

Colour Intensity, Polyphenol Content and Antibacterial Capacity of Unheated and Heat-Treated Sahara Honey

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Abstract

The objective of this research work was to evaluate the effect of heat processing on antibacterial capacity of Sahara honey (SH). Various thermal treatments were carried out at 25°C, 50°C, 75°C and 100°C for 15 min, 30 min, and 60 min, and the parameters were determined: colour intensity, phenolic contents and antibacterial activity of two species of bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*). The total phenolic contents in honey samples varied from 0.55 and 1.54 mg of gallic acid equivalent (GAE) in gram of honey. MIC values respectively 3.12 to 12.5 mg/ml and zone of growth inhibition respectively 2.65 mm to 19 mm. Exposures of SH at 75°C and 100°C, no inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth was detected. Statistical analysis demonstrated positive correlations between color intensity and polyphenol contents and negative correlations with antibacterial capacity. Data from present results revealed that unheated and heat-treated Sahara honey showed growth inhibitory effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with variable degrees.

Keywords: Sahara honey; Antibacterial capacity; Thermal processing

Introduction

Therapy of infections caused by gram-negative and gram-positive negative pathogen is a frequent problem due to the emergence of bacterial strains resistant to numerous drugs [1-3]. Recently many different bacteria now exhibit multi-drug resistance, including *Staphylococci* sp and *Pseudomonas aeruginosa*. Bee's products are believed to be an important source of new natural substances with potential therapeutic effects. Recently, the potent activity of honey against antibiotic-resistant bacteria has further increased the interest for application of honey. Several honeys have been approved for clinical application [4-6]. A review of different antibacterial studies showed that the antibacterial properties of honey depend on the geographical origin of the honey [7] Honey bees has been traditionally recognized as valuable source of energy which contains antibacterial and antifungal proprieties [8]. Several bioactive compounds have been identified in honey which contributed to its antibacterial action. In many studies, the presences of peroxide and non-peroxide antibacterial capacity in honey have been reported [9,10]. The presence of hydrogen peroxide generated by enzymatic activity of glucose oxidase in diluted honey is considered as the major antibacterial factor [11]. Furthermore, heating honey inactivates the glucose and it oxidates phenolic compounds as important factors for the non-peroxide antibacterial activity of honey [10]. The non-peroxide antibacterial activity is insensitive to heat and light [12]. Several authors also studied the correlations between color and antibacterial activities with content of the bioactive compounds of honey. Various studies have been conducted to investigate the antimicrobial effects [13-15]. Limited studies have been done on Algerian honey. Current analysis assessed the antibacterial activities and the content of colour intensity, phenolic contents in 3 honey samples.

Materials and Methods

Sample collection

Three honey samples were collected in Sahara from Algeria during the year 2012. The samples were stored at 4°C until analysis in dark conditions.

Thermal treatment

The SH were heated at 25°C, 50°C, 75°C and 100°C for 15 min, 30 min and 60 minutes. Then they were cooled down to 4°C by immediately plunging the tubes in an ice bath and analyzed. The samples were filled into watertight tubes.

Colour intensity: ABS₇₂₀₋₄₅₀

The net absorbance of the SH was determined by the method of Beretta et al. [16] The SH were diluted to 50% (w/v) with warm (45°C to 50°C) milli Q water and the solution was filtered through a 0.45 µm filter. There was a complete absence of coarse particles in the honey solutions as all the commercial samples were no crystalline liquid honeys. The absorbance was measured using a spectrophotometer at 450 nm and 720 nm and the difference in absorbance was expressed as mAU.

Determination of total phenolic content (TPC)

Foline Ciocalteu method was used to assay total phenolic contents, which was described by Singleton et al. [17]. Thirty microlitre 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. Four hundred and fifty microlitre 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.

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Antibacterial capacity assays

Bacterial strains and culture conditions

Bacterial strains: *S. aureus* (ATCC 25922) and *P. aeruginosa* ATCC 27853 bacteria were selected for antibacterial activity assay. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Preparation of standard inoculums: Bacteria were routinely grown in Nutrient Agar (NA; Merck Germany) slant, incubated at 37°C for 24 h, and kept at 4°C until further use. Bacterial suspensions were prepared by inoculating one loopful of the 24-h-old bacterial colonies into 10.0 ml of sterilized distilled water. The inoculums size was adjusted to match the turbidity of McFarland 0.5 scale (1×10^8 cells/ml) and diluted with sterilized distilled water to the inoculums size of 1×10^7 cells/ml.

Agar well diffusion method: Antibacterial assay was carried out by modified method of Ahmed et al. [18]. Nutrient agar plates (Merck, Germany) were inoculated by rubbing sterile cotton swabs that were dipped into bacterial suspensions (overnight) cultures grown at 37°C on nutrient agar and adjusted to 0.5 McFarland in sterile saline) over the entire surface of the plate. After inoculation 8.2 mm diameter wells were cut into the surface of the agar using a sterile cork borer. 50 µl of test honey was added to each well. Plates were incubated at 30°C for 24 h. Zones of inhibition were measured using a Vernier caliper. The diameter of zones, including the diameter of the well, was recorded. Values are given as mean and standard deviation (SD) of tests performed in triplicate. The results were expressed in terms of the diameter of the inhibition zones: <5.5 mm, inactive; 5.5 -9 mm, very low activity; 9-12 mm, low activity; 12-15 mm, average activity; and >15 mm, high activity.

Minimum inhibitory concentration (MIC): The antibacterial activity of honey was examined by determining the Minimal Inhibitory Concentration (MIC) using the macro dilution broth technique by Eloff [19]. Briefly, Serial 2-fold dilutions of honey were inoculated with 10^6 CFU/ml (final concentrations) for each of the microorganism's tested. A bacterial suspension of approximately 5×10^5 CFU/ml was inoculated into tubes containing honey at different dilutions and incubated at 37°C for 24 h. All MIC values were expressed in % (v/v). Bioassay was performed in duplicate and repeated twice.

Data analysis

Each honey was analyzed in triplicate. Results are shown as mean values and standard deviation. Correlations were established using Pearson's correlation coefficient (r) in bivariate linear correlations ($P < 0.05$). All statistical analyses were performed with the Statistica 7.0 software for Windows.

Results

Color intensity

The color intensity of the Sahara honey samples ranged from 1.26 to 1.44 mAU (Table 1), the color intensity of the Sahara honey induced by thermal treatments ranged from 1.26 to 1.44 mAU (Table 1). The color of honey differ depending on the floral and geographical origin. Honey's color depends on various factors for example: mineral content, ash content. Besides the, honey colour can also be affected by heat, time of storage [20].

Total polyphenol content (TPC)

The results obtained showed that the TPC (mg GAE/100 g honey)

Température (°C)	Time (min)	Sahara honey samples	Color Intensity $A_{450}-A_{720}$		Polyphenol contents (mg/GAE/ 100 g honey)	
			Mean	DS	Mean	DS
Unheated	0 min	SH1	1.26	0.09	0.551	0.002
		SH2	1.37	0.11	0.67	0.01
		SH3	1.44	0.2	1.14	0
25°C	15 min	SH1	1.46	0.2	0.587	0.001
		SH2	1.35	0.03	0.543	0.005
		SH3	1.29	0.15	0.769	0.004
	30 min	SH1	1.34	0.09	0.853	0.002
		SH2	1.52	0.17	0.9015	0.009
		SH3	1.37	0.15	0.734	0.002
	60 min	SH1	1.01	0.56	0.598	0.002
		SH2	1.31	0.11	0.7185	0.002
		SH3	1.38	0.06	0.778	0.016
50°C	15 min	SH1	1.25	0.04	0.8685	0.03
		SH2	1.38	0.06	0.9265	0.03
		SH3	1.54	0.13	0.78	0.01
	30 min	SH1	1.24	0.05	0.8685	0.03
		SH2	1.27	0.1	0.9265	0.03
		SH3	1.45	0.09	0.78	0.01
	60 min	SH1	1.25	0.07	0.668	0.035
		SH2	1.4	0.04	0.6675	0.004
		SH3	1.51	0.16	0.7625	0.016
75°C	15 min	SH1	1.23	0.2	0.705	0.007
		SH2	1.34	0.07	0.849	0.004
		SH3	1.51	0.18	0.8015	0
	30 min	SH1	1.32	0.14	0.669	0.024
		SH2	1.28	0.2	0.731	0.007
		SH3	1.58	0.19	0.88	0.011
	60 min	SH1	1.22	0.04	0.748	0.001
		SH2	1.32	0.07	0.958	0.032
		SH3	1.59	0.19	0.8675	0.006
100°C	15 min	SH1	1.33	0.18	1.274	0.014
		SH2	1.41	0.18	1.247	0.009
		SH3	1.22	0.09	1.2875	0.01
	30 min	SH1	1.64	0.1	1.3985	0.017
		SH2	1.77	0.17	1.541	0
		SH3	1.31	0.19	1.5	0.108
	60 min	SH1	1.81	0.35	1.384	0.025
		SH2	1.88	0.45	1.387	0.016
		SH3	1.43	0.27	1.4595	0.02

Data expressed as mean ± SD (n = 3)

Table 1: Color intensity and TPC before and after heat-treatment of SH.

determined by the modified Folin-Ciocalteu method varied greatly among the honey types, as is apparent from Table 1. In this study the TPC of Sahara honeys is ranged from 0.13 to 1.15 with the mean value of 0.39 g/100 g. The TPC of the Sahara honey induced by thermal treatments ranged from 1.26 to 1.44 mAU. The TPC of SH depends on different parameters for example: environmental conditions, harvest season and storage ability.

Antibacterial capacity assays

Minimum inhibitory concentration (MIC): The MIC of the Sahara honey against the two bacterial strains using the microdilution method is summarized in Tables 2 and 3. Minimum inhibitory concentration (MIC) was used to determine the lowest concentration of honey-in-water solution (w/v) at which the percentage inhibition is almost 100% [21,22]. The MIC before and after heat-treatment of SH

at 25°C and 50°C for 15, 30 and 60 minutes ranged from 3.12-12.5 mm (Tables 2 and 3).

Agar well diffusion method: SH were tested for their ability to inhibit growth of *S. aureus* ATCC 25922 and *P. aeruginosa* ATCC 27853. The results of the *in vitro* antibacterial capacity of SH determined by diameters of inhibition zones (DIZ) are presented in (Table 4). The DIZ produced by the undiluted honeys samples was (14.32 - 16.55) mm. After heat-treatment (at 25°C and 50°C for 15, 30 and 60 min) the DIZ ranged from (11-17.67) mm by *P. auregenosa* to (12.67-27) mm by *S. aureus* (Table 5). The DIZ of SH with 25% concentration varied from (14.33 - 15) mm by *P. auregenosa* to (10.67-12.33) mm by *S. aureus*. The DIZ after heat-treatment at 25°C and 50°C for 15, 30 and 60 minutes ranged from (7-12.67) mm by *P. auregenosa* to (2 - 21.67) mm by *S. aureus* (Table 6). No inhibition (DIZ=not detectable, MIC=not detectable) of bacterial growth was observed when honey at 75°C or 100°C.

Discussion

This is the first report on the changes of colour intensity, polyphenols content and antibacterial capacity of Sahara honeys during thermal processing. Several types of honey are produced in Algeria, where honey production is a traditional practice, well implanted in several regions. The Sahara region is located in the south of Algeria, where, due to its edaphoclimatic conditions and flora diversity, sidr and euphorbe are the principal honey types produced. Large number of honeys from different geographical locations and different botanical origins show growth inhibitory action [23-26]. Several bioactive compounds have been identified in honey which contributed to its antimicrobial proprieties. The intrinsic characteristic of honeys, e.g. high osmolarity, acidity (low pH), low water activity, catalase to hydrogen-peroxide ratio, polyphenols, Maillard reaction products and production of

hydrogen peroxide, although involved in antibacterial action [27-32]. Two important enzymes known to contribute to the major biological proprieties of honey are bee-origin glucose oxidase and floral-origin catalase [33]. Furthermore, heating honey inactivates the glucose oxidates. Molan [34] reported that honey loses its antimicrobial activity when subjected to heat or exposed to light. Radwan et al. [35] showed that heat exposure in a water bath at a temperature that was not clearly specified, but appeared to be 50°C for 10 minutes, had an adverse effect on the antibacterial activity of honey. Moreover, Brudzynski and Kim [36] indicate the antibacterial activity of honey may or may not be affected by heat, age, and storage conditions. Also, Rios et al. [37] studied the effects of extraction, storage conditions and heat treatments on antimicrobial activity of *Zanthoxylum fagara* honey, and found that, at 40°C, the antimicrobial activity of the honey was not affected. In addition, Chen et al. [38] observed a decrease in antimicrobial activity in processed honey (heat to 45°C for 8 h). Polyphenols are another important group of compounds with respect to the appearance and the functional properties of honey [39]. Phenolic content varies between different honeys, and phenolic compounds may contribute to antimicrobial activities in honey [40]. In the studied Sahara honeys, only very small amounts of TPC were present. The literature data indicate that there is a strong positive correlation between honey color and total phenolic content [41,42]. In the present study, relationship between phenolic content and colour of Sahara honeys, possibly due to a highly extended conjugated systems, especially when complexed with minerals.

Escuredo et al. [43] have shown that individual phenolic compounds have growth inhibition on a wide range of Gram positive and Gram-negative bacteria. But Ulusoy et al. [44] found that antimicrobial activity was not linearly correlated with total and individual phenolic compounds. In the present study, no relationship between phenolic

	<i>P. aeruginosa</i> ATCC27853								
	SH1			SH2			SH3		
	15	30	60	15	30	60	15	30	60
Unheated	6.25	6.25	6.25	3.12	3.12	3.12	3.12	3.12	3.12
25°C	6.25	6.25	6.25	3.12	3.12	3.12	3.12	3.12	3.12
50°C	6.25	12.5	12.5	3.12	6.25	12.5	12.5	12.5	12.5
75°C	ND	ND	ND	ND	ND	ND	ND	ND	ND
100°C	ND	ND	ND	ND	ND	ND	ND	ND	ND

(ND=not determined)

Table 2: Values of minimum inhibitory concentration (MIC%) of *P. aeruginosa* at different temperatures for different heating times.

	<i>S.aureus</i> ATCC 25922								
	SH1			SH2			SH3		
	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min
Unheated	6.25	6.25	6.25	3.12	3.12	3.12	3.12	3.12	3.12
25°C	6.25	6.25	6.25	3.12	3.12	3.12	3.12	3.12	3.12
50°C	6.25	12.5	12.5	3.12	6.25	12.5	12.5	12.5	12.5
75°C	ND	ND	ND	ND	ND	ND	ND	ND	ND
100°C	ND	ND	ND	ND	ND	ND	ND	ND	ND

(ND=not determined)

Table 3: Show the values of minimum inhibitory concentration (MIC %) *S.aureus* at different temperatures for different heating times.

Sahara Honey samples	<i>P. aeruginosa</i> ATCC 27853		<i>S.aureus</i> ATCC 25922	
	Undiluted	50%	Undiluted	50%
SH1	16.67	15	19	12.33
SH2	17.33	14.67	2.65	10.67
SH3	15.67	14.33	21.33	11.33

Table 4: Means of zone of growth inhibition (mm) of SH on *P. aeruginosa* and *S.aureus* using agar-well diffusion assays.

Sahara honey samples		<i>P. aeruginosa</i> ATCC								<i>S.aureus</i> ATCC							
		Undiluted				50% diluted				Undiluted				50% diluted			
		25°C	50°C	75°C	100°C	25°C	50°C	75°C	100°C	25°C	50°C	75°C	100°C	25°C	50°C	75°C	100°C
SH1	15 min	16.3	13	ND	ND	15	9.67	ND	ND	27	20	ND	ND	17	21.67	ND	ND
	30 min	15.7	11	ND	ND	12.67	8.67	ND	ND	20	19.67	ND	ND	3	14.67	ND	ND
	60 min	13.5	11	ND	ND	9.33	7	ND	ND	14.33	12.67	ND	ND	10	7.33	ND	ND
SH2	15 min	17.67	12.33	ND	ND	11.67	9.67	ND	ND	23.67	22.67	ND	ND	18.7	21.33	ND	ND
	30 min	13.33	13.33	ND	ND	9.67	10.67	ND	ND	14.33	17	ND	ND	2	16.67	ND	ND
	60 min	17	10.33	ND	ND	11	7	ND	ND	16.67	15.33	ND	ND	9	8.67	ND	ND
SH3	15 min	12.67	13.67	ND	ND	11.67	10.67	ND	ND	26	20.33	ND	ND	18.33	21	ND	ND
	30 min	13.33	12.33	ND	ND	7	10	ND	ND	16	15	ND	ND	2.67	17	ND	ND
	60 min	12	11.33	ND	ND	7.67	7.67	ND	ND	19	12.67	ND	ND	9	5.67	ND	ND

(ND = not determined)

Diameter of the inhibition zones: <5.5 mm: inactive; 5.5-9 mm: very low activity; 9-12 mm: low activity; 12-15 mm: average activity; and >15 mm: high activity.

Table 5: Means of zone of growth inhibition (mm) of SH at different temperatures for different heating times, using agar-well diffusion assays.

	Unheated		25°C		50°C		75°C		100°C	
	Color	TPC	Color	TPC	Color	TPC	Color	TPC	Color	TPC
Color	1	0.896	1	-0.639	1	-0.646	1	0.56	1	-0.958
TPC	0.896	1	-0.639	1	-0.646	1	0.56	1	-0.958	1

Table 6: Matrix of the correlation between color Intensity and TPC before and after heat-treatment of SH.

content, colour and the antibacterial capacity of Sahara honeys was observed. In this study, the assessment of antibacterial capacity was performed using two techniques. The results showed remarkable differences in the nature of the antibacterial capacity when the two methods were compared. The results shown indicate that the bacterial broth macro dilution method presents higher sensitivity compared to the agar-well assay. In the macro dilution test, the liquid medium allows good mobility for both polar and non-polar molecules, since there is no agar barrier to inhibit the diffusion of the non-polar molecules, such as flavonoids and phenolics.

Conclusion

In conclusion, our study can be considered as the first report on the antibacterial capacity of Sahara honeys during thermal processing. This preliminary screening is an interesting evaluation of the potential antibacterial capacity of the SH. These results confirm the potential use of SH in natural medicine for the treatment of numerous infectious diseases. However further studies are required to identify and quantify the biologically active components present in the SH, in addition, further tests are needed to confirm these screening results in other *in vitro* and *in vivo* assays.

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