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ROSEMARY, HEATHER AND HETEROFLORAL HONEYS PROTECT TOWARDS CYTOTOXICITY OF ACRYLAMIDE IN HUMAN HEPATOMA CELLS
LAS MIELES DE ROMERO, BREZO Y MULTIFLORAL PROTEGEN FRENTE A LA CITOTOXICIDAD DE LA ACRILAMIDA EN CÉLULAS DE HEPATOMA HUMANO

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RESUMEN

En abril del 2002 la Agencia Sueca de Seguridad Alimentaria alerta de la presencia de acrilamida (AA) en alimentos ricos en carbohidratos sometidos a temperaturas de fritura elevadas (150-180ºC) como las patatas fritas, las galletas o los cereales de desayuno. La Agencia Internacional para la investigación del Cáncer (IARC) la clasifica como un agente carcinógeno probable, Grupo 2A. En concecuencia, uno de los objetivos prioritarios de la Seguridad Alimentaria ha consistido en reducir la presencia de esta sustancia en los alimentos. Numerosos estudios muestran el efecto inhibidor de los antioxidantes naturales presentes en extractos de frutas y vegetales en la formación de la acrilamida. La miel es también una buena fuente de antioxidantes ya que contiene una gran variedad de compuestos fenólicos. Por ello, el principal objetivo de este trabajo consistió en evaluar el efecto protector de tres mieles de Madrid de distinto origen floral frente a la citotoxicidad de la acrilamida, en células de hepatoma humano (HepG2). Los resultados obtenidos mostraron que la acrilamida a las concentraciones de 1,4 y de 2,8 mg/ml y un tiempo de incubación de 24 horas redujeron significativamente el porcentaje de viabilidad celular (67 y 24%, respectivamente). En los tratamientos simultáneos de acrilamida (2,8 mg/ml) y las correspondientes mieles observamos que la miel de romero a todas las concentraciones evaluadas incrementaba el porcentaje de viabilidad celular en un 40-49%. Mientras que, la miel de brezo y la miel multifloral lo hicieron en un 54 y 66% respectivamente. La miel artificial no atenuó el efecto

citotóxico de la acrilamida. Por tanto, el efecto protector de las mieles evaluadas puede atribuirse a su contenido en polifenoles y no a su contenido en azúcares.
PALABRAS CLAVE: Acrilamida (AA), mieles, origen floral, efecto protector.

ABSTRACT

In April 2002 the Swedish Agency for Food Safety alerts of the presence of acrylamide (AA) in carbohydrate-rich foodstuffs subjected to elevated frying temperatures (150 - 180°C) such as fried potatoes, biscuits or breakfast cereals. The International Agency on Research on Cancer (IARC) classified acrylamide as a probable carcinogen, Group 2A. In consequence, one of the priority objectives of Food Safety is to reduce the presence of this substance in food. Numerous studies show the inhibitory effect of antioxidants present in fruit and vegetable extracts against the formation of acrylamide. Honey is also a good source of antioxidants since it contains a great variety of phenolic compounds. Therefore the main objective of this work was to evaluate the protective effect of three Madrid honeys of different floral origin against the AA-induced cytotoxicity in human hepatoma cells (HepG2). The results showed that the acrylamide in concentrations 1.4 and 2.8 mg/ml and in a 24-hour incubation period significantly reduced the percentage of cell viability (67 and 24 %, respectively). In simultaneous treatment of acrylamide (2.8 mg/ml) and the corresponding honeys we noted that rosemary honey in all concentrations tested increased the percentage of cell survival in 40-49 %, while heather honey and heterofloral honey increased cell viability by 54 and 66% respectively. The artificial honey did not mitigate the AA-induced cytotoxic effect. As a result, the protective effect of the evaluated honeys can be attributed to its polyphenolic content and not the sugar constituents.
KEYWORDS: Acrylamide (AA), honeys, floral origin, protective effect

INTRODUCTION

Acrylamide (AA) is a synthetic chemical product since 1950. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure (Koyama *et al.*, 2006). In April 2002, however, the Swedish National Food Authority and the University of Stockholm jointly announced the determination findings of considerable levels of AA in heat-treated carbohydrate-rich foods (Claus *et al.*, 2008). Subsequently, similar results in other countries have been reported (Imai *et al*, 2005). These findings have challenged investigators worldwide to define more

completely its toxic and genotoxic mechanisms, the exposure and formation rates and methods or materials that could reduce the AA content of foodstuff. The International Agency on Research on Cancer (IARC) classifies AA as a probable human carcinogen, group 2A (Vattem and Shetty, 2003).

This small, water soluble, organic vinyl monomer has multiple chemical and industrial applications including water purification, paper and fabric manufacture, mine industry, soil stabilization, production of contact lenses, chromatography and gel electrophoresis (Sickles *et al*., 2007). Through some of these applications may occur human exposure by inhalation and skin absorption (Dearfield *et al*., 1995). AA exposure is also associated with cigarette consumption (Boettcher *et al*., 2005). In addition to these, the dietary factor must be coestimated, as potato, bakery and cereal products as well as beverages like coffee and tea are important AA sources (Alves *et al*., 2010; Anese *et al*., 2010). Its formation in thermally processed foodstuffs is due to the Maillard reaction during baking, frying, microwave heating and sterilization treatment (Casado *et al*., 2010).

Once ingested, AA is rapidly distributed through in the whole body via the bloodstream (Abramsson-Zetterberg *et al*., 2005). Afterwards, it is readily absorbed and metabolized mainly by two pathways. Either it is inactivated by conjugation with glutathione - the major AA scavenger-followed by excretion of mercapturic acids or it is bioactivated to glycidamide through cytochrome P450 2E1 (CYP 2E1)-dependent epoxidation (Bjellaas *et al*., 2007).

Many scientific initiatives have been launched in order to understand the toxic effects of this compound. It is carcinogenic in multiple tissues and organs of chronically exposed rodents (Rice, 2005) and it presents a potential carcinogenic hazard to humans, although most epidemiologic studies fail to establish an association of AA intake with cancer (Larsson *et al*., 2009). It also appears to have toxic effects in the central and peripheral nervous (Ko *et al*., 2000), reproductive (Parzefall, 2008) and immune (Abramsson-Zetterberg *et al*, 2005) systems as well as in the development, behavior and lifespa (Hasegawa *et al*., 2004) or growth (Takahashi *et al*., 2005).

Therefore the presence of such a toxic compound in foodstuffs has evoked an international health alarm and attempts have been done to reduce AA levels or to mitigate its

toxic results. AA can be removed from the food as steam by choosing suitable temperature and pressure conditions (Anese *et al*., 2010). Biodegradation could also be an effective method to reduce AA concentration (Wakaizumi *et al.,* 2009). Even more promising is the application of additives such as polyphenols, rosemary, substances of natural products (Casado *et al*., 2010; Claus *et al*., 2008). Numerous studies show the inhibiting effect of natural antioxidants present in food and vegetable extracts against AA formation (Cheng *et al*., 2010; Zhang and Zhang, 2008). Honey has also the potential to serve as a significant source of natural antioxidants in human nutrition (Zalibera *et al*., 2008), which might prove useful against the AA-induced oxidative stress. In fact there is a direct relation between the phenolic content of floral origin honeys and its antioxidant activity (Kücük *et al.*, 2007), corroborating the relevance of honey as a healthy alimentary product and as a source of antioxidant substances (Estevinho *et al*., 2008).

Traditionally honey has been a sweetening agent in foodstuffs, as it is a concentrated solution of various sugars prepared by bees mainly from the nectar of flowers or honeydew (Lazaridou *et al*., 2004). However, several aspects of its use indicate that honey it also useful as a food preservative (Nagai *et al*., 2006) and exhibits antioxidant, chemopreventive, antiatherogenic, immunoregulatory, antimicrobial and wound healing properties (Tsiapara *et al*., 2009). On the other hand, the effects of honey on cell viability are largely unknown. Since the composition of honey varies widely in relation to its botanical origin and environmental factors, the aim of the present study was to evaluate the protective effects of honeys from three different floral origin coming from Madrid as well as of an artificial honey towards acrylamide-induced cytotoxicity in human hepatoma cells (HepG2).

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteau's phenol reagent, (+) catechin and acrylamide were purchased from Sigma-Aldrich (St.Louis, MO). Sucrose, maltose, fructose and glucose were purchased from Panreac Chimica, S.A. (Barcelona). All other chemicals and solvents were of the highest grade commercially available. AA solutions were prepared just prior to use by dissolving the compound in sterile phosphate buffered saline. Because AA is a potent carcinogenic agent, safety precautions were taken for proper handling and disposal of the chemical.

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 Maia (1999), 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 heterofloral.

Commercial honeys were obtained from a single experienced producer who provided the three authentic samples: heather and rosemary honeys as unifloral and a heterofloral honey. 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 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose in 17 ml of distilled water and the solution was mixed for 1 hr. The desired amounts of heterofloral, 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).

Honey type	Scientific name (Family)	Common name	Organoleptic Characteristics	Production zone: Autonomic Community of
Unifloral	*Rosmarinus officinalis* (Lamiaceae)	Rosemary honey	Aroma with floral and fresh notes (Arráez-Román *et al*., 2006), Mild flavor, light color (Arráez-Román *et al*., 2006)	El Atazar, Torres de la Ladera, Alcalá de Henares
Unifloral	*Erica arborea* (Ericaceae)	Heather honey	Ripe fruit and spicy aroma (Castro-Vázquez *et al*., 2009), dark color (Fernández-Torres *et al*., 2005)	El Atazar, Prádena de la Sierra, Montejo de la Sierra
Heterofloral		Heterofloral honey		Zarzalejo, La Cabrera, Alcalá de Henares, Torres de la Ladera, Colmenar Viejo, Serranillos, El Vellón , Patones

Table 1. Botanic origin, organoleptic characteristics and production zone of the tested honeys.

HepG2 cells

Human hepatoma cells (HepG2) were obtained from the Biology Investigation Center Collection (BIC, Madrid, Spain). Only cells of passage 10-17 were used in the experiments.

The cells were cultured as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% v/v heat-inactivated foetal calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin and 1% v/v L-glutamine. Culture medium and supplements required for the growth of the cell line were purchased from Gibco Laboratories (Life Technologies, Inc., Gaithersburg, MD 20884-9980). Cell cultures were incubated at 37^{o}C and 100% humidity in a 5% CO_2 atmosphere.

Determination of total phenolic content

Total soluble phenolic contents of the samples were determined with Folin-Ciocalteau reagent according to the method of Slinkard and Singleton (1977) by using ±catechin as a standard. Briefly, 0.1 ml of catechin and sample solutions (different concentrations for the standard and 20% methanolic solutions for the honey samples) was diluted with 5.0 ml distilled water. Folin-Ciocalteu reagent was added, and the contents were vortexed. Following 3 min incubation, 1.5 ml of Na_2CO_3 (2%) was added and after vortexing, the mixture was incubated for 2 hr at 20^{o}C with intermittent shaking. The absorbance was measured at 760 nm at the end of the incubation period. The concentration of total phenolic compounds was calculated as milligrams of catechin equivalents per 100 g of honey sample, by using a standard graph.

Cell Viability assay (MTT)

Cell proliferation kit I (Boehringer Mannheim, GMBH, Germany) was used to test the effect of honeys on HepG2 cell viability. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out in 96-well tissue culture microtiter plates (Nunc, Roskilde, Dennmark). Cell suspensions (100 µl: 10^6 cells/ml) were dispensed in each well, and plates were incubated for 24 hr at 37^{o}C. After incubation, 100 µl of each concentration of honey (0.01-500 mg/ml) or AA (0.35-2.80 mg/ml) were added to the wells, and plates were incubated 24 hr at 37^{o}C in a humidified 5% CO_2 atmosphere. In simultaneous treatments with honey and AA solutions, 50 µl of each concentration of honey and 50 µl of AA (2.8 mg/ml) were added to the wells. After incubation, 10µl of stock MTT solution (0.5 mg/ml) was added to each culture well and plates were incubated for 4 hr at 37^{o}C in a humidified 5% CO_2 atmosphere. In viable cells, the yellow tetrazolium salt, MTT, is converted into a purple formazan substrate by the mitochondrial enzyme succinate dehydrogonase (SDH). To dissolve the dark formazan crystals, 100µl of solubilization solution was added into each well and the plates were incubated overnight at 37^{o}C in a

humidified 5% CO_2 atmosphere. After incubation, the contents of the plates were thoroughly mixed for 5 min on a plate shaker (Heidolph). The optical density (OD) of each well was determined thereafter with an ELISA reader (iEMS Reader MF, Labsystems, Helsinki, Finland) at 620-nm test wavelength and 690-nm reference wavelength.

Honeys, AA and negative controls were evaluated in three independent assays. Values presented in this paper are means ± standard error of the mean. HepG2 cells without honey were considered as negative controls. Cell survival in exposed cultures relative to unexposed cultures (negative control) was calculated and expressed as percentage of survival (%SDH activity) = (A_1 / A_0) x 100, where A_1 is the absorbance of exposed cultures and A_0 is the absorbance of negative control.

Statistical analysis

The Student's t test was used for statistical comparison and the differences were considered significant at $p \leq 0.01$. Tests were performed with the software package Statgraphics Plus 5.0.

RESULTS

The effects of AA exposure on cell survival of the HepG2 cell line at different incubation periods (6-24 hr) and different concentrations (0.35-2.80 mg/ml) were assessed by the MTT assay (**Figure 1**). The results showed that none of the tested concentrations was cytotoxic during 6 hr and 18 hr of exposure. On the other hand, statistical analysis revealed that the cell viability is significantly reduced when the cells are exposed for 24 hr to AA 1.40 mg/ml (67.5%). An even stronger cytotoxic effect was demonstrated with AA 2.80 mg/ml (23.7%). Therefore, the latter concentration was used in order to estimate the possible protective effects of the honeys against AA-induced cytotoxicity.

Previously, the effect of heterofloral, heather, rosemary and artificial honey on HepG2 cell viability was evaluated by the above mentioned cytotoxicity assay. All the honeys were tested at concentrations ranging from 0.01 to 500 mg/ml and incubated for 24 hr. Doses lower than 500 mg/ml did not affect cell viability (**Figure 2**). However, a strong inhibition of HepG2 cell viability was found with 500 mg/ml of rosemary (23 % of survival) and artificial

honey (37%). The maximum reduction on cell viability was observed with heterofloral (2% of survival) and heather honey (3% of survival).

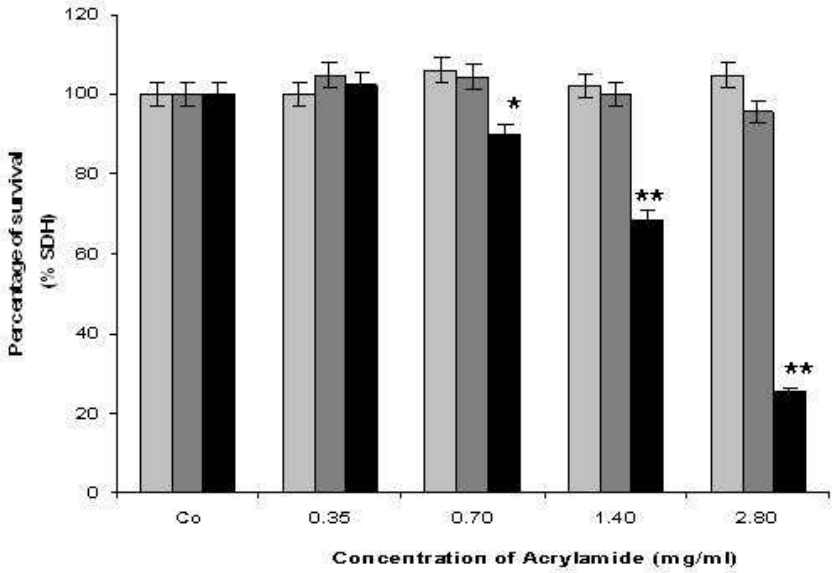

Figure 1. Effect of acrylamide (AA) on HepG2 cell viability by MTT assay. Cells were cultured with different concentrations of AA for 6(□), 18(▨) and 24(■) hours. C_0 HepG2 cells without AA. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. **p≤0.01, *p≤0.05.

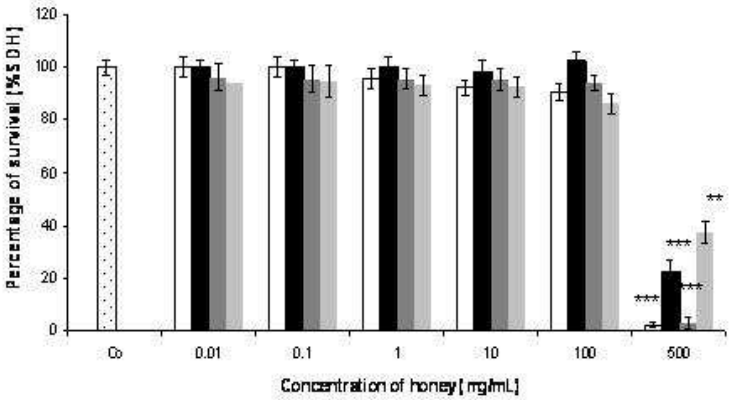

Figure 2. Effect of honeys on HepG2 cell viability tested by the MTT assay. Cells were cultured with different concentrations of each honey type for 24 hours. C_0 (▨), HepG2 cells without honeys, (□) HepG2 cells treated with heterofloral, (■) heather, (▨) rosemary and (▨) artificial honey. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. ** p≤0.01, ***p≤0.001.

The analysis of the phenolic content of the tested natural honeys showed that the total phenolic substances (**Figure 3**) were higher in the heather honey (105 mg catechin/ 100 g of honey), than in the heterofloral (92 mg catechin/100g of honey) and rosemary honey (44 mg catechin/100g of honey). Catechin has been used as a standard for comparison or quantitation in many investigations including those about honeys of various origins (Wei and Zhirong, 2003).

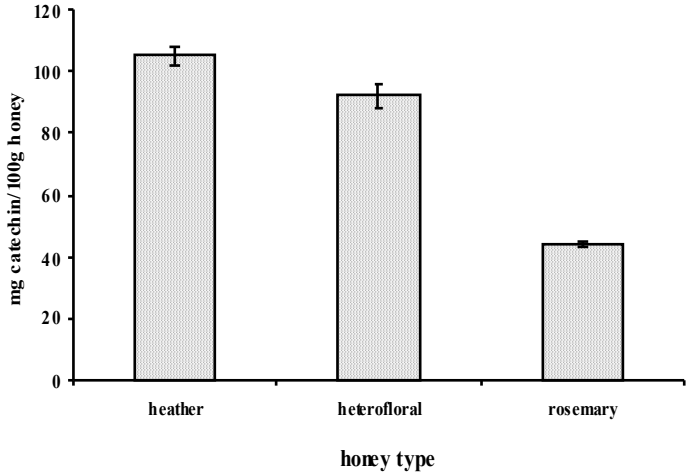

Figure 3. Total phenolic contents of the honey samples expressed in terms of mg catechin/ 100 g honey.

In subsequent experiments HepG2 cells were simultaneously treated with AA in concentration 2.80 mg/ml and honey in concentrations ranging from 0.01 to 10 mg/ml, and incubated for 24 hr. In these experimental conditions, the heterofloral honey at all the concentrations tested showed a significant protective effect against AA-induced cytotoxicity. Treatment of HepG2 cells with AA and heterofloral honey (**Figure 4**) induced an increase of 67-69% in cell viability compared to the positive control (AA, 23.7%). As shown in **Figure 5** the heather honey (0.01-10 mg/ml) also affords protection against cytotoxicity provoked by AA. The obtained results demonstrate a significant raise (55%) in cell survival, although the protective effect is slighter than that of heterofloral honey. As far as rosemary honey is concerned, the simultaneous treatment of HepG2 cells with this honey type and AA verified the protective effect of this sort of honey, as well (**Figure 6**). There was a dose-dependent 40-50% increase in cell viability in the presence of rosemary honey (0.1-10 mg/ml), which demonstrates a significant protective effect, although lower than the former honeys. Finally, **Figure 7** showed the effect of artificial honey on AA-induced cytotoxicity on HepG2 cells.

None of the artificial honey concentrations tested (0.1-10 mg/ml) reduced the cytotoxic effect of AA.

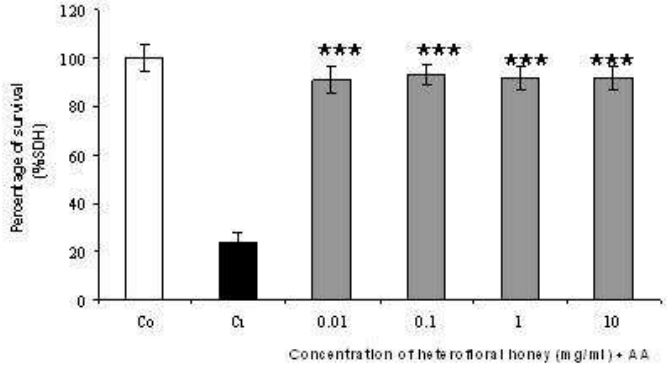

Figure 4. Effect of heterofloral honey on acrylamide-induced cytotoxicity on HepG2 cells. C_0 (□), HepG2 cells without AA or heterofloral honey, C_1 (■), HepG2 cells treated with 2.8 mg/ml AA and (■), HepG2 cells simultaneously treated with 2.8 mg/ml AA and heterofloral honey. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. ***p≤0.001.

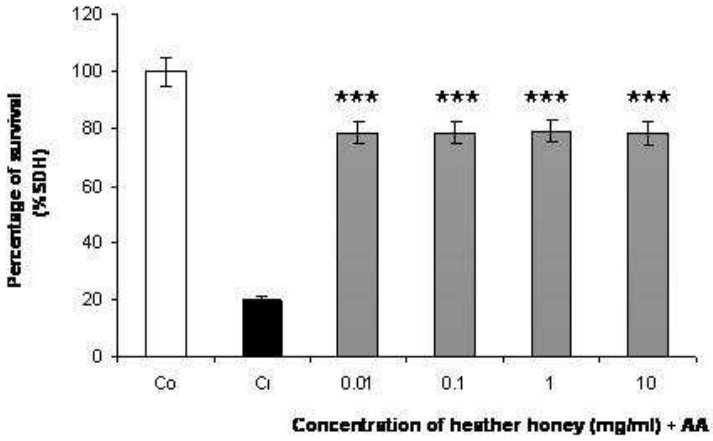

Figure 5. Effect of heather honey on acrylamide-induced cytotoxicity on HepG2 cells. C_0 (□), HepG2 cells without AA or heather honey, C_1 (■), HepG2 cells treated with 2.8 mg/ml AA and (■), HepG2 cells simultaneously treated with 2.8 mg/ml AA and heather honey. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. ***p≤0.001.

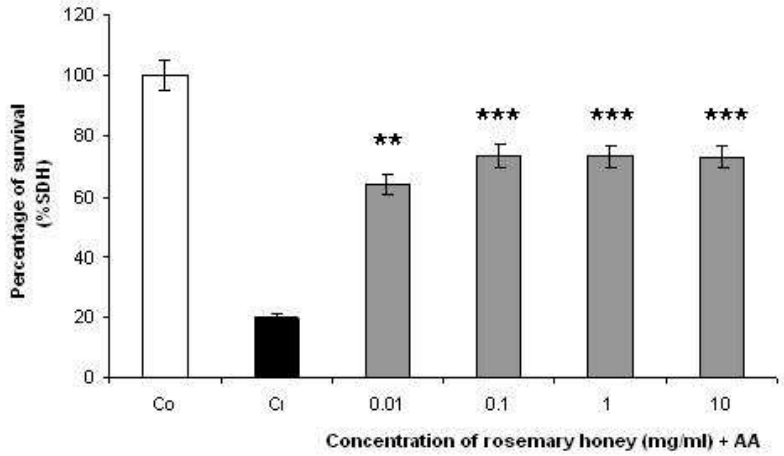

Figure 6. Effect of rosemary honey on acrylamide-induced cytotoxicity on HepG2 cells. C_0 (□), HepG2 cells without AA or rosemary honey, C_1 (■), HepG2 cells treated with 2.8 mg/ml AA and (▣), HepG2 cells simultaneously treated with 2.8 mg/ml AA and rosemary honey. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. ***$p \leq 0.001$.

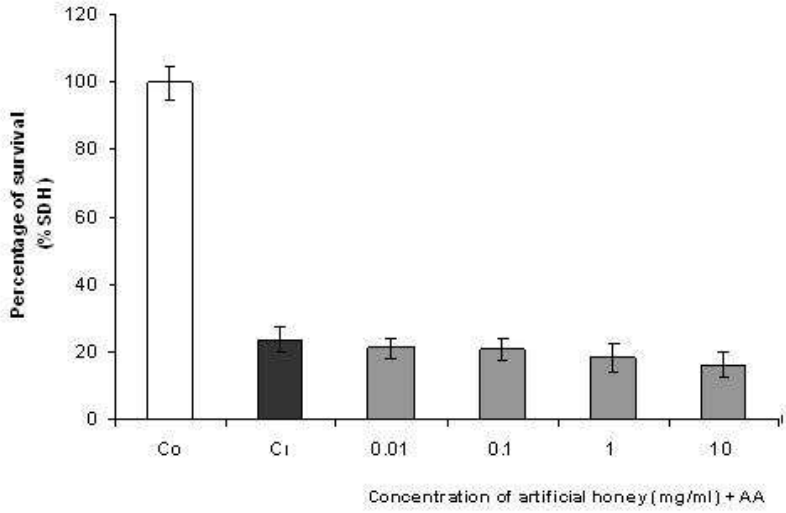

Figure 7. Effect of artificial honey on acrylamide-induced cytotoxicity on HepG2 cells. C_0 (□), HepG2 cells without AA or artificial honey, C_1 (■), HepG2 cells treated with 2.8 mg/ml AA and (▣), HepG2 cells simultaneously treated with 2.8 mg/ml AA and artificial honey. Values are means ± SD of three independent experiments (n=3). Asterisks indicate significant difference from control. ***$p \leq 0.001$.

DISCUSSION

AA is a potent human carcinogen with toxic activity against many tissues and cell types (Claus *et al*., 2008). The recent discovery of AA in a wide variety of commonly consumed foods has energized research efforts worldwide to assess more adequately the hazards linked with its use. A great number of studies about the cytotoxicity of AA in various cell types have been reported. In the present research work, we first investigated its cytotoxic effect in human hepatoma cells (HepG2).

Emphasis was placed on this cell line, as these cells (i) are of human origin and thus may reflect metabolism and effects of xenobiotics in humans better than non-human cells, (ii) retain the activities of both phase I and phase II enzymes and as a result both activation and detoxification reactions take place within the indicator cells (Kassie and Knasmüller, 2000).

The effects of AA exposure on cell viability of HepG2 cells at different incubation periods (6, 18, 24 hr) and different concentrations (0.35, 0.70, 1.40, 2.80 mg/ml) were assessed by the MTT assay (Figure 1). The tested compound in concentrations 0.35-2.80 mg and an incubation period of 6 and 18 hr had no cytotoxic effect. On the other hand, there was increased cytotoxicity in up to 4 hr incubation in mouse lymphoma cells (Mei *et al*., 2008), human lymphoblastoid TK6 cells (Koyama *et al*., 2006) and male rat hepatocytes (Awad *et al*., 1998). In our cell model a significant loss of cell viability was observed only when cells were incubated for 24 hr with 1.40 and 2.80 mg/ml AA (67.5 and 23.7% survival, respectively). This is consistent with previously reported cytotoxicity studies conducted using the same cell line (Jiang *et al*., 2007). Similar results were also obtained by Takahashi *et al.* (2005), who demonstrated that high AA concentrations (>1.5 mg/ml) induce cell death on paramecia, and by Holden and Coleman (2007), who reported that in different astrocytes (U-251 MG, CCF-STTG1 and U-373 MG cells) there were no changes in MTT turnover in response to AA in concentrations lower than 0.7 mg/ml. Neither in Caco-2 (Zödl *et al*., 2007) and V79 cells (Baum *et al*., 2005) was observed a cytotoxic effect in low concentrations of AA upon 24 hr exposure. However, a little augmented concentrations of AA seem to reduce significantly the cell viability of V79 cells, which was attributed to the absence of glutathione –the greater AA scavenger- in this cell type, supporting the role of endogenous glutathione in the mitigation of the toxic effects triggered by AA (Oliveira *et al*., 2009).

On the other hand, the better resistance of HepG2 cells against AA than other cell types in reduced incubation periods or in augmented AA concentration in 24-hr incubation, could be attributed to the increased glutathione levels in this cell type (Huang *et al.*, 2001). Apart from the inactivation of AA by conjugation with glutathione, an indirect action of the above mentioned enzyme could also contribute to the better response of HepG2 cells to AA exposure. Zödl *et al.* (2007) have underlined that depletion of glutathione can favour cellular oxidative stress, which in turn could damage cellular structures. Conversely, the presence of glutathione in great levels in HepG2 could afford an extra protection against AA, which is an agent that increases the oxidative cellular stress (Mei *et al.*, 2008) and can be cytotoxic by decreasing the oxidative defense system in the cells. The results presented here reinforce the role of glutathione in cell protection against AA, but apart from the endocellular defense methods another way to mitigate AA levels is the application of certain additives (Casado *et al.*, 2010). The protective effect of added antioxidants has long been evaluated (Friedman and Levin, 2008). A good source of such substances is also honey (Nagai *et al.*, 2006). It has been used since long time both in domestic and medical needs, but only during the past decade its use for therapeutic purposes was re-evaluated in a more scientific setting and its antioxidant property came to limelight. With increasing demand for antioxidant supply in the food, honey has gained vitality since it is rich in phenolic compounds and other antioxidants like ascorbic acid (vitamin C), amino acids, and proteins (Fiorani *et al.*, 2006). Surveys support the concept that honey consumption can have a positive impact on the antioxidant defence system of healthy human subjects, as it increases the total plasma antioxidant capacity even in very short periods after intake (Scramm *et al.*, 2003). In addition, honey has been shown to ameliorate the unpleasant effects of other food carcinogens (El-Arab *et al.*, 2006).

Therefore, in the present study, the protective effect of three different floral-originated honeys from Madrid against AA-induced cytotoxicity has been investigated. The two unifloral (heather, rosemary) and the heterofloral honey samples as well as the artificial honey (a sugar analogue) have first been evaluated alone for their possible cytotoxic effect in HepG2 cells by the MTT assay. In concentrations 0.01-100 mg/ml there was no decrease in cell viability, while in 500 mg/ml the cell survival was significanlty reduced (Figure 2). The non-cytotoxic effect in low concentrations is in agreement with the findings of Sadeghi-Aliabadi and Kazemi (2009) in MRC-5 cells and Aziz *et al.* (2009) in HepG2 cells, who mention that honey contains amino acids, minerals and vitamins which help in enhancing cell proliferation.

Moreover, honey with its sugar content provides substrates for glycolysis which is the major mechanism for energy production for cell proliferation. Furthermore, low concentrations of extracts of *Rosmarinus officinalis* and *Erica arborea* (the plant sources of our heather and rosemary honey) did not induce cytotoxic effects on Vero cells and A2780 human ovarian cells respectively (Topçu *et al.*, 2009), which is in accordance with our results about the two unifloral honeys. On the other hand, the strong cell growth inhibition observed with 500 mg/ml honey could be attributed to the high apigenin and caffeic acid content, as these polyphenols were found to have an antiproliferative effect in HepG2 cells (Jaganathan and Mandal, 2009).

The non-cytotoxic concentrations of the honeys were chosen for the simultaneous treatments of the HepG2 cells with AA and the corresponding honeys. Among these concentrations the ones that were finally selected for the experiments were 0.01-10 mg/ml, as these values are more likely to be achieved physiologically (Tsiapara *et al.*, 2009). The honey samples were tested in 24 hr incubation with AA 2.80 mg/ml, which was the AA concentration that inhibited cell survival most strongly. Both the unifloral (heather, rosemary) and the heterofloral honey samples were found to exert protection against AA-induced cytotoxicity in all concentrations analyzed (0.01-10 mg/ml, Figures 4-6). The heterofloral honey was found to be the most efficient, by increasing the percentage of survival by 67-69%, while heather and rosemary honey raised it by 55% and 40-50% respectively. On the other hand there was no augmentation in cell survival when the artificial honey was used, indicating that the protective effect is due to non-sugar components. These results are in agreement with those reported earlier regarding gastroprotection (Gharzouli *et al.*, 2001) and hepatoprotection (Kilicoglu *et al.*, 2008). In the latter case it was concluded that the protective effect of honey was due to its antioxidant activity. Antioxidants could exert their beneficial effects by abstracting reactive free electrons from free radical intermediates postulated to be formed in the Maillard reaction (Friedman and Levin, 2008). Since the formation of AA is through the Maillard reaction, antioxidants could prove helpful against it. Besides, antioxidants have already found to attenuate AA-induced toxicity (Cao *et al.*, 2008).

The phenolic compounds in honey may render it a good source of antioxidants (Al-Mamary *et al.*, 2004). Once within the cell, these substances donate electrons to the membrane oxidoreductase to efficiently reduce extracellular oxidants (Fiorani *et al.*, 2006). Moreover, phenolic compounds have been shown to protect HepG2 cells from oxidative

damage by decreasing lipid peroxidation and prevent glutathione depletion (Lima *et al.*, 2006). Furthermore, quercetin - a phenolic compound present in honey was able to increase the intracellular concentration of glutathione by approximately 50% (Myhrstad *et al.*, 2002), which could prove useful against AA. The mean content of total phenolic content obtained for our samples is in good agreement with the total phenolic content of honeys from various floral sources reported in the literature (Beretta *et al.*, 2005; Gheldof *et al.*, 2002). Besides, the two of our natural honeys with the higher phenolic content –heterofloral and heather honey- (Figure 3) were the ones that increased more the cell viability. Previous studies have also shown that rosemary honey has lower phenolic content than heather (Andrade *et al.*, 1997) and heterofloral honey (Martos *et al.*, 1997). However, as far as *Rosmarinus officinalis* extracts are concerned, their hepatoprotective effect has formerly been attributed to the presence of high percentage of phenolic compounds with elevated antioxidant activity (Fawsia *et al.*, 1999).

CONCLUSSION

In the present study we investigated the protective effect of three honeys of Madrid against AA-induced cytotoxicity and found that the three types of honey from different floral origin reduced the negative effects of AA on HepG2 cells. However, the heterofloral honey was found to be the most efficient against the cytotoxic effect of acrylamide. We conclude that this effect of honey might be due to its high phenolic content and antioxidant activity, but further studies are needed to evaluate the exact mechanism of the protective effect of honey and its possible appliance as an additive in foodstuffs in order to mitigate AA-induced toxic results.

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BIBLIOGRAPHY

Abramsson-Zetterberg L., Wong J. and Ibach N.G. 2005. Acrylamide tissue distribution and genotoxic effects in a common viral infection in mice. Toxicology 211: 70-76.

Al-Mamary M., Al-Meeri A. and Al-Habori M. 2002. Antioxidant activities and total phenolics of different types of honey. Nutrition Research 22: 1041-1047.

Alves R.C., Soares C., Casal S., Fernandes J.O., Beatriz M. and Oliveira P.P. 2010. Acrylamide in espresso coffee: Influence of species, roast degree and brew length. Food Chemistry 199 (3): 929-934.

Andrade P., Ferreres F. and Gil M.I. 1997. Determination of phenolic compounds in honeys with different floral origin by capillary zone electrophoresis . Food Chemistry 60 (1): 79-84.

Anese M., Suman M. and Nicoli M.C. 2010. Acrylamide removal from heated foods. Food Chemistry 119: 791-794.

Arráez-Román D., Gómez-Caravaca A.M., Gómez-Romero M., Segura-Carretero A. and Fernández-Gutiérrez A. 2006. Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis–electrospray ionization-mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 41: 1648–1656.

Awad M.E., Abdel-Rahman M. S. and Hassan S.A. 1998. Acrylamide toxicity in isolated rat hepatocytes. Toxicology in Vitro 12: 699-704.

Aziz A., Rady H.M., Amer M.A. and Kiwan H.S. 2009. Effect of some honey bee extracts on the proliferation, proteolytic and gelatinolytic activities of the hepatocellular carcinoma Hepg2 cell line. Australian Journal of Basic and Applied Sciences 3 (3): 2754-2769.

Baum M., Fauth E., Fritzen S., Herrmann A., Mertes P., Merz K., Rudolphi M., Zankl H. and Eisenbrand G. 2005. Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood. Mutation Research 580: 61-69.

Beretta G., Granata P., Ferrero M., Orioli M., Maffei Facino R. 2005. Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. Analytica Chimica Acta 533: 185–191.

Bjellaas T., Stølen L.H., Haugen M., Paulsen J.E., Alexander J., Lundanes E. and Becher G. 2007. Urinary acrylamide metabolites as biomarkers for short-term dietary exposure to acrylamide. Food and Chemical Toxicology 45: 1020-1026.

Boettcher M.I., Schettgen T., Kutting B., Pischetsrieder M. and Angerer J. 2005. Mercapturic acids of acrylamide and glycidamide as biomarkers of the internal exposure to acrylamide in the general population. Mutation Research 580: 167-176.

Cao J., Liu Y., Jia L., Jiang L.P., Geng C.Y., Yao X.F., Kong Y., Jiang B.N. and Zhong L.F. 2008. Curcumin attenuates acrylamide-induced cytotoxicity and genotoxicity in HepG2 by ROS scavenging. Journal of Agricultural and Food Chemistry 56 (24): 12059-12063.

Casado F.J., Sánchez A.H. and Montaño A. 2010. Reduction of acrylamide content of ripe olives by selected additives. Food Chemistry 119: 161-166.

Castro-Vázquez L., Díaz-Maroto M.C. González-Viñas M.A. and Pérez-Coell M.S. 2009. Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. Food Chemistry 112: 1022-1030.

Cheng K.-W., Shi J.-J., Ou S.-Y., Wang M. and Jiang Y. 2010. Effects of fruit extracts on the formation of acrylamide in model reactions and fried potato crisps. Journal of Agricultural and Food Chemistry 58: 309-312.

Claus A., Clare R. and Schieber A. 2008. Acrylamide in cereal products: A review. Journal of Cereal Science 47: 118-133.

Dearfield K.L., Douglas G.R., Ehling U.H., Moore M.M., Sega G.A. and Brusick D.J. 1995. Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. Mutation Research 330: 71-99.

El-Arab A.M.E., Girgis S.M., Hegazy E.M. and Abd El-jhalek A.B. 2006. Effect of dietary honey on intestinal microflora and toxicity of mycotoxins in mice. BMC Complementary and Alternative Medicine 6:6.

Estevinho L., Pereira A.P., Moreira L., Dias L.G. and Pereira E. 2008. Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. Food and Chemical Toxicology 46: 3774-3779.

Fawzia A.F., Amr Y.E., Hoda M.F. and Khaled F.S.H. 1999. Allied studies on the effect of *Rosmarinus officinalis* L. on experimental hepatotoxicity and mutagenesis. International Journal of Food Sciences and Nutrition 50 (6): 413-427.

Fernández -Torres R., Pérez -Bernal J.L., Bello-López M.´A., Callejón-Mochón M., Jiménez -Sánchez J.C. and Guiraúm-Perez A. 2005. Mineral content and botanical origin of Spanish honeys. Talanta 65: 686-691.

Fiorani M., Accorsi A., Blasa M., Diamantini G. and Piatti E. 2006. Flavonoids from Italian multifloral honeys reduce the extracellular ferricyanide in human red blood cells. Journal of Agricultural and Food Chemistry 54: 8328-8334.

Friedman M. and Levin C.E. 2008. Review of methods for the reduction of dietary content and toxicity of acrylamide. Journal of Agricultural and Food Chemistry 56: 6113-6140.

Gharzouli K., Gharzouli A., Amira S. and Khennouf S. 2001. Protective effect of mannitol, glucose-fructose-sucrose-maltose mixture, and natural honey hyperosmolar solutions against ethanol-induced gastric mucosal damage in rats. Experimental and Toxicologic Pathology 53: 175-180.

Gheldof N., Wang XH., Engeseth NJ. 2002. Identification and quantification of antioxidants components of honeys from various floral sources. Journal of Agricultural and Food Chemistry 50: 5870-5877.

Hasegawa K., Miwa S., Tsutsumiuchi K., Taniguchi H. and Miwa J. 2004. Extremely low dose of acrylamide decreases lifespan in *Caenorhabditis elegans*. Toxicology Letters 152: 183-189

Holden L.J. and Coleman M.D. 2007. Assessment of the astrogliotic responses of three human astrocytoma cell lines to ethanol, trimethyltin chloride and acrylamide. Toxicology 241 (1-2): 75-83.

Huang Z.Z., Chen C., Zeng Z., Yang H., Oh J., Chen L. and Lu S.C. 2001. Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. Faseb Journal 15: 19–21.

Imai T., Cho Y.M., Hasumura M. and Hirose M. 2005. Enhancement by acrylamide of N-methyl-N-nitrosourea-induced rat mammary tumor development-possible application for a model to detect co-modifiers of carcinogenesis. Cancer Letters 230: 25-32.

Jaganathan S.K. and Mandal M. 2009. Antiproliferative effects of honey and its polyphenols: A review. Journal of Biomedicine and Biotechnology 2009: 1-13.

Jiang L., Cao J., An Y., Geng C., Qu S., Jiang L. and Zhong L. 2007. Genotoxicity of acrylamide in human hepatoma G2 (HepG2) cells. Toxicology in Vitro 21: 1486-1492.

Kassie F. and Knasmüller S. 2000. Genotoxic effects of allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC). Chemico-Biological Interactions 127 (2): 163-180.

Kılıcoglu B., Gencay C., Kısmet K., Kılıcoglu S.S., Erguder I., Erel S., Sunay A.E., Erdemli E., Durak I. and Akkus M.A. 2008.The ultrastructural research of liver in experimental obstructive jaundice and effect of honey. The American Journal of Surgery 195: 249–256.

Ko M.H., Chen W.P. and Hsieh S.T. 2000. Cutaneous nerve degeneration induced by acrylamide in mice. Neuroscience Letters 293: 195-198.

Koyama N., Sakamoto H., Sakuraba M., Koizumi T., Takashima Y., Hayashi M., Matsufuji H., Yamagata K., Masuda S., Kinae N. and Honma M. 2006. Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells. Mutation Research 603: 151-158.

Kücük M., Kolayli S., Karaoglu S., Ulusoy E., Baltaci C. and Candan F. 2007. Biological activities and chemical composition of three honeys of different types from Anatolia. Food Chemistry 100: 526-534.

Larsson S.C., Akesson A., Bergkvist L. and Wolk A. 2009. Dietary acrylamide intake and risk of colorectal cancer in a prospective cohort of men. European Journal of Cancer 45: 513-516.

Lazaridou A., Biliaderis C.G., Bacandritsos N. and Sabatini A.G. 2004. Composition, thermal and rheological behaviour of selected Greek honeys. Journal of Food Engineering 64: 9-21.

Lima C.F., Fernandes-Ferreira M. and Pereira-Wilson C. 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. Life Sciences 79 (21): 2056-2068.

Maia, M., 1999. Contribuição para a caracterização do mel do Parque Arqueológico do Vale do Côa. Relatório final de Estágio, UTAD, 77.

Martos I., Cossentini M., Ferreres F. and Tomás-Barberán F.A. 1997. Flavonoid Composition of Tunisian Honeys and Propolis. Food Chemistry 45: 2824-2829.

Mei N., Hu J., Churchwell M.I., Guo L., Moore M.M., Doerge D.R. and Chen T. 2008. Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells. Food and Chemical Toxicology 46: 628-636.

Motterlini R., Foresti R., Bassi R. and Green C.J. 2000. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radical Biology and Medicine 28 (8): 1303-1312.

Myhrstad M.C.W., Carlsen H., Nordström O., Blomhoff R. and Moskaug J. Ø. 2002. Flavonoids increase the intracellular glutathione level by transactivation of the γ-glutamylcysteine synthetase catalytical subunit promoter. Free Radical Biology and Medicine 23 (5): 386-393.

Nagai T., Inoue R., Kanamori N., Suzuki N. and Nagashima T. 2006. Characterization of honey from different floral sources. Its functional properties and effects of honey species on storage of meat. Food Chemistry 97: 256-262.

Oliveira N.G., Pingarilho M., Martins C., Fernandes A.N., Vaz S, Martins V., Rueff J., Gaspar J.F. 2009. Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: Role of glutathione modulators. Mutation Research 676: 87-92.

Parzefall W. 2008. Minireview on the toxicity of dietary acrylamide. Food and Chemical Toxicology 46: 1360-1364.

Rice J.M. 2005. Review: The carcinogenicity of acrylamide. Mutation Research 580: 3-20.

Sadeghi-Aliabadi H. and Kazemi Z. 2009. Cytotoxic and genotoxic evaluation of honey in normal human fibroblast and 3 human tumorogenic cell lines. New Biotecnology 25 (1): 284.

Scramm D.D., Karim M., Schrader R.H., Holt R.R., Cardetti M. and Keen C.L. 2003. Honey with high levels of antioxidants can provide protection to healthy human subjects. Journal of Agricultural and Food Chemistry 51: 1732-1735.

Sickles D.W., Sperry A.O., Testino A. and Friedman M. 2007. Acrylamide effects on kinesin-related proteins of the mitotic/meiotic spindle. Toxicology and Applied Pharmacology 222: 111-121.

Slinkard K. and Singleton V.L. 1977. Total phenol analysis: Automation and comparison with manual methods. American Journal of Enology and Viticulture 28:49-55.

Takahashi T., Yoshii M., Kawano T., Kosaka T. and Hosoya H. 2005. A new approach for the assessment of acrylamide toxicity using a green paramecium. Toxicology in Vitro 19: 99-105

Topçu G., Tümen G., Kılıç T. , Gören A.C., Barla A., Türkmen Z. and Kingston D.G.I. 2009. Bioactive Turkish plant extracts and their constituents. Innovations in Chemical Biology 6: 61-81.

Tsiapara A.V., Jaakola M., Chinou I., Graikou K., Tolonen T., Virtanen V. and Moutsatsou P. 2009. Bioactivity of Greek honey extracts on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells: Profile analysis of extracts. Food Chemistry 116: 702-708.

Vattem D.A. and Shetty K. 2003. Acrylamide in food: a model for mechanism of formation and its reduction. Innovative Food Science and Emerging Technologies 4: 331-338.

Wakaizumi M.,Yamamoto H., Fujimoto N. and Ozeki K. 2009. Technical Note: Acrylamide degradation by filamentous fungi used in food and beverage industries. Journal of Bioscience and Bioengineering 108 (5): 391-393.

Zalibera M., Stasko A., Slebodova A., Jancovicova V., Cermakova T. and Brezova V. 2008. Antioxidant and radical-scavenging activities of Slovak honeys – An electron paramagnetic resonance study. Food Chemistry 110: 512-521.

Zhang Y. and Zhang Y. 2008. Effects of natural antioxidants on kinetic behavior of acrylamide formation and elimination in low-moisture asparagine-glucose model system. Journal of Food Engineering 85: 105-115.

Zödl B., Schmidt D., Wassler G., Gundacker C., Leibetseder V., Thalhammer T. and Ekmekcioglu C. 2007. Intenstinal transport and metabolism of acrylamide. Toxicology 232: 99-108.