Journal of
Microbial & Biochemical Technology

Ahmed et al., J Microb Biochem Technol 2012, 4:7
DOI: 10.4172/1948-5948.1000087

Research Article Open Access

Antiradical Activity and Total Phenolics of Algerian Honeys and Antibacterial Effect against Gram-Negative Bacteria

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Abstract

Six Algerian honeys from different floral origins, were examined for potential antibacterial and antiradical activity. The Folin-Ciocalteu assay was used to measure total phenol content (TPC) and the 2,2-diphenyl-picrylhydrazyl (DPPH) assay was used to determine the scavenging activity of the honey samples. An agar well diffusion assay and spectrophotometric method were used to assess antibacterial activity against two Gram negative strains (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 50071).Total phenolic content varied from 63.93 to 95.36 mg/100 g honey as gallic acid equivalent. The DPPH radical scavenging assay was found for an average (30.14 % ± 9.28). The honey samples were found to inhibit all of the tested bacteria. Correlation existed between phenolic content and antiradical activity. Thus Algerian honeys, being a rich source of natural antioxidants, may be used in the prevention of various free radicals related diseases.

Keywords: Algerian honeys; Phenolic compounds; DPPH; Antiradical activity; Antibacterial activity

Introduction

The emergence of resistant Gram negative bacteria presents a major challenge for the antimicrobial therapy of infectious diseases and increases the incidence of mortality and morbidity. Natural products offer an alternative strategy for the discovery of new medications. Honey as most natural products, may have a large variance in therapeutic components depending on its origin. Thus, the floral source of honey plays an important role on its biological properties [1]. Honey ha s been reported to contain about 200 substances (complex mixture of sugars, but also small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals) and is considered to be an important part of traditional medicine [2]. Many authors demonstrated that honey serves as a source of natural antioxidants, which are effective in reducing the risk of heart disease, cancer, immune system decline, different inflammatory processes [3]. The factor contributing to honey antioxidant activity are lysozyme, phenolic acids and flavonoid [4]. The flavonoids in honey are divided into three classes with similar structure: flavonols, flavones and flavonones according to their chemical structure. These are important due to their contribution to honey color, taste and flavour and also due to their beneficial effects on health [5]. Honey is between the Major dietary sources of flavanones, such as Naringenin, which has been shown to possess antioxidant [6], antiproliferative [7], and weakly estrogenic activities *in vitro* [8]. Chrysin a natural flavone commonly found in honey has been shown to possess an anti-proliferative effect on prostate cancer cells [9]. Quercetin is one of the more representative flavonols in honey [10-12]. The best described property of Quercetin is its ability to act as antioxidant. Quercetin seems to be the most powerful flavonoids for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage [13,14].

The phenolic acids are divided in two subclasses: the substituted benzoic acids and cinnamic acids. The actions of flavonoids as immune modulators, radical-scavenging activity, enzyme and hormone action inhibitors are currently of particular interest to medical and nutritionist practitioners and justify the consumption of natural products rich in these phytochemical compounds for their beneficial potential effects [15]. Also the antioxidant activity of honey, however, varies greatly depending on the honey floral source [16]. As well by processing and storage conditions [17,18]. Honey has been shown to efficiently inhibit bacterial growth *in vitro* and *in vivo* [19]. The antimicrobial properties attributed to honey have been related to both the physical properties of osmosis and the antibacterial properties of hydrogen peroxide levels and the presence of some phytochemicals, mainly phenolic compounds including phenolic acids and flavonoids [20,21]. Numerous *in vitro* methods are used to evaluate the antioxidant potential of natural products [22-25] for honey antioxidant capacity determination, DPPH radical scavenging assay is a highly accepted method [26-29]. Algeria is characterized by a richness of polliniferous and melliferous resources. Several types of honey are produced in Algeria, where honey production is a traditional practice, well implanted in several regions. [30,31]. The antibacterial properties of Algerian honey have been reported in many studies [32-34]. In this study, the antioxidant activity (scavenging effect on DPPH radicals) and the antibacterial effects of six natural honeys from different floral origin two Gram negative strains *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 50071 were tested by agar well diffusion method and spectrophotometric assay.

Material and Methods

Honey Samples and their Preparation

Six honey samples from different sources in Algeria Tiaret (H3, H4, H6); Saida (H1); Relizane (H5), and Mascara (H2). The floral origin

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Received October 17, 2012; **Accepted** November 23, 2012; **Published** November 26, 2012

Citation: Ahmed M, Djebli N, Aissat S, Khiati B, Ünal M, et al. (2012) Antiradical Activity and Total Phenolics of Algerian Honeys and Antibacterial Effect against Gram-Negative Bacteria. J Microb Biochem Technol 4: 152-156. doi:10.4172/1948-5948.1000087

of the samples was specified by microscopic analyses of pollen grains at the laboratory Cari, Louvain-la-Neuve, Belgium. All samples were prepared aseptically and were handled such that they were protected from direct sunlight. Honey samples were stored at 4°C in the dark until analyzed.

Antibacterial activity of honey samples

Honey solutions were prepared in two concentrations: 100 and 50 % (by mass per volume). The samples of each honey (10 g) and sterile water were stored at 37°C for 30 min before mixing, to facilitate homogenization. The samples were assayed immediately after dilution. The potential antibacterial activity of six selected natural honeys against two strains of bacteria was studied using the agar well diffusion method and spectrophotometric assay.

Subculturing of test organisms and preparations of the bacterial inoculum

The test organisms were taken from American Type Culture Collections (ATCC): Two bacterial strains were used for the experiment: *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 50071). Bacteria were selected for antibacterial activity assay. Cultures were obtained from the Laboratory of Microbiology, Faculty of SE & SNV, Mostaganem University, Algeria. Stock cultures were maintained on Mueller–Hinton Agar (MHA) at 4°C. Bacterial inocula were prepared by growing cells in Mueller–Hinton Broth (MHB) for 24 h, at 37°C. Cell suspensions were diluted in sterile MHB to provide initial cell counts of about 1×10^6 colony-forming units per ml (CFU/ml).

Total phenolic content (TPC)

The total phenolic content was determined by the Folin–Ciocalteu method [35]. Thirty microliters (µl) of honey solution (0.1 g/ml) was mixed with 2.37 ml of milli Q water and 150 µl of 0.2 N Folin–Ciocalteu reagents. The solution was thoroughly mixed by vortexing and incubated for 2 min at ambient temperature. 450 µl of sodium carbonate solution (0.2 g/ml) was added to the reaction mixture and further incubated for 2 h at ambient temperature. The absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content was determined by comparing with a standard curve prepared using gallic acid (0-200 mg/l). The mean of at least three readings was calculated and expressed as mg of gallic acid equivalents (mg GAE)/100 g of honey.

Determination of antiradical scavenging activity (DPPH)

The DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging effect (H/e- transferring ability) of honey samples was measured as per the method described by [36]. The DPPH was dissolved in absolute ethanol to a 0.2 mM concentration. A 100 µl aliquot of honey solution (0.1 g/ml) was diluted to 500 µl with 70% ethanol, and vigorously mixed with 400 µl of DPPH solution by vortexing. The mixture was incubated at room temperature for 15 min and the absorbance of the solution (T1) was measured at 517 nm. Sample blank (B1) consisted of 600 µl of 70% ethanol and 400 µl of DPPH whereas DPPH blank (B2) contained 100 µl of honey sample, 500 µl of 70% ethanol and 400 µl of absolute ethanol. The DPPH scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity } (\%) = \frac{1-(T_1 - B_2)}{B_1} \times 100$$

where T1, B1, and B2 are the absorbencies of the sample, sample blank and DPPH blank, respectively.

Well diffusion assay

The activity of six stingless bee honeys was assessed against two reference strains *P. aerogenosa* ATCC 50071 and *E. coli* ATCC 25922 using an agar well diffusion assay. Briefly, agar plates (90 mm) containing 20 ml of MHA were inoculated using a swab from a suspension of each organism containing c. 1×10^6 CFU ml^{-1}. An 8-mm diameter well was cut into the agar and 100 µl of 50 and 100% honey solution (w / v,) prepared in sterile distilled water) was aliquoted into the well. The controls were set up with equivalent quantities of water as controls. The plates were incubated at 35 ± 2°C for 24 hours. Zones of inhibition were measured using a Vernier caliper (Draper). The antibacterial potential of test compound was determined on the basis of mean diameter of zone of inhibition around the wells in millimeters. Each assay was performed in duplicate and repeated twice. The antibacterial activity was classified as: no sensitive, for diameters lower than 8 mm; sensitive, for diameters from 8 to 14 mm; very sensitive, for diameters from 15 to 19 mm; extremely sensitive, for diameters higher than 20 mm.

Spectrophotometric assay

The absorbance readings obtained from the dose-response curve were used to construct growth inhibition profiles using the following formula:

$$\%\ \text{growthinhibition} = \frac{A_{\text{control}} - A_{\text{experimental}}}{A\ \text{control}} \times 100$$

Statistical analysis

All analyses were carried out in triplicate and the data were expressed as means ± standard deviations (SD).Statistical analyses were performed using the software Statistica 8.0 (Stat Soft). Differences between means at the 95% ($p < 0.05$) confidence level were considered statistically significant. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlations.

Results and Discussion

The total phenolic content (TPC)

Polyphenols are an important group of compounds regarding the appearance and the functional properties of honey. They are members of a class of natural compounds, recently considered of high scientific and therapeutic interest. In the long human tradition, honey has been used not only as a nutrient but also as a medicine [37]. TPC of the different monofloral and unifloral honeys was investigated by the Folin-Ciocalteu assay and the mean values and standard deviation are shown in table 1. The total phenolic content (mg GAE/100 g of honey) of Algerian honeys was found in the range of 63.93 to 95.36, which was determined using gallic acid as standard (R^2=0.9988) table 1. The total phenolic content of certain honey samples has been previously

Honey	Total polyphenol Content (mg/100 g ± SD)*	DPPH scavenging activity (% ± SD)*
H1	85.62 ± 2.75	26.93 ±3.22
H2	95.36 ± 6.08	22.49 ±11.71
H3	82.85 ± 14.24	29.76 ± 5.36
H4	65.31 ±1.60	42.65 ± 22.34
H5	64.29 ± 1.55	28.95 ± 464
H6	63.93 ± 0.11	30.11 ± 8.45
Mean	75.89 ± 6.20	30.14 ± 9.28

*Values are means of triplicate determinations.
DPPH (2. 2-diphenyl-1-picryl-hydrazyl)

Table 1: Total polyphenol content and DPPH scavenging activity of tested honeys.

determined [38-40] for example Meda et al. [41] reported that total phenols of Burkina Fasan honey were 32.59-114.75 mg GAE/100 g. A similar level of phenolic content was also observed for Romanian honeydew honeys for which the phenolic content varied from 23.0 to 125.0 mg GAE/ 100 g [42]. For Indian and Croatian honeys, the phenolic content ranged from 48 to 99 and 31.72 to 80.11 mg GAE/ 100 g, respectively [43,44].

DPPH scavenging activity

The radical scavenging effects of Algerian honey were tested using methanolic solution of the DPPH free radical which exhibits a deep purple colour with maximum absorption at 517 nm. The DPPH free radical has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition [45]. The results obtained for DPPH radical scavenging activity of these honeys are summarized in table 1. The percentage DPPH scavenging activity ranged from (22.49 ± 11.71) % to (42.65 ± 22.34) %. table1. These values are similar to 23.81%-100% as reported by Wilczyńskal [46] for Polish honey respectively. The activity was also within a lower range of 2.30 % and 90.73 % reported for Turkish honey [47].

Correlation between the polyphenol contents and antiradical activity

The correlation between DPPH activities and total polyphenol contents in 6 honey samples are analyzed in this study and the results are presented in figure 1. The coefficients of correlation between the total phenolic content of six honeys and values obtained with DPPH scavenging activity were calculated.

Inhibitory activity evaluation

The antibacterial activity of the 6 honey samples was first measured by agar well diffusion and spectrophotometric method, which is suitable for a previous screening test**.** In the antibacterial screening, solutions with different percentage of honey were used in the assay. The presence and diameter of zones of inhibition are dependent on the honey concentrations. Figure 2 and 3the inhibitory capacity of honeys of the same botanical origin is variable and the honey effect on the growth of both microorganisms could be different figure 4 and 5

Antibacterial activities of the honey samples with 50 % concentration against *P. auregenosa* and *E. coli* strains are presented in figure 2 and 3. The inhibition zones of honey samples varied from (23-40) mm by *P. auregenosa* to (29-35) mm by *E .coli.* Antibacterial

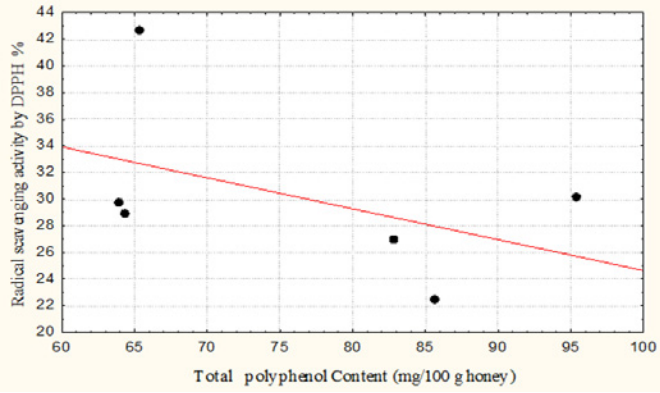

Figure 1: Correlation between DPPH values and total polyphenol contents in honey samples (R^2 = 0 .414, $p < 0.05$).

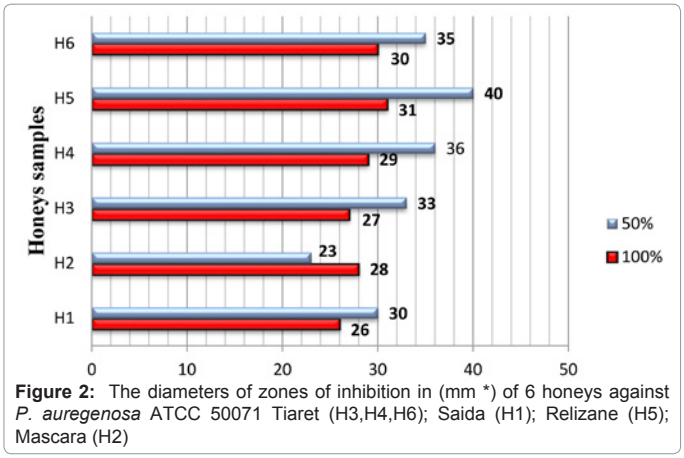

Figure 2: The diameters of zones of inhibition in (mm *) of 6 honeys against *P. auregenosa* ATCC 50071 Tiaret (H3,H4,H6); Saida (H1); Relizane (H5); Mascara (H2)

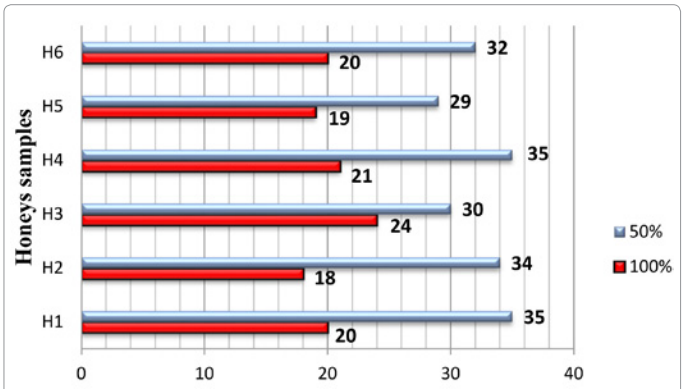

Figure 3: The diameters of zones of inhibition in (mm *) of 6 honeys aigainst *E.coli* ATCC 25922 Tiaret (H3, H4,H6); Saida (H1);Relizane (H5);Mascara …

activities of the honey samples with 100 % concentration against *P. auregenosa* and *E.coli* strains are presented in Figure 2 and 3. The inhibition zones of honey samples varied from (26-31) mm by *P. auregenosa* to (18-24) mm by *E. coli.* The growth inhibition profiles of natural honeys are presented in Figure 4 and 5. A variation in the antibacterial activity with floral source was observed at 50 and 100% concentration, some inhibitory effect was observed on all pathogens. Honey has a broad spectrum of bactericidal and bacteriostatic activities [48,49]. Several bioactive compounds have been identified in honey which contributed to its antibacterial action. The antibacterial property of honey is dependent on several contributing factors. Low water content, high osmolarity (high sugar content), low pH, antibiotic peptides, methylglyoxal, catalase to hydrogen-peroxide ratio, Maillard reaction products , bee defensin-1, production of hydrogen peroxide, although involved in antibacterial action, are common properties for all honeys and could not explain the variability in activity between honeys. From surveys of antibacterial activity in different honeys, it became clear that a phytochemical composition of honeys was responsible for the degree of bacteriostatic and bactericidal action [50-59]. Phenolic compounds originating from plant nectar have been proposed as important factors for the no peroxide antibacterial activity of honey [60]. Several antibacterial phenolic compounds have been identified in honeys [61-63]. Davidson et al. [64] have shown that individual phenolic compounds have growth inhibition on a wide range of Gram-positive and Gram-negative bacteria. In a recent study Ahmed et al. [33] reported that *E. coli* is more sensitive to the action of honey than *P. aeruginosa.* The results of this study clearly show that honey has the

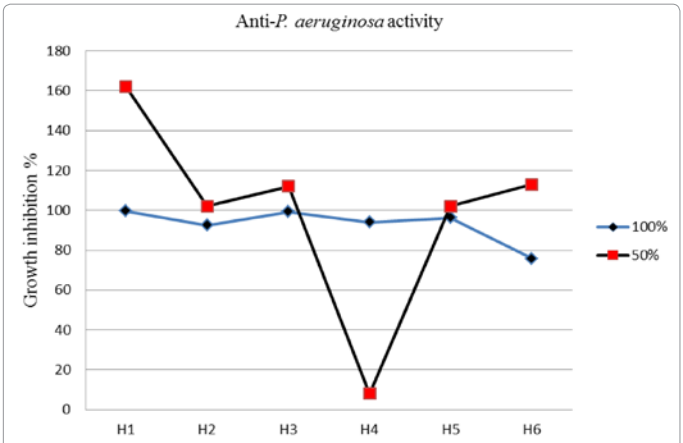

Figure 4: Antibacterial activity of 6 honeys against *P. aeruginosa* ATCC 50071.

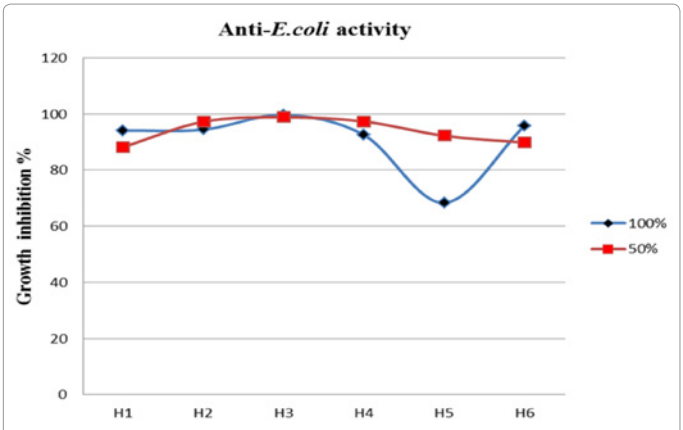

Figure 5: Antibacterial activity of 6 honeys against *E. coli ATCC 25922.*

potential to be used as an antibacterial agent to prevent and control infection with gram-negative bacteria.

Conclusions

This study gave an overview on the antibacterial activity of honey from Algeria and showed that many honeys have potential for therapeutic use as antibacterial agents. Phenolic compounds play a major role in the antibacterial activity of honey and the differences between honey samples in terms of antibacterial and antioxidant activity could be attributed to the natural variations in floral sources of nectar and the different locations. The results obtained, in the inhibitory activity assays, show that the spectrophotometric method is an easy and useful tool in the evaluation of the antibacterial capacity of honeys against *P. aeruginosa and E. coli.*

Nevertheless, further studies including other bacterial strains are necessary in order to confirm the utility of this technique for the evaluation of the non-peroxidic antibacterial activities of Algerian honeys.

Acknowledgments

Authors are thankful to staff of Mostaganem University for providing material.

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