

# ENHANCEMENT OF THE ANTITUMOR EFFECT OF HONEY USING ADIPONECTIN HORMONE

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## Abstract

**Objectives:** To investigate the possibility of modulating the antitumor effects of honey through its combination with adiponectin.

**Design and Methods:** Hepatocellular carcinoma cells (HepG2) were treated with adiponectin, honey and its extracts, individually and in combinations. Cell growth (by MTT test), alkaline phosphatase activity,  $\beta$ -actin, and Bcl-2 gene expression were determined.

**Results:** HepG2 cells treated with adiponectin combined to honey and its extracts showed significant decrease in cell growth. Alkaline phosphatase activity was enhanced in HepG2 cells treated with adiponectin combined with honey ethyl acetate extract. Neither  $\beta$ -actin nor Bcl-2 gene expression was significantly changed by the combined treatment of adiponectin and honey.

**Conclusion:** The results of this study revealed that combined treatment of adiponectin and honey has promising effects on inhibition of cell growth and enhancing cell differentiation. However, this combination did not show significant effects on  $\beta$ -actin or Bcl-2 gene expression.

## Introduction

Honey is a known natural product which has several biological activities. It is considered one of the most complex mixtures of carbohydrates produced in nature. Literatures that report usage of honey as an antitumor are few. Honey was found to down regulate the production of MMP-2 and MMP-9 which have been implicated in the induction of the angiogenic switch in different model systems. In addition, honey also decreased the total extracellular protease and the extracellular gelatinolytic activities of HepG2 cells.

Some authors considered crude honey as a mitogenic agent where it enhances cell proliferation. Enhanced proliferation induced by honey was suggested to be a nutritional effect caused by carbohydrate of honey which provides substrates for glycolysis (the major mechanism for energy production for cell proliferation), and so it does not contradict with its other antitumor effect.

Adiponectin, an adipocyte-secreted hormone, is abundant in human plasma with concentrations ranging from 3 to 30  $\mu\text{g ml}^{-1}$ . It appears to play an important role in the development and progression of various types of cancers where low serum adiponectin levels may be a novel risk factor for cancer. The two putative adiponectin receptors (Adipo R1 and adipo R2) are highly expressed in liver and other metabolically active organs. Adiponectin as well as its analogues may be effective anticancer agents and may have important therapeutic implications. However, incubation of some types of cell lines with increasing doses of adiponectin did not cause any significant change in the proliferation rate of the tumor cells. This discrepancy in results among different studies may be due to the different doses of adiponectin or different types of cancer cell lines used in experiments.

## Aim of the work

This study was conducted to evaluate the antitumor effects of honey, its ethyl acetate and chloroform/methanol extracts as well as their combinations with adiponectin on hepatocellular carcinoma cells (HepG2) *in vitro*. The possibility of modulating HepG2 cell growth, differentiation, metastasis, and apoptosis was investigated through measuring the MTT assay,  $\beta$ -actin, and Bcl-2 gene expressions respectively. We tried to overcome the undesired well established proliferative effect of honey through its combination with adiponectin.

## Material and Methods

Sider Kashmiry bee honey (K), brought from the kingdom of Saudi Arabia markets, and Madina bee honey (M), brought from the Egyptian market were selected on the basis of the adulteration tests (diastase activity, 5-hydroxymethyl furfural (HMF) content and detection of commercial glucose). Recombinant adiponectin was purchased from Linco Research (USA). HepG2 cells were treated with honey and/or adiponectin at concentrations of 100  $\mu\text{g/ml}$  and 100  $\mu\text{M/ml}$  respectively.

### Preparation of honey extracts

The selected honey sample was successively fractionated using five solvents with ascending polarity (petroleum ether, diethyl ether, ethyl acetate, chloroform and chloroform/methanol (6:4), respectively). Ethyl acetate and chloroform/methanol extracts (dried at room temperature) were chosen for further investigations depending on our previous studies.

### Cell culture

Tumor Liver cell line, hepatocellular carcinoma (HepG2), was supplied by Naval American Research Unit-Egypt (NAMRU). 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, and 1% antibiotic mixture (20 units/ml of penicillin G sodium and 20 mg/ml streptomycin sulfate, Gibco).

### Cytotoxicity test (MTT)

The cytotoxic effects of adiponectin, crude honeys and their ethyl acetate and chloroform/methanol fractions on hepatocellular carcinoma (HepG2) cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann, (1983). Briefly, cells were seeded in 96-well plate (3X10<sup>3</sup> cells/well), and incubated at 37°C with the various treatments for 24 hrs in complete RPMI-1640 medium. After incubation, cells were washed with sterile PBS, and 100  $\mu\text{L}$  of MTT (0.5 mg/ml) were added to each well and then incubated for additional 3hrs. Formazan crystals were solubilized by 200  $\mu\text{L}$  DMSO. Absorbance was measured at 590 nm using a microplate ELISA reader (Biorad, U.S.A.).

### Alkaline phosphatase enzyme activity

HepG2 cells were seeded in 6-well plate at a density of 0.5X10<sup>6</sup> cells/ml. The enzyme was extracted using a modified method of Chang *et al.*, (1994). Briefly, the cells were washed twice with PBS and suspended in 0.1 ml of extraction prepared buffer (20 mM Tris/ HCl, 1.0 mM MgCl<sub>2</sub>, 150 mM NaCl, pH 8.0). The cells were solubilized by adding Triton X-100 to a final concentration of 1%. The enzyme extracts were transferred into 96-well plate. Alkaline phosphatase activity was measured by the method of Singh and Singh, (2005).

### Messenger RNA expression analyses for $\beta$ -actin and BCL-2 genes

HepG2 cells were seeded in 6 well tissue culture plate at a density of 0.5X10<sup>6</sup> cells/ml. Total RNA was extracted from the HepG2 cell line using PqGold Trifast (Biotechnologie GmbH, USA) according to the manufacturer instructions. The primer sequence for the  $\beta$ -actin gene was as follows: Forward, cctctggcagatgagctct; Reverse, ggagcaatgatctgtcttc. The primer sequence for Bcl-2 gene was as follows: Forward, cctctggcagacacatcgc; Reverse, aatcaaacagagccgatgc.

Qiagen on step RT-PCR kit (Qiagen Inc, USA) was used for RNA reverse transcription and subsequent amplification. PCR reaction was performed separately for  $\beta$ -actin and BCL-2 by adding 2  $\mu\text{g}$  RNA to the PCR mixture and completing the volume to 50  $\mu\text{L}$ . The PCR mixture contained 2mM Tris Cl, 10mM KCl, (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 1.25 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol; PH 8.7, 0.4 mM dNTPs mixture, Qiagen one step RT-PCR enzyme Mix (contains OmiscriptTM Reverse transcriptase, SensiscriptTM reverse transcriptase and Hot star TaqR DNA polymerase), and 0.6  $\mu\text{M}$  of each specific primer. The reaction mixture was subjected to reverse transcription at 50°C for 30 minutes then to 35 cycles of PCR amplification as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The PCR products were separated on 1% agarose gel and visualized by gel documentation system. The genes expressions were semi-quantified using LabImage analysis (LabImage2.7.0, Kapelan GmbH) software.

### Statistical Analysis:

The data were analyzed using version 11.0 of the computer based statistical package of Statistical Product and Service Solutions. Results are expressed as means  $\pm$  SD from three experiments per group. Statistical significance of difference was determined using analysis of variance (one-way ANOVA). Further statistical analysis for post hoc comparisons was carried out using the LSD test. Differences with P<0.05 were considered as significant.

## Results

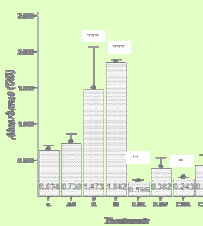


Fig (1): Effect of adiponectin, honey and its extracts on cell growth of HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

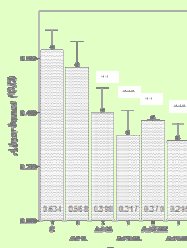


Fig (2): Effect of combined treatments of adiponectin with honey and its extracts on cell growth of HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

## Results

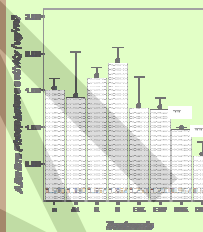


Fig (3): Effect of adiponectin, honeys, and their extracts on alkaline phosphatase activity of HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

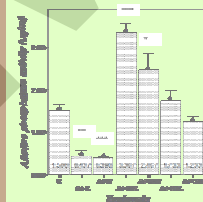


Fig (4): Effect of adiponectin combinations with honey, as well as its extracts on alkaline phosphatase activity in HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

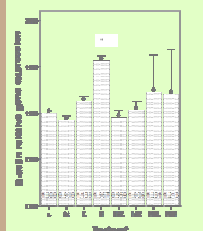


Fig (5): Effect of adiponectin, honey and its extracts on  $\beta$ -actin gene expression in HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

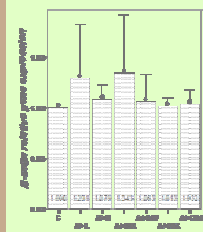


Fig (6): Effect of combination of adiponectin with honey and its extracts on  $\beta$ -actin gene expression in HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

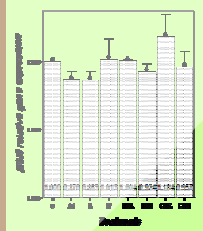


Fig (7): Effect of adiponectin, honey and its extracts on Bcl2 gene expression in HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

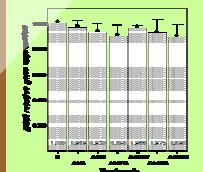


Fig (8): Effect of combination of adiponectin with honey and its extracts on Bcl2 gene expression in HepG2 cells. C, control; Ad, adiponectin; K, Kashmiry honey; M, Madina honey; EAK, ethyl acetate extract of honey K, EAM, ethyl acetate extract of honey M, CMK, chloroform/ methanol extract of honey K, CMM, chloroform/ methanol extract of honey M.

**Conclusion:** We may conclude that the proposed combinations of adiponectin with honey, and honey extracts exerted their antitumor effects on HepG2 cells through the inhibition of cell growth and the enhancement of alkaline phosphatase activity rather than through acceleration of apoptosis via bcl-2 down regulation. Adiponectin and honey may exert their known anti-metastatic effect on tumor cells through other pathways which do not involve  $\beta$ -actin down regulation.