ORIGINAL ARTICLE

Cytotoxic Activity of Honey in Hepatoma Cells: In Vitro Evaluation

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ABSTRACT

Background: Honey is used extensively in south East Asia, Middle and Far Eastern countries as a sweetener as well as medication for its anti-bacterial, anti-fungal, and anti-viral properties with no risk of resistance or side effects. Recent studies suggest that honey can modulate tumor growth by reducing cell proliferation and increasing apoptosis susceptibility.

Objectives: To find out the apoptotic effect of honey on hepatoma cancer cell lines.

Methods: The growth of hepatoma cell lines (*Huh-7*) was checked by adding Honey to the cells before culturing in a 24 well plate. Wells were selected and labeled for each of the variables (controls, honey). After 2 days, cells were studied under an inverted phase contrast microscope and fields were recorded. Approximately four fields per slide of higher intensity were selected randomly to determine the dead cell density, and the procedure was repeated 10 or more times. Frequency and percentages were calculated for dead or alive cells in controls, honey and their mixture. Odds Ratio was used to compare the qualitative variables.

Results: Honey was found to induce cell death in hepatoma cell lines (Huh-7). At a magnification of 40x, the dead cells were 1% in controls whereas, 70.8% in honey. At magnification of 20x and 10x the dead cells density was 52.3% and 82.4 % respectively in honey treated cells compared to 4% in controls.

Conclusion: Honey induced cell death in *Huh-7* cells. *Huh-7* exposed to honey have high probability of apoptosis/necrosis. Honey should be considered as co-adjuvant treatment against cancer.

KEY WORDS: *Traditional Medicine; Honey; Hepatoma Cells (Huh7).*

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INTRODUCTION

Hepatocellular carcinoma (HCC), or malignant hepatoma is the fifth most common type of cancer worldwide and is the third leading cause of cancer deaths (598,000) globally including both genders.[11] In developing countries, especially Asia and Africa, where the endemic high prevalence of hepatitis B and hepatitis C strongly predisposes to the development of chronic liver disease and subsequent development of hepatocellular carcinoma with grave prognosis, the treatment against it remains a major challenge.[2] Several studies have shown natural products such as honey contain ingredients with medicinal effects that include, anti-bacterial, anti-fungal, anti-viral and anticancerous effects.

Honey, a well known broad spectrum antimicrobial agent, has been shown to enhance wound healing as well as exert beneficial effect in various cancers in cell cultures and in animal studies. Follow up studies (for 25 years) on subjects who consumed honey regularly showed low mortality and morbidity.[3] Honey, being tested on many cancers have been found to have a sustainable inverse relationship with various cancers in the setting of developing nations where resources for cancer prevention and treatment are limited.[4]

On account of the high morbidity and mortality of HCC and problems encountered in their treatment and the toxic effects of chemotherapy this study was undertaken to reveal the anticancer effects of the relatively safe edible herbal products which can replace the currently used cytotoxic drugs in the treatment of HCC.

METHODOLOGY

This descriptive, comparative, controlled *in-vitro* study was conducted at the Aga Khan University Molecular Biology Laboratory. Hepatoma cell lines (Huh-7) were kindly provided by them for research. The Huh-7 cell lines were cultured according to given protocol by Ryan.[5] The medium for cell culture contained all the additives mandatory by Huh 7. Magnesium-free phosphate-buffered saline CMF-PBS (10 mL) and Calcium with trypsin solution 0.1% were used. When the flasks of actively growing cells were 80-90% confluent they were selected for experiment. Prior to sub-cultivation the cultures were examined under a microscope (100-200x) to check any signs of microbial contamination. After harvesting the Huh 7 cells using the standard protocol, the cells were cultured in a 24 well plate using the additives. All wells were labeled for two variables; controls and honey). Since the base of wells in the 24 well plate are of same size as slide covers therefore, glass slide covers were inserted in all the wells for the growth of cells. Samples were prepared as 1µg/1µl with sterilized water and 10 µl was used for each well. In brief hepatoma, cells were seeded at a density of 0.05 x 106 cells per well of 24-well cell culture plates in the presence of minimum essential medium (Invitrogen, Carlsbad, CA), with 10 µl prepared sample of honey wells, whereas, 10µl of H2O each in the wells labeled as controls. After 2 days, the slide covers from 24 well plate were removed and placed over a glass slide containing a drop of accridine orange and were studied under a immunoflorecent microscope (inverted phase contrast microscope) and fields recorded. Two to four fields per slide of higher intensity were selected randomly to determine the dead cell density, and the procedure was repeated 10 or more times. Frequency and percentages were calculated for dead or alive cells in controls, and Honey. Odds ratio for dead and alive Huh 7 cells was calculated.

RESULTS

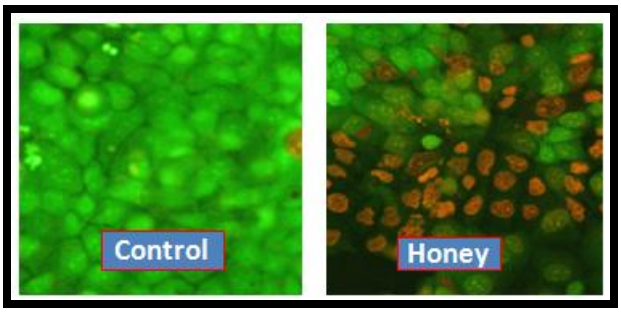
The effects of honey on Huh-7 cells at various magnifications are described in Table I. At magnification of 10X, 20X and 40X dead cells in controls were 1.7%, 1.6% and 0.5% compared to 82.4%, 52.3% and 70.8% in cells containing honey respectively.

The cell cultures showed death in large groups of confluent cells with cellular detachment as well as individual cell death or in small groups. Figure 1 shows various patterns of cellular death at 40 x magnification induced by honey. There was no cell death seen in controls at this magnification and odds ratio shows that odds of *Huh-7* exposed to honey has approximately 464 times the probability of apoptosis/necrosis (Table 1). Odds ratio was calculated which was highly significant.

Table 1: Comparison of alive and dead cell between Honey and controls at various magnifications.

Specimens	No. of Fields	Type of Magnification	Hepatoma cell line (Huh7)					*Mean Dead Cells*
			Cells	Alive		Dead		
				No.	%	No.	%	
Control: H_2O	N=11	10x	4586	4506	98.3	80	1.7	**29**
	N=11	20x	364	358	98.3	6	1.6	
	N=6	40x	195	194	99.5	1	0.5	
Honey:	N=8	10x	1996	352	17.6	1644	82.4	**675.3**
	N=11	20x	329	153	47.7	176	52.3	
	N=10	40x	292	86	29.2	206	70.8	

Figure 1: Patterns of Cellular Death at 40x



DISCUSSION

This is the first time that the effect of honey has been studied on hepatoma cell lines. The study provides exceptional experimental evidence that honey inhibits the growth of hepatoma cells by inducing apoptosis and necrosis. Few researchers studied the effect of natural honey on cancer cell lines. Honey with its low pH and abundant phenolic content limit oxidant-induced cell death more effectively through its antibacterial property of supplying free radicals.[6] Cytotoxic studies of honey on a selected sample of human colon cancer cell illustrated the apoptosis inducing ability of the honey. Cell cycle analysis showed that Honey induced apoptosis in human colon cancer cells by arresting the cells at subG1 phase, displayed by increased accumulation of cells. Further the researchers confirmed apoptosis through DNA fragmentation assay in HT 29 cells which showed typical ladder pattern. Finally PARP cleavage and Caspase-3 activation was observed. [7]

Necrosis and apoptosis observed in Huh7 cells induced by honey in this study are two different processes. Necrosis is cell death in response to acute inflammation. Whereas, apoptosis is a programmed cell death in which a cell undergoes morphological changes that include cell blebbing, nuclear fragmentation, chromatin condensation and DNA fragmentation. In normal state cell replication and apoptosis is always maintained, whereas, in cancer, cells divide continuously causing damage to the organs. Honey, in cancer cells, causes morphological fragmentation and appearance of nuclear chromatin condensation showing a significant growth inhibitory effect. [8] Ghashm etal observed this early apoptosis outcome by flow cytometry in OSCC and HOS cell when doses of honey were added. [9] Similarly, work on mammary carcinoma by Kadir et al in DMBA-induced rats also showed inhibitory effects on the initiation and promotion of cancer cells by honey. Basically, honey modified the incidence and severity of cancerous tumor development in DMBA-induced mammary carcinoma in rats.[10] The anti-proliferative actions of honey has been elaborated through MTT assay by testing on the growth of colon cancer cell (HCT-15 and HT-29). Honey stimulates the apoptotic signal via increasing the reactive oxygen species (ROS) and up-regulating the p53, modulating the expression of pro and anti-apoptotic proteins, clearly defining honey as a potential chemotherapeutic agent especially against colon cancer.[11] Cytotoxic studies of a selected sample on a breast cancer cell displayed growth inhibition, depending on the concentration of honey used. Cell cycle analysis indicated increasing accumulation of cells at the sub-G(1) phase.[12] Antiproliferative and apoptotic effects of honey also were observed on HL-60 cells at various concentrations. The percentage of cell viability was evaluated by MTT assay, apoptotic cells were identified and ROS production was determined. The researchers concluded that antiproliferative and apoptotic effects of Honey

are attributed to the presence of polyphenols which have been proven to be beneficial effects against numerous diseases through their antioxidant activity. Honey is a good source of antioxidants since it contains a great variety of phenolic compounds.[13] Many other studies have also attributed the apoptotic performance of honey is to its abundant phenolic acids and flavonoids and other antioxidants including glucose oxidase, catalase, ascorbic acid, carotenoid derivatives, organic acids, amino acids and proteins content. Some simple and polyphenols found in honey, namely, caffeic acid (CA), caffeic acid phenyl esters (CAPE), Chrysin (CR), Galangin (GA), Quercetin (QU), Kaempferol (KP), Acacetin (AC), Pinocembrin (PC), Pinobanksin (PB), and Apigenin (AP), have evolved as promising pharmacological agents in treatment of cancer.[14]

Makedou et al[15] examined Honey for its in vitro oxidation of human serum lipoproteins and isolated plasma low density lipoproteins (LDL) and found that it exerts strong antioxidant activity on both human LD and serum total lipoprotein oxidation in vitro by postponing LDL oxidation. However, the effective quantity to produce that result is yet to be determined. Honey has also been famous for topical treatment of various wounds infections and burns hence, Mohamed et al[16] tested honey on diabetic foot ulcer and found that a daily dressing with honey with strict offloading can heal ulcer completely by 16 weeks. A systemic review on topical treatment of honey, found that the data in this area is extremely scarce. It concluded that a limited research has been done on its deodorizing, debridement, anti-inflammatory and analgesic properties. Therefore, the researchers recommended and suggested more studies on honey should be conducted regarding modern wound care. [17]

In cancer management and treatment, research has also shown the importance of co-adjuvant therapies. Honey in combination with aloevera showed probable inhibition of tumour growth while aloevera reduced tumor mass and metastasis rates in Wistar rats. The apoptosis process in this study was established by evaluating the cancer markers Ki67-LI (cell proliferation rate) and Bax/Bcl-2 gene expression at 7, 14 and 20 days in comparison to control group.[18]

Cancer treatment has various toxic side effects especially chemotherapy The studies discussed above have shown that consuming natural products, especially honey for the treatment of cancer carry low or no risk of side effects or toxicity.[19] Until now people have been using natural food products as remedies for minor ailments such as colds simply because they are harmless and safe. Now people with terminal illnesses are reverting back to natural remedies because they are now being proven effective through modern equipment and expertise. Thus, many patients with cancer or other chronic conditions are using alternative therapies and Honey; amongst them is one of the most complex mixtures produced in nature, with an unparalleled medicinal history. It is a known natural product with several biological activities.[20]

CONCLUSION

Honey, known for centuries for a wide range of ailments from sore throat to treatment of wounds, has recently found a place in the modern research. In the emerging era of Preventive healthcare strategies, honey and its antimicrobial, anti-inflammatory, antioxidant, antimutagenic, antitumor, and antidiabetic properties, in addition to wound-healing attributes is like a breath of fresh air. Although the available evidence is rather limited yet it proves the role of honey in contemporary health care. Its emergence and revival in the days when modern medicine has exhausted the mankind with side effects recommendations are suggested for future research.

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