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The Evaluation of Phenolic Constituents as Markers to Differentiate between Clover and Alfalfa Honeys Collected from Different Regions of Egypt

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ABSTRACT

Authentic samples of clover and alfalfa honeys were selected and extracted from their hives placed in clover and alfalfa farms, and honey collected from two regions, Al-Wadi Al-Jadeed Governorate as a source of alfalfa honey and Al-Sharqiya Governorate as a source of clover honey. Their botanical origin was assured then they were extracted and analyzed for flavonoids and phenolic acid contents by using HPLC device. Results showed that there were 19 phenolic compounds found in clover honey samples, while 14 phenolic constituents were found in alfalfa honey samples. The highest amount of phenolic compounds in the main Egyptian honeys was in clover honey, while phenolic acid was the dominant compound in both alfalfa and clover honeys. There were clear variations in phenolic compounds between honey samples; perhaps due to different plant sources (botanical origin).

Key words: Authentic honey, flavonoids, Honey, phenolic acid.

Introduction

Honey is a natural product consisting of a high concentrated solution of a complex mixture of sugar, and minute quantities of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes, and volatile compounds (Al-Mamary *et al*., 2002 and Khalil *et al*., 2011). The quantity of these different compounds varies depending on the plant source and the geographical origin of the honey (Wang *et al*., 2002 and Bertoncelj *et al*., 2007). Honey has been used traditionally over the years by Egyptians as food and traditional medicine in the treatment of several diseases. Phenolic compounds in their many forms are the main components responsible for the functional properties associated with many foods, such as antioxidant capacity (Kerem *et al.,* 2006 and Almaraz *et al.,* 2007), antibacterial capacity (Huang *et al.*, 2006 and Theodori *et al.,* 2006), antiviral capacity (Evers *et al.,* 2005 and Ozcelik *et al.,* 2006), anti-inflammatory capacity (Harris *et al.,* 2006 and Wu *et al.,* 2006), cardio-protective effects (Moon *et al.,* 2003 and Celle *et al.,* 2004) as well as the prevention of enzymatic browning (Chen *et al.,* 2000 and Jeon and Zhao 2005). Together with the honey itself propolis and royal jelly could be included since they contain phenolic compounds collected by the bees from the plants where they gather nectar (Marcucci *et al.,* 2001 and Fiorani *et al.,* 2006). Phenolic compounds are found mainly in fruits, to which in many cases they contribute color and taste (Belitz and Grosh 1997). Chemically, phenols can be defined as substances that possess an aromatic ring bound with one or more hydrogenated substituent, including their functional derivate (Marin *et al.,* 2001).

The main groups of phenolic compounds present in plants, whether in free form or as glycosides, are derivatives of cinnamic acid, cumarins, and flavonoids (Manthey and Grohmann 2001).In honey, propolis, and royal jelly, most of the phenolic compounds are in the form of flavonoids, whose concentration depends on various factors, including plant species used by the bees, health of the plant, season and environmental factors (Kucuk *et al.,* 2007).

Traditionally, the floral source of honey has been identified by sensory and pollen analysis of honey. However, the use of phenolic compounds in the identification of honey has been suggested and has since been used as a tool for studying the floral and geographical origins of honeys (Ferres *et al*., 1992). Martos *et al*., (2000) mentioned that European Eucalyptus honeys showed a common and characteristic HPLC profile in which the flavonoids myricetin, tricetin, quercetin, luteolin and kaempferol were identified. Their contents, and relative amounts, in the analyzed honey samples were quite constant and supported their floral origin. Myricetin, tricetin, and luteolin had not been identified as floral markers in any other honey sample previously analyzed in their laboratory (chestnut, citrus, rosemary, lavender, acacia, rapeseed,

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sunflower, heather, lime tree, etc.) or reported in the literature, suggesting that these could be useful markers.

This study aimed to determine the flavonoids and phenolic acid contents of clover and alfalfa floral honeys which are the main botanical sources of honey in Egypt.

Materials and Methods

The present investigation was carried out at the Beekeeping Research Section, Plant Protection Research Institute, Giza, during 2015 to study the phenolic compound characteristics of the Egyptian honeys which were collected from Al-Wadi Al-Jadeed Governorate as a source of alfalfa (*Medicago sativa*) honey and Al-Sharqiya Governorate as a source of clover (*Trifolium alexandrinum*) honey .

The analysis of phenolic components was carried out for the two studied Egyptian honeys (clover and alfalfa) to study their potential for floral authentication. The analysis included 23 standard flavones (daidzin, b-oH benzoic, caffeic, gallic, kaempherol, pyro gallic, ferulic, alicylic, vanillin, genstin, p-coumaric, quercetin, chrysin, galangin, phenol, Cinnamic, Dadazien, 3, 5 di methoxy benzyl, genstein, catechine, euganol,rutin and Pinostrobin). These components were separated by High Performance Liquid Chromatography (HPLC) from three samples of each honey type.

Preparing of 10 % honey solution, one gram of honey was dissolved in 10ml ethyl alcohol 70%, and then kept in closed glass tubes for analysis. Estimation of weight % of phenolic compounds, the scanning of identified phenolic compounds extracted from honey samples by (HPLC) analysis were estimated and calculated as follows:

$$\text{Weight \% phenolic} = 100 \text{ X } (PH/PH^*) \text{ X } (v/v^*) \text{ X } (w^* \text{ x } w)$$

Where: PH: area for sample, PH*: area of standard, V: volume of sample, V*: volume of standard, W*: weight of standard, W: Weight of sample.

HPLC Identification:

Identification of phenolic compounds of the honey samples was performed by a JASCO, using a hypersil C18 reversed- phase column (250 X 4.66 mm) with 5 µm particle size. Injection by means of a Rheodyne injection valve with 50 µl fixed loop was used. A constant flow rate of 1 ml min^{-1} was used with two mobile phases (A) 0.5 % acetic acid in distilled water at pH 2.65; and solvent (B) 0.5 % acetic acid in 99.5 % acetonitcile. The elution gradient was linear starting with (A) and ending with (B) over 35 min, using a µv detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standards mixture chromatogram. The concentration of individual compound was calculated on the basis of the peak area measurements, and then converted to µg phenolic g^{-1}dry weight. All chemicals and solvents used were in HPLC spectral grade.

Results and Discussion

The phenolic contents of the 6 Honey samples were analyzed, and the mean numbers were tabulated in Table (1). Phenolic compounds found in clover honey samples were 19, while 14 phenolic compounds were found in alfalfa honey samples. From data in Table (1) phenolic acid was the dominant compound in both alfalfa and clover honeys,it was represented by 22.5mg/100g and 96.31mg/100g,respectively.It was cleared that Caffeic acid Genistein acid were the characteristic of clover honey whereas these compounds were absent in alfalfa honey samples Fig (1). The variation in phenolic compounds between honey samples, it may be due to differences in botanical origin (plant source).

Data presented in Fig. (2) show that phenol, daidzin, cinnamic are represented in both types of honey but in different quantities, i.e. all the three components are significantly higher in clover than in alfalfa honey. Phenolic acid was 96.31 and 22.50 mg/100g, daidzin was 22.01 and 2.74 mg/100g, and cinnamic was 14.24 and 2.64 mg/100g in clover and alfalfa, respectively. It indicates that beside presence and absence of some compounds to differentiate between different types of honey, the quantity also could be helpful in that identification.

Phenolic compounds are a widespread group of antioxidants present in the plants and their derived products. Some of these compounds are taken over from plants to honey by bees (*Apis mellifera)*. Few phenolic compounds were used as the honey authenticity indicators. Comparing of hydroxyl-benzoic and cinnamic acid hydroxyl derivatives concentration can be used to differentiate various kinds of mono-floral honeys (Jorg and Sontag, 1992). Useful markers of heather honey could be cis, trans-abscisic acid and

trans, trans-abscisic acid (Ferrere s*et al*., 1996). The major source of kaempferol and its derivatives in rosemary honey is not rosemary pollen but rosemary nectar only. These results suggest that phenolic markers of the botanical origin honey should be addressed to the identification of nectar flavonoids (Ferreres *et al*., 1998). Phenolic compounds can be useful markers for the floral origin of some honey types, particularly in heather, chestnut, eucalyptus, rapeseed and lime-tree honeys. The role of particular markers was confirmed, for example hesperetin for citrus honey, kaempferol for rosemary honey and quercetin for sunflower honey. Abscisic acid, which was indicated as a marker for heather honey, is also present in significant amounts in rape seed; lime-tree and acacia honeys .The results of comprehensive study of phenolic acids in 49 honey samples confirm significant differences of phenolic acids content depending on the floral origin (Tomas-Barberan *et al*., 2001).

Table 1: Phenolic constituents in Egyptian honeys (clover and alfalfa) collected from two different regions (mg EGA/100g)

Phenolics comp.	**Alfalfa honey (*Medicago sativa*)**	**Clover honey (*Trifolim alexandrienum*)**
Daidzin	2.74	22.01
Benzoic acid	2.08	2.61
Caffeic acid	**0.00**	**19.69**
Gallic acid	9.86	0.00
Kaempherol	0.00	3.48
Pyrogallic	0.06	12.85
Ferulic acid	1.23	8.92
Salicylic acid	0.00	73.12
Vanillin	0.03	0.00
Gestin	0.86	1.83
p-Coumaric	0.00	0.48
Quercetin	0.00	17.54
Chrysin	0.02	0.38
Galangin	0.00	0.07
Phenol	**22.50**	**96.31**
Cinnamic acid	2.64	14.24
Dadazien	1.64	29.71
3,5 di methoxy benzyl	0.01	0.01
Genistein	**0.00**	**5.67**
Catechin	0.42	6.70
Euganol	0.18	0.72

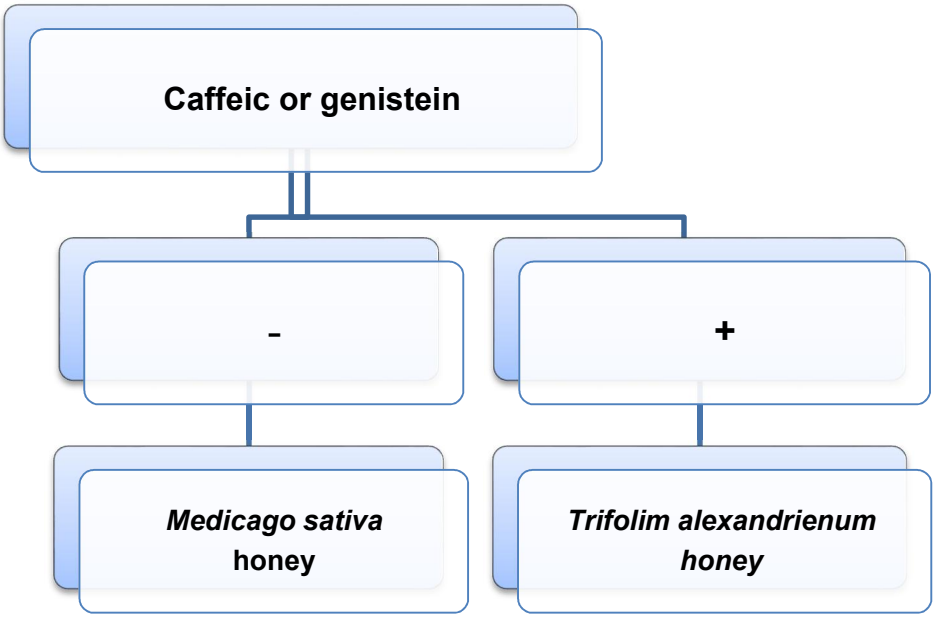

Fig. 1: Differentiation between *Trifolim alexandrienum* honey and *Medicago sativa* honey on the basis of presence or absence of some phenolic and flavonoid components as a phenolic compound marker.

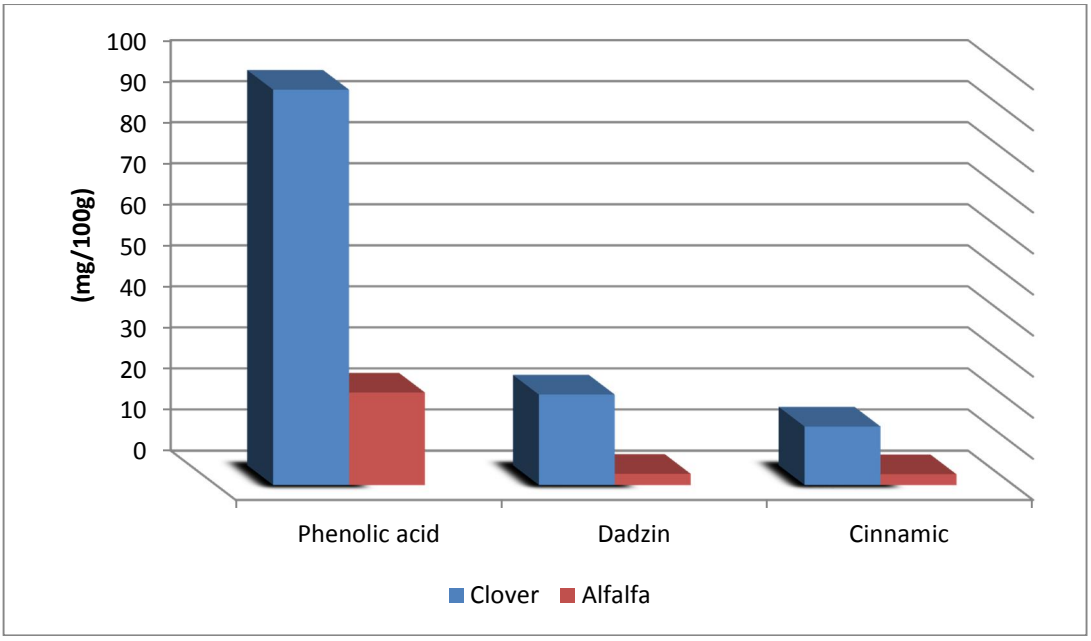

Fig. 2: Three common Phenolic components in the two studied types of honey (mg/100g) collected from clover and alfalfa

It is very likely that some phenolic compounds could be used also as the indicator of mead quality and composition. Ferreres *et al.,* (1996) reported that the floral source can be reliably authenticated on presence of phenolic constituents such as volatile compounds, abscissic acid, myricetin and quercetin. Also, Hausler and Montage (1990) found that heather honey, (*Calluna vulgaris* and *Erica arborea*) could be distinguished from clover, lime tree and acacia honeys by their high phenylacitic acid and benzoic acid contents. Yoa, *et al*., (2004a) analyzed the flavonoids in Australian honeys from five botanical species and suggested that those honeys of various floral species can be differentiated by their levels of total flavonoid being 2.12mg/100g for heather and 6.35mg/100g for tea tree honey. In the similar and previous work, Guyat, *et al.,* (1999) stated that heather honeys could be distinguished from non-heather samples on the basis of their content in benzoic acid which was present in heather honeys at concentrations ranging from 2 to 64 µg/g as opposed to less than 1.3 µg/g in the non-heather samples. BesidesYao, *et al.*, (2003) found that in Australian jelly bush honey (*Leptospermum polygalifolium*) the content of total phenolic acids averaged 5.14mg/100g honey, with gallic acid (23.6%) and coumaric acid (22.2%) as the main components while caffeic acid represented 9.7% of the total phenolics. Soria *et al.* (2004) stated that, forty-six artisanal honey samples, from different places of Madrid province (Central Spain), were characterized on the basis of their volatile composition data. Among the volatile components, concentrations of borneol, 1-(2-furanyl)-ethanone and 3-hydroxy-2-butanone were the most discriminant variables. In the differentiation of honey samples from mountain and plain zones, 2, 3-butanediol and 1-(2-furanyl)-ethanone were the most significant volatiles. Yao *et al.* (2004b) reported that, flavonoids of nine Australian mono-floral Eucalyptus honeys have been analyzed and related to their botanical origins. The mean content of total flavonoids varied from 1.90 mg/100 g of honey for stringybark (*E. globoidia*) honey to 8.15 mg/100 g of honey for narrow-leaved ironbark (*E. crebra*) honey, suggesting that species-specific differences occur quantitatively among these Eucalyptus honeys. All of the honey samples analyzed in this study have a common flavonoid profile comprising tricetin (5,7,3',4',5'-pentahydroxyflavone), quercetin (3,5,7,3',4'-pentahydroxyflavone), and luteolin (5,7,3',4'-tetrahydroxyflavone), which, together with myricetin (3,5,7,3',4',5'-hexahydroxyflavone) and kaempferol (3,5,7,4'-tetrahydroxyflavone), were previously suggested as floral markers for European Eucalyptus honeys.

Blasa *et al.* (2006) pointed that total polyphenols, flavonoids and antioxidant power of raw honey samples from two of the most common Italian varieties, i.e., Millefiori and Acacia, were evaluated. Phenolic content, expressed as caffeic acid equivalents, ranged from 12.5 to 17.5 mg/100 g and from 3 to 11 mg/100 g in Millefiori and Acacia honeys, respectively. All Millefiori samples exhibited the highest flavonoid concentration being between 1.23 and 2.93 mg catechin equivalents (CE)/100 g honey. Total flavonoids in 100 g Acacia honeys were in the range of 0.45–1.01 mg CE. Acacia honeys had lower total antioxidant power, as assessed by ferric reducing/antioxidant power assay, than Millefiori.

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