

The Relationship between Bioactive Compounds with Diastase Activity and Antibacterial Synergy of Honey and Potato Starch Combinations against *Klebsiella pneumoniae*

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Abstract

Honey samples produced by *Apis mellifera*, both unifloral and multifloral from different sources in Algeria were examined for their antibacterial capacity and their synergism with potato starch. An agar incorporation technique was used to assess the minimum inhibition concentration (MIC) and the minimum inhibitory concentration (MIAC) of honey against *K. pneumoniae* ATCC 27736. The physicochemical properties, α -amylases activity, total phenolic and flavonoid content, of six representative honey samples were determined. The MIC for the six varieties of honey without potato starch against *K. pneumoniae* ranged between 14% and 24% (v/v). When starch was incubated with honey and then added to media, an MIC drop was noticed with each variety and it ranged between 5.55% and 16.66%. The total phenolic content of honey samples was between 1.50–108.21 mg GAE/100 g honey as gallic acid equivalent, total flavonoid content varied from 5.41 to 9.94 mg Catechin/kg. Mean value for diastase was 16.55 ± 2.8 (range 7.3–23.5) expressed as diastase number in Gothe's scale. No significant correlation was established between α -amylases activity and bioactive compounds. Honey: potato starch combinations show real potential for future use as a treatment for infections caused by *K. pneumoniae*.

Keywords: Algerian honey; Antibacterial activity; Potato starch; Synergism; Bioactive compounds; Diastase activity

Introduction

Klebsiella pneumoniae, an important human pathogen, is the most common cause for pneumonia, bacteremia, and septicemia, is also involved in urinary tract infections (UTI) [1]. Treatment of *K. pneumoniae* infections has become increasingly difficult because of the predominance of multiple-antibiotic-resistant strains [2,3]. Antibiotic resistance has increased substantially in recent years and is posing an ever-increasing therapeutic problem [4,5]. Honey is a natural, nontoxic, and inexpensive product for the need of novel therapies against bacterial infections. The clinical use of honey has an enormous potential, especially in the fight against antibiotic-resistant strains [5,6]. Several bioactive compounds have been identified in honey which contributed to its antibacterial action. The main factor is the high osmotic activity of honey, which does not allow bacterial growth [7]. Recent experimental finding indicated that the amylase present in honey increases the osmotic effect in the media by increasing the amount of sugars and consequently increasing the antibacterial activity [8]. 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 [9]. Diastase is a natural enzyme of honey. Alpha-amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose and oligosaccharides known as dextrin's [10]. Also phenolic compounds may contribute to antimicrobial activity [11]. The inhibitory effects of polyphenols for α -amylases have attracted great interest among researchers [12,13]. In this study, we evaluated the antibacterial activity of 6 varieties of honey against *K. pneumoniae* and their combination with potato starch in correlation with diastase number (α -amylase), and Bioactive Compounds (total flavonoid and polyphenol content).

Materials and Methods

Honey samples

Six (n=6) commercial honeys of different floral sources and

geographical origins where purchased from local market and left at room temperature until further 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.

Bacterial culture and inoculum preparation

Pure culture of *K. pneumoniae* 27736 was obtained from the Department of Biology, Faculty of Sciences, Mostaganem University, Algeria. The bacteria was grown on Nutrient Agar (NA; Merck Germany) slant, incubated at 37°C for 24 h, and kept at 4°C until further use. Bacterial suspension was prepared by inoculating one loopful of the 24-h-old bacterial colonies into 10.0 ml of sterilized distilled water. The inoculum 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 inoculum size of 1×10^7 cells/ml.

Diastase activity (Diastase number)

Diastase activity was measured with Phadebas, according to the Harmonized Methods of the European Commission of Honey [14].

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An insoluble blue dyed cross-linked type of starch is used as the substrate. This is hydrolysed 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:

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

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

Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method [15]. Thirty microliters (μ l) of honey solution (0.1 g/ml) were mixed with 2.37 ml of milli Q water and 150 μ l of 0.2 N Folin–Ciocalteu reagent. The solution was thoroughly mixed by vortexing and incubated for 2 min at room temperature. Four hundred and fifty microliters (μ l) of sodium carbonate solution (0.2 g/ml) were 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 results were mean values \pm standard deviations and expressed as milligrams of gallic acid equivalents (mg GAE)/100 g of honey

Determination of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was assessed using aluminium-chloride assay [16]. A 10 μ l volume of a 10% (v/v) honey solution was added to the wells of a 96 well plate; then 30 μ l of a 2.5% sodium nitrite, 20 μ l of 2.5% aluminium chloride solutions and then 100 μ l of a 2% sodium hydroxide solution were sequentially added. The samples were mixed well and Abs 450 nm was measured. Total flavonoid content was expressed as mg of (+)-catechin equivalents (CEQ) per kg of honey.

Antibacterial activity of honey alone

Increased concentrations (5%-50% vol/vol) were incorporated into media to test their efficiency against *K. pneumoniae* 27736. Each plate with final volume of honey and media of 5 ml was inoculated and incubated at 37°C for 24 h. The MICs 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 % (v/v).

Antibacterial synergism of honey and potato starch toward *K.pneumoniae*

To evaluate the effect of starch on the antibacterial 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 *K. pneumoniae*. 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% (w/v). 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 bioassay was performed in duplicate and repeated twice.

Statistical analysis

Analyses are representative of 3 independent experiments, and the

data are presented as means \pm standard deviations. 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 (Diastase number)

The major enzymes present in honey are invertase, glucose oxidase and diastase, a mixture of α -amylase and β -amylase. The honey samples analyzed in the present work show a range of values, between 22.1 and 7.3 Schade units. One sample (H5) has been shown value below 8 Schade units (Figure 5). The explanation for the low content of diastatic activity found in this one sample could be accounted for an inadequate processing or storage conditions.

Total Phenolic Content (TPC)

The amount of total phenolic content estimated using the Folin–Ciocalteu reagent in the different samples ranged from 63.93 to 95.36 mg (mg GAE/100 g honey) (Figure 4). The total phenolic content of certain honey samples has been previously determined [17,18]. For example, Dong, Zheng and Xu [19] has been reported that total phenols of in Chinese honey were 9.41 to 102.1 mg GAE/100 g. The concentration and type of polyphenolic substances depend on the floral origin of honey and are major factors responsible for biological activities, including antimicrobial activities [20].

Total Flavonoid Content (TFC)

The TFC of honey samples ranged from 5.41 to 9.94 mg CE/100 g (Figure 6) and these values are higher than that of 0.25–4.27 mg CE/100 g as reported for Brazilian honeys [21]. Flavonoid contents in Tualang honey samples studied by Khalil et al. [22] were 4.74–22.76 mg CE/100 g of honey.

Antibacterial activity of honey alone and in combination with potato starch toward *K. pneumoniae*

In the present study, it was assessed the *in vitro* antibacterial action of honey alone and in combination with potato starch against *K. pneumoniae*, determined by agar incorporation method. The determination of all the MICs and MIACs was performed in duplicate. All varieties of honey were effective against the tested strain. Without starch, the MICs of the six varieties ranged between 14% (v/v) and 24% (v/v). When starch was incubated with honey and added to media, the MIACs of the six varieties ranged between 8% (w/v) and 20% (w/v) which represents an MIC drop between 65.55% and 50% (Figures

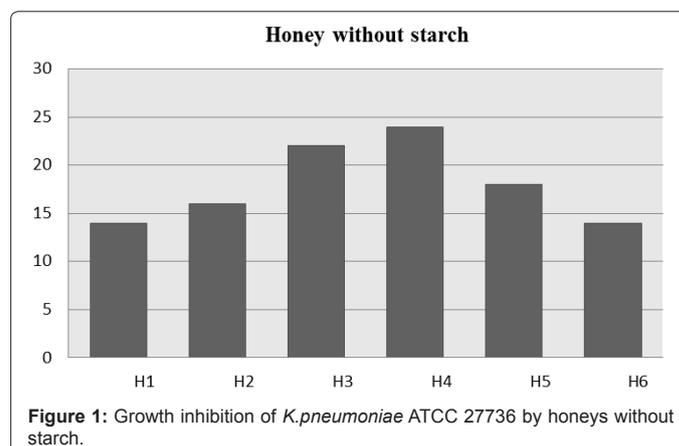
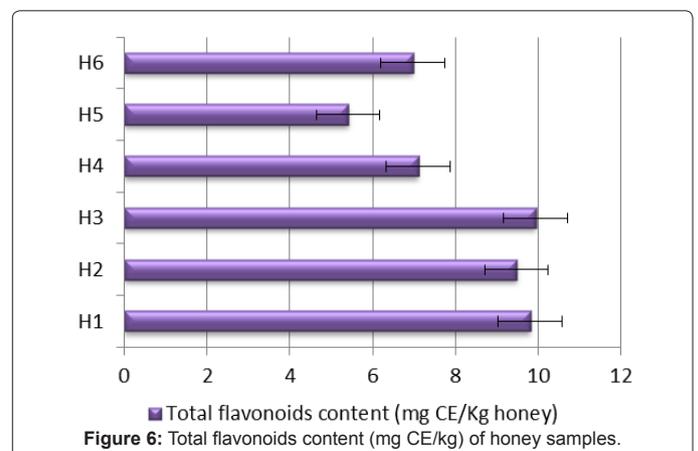
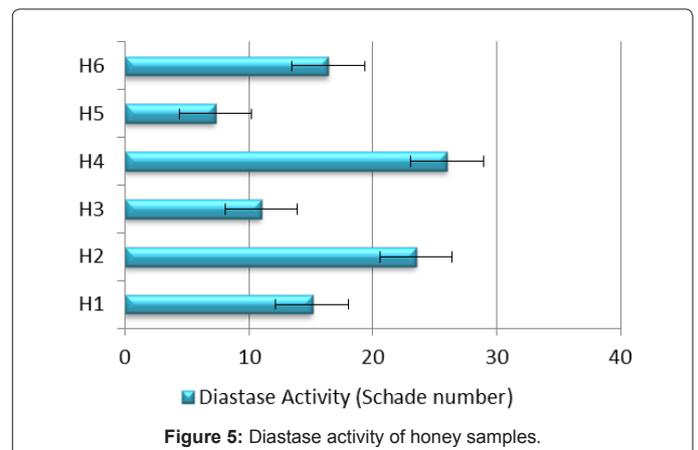
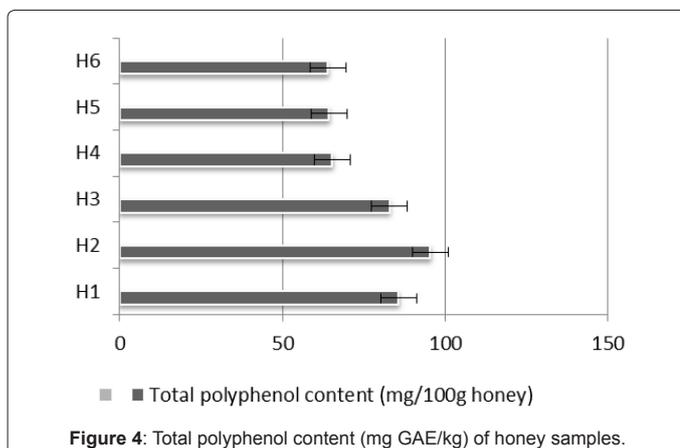
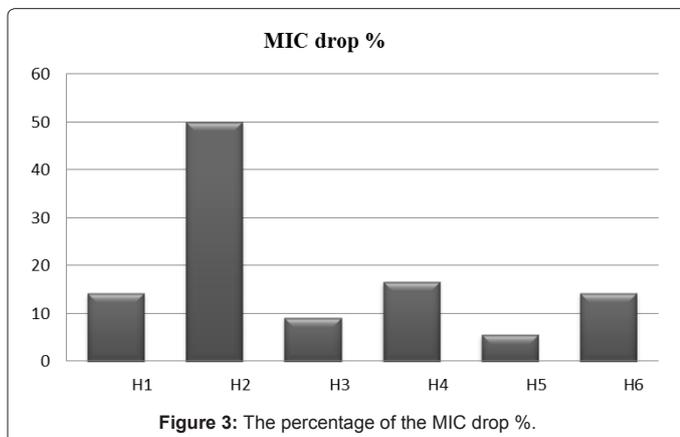
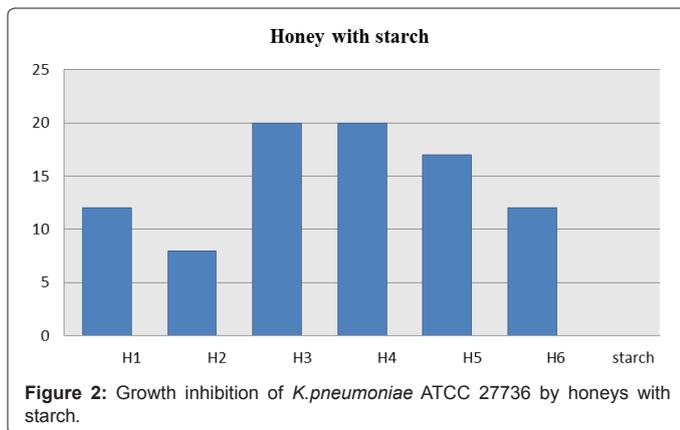


Figure 1: Growth inhibition of *K.pneumoniae* ATCC 27736 by honeys without starch.

1-3). The antimicrobial activity of honey against several pathogens and its dependence on the floral origin has been widely reported [23,24]. Several activities of honeys that contribute to their antibacterial effect are now well known. Osmolarity is an important factor in the efficacy of honey when used as an antibacterial agent for skin wounds [25]. A recent study by Ahmed et al. [26] has been demonstrated that osmosis determined the bactericidal effects of starch and honey towards *Escherichia coli* and *Pseudomonas aeruginosa*. The use of sugar for the treatment of infected wounds was investigated *in vitro* experiments with bacteria pathogenic to humans, such as *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumoniae* [27]. Amylases present in honey were expected to split starch chains to randomly produce



dextrin and maltose and probably increase the osmotic effect in the media by increasing the amount of sugars and consequently increase the antibacterial activity [28]. The mechanism of honey antibacterial activity is complex and might be attributed to the synergistic activity between its various potent biological ingredients such as polyphenols and flavonoids [29,30]. Flavonoids are the most common polyphenols and are widely studied as α -amylase inhibitors [31]. However, in the present study, No significant correlation was established between α -amylases activity and bioactive compounds.

Conclusions

In conclusion, the results of this preliminary study highlight synergy between honey and potato starch, when used against *K. pneumoniae* which could be harnessed to improve their antibacterial capacity.

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Conflict of Interest

We declare that we have no conflict of interest.

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