examined for their cytotoxicity using the MTT assay, and for their antimetastatic and antiangiogenic effects using the total protease activity and the gelatin zymography assays. Crude honeys and the residues were applied at a final concentration of 100 μg/ml assay, while the extracts of one gram of each crude honey were applied per one milliliter assay.

MTT Cytotoxicity Assay.

The cytotoxic effect of crude honeys and their extracts was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay according to Mosmann (1983). Cells were dispensed in a 96 well sterile microplate (3x10⁵ cells/well), and incubated at 37°C with the various treatments for 24 hours in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, cells were washed with sterile PBS, and 100 μL of MTT (0.5 mg/mL) were added to each well and then incubated for an additional 3 hours. Formazan crystals were solubilized by the addition of 200 μL of acidified isopropanol. The absorbance was measured at 590 nm using a microplate ELISA reader (Biorad, U.S.A).

Total Protease Activity Assay:

Protease assay is performed according to (Khan et al., 2000) using azocasein protease assay. 200 μl of 1mg/ml azocasein was incubated with 100 μl of conditioned media of HepG2 cells (containing 10 μg protein) for 60 min. Reactions were stopped by adding 300 μl of 10% trichloroacetic acid (TCA); mixtures were left for 15 min and then centrifuged. 150 μl of supernatant was added to 150 μl of 1 M NaOH. Absorbance was determined at 440 nm and then converted to units of protease activity by the equation (absorbance/extinction coefficient) × 10³ = micromoles of dye, and micromoles of dye are converted to units of enzyme by the equation 1 U of enzyme activity = 1 μmol of substrate converted per min. In negative controls, conditioned medium was added immediately prior to the addition of TCA.

Azocasein standard curve was plotted in order to determine the extinction coefficient according to (Melamane, 2003).

Gelatin Zymography:

Gelatin zymography was carried out according to the method described by Rao et al. (2004). Gelatin zymography was performed on 10% polyacrylamide gel copolymerized with 2 mg/ml gelatin (Merck). Samples containing 5μg protein were loaded into the lanes of the gel and run with 1X Tris-glycin electrophoresis buffer at 10 mA under nonreducing conditions. Negative control was a fresh RPMI-1640 medium that was not conditioned with cells. After electrophoresis, gels were washed twice, 30 minutes each, in order to remove SDS allowing proteins to renature. Then, gels were immersed in the reaction buffer overnight at 37°C. The gels were stained with Coomassie Blue, and then destained in destain I for five minutes then in destain II until the clear bands appeared. Clear zones of gelatin lysis against a blue background stain indicated enzyme activities. To characterize the extracellular gelatinase activity produced by HepG2 cells according to its sensitivity to EDTA, an inhibitor of metalloproteinases, each sample was mixed with 1 mM, 5 mM and 10 mM of EDTA (equal volumes) and incubated for 15 minutes (Liu et al., 1998). After electrophoresis, gels were
washed with Triton-buffer, then incubated in reaction buffer containing 10mM EDTA (Huang et al., 1999). Then, gels were stained and destained as mentioned previously. A sample that was not treated with EDTA was prepared as a control.

Data were analyzed using SPSS program version 11.0 to evaluate the above determinations. Data were analyzed for statistical significance using One-way ANOVA test. Results were expressed as the mean ± SD, P values less than 0.05 were considered significant and that less than 0.01 were considered highly significant. Each test was repeated three times in triplicates separately.

RESULTS AND DISCUSSION

**MTT Cytotoxicity Assay:**

The cytotoxic effect of the different crude honeys and their extracts is illustrated in the photos shown in fig. 1. Crude honey, petroleum ether and the residue fractions of H1 enhanced the proliferation of HepG2 cells with high significant values where the OD were 2.16 ± 0.34, 1.8 ± 0.14 and 2.18 ± 0.18, respectively against a control of 0.81± 0.044 (P< 0.01). Ethyl acetate and chloroform/methanol extracts had high significant cytotoxic effect on HepG2 cells (OD, 0.34 ± 0.04 and 0.43 ± 0.01, respectively against a control of 0.81± 0.044, P< 0.01). Chloroform extract showed significant cytotoxic effect on HepG2 cells (OD, 0.59 ± 0.07 against a control of 0.81± 0.044, P< 0.05). Ether extract had nonsignificant cytotoxic effect on HepG2 cells (OD, 0.64 ± 0.016 against a control of 0.81± 0.044, P > 0.05) (fig. 2). In case of H2, crude honey, petroleum ether, chloroform extracts and the residue enhanced the proliferation of HepG2 cells with high significant values (OD, 1.77 ± 0.19, 1.80 ± 0.29, 1.12 ± 0.02 and 2.17 ± 0.08, respectively against a control of 0.81± 0.044, P< 0.01). Ether, ethyl acetate and chloroform/methanol extracts had high significant cytotoxic effect on HepG2 cells (OD, 0.32 ± 0.03 against a control of 0.81± 0.044, P< 0.01), (fig. 3). Crude clover honey (H3) and its petroleum ether, chloroform residue extracts accelerated the proliferation of HepG2 cells with high significant values where the OD were 1.9 ± 0.28, 1.30 ± 0.11 and 2.43 ± 0.14, respectively against a control of 0.81± 0.044 (P< 0.01). Ether extract reduced the viability of HepG2 cells with nonsignificant value where the OD was 0.68 ± 0.062 against a control of 0.81± 0.044 (P > 0.05). Ethyl acetate and chloroform/methanol extracts exhibited significant cytotoxic effect on HepG2 cells where the OD were 0.58 ± 0.018 and 0.54 ± 0.06, respectively against a control of 0.81± 0.044 (P< 0.05).

**Total Protease Activity Assay:**

H1 crude honey and all its extracts decreased the total protease activity. Crude honey, petroleum ether, ethyl acetate, chloroform, chloroform/methanol extracts and the residue had high significant values where the units of protease were 0.90 ± 0.01, 0.73 ± 0.04, 0.55 ± 0.12, 0.81 ± 0.04, 0.38 ± 0.08 and 0.93 ± 0.04, respectively against a control of 1.18 ± 0.038, P< 0.01. Ether extract exhibited significant effect where the units of enzyme were 0.97 ± 0.05 against a control of 1.18 ± 0.038, P< 0.05 (fig. 6). Crude honey and all extracts of H2 decreased the total protease activity with high significant values. The units of enzyme were 0.96 ± 0.12, 0.88 ± 0.04, 0.45 ± 0.08, 0.58 ± 0.12, 0.76 ± 0.08, 0.63 ± 0.09, 0.99 ± 0.014 for crude honey, petroleum ether, ether, ethyl acetate, chloroform, chloroform/methanol extracts and the residue fraction, respectively against a control of 1.18 ± 0.038 (P< 0.01) (fig. 7). Crude honey and residue of H3 exhibited high significant stimulatory effect on protease activity of HepG2 cells where the units of protease were 1.87 ± 0.08 and 1.39 ± 0.05, respectively against a control of 1.18 ± 0.038, P< 0.01. Petroleum ether, ether, ethyl acetate, chloroform, chloroform/methanol of H3 had nonsignificant stimulatory effect on protease activity (1.3 ± 0.09, 1.3 ± 0.11, 1.28 ± 0.17, 1.26 ± 0.12 and 1.2 ± 0.07, respectively against a control of 1.18 ± 0.038, P> 0.05) (fig. 8). H1 crude honey and all its extracts decreased the total protease activity. Ethyl acetate, chloroform and Chloroform/methanol extracts of H3 had high significant inhibitory effect on HepG2 proteases (0.83 ± 0.06, 0.78 ± 0.09 and 0.61 ± 0.08, respectively against a control of 1.18 ± 0.038, P< 0.01), while crude honey,
petroleum ether and ether extracts exhibited significant inhibitory effect on HepG2 proteases (1.02 ± 0.1, 1.00 ± 0.11, 1.00 ± 0.12, respectively against a control of 1.18 ± 0.038, P < 0.05). The residue fraction exhibited non significant inhibitory effect on HepG2 proteases where the units of protease were 1.1 ± 0.09 against a control of 1.18 ± 0.038 (P > 0.05) (fig. 9).

Fig. 2: Effect of Sider Kashmiry honey (H1) and its extracts on the viability of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).

b: Significant (P< 0.05).

c: Nonsignificant (P> 0.05).

Fig. 3: Effect of Magarian honey (H2) and its extracts on the viability of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).
Fig. 4: Effect of clover honey (H3) and its extracts on the viability of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).  

b: Significant (P< 0.05).  

c: Nonsignificant (P>0.05).

Fig. 5: Effect of El-Madina El-Monawara honey (H4) and its extracts on the viability of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).  

b: Significant (P< 0.05).  

c: Nonsignificant (P>0.05).
Fig. 6: Effect of Sider Kashmiry honey (H1) and its extracts on the total extracellular proteolytic activity of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).
b: Significant (P< 0.05).

Fig. 7: Effect of Magarian honey (H2) and its extracts on the extracellular proteolytic activity of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P< 0.01).
Fig. 8: Effect of Clover honey (H3) and its extracts on the extracellular proteolytic activity of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P < 0.01).  
b: Significant (P < 0.05).  
c: Nonsignificant (P > 0.05).

Fig. 9: Effect of El-Madina Elmonawara honey (H4) and its extracts on the extracellular proteolytic activity of hepatocellular carcinoma (HepG2) cell line. H: crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, C: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

a: High significant (P < 0.01).  
b: Significant (P < 0.05).  
c: Nonsignificant (P > 0.05).
Gelatin Zymography:

The zymogram of HepG2 cells showed a single gelatinolytic band at a position corresponding to MW < 14 KD. This band was not inhibited by different concentrations of the metalloproteinase inhibitor, EDTA, (1 mM, 5 mM & 10 mM) as shown in figure 10.

Crude H, and all its extracts had inhibitory effect on the gelatinolytic activity of HepG2 cells where the percentages of control were 75%, 74%, 77%, 71%, 73%, 71% and 82% for the crude honey (H), petroleum ether (PE), ether (E), ethyl acetate (EA), chloroform (C), chloroform/methanol (6:4) (CM), and residue extracts, respectively (fig. 11). Crude honey and all extracts of H, PE, E, EA, C, CM and R, decreased the gelatinolytic activity of HepG2 cells (93%, 88%, 72%, 78%, 79%, 77% and 94%, respectively) (fig. 12). The gelatinolytic percentages of HepG2 cells treated with H, honey and its extracts were 117%, 114%, 113%, 116%, 115%, 114% and 116%, for crude honey, PE, E, EA, C, CM and R, respectively (fig. 13). The gelatinolytic percentages of HepG2 cells treated with H, honey and its extracts were 97%, 94%, 86%, 82% and 87, 86% and 95 for crude honey, PE, EA, C, CM and residue extracts, respectively (fig. 14). Data are summarized in table 1.

Fig. 10: Effect of different concentrations of EDTA on the extracellular gelatinolytic activity displayed by the hepatocellular carcinoma (HepG2) cells. 1: sample treated with 10 mM EDTA, 2: sample treated with 5 mM EDTA, 3: sample treated with l mM EDTA, 4: control sample (not treated with EDTA).

Fig. 11: Effect of Sider Kashmiry honey and its extracts on the extracellular gelatinolytic activity of the hepatocellular carcinoma (HepG2) cells. M: molecular weight marker, NC: negative control (fresh not conditioned medium), C: control (conditioned medium of untreated cells), H: Crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, CH: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.
Fig. 12: Effect of Magarian honey and its extracts on the extracellular gelatinolytic activity of the hepatocellular carcinoma (HepG2) cells. M: molecular weight marker, NC: negative control (fresh not conditioned medium), C: control (conditioned medium of untreated cells), H: Crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, CH: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

Fig. 13: Effect of clover honey and its extracts on the extracellular gelatinolytic activity of the hepatocellular carcinoma (HepG2) cells. M: molecular weight marker, NC: negative control (fresh not conditioned medium), C: control (conditioned medium of untreated cells), H: Crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, CH: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.

Fig. 14: Effect of El-Madina Elmonawara honey and its extracts on the extracellular gelatinolytic activity of the hepatocellular carcinoma (HepG2) cells. M: molecular weight marker, NC: negative control (fresh not conditioned medium), C: control (conditioned medium of untreated cells), H: Crude honey, PE: Petroleum ether extract, E: Ether extract, EA: Ethyl acetate extract, CH: Chloroform extract, CM: Chloroform/Methanol extract & R: Residue.
The proliferative effect 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).

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; Silvestri and Rivera, 2005). One of these strategies is the prevention of cancer metastasis and angiogenesis using protease inhibitors which were found to be very effective in their ability to suppress carcinogenesis in many different in vivo and in vitro assay systems (Kennedy, 1994; Wall et al., 2003).

Discussion:
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).

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Natural products are perceived as pure, and without side effects medication products (Montbriand, 2004). Many patients with cancer or other chronic conditions use alternative therapies, often herbal or natural products (Eisenberg et al., 1993, 1998; Montbriand, 1994, 1995a, 1995b, 1997, 2000). Honey, which is one of the most complex mixtures of carbohydrates produced in nature (Swallow and Low, 1990), has a long history as a medicinal substance (Molan, 1995). It is a known natural product with several biological activities (Molan and Allen, 1996; Sesta et al., 2006). Some bioactive compounds have been found in honey such as chrysin which have been used to prevent cancer, in a similar fashion as anastrozole (a breast cancer drug), and treat conditions such as anxiety and inflammation (Montbriand, 2004; Galijatovic et al., 2000; Galijatovic et al., 2001). Honey is also known as a dietary source for flavonoids (Sabatier et al., 1992) which have been demonstrated to have anti-carcinogenic and anti-inflammatory activities (Middleton and Harborne, 1986).

Although crude honey was reported by some authors as a proliferative agent that enhances the proliferation of both normal and malignant cells (Abuharfeil et al., 1999; Tonks et al., 2001; Rady, 2005), it was also reported as a promising antitumor agent with pronounced antimitastatic effect (Nada and Ivan, 2004; Orsolic et al., 2005; Rady, 2005). The proliferative effect of honey on tumor cells was suggested to be a nutritional effect rather than a carcinogenic effect (Rady, 2005), and the antitumor effect was reported to result from many activities such as the inhibition of DNA synthesis with no signs of cytotoxicity (Rady, 2005), and down regulation of MMP-2 and MMP-9 which have been implicated in the induction of the angiogenic switch in different model systems (Egeblad and Werb, 2002; Rady, 2005).

According to the results of MTT cytotoxicity test, all the tested crude honeys and their petroleum ether extracts and residues increased the proliferation of HepG2 cells significantly. Chloroform/methanol (6:4) extracts of all honeys exhibited antiproliferative effect on HepG2 cells with no significant differences between their inhibition values. The proliferative effect of the four crude honeys as well as their residual parts was concomitant with some authors who reported that honey enhances cell proliferation (Abuharfeil et al., 1999; Tonks et al., 2001; Rady, 2005). This may be explained by the fact that honey with its sugar content provides substrates for glycolysis which is the major mechanism for energy production for cell proliferation (Ryan and Majno, 1977). Therefore, the proliferative effect of the four tested crude honeys as well as their residual fractions which retained most of honey sugars may be mainly due to their sugar content.

Beside sugars, honey contains amino acids, minerals and vitamins which help in enhancing cell proliferation (Abuharfeil et al., 2000). These amino acids, minerals and vitamins contents of honey may explain the proliferative effect of honey as well as the extracts that retained its proliferative effect (residues, petroleum ether extracts of all honeys and chloroform extracts of H2, H3, and H4).

The proliferative effect of petroleum ether extracts of all tested honeys on HepG2 cells, and the antiproliferative effect of ether extracts of all honeys (except that of H1, honey which had no effect on the proliferation of HepG2 cells) may be explained by the fact that these two solvents, petroleum ether and ether, extract HMF present in honey (Xu et al., 2007).

Table 1: Extracellular gelatinase activities of the hepatocellular carcinoma (HepG2) cells treated with Sider K ash miry (H1), Magarian (H2), Clover (H3), and El-Madina El-Monawara (H4) honeys and their extracts in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
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<tr>
<td>H</td>
<td>75%</td>
<td>93%</td>
<td>117%</td>
<td>97%</td>
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<td>PE</td>
<td>74%</td>
<td>88%</td>
<td>114%</td>
<td>94%</td>
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<td>E</td>
<td>77%</td>
<td>72%</td>
<td>113%</td>
<td>86%</td>
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<td>EA</td>
<td>71%</td>
<td>78%</td>
<td>116%</td>
<td>82%</td>
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<td>C</td>
<td>73%</td>
<td>79%</td>
<td>115%</td>
<td>87%</td>
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<td>CM</td>
<td>71%</td>
<td>77%</td>
<td>114%</td>
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<tr>
<td>R</td>
<td>82%</td>
<td>94%</td>
<td>116%</td>
<td>95%</td>
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The cytotoxic effect of the ethyl acetate extracts can be explained by the fact that honey is a dietary source of phenolic compounds that are referred to as flavonoids. These compounds have been isolated from several types of honey (Russo and Neri, 2002), and they have been reported as potent anticancer agents (Moein et al., 2007; Yang et al., 2008) that can be extracted from their sources mainly by ethyl acetate (Russo and Neri, 2002).

The cytotoxic effect of chloroform extract of H. may be attributed to the fact that honey may contain pyrrolizidine alkaloids which considered as toxic compounds that were extracted by chloroform (Deinzer et al., 1977). The increase in cell proliferation of HepG2 cells treated with other chloroform extracts indicates that they lack the cytotoxic compounds extracted from H. with chloroform and, in addition, they may contain proliferative compounds.

Chloroform/methanol solvent was reported to extract lipids, including unsaturated fatty acids (Gao et al., 2001). It was reported that unsaturated fatty acids possess a broad spectrum of biological properties in both animals and plants and some of these compounds show biological activity, e.g., 9,10-dihydroxy-8-oxo-12Z-octadecenoic acid exhibits cytotoxicity against HeLa cells and an inhibitory effect on tea pollen growth (Gao et al., 2001); it was also reported that the lipid fraction extracted from Agaricus blazei by chloroform/methanol retarded tumor growth (Takaku et al., 2001). Thus the cytotoxic effect of the chloroform/methanol extract of all tested honeys may be attributed to the presence of cytotoxic lipid contents of honeys and, probably, to other cytotoxic component(s).

In the present work, zymogram of HepG2 cells showed a single gelatinolytic band at a position corresponding to MW < 14 KD. The activity of this gelatinolytic band was not inhibited by EDTA (a metalloprotease inhibitor). Therefore, this band was considered to represent an enzyme that belongs to a non-matrix metalloproteinase class of proteases. This result is consistent with the fact that HepG2 cell line does not produce MMP-2 and MMP-9 gelatinases in both conditioned media and membrane preparation. However, HepG2 cells showed in vitro migratory ability on Ln-1 and coll IV components of the extracellular matrix (Giannelli et al., 2001), and their migration was reported to proceed as an MMP-independent chemotactic migration (Yang et al., 2003). Moreover, many gelatinases are becoming known such as tryptase (Fajardo and Pejler, 2003) and Seprase which has gelatinase activity and may be involved in cancer invasion and metastasis (Okada et al., 2003).

Hepatocellular carcinoma cells (HCC), including HepG2 cells, interact with several different extracellular matrix components to migrate and invade the surrounding tissues. Such interactions are ensured by integrins, a class of heterodimeric transmembrane receptors composed of one α and one β chain; α, β, integrin is expressed by the MMP-dependent invasive HCC cells but not expressed by the noninvasive HepG2 cells. α, β, is implicated in the production and/or activation of matrix metalloproteinases MMP-9 and MMP-2 which promote invasion and migration of invasive cells.

In the present work, H, H, and H honeys and all of their extracts exerted an inhibitory effect on both of the total extracellular protease activity and extracellular gelatinase activity, but they exhibited different levels of potency. H, H, and all of their extracts had highly significant inhibitory effect on the total extracellular protease activity. H and its extracts (except the residue which had nonsignificant effect) exhibited significant effect on the total extracellular protease activity, but its effect was less potent than those of H and H; the effect of H and its extracts on the extracellular gelatinase activity was lower than that of H and H. The inhibition of total extracellular protease activity and extracellular gelatinase activity by H, H, and H honeys was approved by Rady (2005) who reported that bee honey down regulates the production of the gelatinases MMP-2 and MMP-9, and this may indicate that bee honey contains protease and gelatinase inhibitors that play a role in its antimetastatic and antiangiogenic activities against cancer.

On the contrary, H crude honey and its residue showed significant stimulatory effect on the total extracellular protease activity, while its extracts had nonsignificant stimulatory effect on the total extracellular protease activity. In addition, the percentages of the gelatinase activity of cells treated with H and its extracts exceeded that of the control, these results were approved by the results of adulteration tests that reported H as an adulterated honey.

Although crude honey (100 μg/ml), petroleum ether and the residue fractions of H, H, and H, as well as chloroform extract of H and H, increased the proliferation of HepG2 cells, they decreased the protease and gelatinase activities of HepG2 cells, this is consistent with the fact that some non-cytotoxic compounds can be considered as antimetastatic agents (Thein and Lotan, 1982), for example, fumagillin (Griffith et al., 1998) and squalamine (Sills et al., 1998).

Ether, ethyl acetate and chloroform/methanol extracts of the unadulterated honeys (H, H and H) as well as chloroform extract of H inhibited the total extracellular protease and extracellular gelatinase activities, and
at the same time inhibited the proliferation of HepG2 cells. These results can be explained by the fact that proteolytic activity of ECM-degrading proteases generates biologically active protein fragments that can affect tumor cell proliferation, survival, spread and plasticity, and inhibition of these enzymes has shown impressive therapeutic effects on tumors (Lee et al., 2004; Keld et al., 2005).

In conclusion, some bee honey extracts (ether, ethyl acetate and chloroform/methanol) can be considered as promising antitumor agents where they inhibited the proliferation, protease activity and gelatinase activity of HepG2 cells in an independent manner. Further investigations are needed to study in details the composition and mechanism of action of these extracts on HepG2 cells and other cell lines. It was found that the extracts of H1, H2 and H3 that enhanced cell proliferation (petroleum ether extracts and the residues) inhibited the protease activity and gelatinase activity of HepG2 cells, thus they can be considered to contain the proliferative components of honey, and these components are nutritional factors of honey; in addition, they may also contain protease and gelatinase inhibitors that need further separation and identification.

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