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Characterization of Algerian Honey from Tiaret Region and Immunoassay Study of Its Immunomodulatory Effect in BALB/c Mice

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Abstract

Honey is a food that possesses several antiseptic, antibacterial, anti-inflammatory and immunomodulatory properties. In this study, the immunomodulatory effect of honey was evaluated by Enzyme Linked Immunosorbent Assay (ELISA) using ovalbumin as an allergen model. To compare the honey quality, we conducted a range of physicochemical analyses on four different samples from the Tiaret region (Algeria) using the immunosuppressive or immunomodulatory effect in Balb/c mice. Our results show that the injection of 100 µL of honey before 6 hours, 6 hours after and at the same time as the injection of antigen (ovalbumin) causes a significant suppressive activity on production of the IgG isotype by Balb/c mice. This result corroborates this therapeutic virtue ascribed to honey which has resulted in a suppressive demonstration of honey on the humoral immune response. This opens an interesting perspective in the clinical area, as immunosuppressive agents play an important role in the transfer of various organs and immune system diseases.

Keywords: Honey, immunomodulatory effect, IgG, ELISA

1. Introduction

The immune system plays a crucial role in natural defence, but its excessive or inappropriate activation may have adverse consequences for the host. The modulation of the immune system or "immunomodulation" can be applied to reduce excessive responses or, conversely, to strengthen the inadequate response of the system (Labro, 2006). Honey is among the ancient foods of mankind, and is one of the few natural products that can interfere with the immune system by its immunosuppressive agents. It was found to be a suitable alternative for healing wounds and burns, and a product that can be used in oral health treatments (Molan, 2001; Lusby et al., 2002; Gallardo-Chacon et al., 2008; Lavflurrie, 2008). It also has a potential role in cancer care and shows antimicrobial properties (Bardy et al., 2008). Furthermore, it is used much more as a food than for therapeutic purposes because of its properties. This allows it to be a noble and precious product. The main purpose of this study was to investigate some properties of various honey samples collected from Tiaret region of Algeria by using different honey analysis tests such as moisture, ash, pH, free acidity, electrical conductivity, and hydroxymethylfurfural. The determinations of the frequency of pollen grain classes were also determined in these honey samples, in order to verify the floral origin and to obtain a complete pollen spectrum. In the second part of our work, the best honey sample was used to study the immunomodulatory effect of honey in the Balb/c mice using an ELISA immunochemical technique and ovalbumin as an allergen model.

2. Material and Methods

2.1 Sample Collection

Four different honeys samples were obtained from individual beekeepers in the Tiaret Region (E1: Sebt, E2: Sougueur, E3: Sidi Ali Mellel, E4: Dahmouni) of Algeria. All samples were collected in their original packages

and were transferred to the laboratory and kept at 4–5°C until analysis. They were labelled using a code number indicating their geographical origin, harvest date, and their extraction method (Table 1).

Table 1. Description of the sampled honey

Honey samples	Harvested period	Locality	Extraction mode
1	June 2008	Sebt	Manual
2	June 2009	Sougueur	Mechanic
3	June 2009	Sidi Ali Mellel	Manual
4	July 2009	Dahmouni	Manual

2.2 Physicochemical Analysis

The physical and chemical parameters of honey samples were studied using the methods harmonized by the International Honey Commission (Bogdanov et al., 1997). All analyses were performed in triplicate. Electrical conductivity of honey was determined from a honey solution containing 20% of honey dry matter in 100 mL distilled water at 20°C. Honey density was determined by weighing 10 mL of honey compared to the same volume of distilled water using a pycnometer (Bogdanov et al., 1995). The pH value was measured with a pH meter in a 10% (w/v) solution of honey in distilled water (French Official Journal, 1977). Water content (moisture) was determined using refractive index (RI) referring to the table of Chataway reported in Gonnet (1982). Acidity was estimated using the official method of analysis (AOAC, 1990). HMF (hydroxymethylfurfural) was performed using the Winkler method. The resulting colour was measured with a UV-spectrophotometer at 550 nm. Ash was evaluated by incineration of honey samples in a muffle furnace at 600°C (International Honey Commission, 2009).

2.3 Immunomodulatory Effect of Honey

2.3.1 Chemicals and Reagents

Ovalbumin (OVA) used to induce anti-OVA response in mice was obtained from Sigma. Multifloral honey of Africanized bees *Apis mellifera* (E3) was obtained from the Tiaret region of (Algeria).

2.3.2 Animals

Eight week old Balb/c mice were obtained from the Pasteur National Institute of Algeria. Six mice were used in each group for determination of antibody response.

2.3.3 Immunization Protocol

Different groups of mice were intraperitoneally (i.p.) injected with 10µg OVA or OVA plus 100 µL of honey on days 0, 21 and 35 and were bled on day 42 (Duddukuri et al., 2001). The antisera was separated and used for the estimation of total immunoglobulin IgG. The suppressive effect of honey on OVA-specific IgG antibody response was further studied by administering honey at different time intervals (0, 6h, 24h) prior to and after immunisation with OVA.

2.3.4 Determination of Immunoglobulin Levels by ELISA

Total IgG Anti-OVA levels in the sera from both control and test groups of mice were assayed by ELISA. Briefly, the 96-well microtitre plates (Nunc, n°167008, Lot: 049283) were coated with 100 µL of OVA at a concentration of 5µg/mL in carbonate-bicarbonate buffer (0.1 M, pH 9,5) and incubated for 2 h at 37°C (or overnight at 4°C). After incubation, the wells were washed three times with PBS containing 0.1% of Tween-20 (PBS-T). One mL of 5% pork gelatine (Fluka, n: 2325546, Lot: 387213/1 40101) in 9 mL of PBS-Tween was used to block the nonspecific binding sites for 1 h at room temperature (ELISA incubator: THERMOSTAR BMG). After washing the plates, the wells were further incubated with 100 µL of diluted sera (1:300 in PBS-Tween-Gelatine) for 2 h at 37°C. The unbound serum constituents were washed off and the levels of total bound IgG were measured by incubating with 100 µL of horseradish peroxidase (HRPO) conjugates of goat anti-mouse IgG (Sigma, 5 CODE 026K4846 A9917-1 mL) at a dilution of 1:2000 for one hour at 37°C. Finally, the unbound conjugates were washed with PBS-T five times and 100 µL of freshly prepared substrate solution [6 mg of orthophenylen diamine (OPD) in 12 mL of citrate sodium-citric acid buffer 0.1 M, pH 5.5 with addition of 100 µL H_2O_2 at 3%] was added per well. The reaction was stopped after 15 to 30 min by adding 50 µL of 2N H_2SO_4. The developed colour was read at 492 nm using automatic ELISA reader (TECAN SUNRISE). The data

expressed was the mean of the optical density (OD) of the triplicates after subtracting the absorbance for the pre-immune serum.

2.4 Statistical Analysis

All results were statistically analyzed by Microsoft Excel 2007 using Student's test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1 Quality of Honey Samples

The results of physicochemical parameters of honey samples from the Tiaret region were presented in Table 2. All honey samples were acidic in nature and the pH values varied between 3.83 and 4.34. Total acidity of analyzed honey samples was between 20 and 32 meq/kg.

The electrical conductivity was less than 0.8 mS/cm. Sidi Ali Mellal honey sample (E3) had the highest conductivity (Table 2). The EC found in all of the samples was typical for floral honey. The HMF content in four honey samples was lower than the allowed maximum limit of 40 mg/kg recommended by the European Honey Commission (2009).

Table 2. Physicochemical characterization of honeys

	Honey samples			
Physicochemical parameters	E1	E2	E3	E4
Density	1.343 ±0.015	1.477 ±0.012	1.390 ±0.010	1.403 ±0.015
Electrical Conductivity (ms/cm)	0.453 ±0.008	0.382 ±0.003	0.484 ±0.007	0.484 ±0.007
pH	4.343 ±0.060	3.830 ±0.085	4.293 ±0.025	3.963 ±0.140
Moisture (%)	17.800 ±0.265	18.600 ±0.300	16.200 ±0.265	16.600 ±0.265
Ash (%)	0.456 ±0.060	0.059 ±0.003	0.181 ±0.022	0.044 ±0.002
Acidity (meq/kg)	20 ±1.00	32 ±1.00	21 ±1.00	32 ±0.00
HMF (hydroxymethylfurfural) (mg/kg)	36.483 ±1.456	7.683 ±0.495	5.760 ±0.288	15.363 ±0.638

*According to the ANOVA test the relationship between the different honeys and parameters was statistically significant ($P < 0.001$).

Table 3. Quantitative melissopalynological analyses

Honey samples	Pollen number GP/g	Classes	Interpretation
E1	314034	V	Rich
E2	79711	IV	Rich
E3	697452	V	Rich
E4	15478	III	Rich

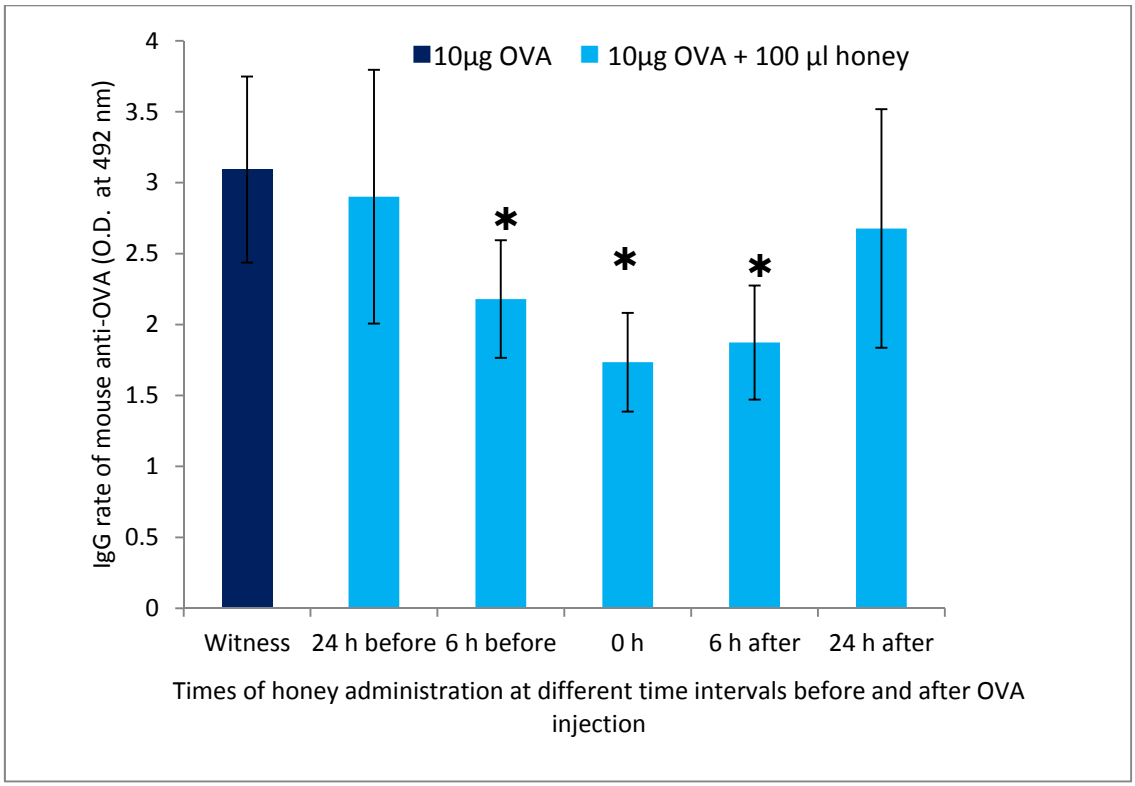
Table 4. Qualitative melissopalynological analyses

Honey samples	Origin place	Predominant and secondary pollen types
E1	Sebt	*Eucalyptus sp*+ *Daucus carota*
E2	Sougueur	Multifloral+ *Eucalyptus sp*+*Brassica napus*
E3	Sidi Ali Mellel	Multifloral + *Hedysarum coronarium* + *Daucus carota* + *Brassica napus*
E4	Dahmouni	Multifloral +*Eucalyptus sp*

3.2 Immunomodulatory Effect of Honey

Different groups of mice were treated with honey at different time intervals (in hours) prior to and after immunization with OVA on days 0, 21 and 35. The sera collected on day 42 were used to evaluate the OVA-specific IgG antibody responses ($P< 0.01$).

In order to examine the possible immunomodulatory activity of honey, the antibody response was induced in mice by intraperitoneal injection of 10 µg of OVA in the absence or presence of honey. As seen in figure 1, the administration of 100 µL of honey at different time intervals (0, 6h, 24h) prior to and after immunization with OVA was found to exhibit a suppression of anti-OVA IgG antibody response. This suppressor effect of honey was significant ($P<0.01$) after administration of honey 6h prior to and after immunization with OVA. This confirms the immunosuppressive effect of honey.



* $p<0.01$.

Figure 1. Influence of honey administration on OVA-specific IgG antibody response

4. Discussions

4.1 Physicochemical Analysis

To ensure the authenticity of honey samples, it is necessary to study in detail the physicochemical properties which are important for the certification process that determines honey quality.

4.1.1 Electrical Conductivity

This parameter is a good criterion related to botanical origin of honey (Bogdanov et al. 2004) and this is very often used in routine honey control instead of the ash content. Ash and electrical conductivity are two parameters bound to honey minerals content. The EC found in all of the samples was typical for floral honey. Its value varied from 0.38-0.48 mScm-1. The lowest value was obtained from honey samples from the Sougueur area and the highest value of EC obtained from Sidi Ali Mellel area of the Tiaret region.

4.1.2 Density

This parameter depends on the water content; the sample E1 has a slightly lower density than other honey samples because it has high water content (17.8%).

4.1.3 The pH

The pH values of the studied honeys were consistent with the result of Algerian honeys, the pH range of which was 3.49-4.53; these results are in the range reported by White (1975), who mentioned that honey is characteristically quite acidic.

4.1.4 Water Content (Moisture)

Honey moisture content depends on the environmental conditions and the manipulation from beekeepers at the harvest period, and can vary from year to year (Acquarone et al., 2007). High moisture content could accelerate crystallization in certain types of honey and increase its water activity to values where certain yeasts could grow. Moisture contents of honey samples ranged from 15.9 to 17.2, which are well below the imposed limit of 21% (EU, 2001). These results are indicative of good storage ability of these honeys, since high moisture content could lead to fermentation during storage.

4.1.5 Acidity

The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones. High acidity can be indicative of the fermentation of sugars into organic acids. In fact, the presence of gluconic acid in all honey originates largely from the activity of glucose oxidase which the bees add during ripening (Ruiz-Argueso et al., 1973). None of the samples exceeded the limit recommended by White et al. (1962) (8.68-59.40 meq/kg), which may be taken as indicative of the freshness of all honey samples.

4.1.6 HMF

The HMF content is indicative of honey freshness (Terrab et al., 2002), because it is absent in fresh honeys and tends to increase during processing and/or aging of the product. Several factors influence the levels of HMF, such as temperature and time of heating, storage conditions, pH and floral source; thus, it provides an indication of overheating and storage in poor conditions (Fallico et al., 2006), and from this point of view most of the analyzed samples are fresh. The spectrophotometric analysis of our samples revealed that all of them had levels below the limits HMF (40 mg/kg) suggested by the European Commission of honey.

4.1.7 Ash

The ash values of the honey samples evaluated ranged from 0.04 to 0.45% with an average of 0.18±0.19. These results are good agreement with those of June et al. (2012) (maximum and minimum limits are 0.01 and 0.65%, respectively). This variability in the ash content may be attributed to different floral sources or to factors related to honey sampling such as different geographical locations of various honey types or different environmental conditions of producing regions (Turgay, 2009).

The physicochemical characteristics of honey samples analyzed in this study completely agree with the European Commission and the Codex Alimentarius indicating adequate processing, good maturity and freshness that open the door for the commercialization of Algerian honey from the Tiaret region.

4.2 Immunomodulatory Effect of Honey

In this study, it was found that the OVA-specific IgG antibody response was suppressed ($P<0.01$) by the intraperitoneal administration of honey (100µl) at different time intervals (0, 6 h) prior to and after immunization with OVA. Many animals' experimentations confirm the immunosuppressor effect of honey; Duddukuri et al. (1997) found that honey exerted an immunosuppressive effect on the induction of allergen-specific humoral antibody response in mice as evaluated by passive cutaneous anaphylaxis and Ouchterlony double immunodiffusion methods. The same authors reported that OVA-specific IgG antibody response was significantly suppressed by honey ($p < 0.01$) in Balb/c mice, as measured by ELISA. However, honey does not

show an immunosuppressive effect on antibody responses of other IgG subclasses such as IgG2a and IgG3. Furthermore they showed that the total IgG antibody response was significantly suppressed by the administration of honey 12 h before and after immunization with OVA. These results are in contradiction with those of herbal Chinese medicine called "Hochu- ekki-to" which suppresses the IgE antibody response without affecting the levels of IgG1 and IgG2a. Also, the dexamethasone selectively inhibits IgE and IgA but does not influence IgG and IgG1 levels (Puignerb et al., 1995). These immunosuppressive presentations in honey may come from medicinal plants from which bees collect pollen and nectar; therefore, honey has some healing properties.

5. Conclusion

Honey is a natural complex and is diverse. However, whatever its origin, the presence of enzymes secreted by the hypopharyngeal glands of bees makes it fragile and it has evolved physicochemically over time. Such variations have allowed many authors to describe honey as a "living product". This food presents all of the qualities required to enter the list of natural therapeutic samples, either in the cure or prevention, where the composition and characteristics present important variations related to its geographical and botanical origin. The sensitivity of the ELISA test chosen for the determination of immunoglobulins in BALB/c mice immunized with ovalbumin, which showed very highly significant suppression ($p<0.01$) after the administration of honey 6 h before and after injection with OVA. This confirms the immunosuppressive effect of honey. This immunomodulatory activity can be used for clinical applications such as autoimmune diseases and allergies. Further investigation, in order to identify and purify these immunosuppressants, are present in honey. Moreover, the characterization of different honeys from different regions of our country can be an interested, certain especially when the various active principles specific to each honey will be specified. Composition tables for this purpose will be beneficial to the various uses of this product.

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