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EVALUATION OF ANTI-UROPATHOGENIC BACTERIA ACTIVITY OF ALGERIAN HONEY

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ABSTRACT

The study investigated the antibacterial effect of honey against uropathogenic bacteria isolated from clinical samples (*Escherichia coli*, *Morganella morgani*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Serratia fonticola, Proteus mirabilis* and *Pseudomonas aeruginosa*). A total of four natural Algerian honeys (NAHs) were investigated for their antibacterial activity against some uropathogenic isolates. They were analyzed for their color, total phenolic content (TPC) and total flavonoid content (TFC). Two different assays were performed to evaluate the antibacterial potential of the NAH samples: agar-well diffusion and disc diffusion methods. Undiluted and two-fold serial dilutions of NAH (50, 25 and 12.5%) were tested to determine zone of inhibition diameters (ZID). Antibiotic susceptibility profiling were performed according to CA-SFM (Antibiogram committee of French society of microbiology). Results showed that TPC values ranged from 0.682 mg GAE/g for NAH1to 0.510 mg GAE/g for NAH2. TFC ranged from 0.166 mg QE/g (NAH1) to 0.102 mg QE/g (NAH4).The color intensity of the honey samples ranged from 1.10±0.11 to 1.44±0.03 mAU. The diameter of ZDI ranged from 7.5 to 13 mm for *Escherichia coli*, 8–13 mm for *Morganella morgani*, 6.8-13.40 mm for *Klebsiella pneumoniae,* 8-13.4 mm for *Enterobacter cloacae*, 8-13.5 mm for *Serratia fonticola,* 7.4-14.5 mm for *Proteus mirabilis* and 7.6-13.2 for *Pseudomonas aeruginosa.* The highest activity was induced by NAH3, followed by NAH1, NAH4, and NAH2. The clinical isolates of enterobacteriaceae had a higher resistance profile than other Gram-negative bacteria for most commonly prescribed antibiotics. The overall results of this study indicated that Algerian honey could be used to treat urinary tract infections (UTIs) caused by the tested bacteria.

Keywords: Honey; antibacterial potential; uropathogenic bacteria; antibiotic; susceptibility.

INTRODUCTION

Gram-negative bacteria have become the main problem of UTIs in the last years. The epidemiology of these infections varies with age, sex and the presence of underlying genitourinary abnormalities [1].

The primary etiological agents are the gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, and the Gram-positive bacteria *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Staphylococcus aureus* [2-3]. Frequent antibiotic treatment of urinary tract infections has resulted in the emergence of antimicrobial resistance, necessitating alternative treatment options [4-5]. This situation has forced scientists to research and test new antibacterial substances from various sources including bee natural antibacterial products (BNAP) [5].

BNAP represents a rich source of antimicrobial agents and less toxic option

compared to most conventional therapies. A large number of researchers in different countries has studied the effects of BNAP [6-7]. Several research studies of natural honey have confirmed its biological properties, such as anti-bacterial and anti-biofilms properties [8]. Algerian Sahara honey has recently shown excellent antibacterial activity against multi-drug resistant clinical isolates from community-acquired UTIs [9]. In this context, the aim of the present study was to evaluate the *in vitro* antibacterial effect of four NAHs against *Enterobacteriaceae* and *Pseudomonas aeruginosa* (*P.aeruginosa*) uropathogens isolated from clinical UTIs, for a possible use as an alternative treatment.

MATERIALS AND METHODS

Four (04) natural multifloral honey samples were collected from different geographical areas of Algeria. The samples were stored at 4°C in dark conditions until analysis.

Color intensity: ABS $_{720-450}$

The color intensity of the NAH samples was determined using the method described by Beretta et al. [10]. Honey samples were diluted to 50% (*w/v*) with warm milli Q water (45– 50°C). Solutions were then filtered through a 0.45 lm filter. As all the commercial samples were no crystalline liquid honeys, there was a complete absence of coarse particles in the honey solutions. The absorbance was measured using a spectrophotometer at 720 and 450 nm and the difference in absorbance was expressed as mAU.

Determination of the Total Phenolic Content (TPC)

To determine the TPC of the honey extracts, the Folin-Ciocalteu method was applied [11-13]. Briefly, 200 µl of honey solution was mixed with 500 µl of Folin–Ciocalteu reagent (10%). The solution was thoroughly mixed by vortexing, and incubated for 5 min at ambient temperature. Before using, 1500 µl of sodium carbonate solution (7.5%) was added to the reaction mixture and further incubated for 30 min 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 (0–0.1 mg/l). The mean of at least three readings was calculated and expressed as mg of gallic acid equivalents (GAE)/g of honey.

Determination of the Total Flavonoid Content (TFC)

The total flavonoid content was measured by a colorimetric assay, based on the method of Ordonez et al. [14], with some modifications. An aliquot (1.5 mL) of the appropriately diluted honey extracts (1/10 g/v) was mixed with 1.5 mL of 2% aluminum chloride. After 60 min of incubation at room temperature, the formation of the complex was measured at 420 nm. The TFC was expressed as mg cateching equivalent (mg)/100 g of honey.

Preparation of Honey Solutions

Immediately before the use, honey solutions were prepared to the required concentrations (undiluted, 50%, 25% and 12.5% *v/v*). All samples were then incubated for 30 minutes at 37°C in a shaking water bath that allowed aeration of the solutions. Incubation was carried out in the dark giving that both hydrogen peroxide and glucose oxidase are light sensitive [15].

Antimicrobial Screening

Test organisms

The NAH samples were tested against six uropathogenic bacteria: *Escherichia coli (E.coli)* (n=10), *Morganella morganii (M. morganii)* (n=1), *Klebsiella pneumoniae (K. pneumoniae)* (n=2), *Enterobacter cloacae* (*E. cloacae)* (n=2), *Serratia fonticola (S. fonticola)* (n=1), *Proteus mirabiis (P. mirabilis)* (n= 1) and *Pseudomonas aeruginosa (P. aeruginosa)* (n=1). Bacterial strains were provided by a private laboratory in Tiaret district, Algeria.

Preparation of standard inocula

Prior to the experiment, the bacterial strains were inoculated onto the surface of nutrient agar media. The inoculum suspensions were obtained by taking five isolated colonies from 24 h cultures. The colonies were suspended in 5 mL of sterile saline (0.85% NaCl) and shaken for 15 seconds. The density was adjusted to the turbidity of a 0.5 McFarland Standard (equivalent to 1–5 × 10^8 cfu/mL).

Agar well diffusion method

Antibacterial assay was carried out by modified method of Moussa et al. [16]. Nutrient agar plates (Merck, Germany) were inoculated over the entire surface of the plate by rubbing sterile cotton swabs that were dipped into bacterial suspensions cultures grown at 37°C on nutrient agar and adjusted to 0.5 McFarland in sterile saline. After inoculation, 8 mm diameter wells were cut into the surface of the agar using a sterile cork borer. 50 µl of the corresponding honey was added to each well. Plates were incubated at 30°C for 24 h. Zones of inhibition were measured by holding a ruler on the underside of the petri dish. 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.

Agar disc diffusion method

All honey samples were tested by agar disc diffusion assay as described by Ahmed et al. [17]. Briefly, 100 µL of fresh culture suspension of tested microorganisms was spread on the respective media nutrient agar plates. Sterile filter paper discs (5 mm diameter) were impregnated with 10 µL of corresponding NAH equivalent to 0.1 mg of honey after being placed on the surface of the inoculated media agar plates. The plates were kept at 4°C for 2 h before being incubated under optimum conditions at 37°C for 24 h. Clear inhibition zones around the discs indicated the presence of antibacterial activity. The diameters of the inhibition zones were measured in millimeter, including the diameter of the disc. Negative controls were set up with equivalent quantities of water.

Antimicrobial susceptibility test

Antibiotic susceptibility profiling were performed according to CA-SFM (Antibiogram committee of French society of microbiology) [18]. The drugs that were tested include: Ampicillin (AMP), Amoxicillin/Clavulinicacid (AUG), Ticarcillin (TIC), Piperacillin/Tazobactam (TZP), Cephalothin (CTH), Cefoxotin (CXT), Cefotaxim (CTX), Ceftazidim (CAZ), Ertapenem (ETP), Imipenem (IMI), Amikacin (AMK), Gentamicin (GEN), Tobramycin (TOB), Nalidixic acid (NAL), Ciprofloxacin (CIPRO), Ofloxacin (OFX), Nitrofurantoin (NIT) and Sulfamethoxazole/Trimethoprim (SXT).

RESULTS AND DISCUSSION

This study examined the physicochemical properties and the antibacterial activity of a number of Algerian honeys.

Color Intensity

The color intensity of the honey samples ranged from 1.1 to 1.44 mAU (Fig. 1).

The ABS_{450} is a reliable index for confirming the presence of pigments with antioxidant activities, such as carotenoids and some flavonoids [19]. The color depends on its botanical origins. In addition, honey color is usually correlated with its mineral content [20].

Total Phenolic Content and Total Flavonoid Content

The TPC and the TFC ranged from 0,682 mg GAE/g to 0,510 mg GAE/g and 0,166 mg QE/g to 0,102 mg QE/g, respectively. TPC and TFC were highest in NAH1 (Fig. 2).

The results herein are in accordance with those of Khalil et al. [21] and Aissat et al. [22], who found a high level of polyphenols and flavonoids in the studied Algerian honeys.

TPC and TFC have been considered as the potential markers of honey botanical origins that contribute to honey color, taste and flavor as well as to their beneficial effects on health [23].

Antimicrobial Screening

In this study, initial screening with the agar-well diffusion assay demonstrated that all tested honeys had an antibacterial activity against uropathogenic isolates. All of the different types of honey were found to inhibit the growth of all tested bacteria.

The Diameter of zone of inhibition (mm) in different concentration of honey is displayed in Table 1.The antibacterial activity of NAHs at 100% concentration against *P.mirabilis* is higher when compared to previously published results on the flower honey at the same concentration.

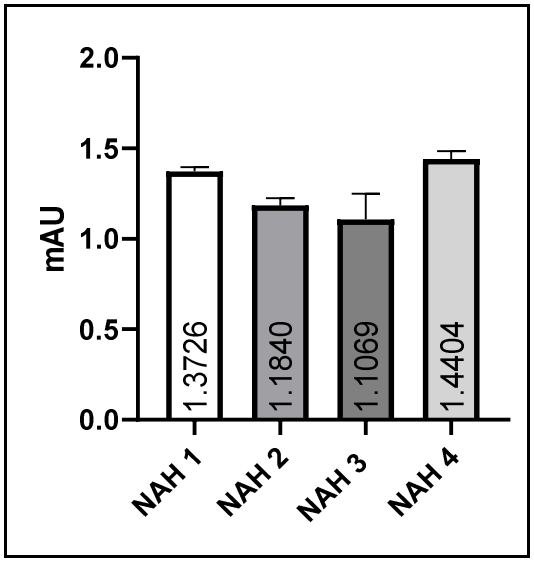

Fig. 1. Color intensity of different honey samples

These differences could be due to the origins of the honey samples and the content of their bioactive compounds (Phenolic compounds, hydrogen peroxide (H_2O_2), non-peroxide components including methylglyoxal, leptosin, melanoidins and the antimicrobial peptide bee defensing-1. [24-27]. *P. aeruginosa* was reported to be resistant to honey by Efem [28]; in contrary to this result however, the bacteria was sensitive to all honeys tested in our study. Result was also supported by the study done in other part of Algeria by Ahmed et al. [29]. The results concerning *E. coli* are

Table 1. Diameter (mm) of zone of inhibition produced by various NAH

Honey samples		*E. coli (N=10)*		*M. morganii (N=01)*		*K. pneumoniae (N=02)*		*E. cloacae (N=02)*		*S. fonticola (N=01)*		*P. mirabilis (N=01)*		*P. aeruginosa (N=01)*	
		Well	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**
NAH1	**100%**	13	11.4	12.5	11	13.16	10.6	13	12	13.5	11.5	14	12	12.8	10.4
	50%	10.8	9.8	11	10	11.10	8.8	11.2	10.2	11.5	10.5	11.2	9.6	11.2	9.4
	25%	9.5	8.8	10	9	9.6	7.6	10	9	10.5	9.5	9.6	8	9.8	8.4
	12.5%	8.8	7.6	9.5	8.5	8.2	6.8	9.2	8.4	10.5	9	8..8	7.6	8.2	7.6
NAH2	**100%**	13	11.8	12.5	10.5	12.8	11	12.8	11	13.5	11	14	11.8	12.2	11
	50%	11	10.5	11	10	10.4	9.4	10.8	9.8	11.5	10	11.8	10	10.6	9.8
	25%	9.6	9.2	10	8.5	9.4	8	10	8.8	10.5	9	10	8.8	9.6	8.8
	12.5%	8.4	8	9.5	8	8.2	7.2	9.6	8.4	9.5	8.5	8.8	7.6	9.2	7.8
NAH3	**100%**	12.5	12.8	13	11	13.4	10.6	13.4	11.6	12.5	10.5	14.5	12.4	13	11.2
	50%	10.6	10.8	11.5	9.5	10.8	9	11.6	10.2	11.5	9.5	11.8	10.4	11.2	9.8
	25%	9.2	9.2	10.5	8.5	9.4	8	10.2	9	10.5	9	10	8.8	10	8.8
	12.5%	8.2	8.2	9.5	8.5	8.8	7.4	9	8.4	10	8	8.8	7.4	8.6	8.4
NAH4	**100%**	12	12.4	12	11	13	10.8	13.4	11.4	12.5	11	13.2	11.8	13.2	10.8
	50%	10.8	8.8	10.5	9.5	10.8	9.2	11.4	10	11	9.5	11	10	10.8	9.4
	25%	9.6	9.4	10	8.5	9.2	7.8	10.6	9	10.5	9	9.6	8.6	9.8	8.4
	12.5%	8.6	8.6	9	8	8.2	7.4	9.6	8	10	8	8.8	7.6	8.8	8

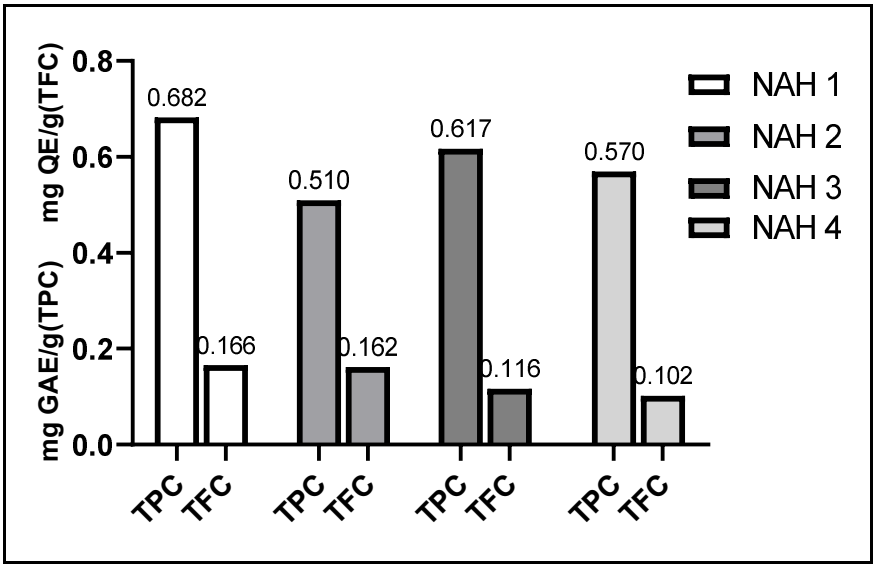

Fig. 2. Total phenols (TPC) and flavonoids content (TFC) in honey samples.

similar to those reported by Ahmed et al. [30], which found that different Algerian honey samples varied in the inhibitory effects on *E. coli*. The diameter of zones of inhibition are dependent on the honey concentrations.

Ahmed et al. [30] described the synergistic antibacterial effect of Sahara honey and curcuma starch considered the advantage of honey over other antibacterial agents. This is in accordance with what has been reported by other authors, who used honey as an agent to inhibit biofilm formation by *E. coli, P. aeruginosa*, *P. mirabilis* and *K. pneumoniae* [30-33].

The recent research indicates that the effectiveness of honey in many of its medical uses is due to its antibacterial activity that is capable of inhibiting Gram-positive and Gram-negative bacteria [34].

In addition, Wasihun and Kasa [35] compared *in vitro* antibacterial activity of several types of honey evaluated against multidrug resistant human pathogenic bacterial isolates (*Staphylococcus aureus*, *E. coli*, *P. aeruginosa*, *P. mirabilis*, coagulase-negative *Staphylococcus sp.*, *Streptococcus pyogenes* and *K. pneumoniae*). Enterobacteriaceae isolates had a higher resistance profile than other gram-negative bacteria for most commonly prescribed antibiotics. *E. coli* showed high percentage of resistance to ampicillin (96%) and sulfamethoxazole/trimethoprim (70%), and was sensitive to amikacin and imipenem. *P. mirabilis* showed high percentage of resistance to ofloxacin (100%), ampicillin (92%), ticarcillin (80%) and cephalothin (76%). *K. pneumoniae, E. cloacae, P. aeruginosa* and *M. morganii* showed high percentage of resistance to ampicillin (100%).

CONCLUSION

This study proved that the tested NAHs could be used as an alternative anti-

bacterial agent to prevent and treat some urinary infectious diseases limiting consequently the expansion of antibiotic resistance. However, before any *in vivo* application, it will be necessary to conduct clinical trials and further *in vitro* tests on urinary cell cultures.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Noureddine Djebli designed the study. Abdelmalek Meslem performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Idir Benbelkacem managed the analyses of the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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