Research Article

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In Vitro Synergistic Antibacterial Activity of Natural Honey Combined with Curcuma Starch and their Correlation with Diastase Number, Flavonoid and Polyphenol Content

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Abstract

Honey has been acknowledged worldwide as a good source of natural carbohydrates and sweetener. Six honey samples of *Apis mellifera* forged on plants from western Algeria were analyzed to determine Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Diastase Number (DN), Hydroxy Methyl Furfural (HMF) content and antibacterial activity alone and in combination with Curcuma Starch. The total phenol content was determined by using the Folin-Ciocalteu method, and the flavonoid content was analyzed using the aluminum chloride method. The HMF and DN were determined by harmonized methods. An agar incorporation technique was used to assess the Minimum Inhibition Concentration (MICs) and Minimum Inhibition Additive Concentration (MIACs) of honey against two strains of Gram-Negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 2154). Total phenolic content of honeys ranged from 63.93 to 95.36 mg GAE/100 g honey, while Total Flavonoid Content (TFC) ranged from 5.41 to 9.94 mg CE/100 g. Hydroxy Methyl Furfural (HMF) content shows values between 3.8 and 78.4 mg kg⁻¹; diastase values were between 7.3 and 26. The MAIC for the six varieties of honeys tested ranged between 12 and 18% (vol/vol) and 11 and 15% (vol/vol) against *E. coli* and *P. aeruginosa*, respectively. The MIC range for honey alone was 5-70% (vol/vol) and 5-40 % (vol/vol) against *E. coli* and *S. aureus*, respectively. A positive correlation was observed between total phenolic content and diastase activity ($r=0.248$) and between diastase activity and total flavonoid content ($r=0.240$). No clear correlation has been established between the MIC drop and the Diastase Number. The use of curcuma starch allows honey benefit and would constitute a synergetic effect to the antibacterial activity of honey.

Introduction

Microbial infections are the cause of a large burden of diseases and bacteria are listed in the first position among the common microorganisms responsible for opportunistic diseases occurring associated with AIDS. Therapy of bacterial infections is a frequent problem due to the emergence of bacterial strains resistant to numerous antibiotics [1,2]. These shortcomings lead to an urgent global call for new antibacterial drugs, particularly from natural resources. Honey is a natural product, well known for thousands of years for its high nutritive value and healing properties. Also Honey has been considered as an important part of traditional medicines since ancient times [3]. Its functions in the treatments of burns, gastrointestinal disorders, asthma, infections, and skin ulcers [4,5]. Honey has an established function as an antibacterial agent that has a broad spectrum of activity against gram-positive and gram negative bacteria [6-8]. More recently maillard reaction products was discovered to contribute to the activity of Canadian honeys [9], bee defensin-1 was detected in a Dutch honey [10] catalase to hydrogen peroxide ratio [11], antibiotic peptides [12], methylglyoxal [13] and melanoidins [9]. Phenolic acids and flavonoids in honey have also been found to inhibit the growth of a wide range of Gram-negative and Gram-positive bacteria [14,15]. Honey is a supersaturated solution of sugars, of which fructose (38%) and glucose (31%) are the main contributors, with phenolic compounds, minerals, proteins, free amino acids and vitamins acting as minor components [16]. Also honey contains small amounts of different enzymes, notably, diastase (α - and β -amylase), invertase (α -glucosidase), glucose-oxidase, catalase and acid phosphatase, which come from nectar sources, salivary fluids and the pharyngeal gland secretions of the honeybee [17]. A diastase is any one of a group of enzymes that catalyze the breakdown of starch into maltose [18]. Alpha amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose

and oligosaccharides known as dextrans. Diastase activity is expressed as the Diastase Number (DN) in Schade units and is defined as follows: one diastase unit corresponds to the enzyme activity of 1 g of honey, which can hydrolyse 0.01 g of starch in 1 h at 40°C [19], the activity of this enzyme decreases with the time of storage and that of heating. Alpha amylase inhibitory activity has been reported previously for various plant extracts [20]. The inhibitory effects of polyphenols for α -amylases have attracted great interest among researchers [21-26]. Tadera et al. [27] tested several flavonoids compounds for their inhibitory activity against α -amylase. Also Lo Piparo et al. [28] investigated the interactions between flavonoids and human α -amylase in order to understand the molecular requirement for enzyme inhibition. The antimicrobial properties of Algerian honey available on the market differ on account of various factors like geographical and botanical source [29,30]. In this study, we evaluated the antibacterial activity of honey combined with curcuma starch and their correlation with diastase number, flavonoid and polyphenol content.

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Materials and Methods

Honey samples

A total of 6 honey samples were collected from various botanical and geographical sources in Algeria were collected from bee keepers and obtained from local markets. All samples were transferred to the laboratory, stored in amber flasks, and kept at 4°-5°C until analysis.

Preparation of the stock starch solution

The stock starch solution was prepared by dissolving 0.5 g of dried soluble starch in deionised water in a volumetric flask. After heating and stirring the solution for approximately ten minutes, starch was completely dissolved, and the volumetric flask was filled with deionised water to the mark.

Diastase activity (Diastase number)

The determination of Diastase activity or Diastase Number (DN) was carried out according to Phadebas method recommended by Harmonized methods of the International Honey Commission [31]. An insoluble blue dyed cross-linked type of starch is used as the substrate. This is hydrolyzed by the enzyme, yielding blue water-soluble fragments, determined photometrically at 620 nm. The absorbance of the solution is directly proportional to the diastatic activity of the sample. The diastase activity, expressed as DN or diastase number, was calculated from the absorbance measurements using Eqs. (1) and (2) for high (8-40 diastase units) and low (up to 8 diastase units) activity values, respectively:

$$DN=28.2 \times \Delta A_{620} - 2.64 \quad (1)$$

$$DN=35.2 \times \Delta A_{620} - 0.46 \quad (2)$$

Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) was detected using a technique based on the method described by Winkler [32]. Five grams of honey were dissolved, without heating, in oxygen free distilled water and transferred to a 125 ml graduated flask and diluted to volume with oxygen free distilled water. Two milliliters of honey solution was pipetted into two tubes and 5 ml of P-toluidine solution was added to each. Into one test tube, 1 ml of water was pipetted and into the other 1 ml of barbituric acid solution was added; both mixtures were then shaken. Absorbance was read using a spectrophotometer against a blank at a wave length of 550 nm. Calculation: mg/100 g

$$\text{hydroxymethylfurfural} = \text{absorbance/test} \times 192 \quad [33].$$

Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu (F-C) method [34]. 30 ml 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 reagent. The solution was thoroughly mixed by vortexing and incubated for 2 min at ambient temperature. 450 ml 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.

Total flavonoid content

The Total Flavonoid Content (TFC) was determined using the aluminium chloride assay according to Amaral et al. [35]. A 10 µl

volume of a 10% (v/v) honey solution was added to the wells of a 96 well plate; then 30 µl of a 2.5% sodium nitrite, 20 µl of 2.5% aluminium chloride solutions and then 100 µl of a 2% sodium hydroxide solution were sequentially added. The samples were mixed well and Abs 450 nm was measured. TFC was expressed as mg catechin equivalents (CE)/100 g.

Determination of antibacterial activity

Bacterial stains: Two Gram-negative *Escherchia coli* ATCC 25922 and *Pseudomonas aeruginosa* (ATCC2154). All the strains were cultured at 37°C on nutrient agar (NA; Merck, Germany) medium, and the Mueller-Hinton broth medium (MHB; Merck, Germany) has been used in the antibacterial activity assay. All the test microorganisms were purchased from the American Type Culture Collection. 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: Fresh microbial cultures were prepared by streaking loopful of bacterial suspension into organism specific selective media (Merck, Germany) and incubated at optimal temperature in order to maintain approximately uniform growth rate of each organism. The bacterial cultures from fresh media were compared with 0.5 McFarland turbidity standards, which is equivalent to approximately 1×10^8 bacterial cell counts per ml and it was maintained throughout the experimentation.

MIC and MICAC determinations

Minimum inhibitory concentration (MIC): Increased concentrations (1%-50% vol/vol) were incorporated in to media to test their efficiency against *P. aeruginosa* and *E. coli*. Each plate with final volume of honey and media of 5 ml was inoculated and incubated at 37°C for 48 h. The MIC was determined by finding the plates with the lowest concentration of honey on which the strain would not grow. All MIC values were expressed in % (vol/vol).

Minimum additive inhibitory concentration (MAIC): To evaluate the effect of starch on the antifungal action of honey, 1% starch solution was prepared using sterile water. Different volumes from the stock solution were added to arrange of honey concentrations lower than the MIC. The same volume of starch solution that has given inhibition with honey was added alone to media as control to check whether or not starch alone has an inhibition effect against *P. aeruginosa* and *E. coli*. An equivalent volume of water was added to honey instead of starch solution to confirm that additive inhibition is not due to the dilution of honey. The final volume in each plate was 5 ml. Starch content in media ranged between 1% and 8% (wt/vol). Honey and starch as well as honey and water were incubated for 24 h at 37°C before being incorporated into media. Plates were inoculated and incubated at 37°C for 24 h bioassay was performed in duplicate and repeated twice.

Statistical analysis

Analyses were made in triplicate, and the data are presented as mean $\pm$ standard deviation. Correlations were established using Pearson's correlation coefficient (r) in bivariate linear correlations ($p < 0.01$). All statistical analyses were performed with the Statistica 7.0 software for Windows.

Results and Discussion

Diastase activity and HMF are widely recognized as parameters for the evaluation of honey freshness and/or overheating. International regulations set a minimum value of 8 on Gothe's scale for diastase

activity, and a maximum HMF content of 40 mg kg⁻¹. The mean of HMF was found 31.2 mg/kg with the range of 7.68 to 52.6 mg/kg (Table 1). One of the samples (H1) showed levels of HMF higher than the allowed limits of 40 mg/kg, which are indicative of temperature abuse during processing and/or bad storage practices. Several factors influence HMF levels, such as temperature and time of heating, storage conditions, pH and floral source, so it provides an indication of overheating and storage in poor conditions [36]. The mean of diastase activity was 9.80 in all samples (Table 1). The variation in the activity of diastases and HMF may be related to source of honey as well as climate of region [37]. Polyphenols are an important group of compounds which influence the appearance and the functional properties of honey [38]. The total phenolic content (mg GAE/100 g of honey) of Algerian honeys was found in the range of 63.93 to 95.36 (Table 1) which was determined using gallic acid as standard ($R^2=0.9988$). 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 [39]. The concentration of polyphenols varies in honeys of different botanical origin and ranges from 46.0 to 456 mg/j/kg [40] and is major factors responsible for biological activities, including antioxidant, antimicrobial, antiviral and anticancer activities [41]. The TFC of honey samples ranged from 5.41 to 9.94 mg CE/100 g and these values are higher than that of 0.25-8.38mg CE/100 g as reported for Brazilian honeys [42]. Flavonoid contents in Burkina Faso honey samples studied by Meda et al. [43] were 0.17-8.35 mg CE/100 g of honey. In samples from Chile, flavonoid content ranged from 0.014 to 13.8 mg CE/100 g of honey [44]. In the analysis of Algerian honey samples, a low correlation ($r=0.878$) (Table 3) between the total flavonoid and total phenolic contents was observed. Islam et al. [42] also described a low correlation ($r=0.959$) between the total amount of flavonoids and the total amount of phenolic compounds.

The antibacterial properties of honey have been reviewed

	Phenolics (mg Gallic acid/kg)		Flavonoids (mg Catechin/kg)		Diastase Number (Schade Number*)		HMF (mg/kg)	
	Mean	DS	Mean	DS	Mean	DS	Mean	DS
H1	85.62	2.75	9.81	0.07	15.1	2.8	78.4	1.9
H2	95.36	6.08	9.48	0.20	23.5	2.8	28.2	1.9
H3	82.85	14.24	9.94	0.54	11	2.8	11.1	1.9
H4	65.31	1.60	7.10	0.04	26	2.8	9.8	1.9
H5	64.29	1.55	5.41	0.04	7.3	2.8	7.6	1.9
H6	63.93	0.11	6.97	0.00	16.4	2.8	3.8	1.9

*Schade number corresponds to Gothe number or 0.01 g starch hydrolysed 1h at 40°C per 1 g honey.

HMF: Hydroxymethylfurfural.

Table 1: Total polyphenol content (TP), total flavonoids content (TFC), diastase Number and HMF results represent the average of four measurements $\pm$ SD (n=3).

Honey samples	Honey only		Curcuma starch and honey			
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 2154	<i>E. coli</i> ATCC 25922		<i>P. aeruginosa</i> ATCC 2154	
	MICs % (vol/vol)	MICs % (vol/vol)	MIACs % (vol/vol)	MIC drop%	MIACs % (vol/vol)	MIC drop%
H1	16	12	12	25	11	8.33
H2	14	12	12	14.28	11	8.33
H3	20	16	18	10	15	6.25
H4	16	14	14	12.5	13	7.14
H5	16	12	14	12.5	11	8.33
H6	10	12	09	10	11	8.33

Table 2: Minimum inhibitory concentration (MIC), minimum additive inhibitory concentration (MAIC) and minimum inhibitory concentration drop (MIC drop) of tested honeys.

	Phenolics	Flavonoids	Diastase Number	HMF
Phenolics	1.000	0.878*	0.248*	0.577*
Flavonoids	0.878*	1.000	0.240*	0.585*
Diastase Number	0.248*	0.240*	1.000	0.137
HMF	0.577*	0.585*	0.137	1.000

(*) Significant P values ($P<0.01$)

Table 3: Correlation showing the interrelation among phenolics, flavonoids, diastase number and HMF.

	Phenolics	Flavonoids	Diastase Number	HMF
MIC drop <i>E. coli</i> ATCC 25922	0.437*	0.414*	0.042	0.978*
MIC drop <i>P. aeruginosa</i> ATCC 2154	-0.013	-0.308*	0.009	0.325*

(*) Significant P values ($P<0.01$)

Table 4: Pearson's correlation coefficients obtained when correlations between MIC drop honey and different characteristics of honey were studied.

extensively during the last years in multiple studies all over the world. The results of our work indicated that the six varieties of honeys tested have an antibacterial property. The intensity of effect on the growth of germs, varied according to the botanical honey origin and the type of germ tested. As a whole, the six varieties presented antibacterial activities against *E. coli* at concentrations from 14 to 20% (vol/vol) and 12 to 16% (vol/vol) for *P. aeruginosa* (Table 2). When starch was incubated with honey and added to media, a MIC drop was noticed with each variety and the MAICs of the six varieties ranged between 11 to 15% and 9 to 18% (vol/vol) (Table 2). The inhibitory action was seen neither in the media containing starch only nor in media with water and starch. Infection with *P. aeruginosa* is the most serious complication in burns patients [45,46], followed by infections with *E. coli* and other pathogen microorganisms [45]. With increasing interest in the use of alternative therapies and as the development of antibiotic resistant bacteria spreads. Many works was interested, during this last decade, with the products of the hive and in particular honey, efficient product against the germs secreted by the bees as a possible source of new pharmaceutical and medical agent. Honey saturated or super-saturated sugar, containing about 95% sugars [47]. Such a high concentration of sugar has antimicrobial activity since it cause osmotic stress [48, 49]. The high osmolarity of honey is due to the high content of sugar (average over 85% of honey) including fructose, glucose, maltose, sucrose and other types of carbohydrates [50]. Sugar paste was reported as being used successfully in 605 patients with wounds, burns and ulcers, with lower requirements for skin grafting, antibiotics, and lower hospital costs [51]. Recent experimental finding indicated that the ginger and potato starch and amylase present in honey increases the osmotic effect in the media by increasing the amount of sugars and consequently increasing the antimicrobial activity [52-55]. In recent years, a large number of studies have been done on the antibacterial activity of phenolic compounds of natural origin. Phenolics compounds of honey have been shown to exhibit antibacterial activities [16, 56-58]. Davidson et al. [59] have shown that individual phenolic compounds have growth inhibition on a wide range of Gram-positive and Gram-negative bacteria. But Ulusoy et al. [60] found that antimicrobial activity was not linearly correlated with total and individual phenolic compounds. In the present study, the relationship between phenolic, Flavonoids content, HMF and the MIC drop of honey was observed (Table 4). No correlation has been established between the MIC drop and the Diastase Number.

Honey is one of the natural products widely used to treat infections

that resist conventional drugs. But its price makes it an unaffordable substance in developing countries. Our results show that adding starch to honey could contribute to reducing the quantity of honey to be used without losing the expected effect. In the present study, the antibacterial activity of honey on *E. coli* and *P. aeruginosa* strains were confirmed and synergism was possible with curcuma starch tested. These data encourage further *in vivo* studies to validate these interesting results before clinical trials can proceed.

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