Antiproliferative and apoptotic effects of spanish honeys

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ABSTRACT

Background: Current evidence supports that consumption of polyphenols has beneficial effects against numerous diseases mostly associated with their antioxidant activity. Honey is a good source of antioxidants since it contains a great variety of phenolic compounds. **Objective:** The main objective of this work was to investigate the antiproliferative and apoptotic effects of three crude commercial honeys of different floral origin (heather, rosemary and polyfloral honey) from Madrid Autonomic Community (Spain) as well as of an artificial honey in human peripheral blood promyelocytic leukemia cells (HL-60). **Material and Methods:** HL-60 cells were cultured in the presence of honeys at various concentrations for up to 72 hours and the percentage of cell viability was evaluated by MTT assay. Apoptotic cells were identified by chromatin condensation and flow cytometry analysis. ROS production was determined using 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA). **Results:** The three types of crude commercial honey induced apoptosis in a concentration and time dependent-manner. In addition, honeys with the higher phenolic content, heather and polyfloral, were the most effective to induce apoptosis in HL-60 cells. However, honeys did not generate reactive oxygen species (ROS) and N-acetyl-L-cysteine (NAC) could not block honeys-induced apoptosis in HL-60 cells. **Conclusion:** These data support that honeys induced apoptosis in HL-60 cells through a ROS-independent cell death pathway.Moreover, our findings indicate that the antiproliferative and apoptotic effects of honey varied according to the floral origin and the phenolic content.

Key Words: Antioxidants, Antiproliferative, Apoptosis, Honey, Reactive oxygen species

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INTRODUCTION

Consumption of certain dietary components have been related to several protective effects against certain forms of cancer and cardiovascular disease likely because of their antioxidant content.[1] Traditionally honey has been a sweetening agent used since long time both in medical and domestic applications.[2] However, several aspects of its use indicate that honey also functions as a food preservative and exhibits antioxidant, chemopreventive, antiatherogenic, immunoregulatory, antimicrobial and wound healing properties.[3,4]

The components in honey responsible for its antioxidative effects are mainly flavonoids, phenolic acids, catalase, peroxidase, carotenoids and non-peroxidal component.[5] The quantity of these components varies greatly according to the floral and geographical origin, processing, handling and storage.[6] However, the botanical origin of honey has the greatest influence on its antioxidant activity. In fact, considerable differences in both composition and content of phenolic compounds have been found in different unifloral honeys. Also, the phenolic and flavonoid contents of honey have been reported as a specific marker for the botanical origin.[7-9]

Most of the drugs used in the cancer treatment are apoptotic inducers and polyphenols were reported to have antiproliferative potential.[10] Apoptosis or programmed cell death, is now recognized as a vital process in the regulation of tissue development and homeostasis.[11] The induction of apoptosis in tumor but not in normal cells is considered very useful in the management and therapy of cancer.[12] Thus, apoptotic screening *in vitro* provide important preliminary data to help select natural product with potential antineoplastic properties for future study.

Many studies have supported that apoptosis can be initiated by oxidative stress, which is mediated by the generation of reactive oxygen species (ROS). Moreover, alterations in the redox status of the cell to a more oxidizing environment occurs prior to the final phase of caspase activation in many model of apoptosis.[13] Recent results showed

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honey as a plausible candidate for induction of apoptosis through ROS and mitochondria-dependent mechanism in colon cancer cells.[14]In addition, since the composition of honey varies widely in relation to its botanical origin and environmental factors, it can be reasonably expected that honey properties from different floral sources are different.

The aim of the present study was to evaluate the antiproliferative and the apoptotic effects of three crude commercial honeys of different floral origin from Madrid Autonomic Community (Spain) as well as of an artificial honey, using a human tumor leukemia cell line (HL-60) as a model system. To our knowledge this is the first time that Madrid Autonomic Community honeys have been tested against HL-60 cells. Further we evaluated the possible molecular mechanism of honey induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Chemicals

Sucrose, maltose, fructose and glucose were purchased from PanreacChimica, S.A. (Barcelona). Etoposide, N-Acetyl-L-cysteine (NAC) and ethidium bromide were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Hoechst 33342 and 2´, 7´-dichlorodihydroflourescein diacetate (H_2DCFDA) were obtained from Molecular Probes (Eugene, Oregon, USA). All other chemicals and solvents were of the highest grade commercially available.

Honey samples

The type and region of the honey samples, as well as the family, scientific and common name of the plants that form the basic flora of the honey samples, are shown in Table 1. According to Sereia *et al.*[15] a honey is classified as unifloral if it contains pollen in quantities exceeding 45% on the remaining pollen identified. In any other case a honey sample is characterized as polyfloral. Commercialized honeys (Honey Antonio Simon) were obtained from a single experienced producer who provided the three authentic samples: Rosemaryand heather honeys as unifloral and a polyfloral honey.[16-18] A sugar analogue (an artificial honey whose composition reflects the approximate sugar composition of honey) was used to check whether the main sugar components interfere in the assays. The artificial honey (100g) was prepared by dissolving 1.5g sucrose, 7.5g maltose, 40.5g fructose and 33.5g glucose in 17ml of distilled water and the solution was mixed for 1hour. The desired amounts ofpolyfloral, heather, rosemary and artificial honey (w/v) were weighed and diluted in sterile distilled water. The honey solutions were made up to 1% (w/v) and rendered sterile by Millipore filtration (0.2 μm).

HL-60 cells

Human peripheral blood promyelocytic leukemia cells (HL-60) were obtained from the Biology Investigation Centre Collection (BIC, Madrid) and maintained in RPMI 1640 medium supplemented with 10% v/vheat-inactivatedfoetalcalfserum, 50 g/mL streptomycin, 50 UI/mL penicillin and 1% v/v L-Glutamine at 37ºC in a humidified atmosphere of 5% CO_2. Culture medium and supplements were purchased from Gibco Laboratories (Life Technologies, Inc., Gaithersburg, MD 20884-9980). Controls included a medium control without honey as negative control. Etoposide has been extensively studied and was used in this study as a positive control (5 μM) of apoptosis.[19]

Cell proliferation assay (MTT)

Viability of honeys treated HL-60 cells was assessed by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay(Cell Proliferation Kit I, Roche, Indianapolis, USA). Briefly, HL-60 cells were plated at a density of 1×10^6cells/mL culture medium. After seeding, concentrations of honeys (1-250mg/mL) were added and plates were incubated for 24, 48 and 72hours. The optical density (OD) of each well was read at 620nm (test wavelength) and 690nm (reference wavelength) by an ELISA with a built-in software package for data analysis (iEMS Reader MF, Labsystems, Helsinki, Finland). Values presented in this paper are means ± standard error of the mean. Cell survival in exposed cultures relative to unexposed cultures (negative control) was calculated and expressed as percentage of

Table 1: Honey Samples

Honey Type	Scientific name (Family)	Common name	Organoleptic Characteristics	Production zone: Autonomic Community of Madrid
Unifloral	*Rosmarinus officinalis* (Lamiaceae)	Rosemary Honey	Aroma with floral and fresh notes, mild flavor, light color [16]	El Atazar, Torres de la Ladera, Alcalá de Henares
Unifloral	*Erica arborea* (Ericaceae)	Heather Honey	Ripe fruit and spicy aroma, dark color [17,18]	El Atazar, Prádena de la Sierra, Montejo de la Sierra
Polyfloral		PolyfloralHoney		Zarzalejo, La Cabrera, Alcaláde Henares, Torres de la Ladera, Colmenar Viejo, Serranillos, El Vellón, Patones

survival (%SDH activity) = $(A_1/A_0) \times 100$, where A_1 is the absorbance of exposed cultures and A_0 is the absorbance of negative control. All the concentrations were tested in 16 replicates and the experiments were repeated three times.

Chromatin condensation assay

HL-60 cells (1×10^6/mL) were treated with 25 or 50 mg/mL of each honey for 24 and 48hours. After treatments, cells were stained with Hoescht 33342 (100 mg/mL) and ethidium bromide (20 mg/mL) for 5minutes and observed by fluorescence microscopy (Axiostar plus microscope, Zeiss).[20] A total of 200 cells were counted in multiple randomly selected fields, and the percentage of apoptotic cells was then calculated.

Annexin V/propidiumIodide (PI) assay

Apoptotic cells were detected using Vibrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, Oregon, USA). HL-60 cells were treated with 25 or 50mg/mL of each honey for 24 and 48hours. When N-Acetyl-L-cysteine (NAC) was used, cells were pre-incubated with 20mM NAC for 1hour and then exposed to 50mg/mL of each honey for 48hours. Briefly, after treatment 1.5×10^5 cells were resuspended in 100 mL 1´annexin binding buffer and incubated with 4 mL of Alexa Fluor 488 annexin V and 8 mL of PI (10 mg/mL) for 15minutes at room temperature. After the incubation period, the cells were washed with 300 mL of 1× annexin-binding buffer, mixed gently and kept the samples on ice. The cells were analysed by flow cytometry using a FACS Calibur flow cytometer (Beckton Dickinson) and the CellQuest software. For each experiments 10^4 cells were analyzed.

Reactive oxygen species (ROS) assay

ROS production was determined using 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA). For these experiments, HL-60 cells were cultured in RPMI 1640 without phenol red and without foetal calf serum and subsequently were treated with 50 mg/mL of each honey or NAC (20mM) for different time intervals (0.25-24 hours).Then, 3×10^5cells were washed with PBS loaded for 30minutes with H_2DCFDA (10 mM) and incubated in a waterbath (37°C). The cells were kept on ice and fluorescence intensity was read immediately with FACS Calibur flow cytometer (Becton andDickinson) and the CellQuest software. For each experiment 10^4 cells were analyzed.

Statistical analysis

The Student's t test was used for statistical comparison and the differences were considered significant at $P \leq 0.05$. Tests were performed with the software package Statgraphics Plus 5.0.

RESULTS

Antiproliferative activity of honey samples in HL-60 cell line

The effects of heather, rosemary, polyfloral and artificial honey exposure on HL-60 cell survival at different incubation periods (24-72 hours) and different concentrations (1-250 mg/mL) were assessed by the MTT assay [Figure 1]. Doses lower than 25 mg/mL did not affect cell viability. However, treatment of HL-60 cells with 50 mg/mL for 72hours caused a significant inhibitory effect on the proliferation of HL-60 cells greater than 70% for heather honey [Figure 1a] and 60 % for polyfloral honey [Figure 1c].

The highest antiproliferative activity was found after treatment for 72hours with 100-250 mg/mL of heather [Figure 1a, 11.9-7.1% of survival, respectively), rosemary (Figure 1b, 22.4-21.7% of survival, respectively), polyfloral (Figure 1c, 23.8-2.4% of survival, respectively) and artificial honey (Figure 1d, 29.0-22. 0% of survival, respectively).

Analysis of morphological changes induced by honey samples

As shown in Table 2, nuclear chromatin condensation was observed in 44.6% cells treated for 24hours with 50mg/mL of heather honey. After 48hours treatment the percentage of apoptotic cells treated with 25 mg/mL of heather honey was 45.1% and 48.1% for polyfloral honey. When the cells were incubated with 50 mg/mL of rosemary or artificial honey for 48hours, the percentage of apoptotic cells was around 50.0%-31.1%, respectively. Finally, the percentage of apoptotic HL-60 cells obtained with the highest concentration (50 mg/mL) of heather or polyfloral honey for 48 hours (70.4%-78.5%, respectively) was similar to that obtained with etoposide (5 μM, 72.3%).

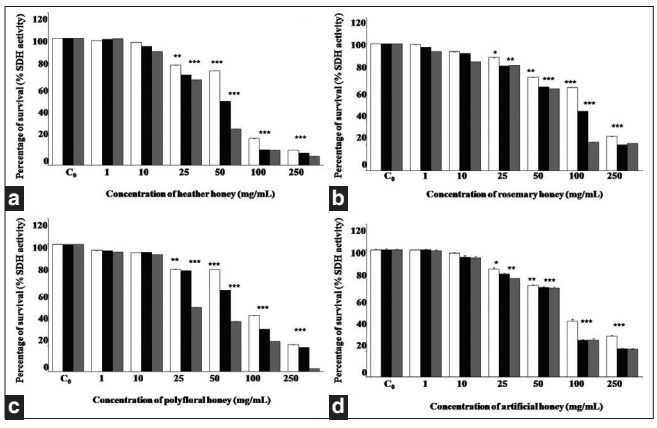

Figure 1: Antiproliferative effects of heather (a), rosemary (b), polyfloral (c) and artificial honey (d) in HL-60 cells by MTT assay. Cells were cultured with different concentrations of each honey type for 24 (□), 48 (■) and 72 (■) hours. C0, HL-60 cells without honeys. Asterisks indicate significant difference from control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Annexin V/PI assay

HL-60 cells were exposed to 25 and 50 mg/mL honeys for 24 and 48 hours and then labelled with Alexa Fluor 488 annexin V and PI. The results are expressed as percentage of apoptotic cells (annexin V-positive and PI-negative) over the total cells. The short time treatment (24hours) with 50mg/mL of heather honey [Table 3] induced a 40.5% of apoptotic cells. After 48hours treatment a marked percentage of apoptotic cells was noted with 5 μM etoposide (57.8%) and 25 mg/mL of heather or polyfloral honey (41.9%-55.8%, respectively). The 48 hours treatment with 50 mg/mL of rosemary or artificial honey significantly elevated the proportion of apoptotic cells (45.5%-36.2%). According to Table 3, the major increase in the number of apoptotic cells was apparent with 50mg/mLheather or polyfloral honey (about 74%) after 48hours incubation.

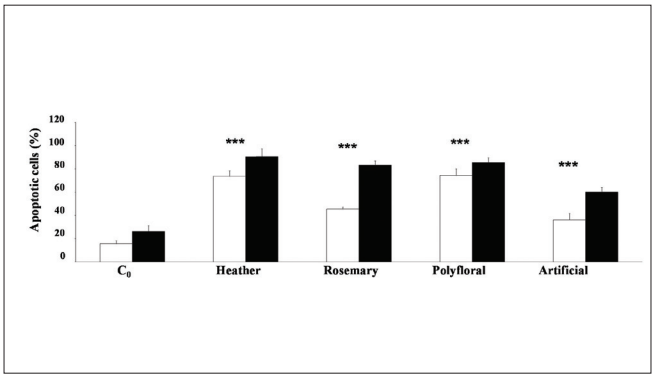

Figure 2: Effect of NAC on honeys-induced apoptosis. Apoptotic cells were measured using annexin V/PI assay. C_0, HL-60 cells without honeys. Cells were pretreated with (■) or without (□) NAC at 20 mM for 1 h and then incubated in the presence of honeys (50 mg/mL for 48 h). Asterisks indicate significant difference from control ***$P < 0.001$

Effect of NAC on honeys-induced apoptosis

To explore the role of ROS on apoptosis induced by honeys, HL-60 cells were pre-incubated with the antioxidant NAC (20 mM) for 1hour before exposing to 50 mg/mL of each honey for 48hours and the percentage of apoptotic cells was measured by flow cytometry using Annexin V and PI.As shown in Figure 2, NAC pretreatment caused a significant increase in the percentage of apoptotic cells (26.2%), and therefore the percentage of apoptosis was not reduced in the subsequent combined treatment with the honeys. For instance, in combined treatments the percentage of apoptotic cells increased from 73.9% to 90.7% (heather honey) from 45.5% to 83.4% (rosemary honey), from 74.4% to 85.6% (polyfloral honey) and from 36.2% to 60.3% (artificial honey). These results suggest that there might be not ROS production in honeys-treated cells.

To confirm this, after treatment of HL-60 cells with the honeys (50 mg/mL) or NAC, DCF fluorescence was measured by flow cytometry and expressed as percentage of control. As shown in Figure 3, honeys did not significantly increase the intracellular ROS levels at the indicated ranges of concentrations and times. On the contrary, a significant time-dependent decrease of ROS levels was observed in honeys and NAC treated cells compared with the untreated cells, reaching the minimum signal after 24hours. A slight increase of ROS levels was only found in rosemary treated

Table 2: Effects of honey samples on apoptosis in HL-60 cell line evaluated using Hoescht 33342 and Ethidium Bromide

Treatment (hours)	Control[a]	Etoposide[b]	% Apoptotic cells							
			Concentration of Honey (mg/mL)							
			Heather		Rosemary		Polyfloral		Artificial	
			25	50	25	50	25	50	25	50
24	9.8±2.1	3.5±3.9***	19.0±1.0	44.6±2.9***	14.3±1.1	23.1±1.8*	28.3±2.6**	30.0±0.4***	18.9±0.8	29.5±1.7**
48	12.7±1.7	72.3±2.3***	45.1±2.3***	70.4±2.5***	17.8±3.1	50.0±3.2***	48.1±2.9***	78.5±2.9***	21.8±0.6*	31.1±3.6***

Data shown are mean ± SD (n=4), [a]HL-60 cells without honey, [b]Etoposide treated HL-60 cells (5 μM), Asterisk indicate significant difference with respect to the control: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

Table 3: Effects of honey samples on apoptosis in HL-60 cell line evaluated using Anexin V/P assay

Treatment (hours)	Control[a]	Etoposide[b]	% Apoptotic cells							
			Concentration of Honey (mg/mL)							
			Heather		Rosemary		Polyfloral		Artificial	
			25	50	25	50	25	50	25	50
24	13.0±1.3	31.1±2.5***	19.4±1.1	40.5±1.0***	15.8±0.4	25.7±2.2*	24.6±2.6*	34.5±2.9***	15.1±2.0	27.1±4.3**
48	15.7±0.6	57.8±2.8***	41.9±2.3***	73.9±4.8***	29.9±1.2	45.5± 1.9***	55.8±0.7***	74.4±2.9***	21.3±0.6	36.2±2.9***

Data shown are mean ± SD (n=4), [a]HL-60 cells without honey, [b]Etoposide treated HL-60 cells (5 μM), Asterisk indicate significant difference with respect to the control: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

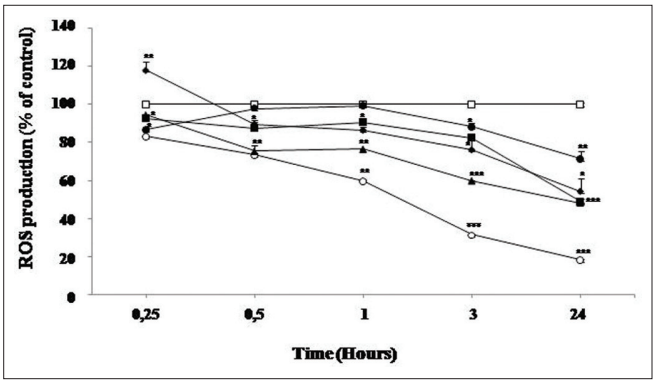

Figure 3: Time course of ROS production in untreated HL-60 cells (□), treated with NAC at 20 mM for 1 hour (○) and treated with 50 mg/mL of heather (▲), rosemary (◆), polyfloral (■) and artificial honey (●). Asterisks indicate significant difference from control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

cells for 0.25 h and it was reduced after 0.5 hours. The lowest ROS levels were found in honeys (heather and polyfloral, 52% of reduction; rosemary, 46% of reduction) and NAC (82% of reduction) treated cells for 24hours.

DISCUSSION

The present study was undertaken with the goal to determine the antiproliferative and the apoptotic effects, of three crude commercial honeys of different floral origin from Madrid Autonomic Community (Spain) as well as of an artificial honey (sugar analogue) in a leukemia cell line (HL-60). To our knowledge, no previous investigation has been done on the anticancer properties of these honeys towards HL-60 cell line.

Artificial honey was found to have a weaker anticancer effect than the three commercial honeys.Thus, sugars are not the only anticancer honey components. Moreover in our study, the ability of the three types of honey of different floral origin to induce apoptosis was different. The heather (unifloral) and the polyfloral honey samples were the most effective to induce apoptosis [Table 3]. At 48hours, 50 mg/mL heather or polyfloral honey induced about 74% of apoptotic cells while rosemary and artificial honeys induced 46%-36% of apoptotic cells, respectively. In a previous work, we determined the total soluble phenolic contents of these tested commercial honeys with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton by using ± catechin as a standard.[21,22] Our results showed that the total phenolic substances were higher in the heather honey (105 mg catechin/100 g of honey), than in the polyfloral (92 mg catechin/100 g of honey) and rosemary honey (44 mg catechin/100 g of honey). Previous studies have also shown that rosemary honey has lower phenolic content than heatherand heterofloral honey.[23,24]

Our results clearly demonstrated that the three types of honey from different floral origin induced apoptosis in a concentration and time dependent-manner in HL-60 cells. This data is consistent with previous studies that indicate that honey induces apoptosis in human bladder (T24, RT4, 253J, and MBT-2), colon (HCT 15 and HT-29) and prostate (PC-3) cancer cell lines.[25-28] *Tualang* honey caused time and dose dependent cell death of human breast (MCF-7 and MDMA-MB-231) and cervical (HeLa) cancer cells.[29] Greek honey extracts also inhibited cell viability on human prostate and endometrial cancer cells.[30]

The results obtained imply that phenolic substances present in the honey samples, may act as potential chemopreventive agents with respect to inhibition of the proliferation of human leukemia cells through the induction of apoptosis *in vitro*. These findings are in agreement with Jaganathan and Mandal who reported that honeys with higher phenolic content were more potent in apoptosis induction in colon cancer cells.[26] Also, honey containing higher phenolic content was found to significantly inhibit the growth of Ehrlich ascites carcinoma as compared to other samples.[31] Acacia honey-induced cytotoxicity in human (A375) and murine (B16-F1) melanoma cell lines was suggested to be attributed to the presence of chrysin (5, 7-dihydroxyflavone).[32] Therefore honey phenolic compounds such as caffeic acid, caffeicacid phenyl ester, chrysin, galangin, quercetin, acacetin, kaempferol, pinocembrin, pinobanksin and apigeninare an important group of substances regarding the anticancer properties of honey.[33] Recently, it has been shown that honey contains bioactive compounds that inhibit the proliferation of a human prostate cell line (PC-3) through induction of apoptosis. These results also suggest that the antiproliferative effects of honey are mainly due to chrysin.[28]

A role for oxidative stress in apoptosis has been shaped by the ability of cellular antioxidants to block apoptosis. NAC has been recognized as potential antioxidant capable of inhibiting apoptosis induced by ROS in HepG2 cells.[34] Honeys did not generate ROS and therefore NAC did not block apoptosis in HL-60 cells.These data indicate that the apoptosis induced by honey may be independent of ROS generation. However, Jaganathan and Mandal showed that honeys from India induced apoptosis through ROS and mitochondria-dependent mechanism in colon cancer cells.[14] Recently, Fauzi *et al.* have revealed the involvement of mitochondrial pathway in *tualang* honey-induced apoptosis of breast and cervical cancer cells.[29] Honey induced apoptosis in human colon cancer cell lines was associated with the activation of caspase-3 and DNA laddering.[26] The sensitization effect of chrysin ontumor necrosis factor-alpha (TNF-α)-induced apoptotic cell death is mainly achieved through enhanced activation of caspase 8, the initial caspase in the death receptor signaling pathway that typically induces apoptosis.[35]

Numerous studies *in vivo* have demonstrated the benefits of bee honey in cancer.[36] Animal studies indicate that honey possesses moderate antitumor and pronounced antimetastatic effects. These effects may be related to the biologically active compounds of honey, that inhibit tumor cell proliferation and transformation by the down regulation of many cellular pathways.[37] The clinical value of honey in cancer patients was recently reviewed. Honey has been found to be effective for radiation-induced oral mucositis, stomatitis, periodontal, gum disease, radiotherapy-induced skin reactions, malignat ulcers, external surgical wounds and infected lesions in pediatric oncology patients.[38]

Taken together our results support that the anticancer effect of honey varied according to the floral origin and the phenolic content. Moreover, our data indicate that honeys induce apoptosis in HL-60 cells through ROS-independent pathway. We suggest that honeys from Madrid Autonomic Community (Spain), especially heather (unifloral) or polyfloral honey, may be used as an alternative to sugar promoting the health of consumers. Detailed investigation of mechanism behind the honey-induced apoptosis in HL-60 cells is in progress in our laboratory.

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