ORIGINAL ARTICLE

Evaluation of the microbiological quality of some natural honeys and determination of their antibacterial activity

Evaluation de la qualité microbiologique de certains miels naturels et détermination de leur activité antibactérienne

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Abstract

Antibiotic resistance is a major concern for health systems worldwide, due to the irrational use of antibiotics. Honey can be considered an alternative therapy to antibiotics. Our study aims to evaluate the microbiological quality and presumed antibacterial activity of 7 natural honeys of different floral origins. Aerobic bacteria grew in 3 samples of honey: (jujube honey (*Bacillus subtilis*), fig honey (*Staphylococcus hominis, S. epidermidis, and S. pettenkoferi*) and holm oak honey (Aerococcus viridans)). Only holm oak honey contained a yeast (*Cryptococcus neoformans*), while the other honeys were free of yeasts and molds. Holm oak honey was most effective on most bacterial strains studied, particularly S. pyogenes. The other honeys were mainly active against *S. pyogenes*. None of the honeys showed activity against *P. aeruginosa, K. pneumoniae, S. aureus and C. albicans*. The MIC was 25% for holm oak honey against the 5 bacterial strains which showed a response. For fig honey, the MIC varied between 50% (*S. pyogenes, S. aureus and S. epidermidis*) and 100% (*S. hominis and E. coli*). For most other honeys, the MIC was 100%, meaning only the pure form of honey could prevent bacterial growth. In this study, honey, consistent with its traditional use in the ENT sphere, was found to be effective against *S. pyogenes*, a bacteria often responsible for bacterial tonsillitis which can limit the inappropriate use of antibiotics. These results could guide pharmaceutical research aimed at extracting active ingredients from honey to develop new antibacterial agents.

Keywords: antibiotic resistance, honey, microbiological quality, antibacterial activity.

Résumé

L'antibiorésistance est une préoccupation majeure des systèmes de santé dans le monde entier, elle est due à l'usage irrationnel des antibiotiques. Le miel peut être considéré comme une alternative thérapeutique aux antibiotiques. Notre étude vise à évaluer la qualité microbiologique et l'activité antibactérienne présumée de 7 miels de différentes origines florales. Des bactéries aérobies ont poussé dans 3 échantillons de miel : (miel de jujubier (*Bacillus subtilis*), miel de figue (*Staphylococcus hominis, S. epidermidis et S. pettenkoferi*) et miel de chêne vert (*Aerococcus viridans*)). Seul le miel de chêne vert contenait une levure (Cryptococcus neoformans), tandis que les autres miels étaient exempts de levures et de moisissures. Tous les miels étaient surtout actifs sur S. pyogenes. La CMI était de 25% pour le miel de chêne vert vis-à-vis des 5 souches bactériennes qui ont montré une réponse. Pour le miel de figue, la CMI variait entre 50% (*S. pyogenes, S. aureus et S. epidermidis*) et 100% (*S. hominis et E. coli*). Pour la plupart des autres miels, la CMI était de 100%, ce qui signifie que seule la forme pure du miel pouvait empêcher la croissance bactérienne. Dans cette étude, le miel, conforme à son utilisation traditionnelle en sphère ORL, s'est révélé efficace contre S. pyogenes, une bactérie souvent responsable des angines bactériennes ce qui pourrait limiter le recours inapproprié aux antibiotiques. Ces résultats pourraient orienter des recherches pharmaceutiques visant à extraire des principes actifs à partir du miel pour développer de nouveaux agents antibactériens.

Mots clés : Antibiorésistance, miel, qualité microbiologique, activité antibactérienne.

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Introduction

Antibiotic resistance is a major concern for health systems throughout the world. This public health problem is essentially due to the irrational use of antibiotics, some of which have become ineffective against several bacterial strains, hence the interest in finding effective therapeutic alternatives to limit and optimize the use of antibiotics which must be reserved for documented bacterial infections and after an antimicrobial susceptibility testing. Among the therapeutic alternatives to overcome this scourge we find antimicrobial peptides (1), the use of bacteriophage viruses having lytic cycles to destroy pathogenic bacteria (2), or the use of monoclonal antibodies directed against specific antigens of certain bacteria (3), but all these means of antibacterial control remain expensive means, while there are other cheaper products with fewer adverse effects such as plants (phytotherapy) (4), essential oils (aromatherapy) (5) or even honey which is a beehive product used for a long time to treat some benign ailments such as tonsillitis or sore throats (6). Honey is a natural sweet substance produced by bees from the nectar of flowers after chemical transformations followed by a process of dehydration and maturation at the hive level, the composition of honey is conditioned by several factors, namely the origin of floral nectar, water content, enzymatic arsenal of foraging bees, temperature and ventilation of the hive, physiological state of the colony, as well as weather conditions during harvest (7). Honey is essentially composed of carbohydrates, the two main ones being glucose and fructose, water, and organic acids, the most important of which is gluconic acid derived from glucose under the effect of a bacterium, gluconobacter (8) hence the acidic pH of honey (3.2 to 4.5), honey contains flavonoids which give it its specific color, honey also contains enzymes, the most important of which are invertase and amylase, Honey also contains minerals, the main one being potassium, as well as trace elements. Proteins, amino acids, lipids and vitamins are in small quantities (9). Honey has its own organoleptic (color, appearance, odor, texture and taste) and physicochemical (pH, electrical conductivity, solubility, density, viscosity, fluorescence and crystallization) characteristics (10). The sources of honey contamination are multiple, we can subdivide them on the one hand into microbiological contamination coming either from the environment or human practices, especially at the time of harvest where the honey can be contaminated by the beekeeper himself and on the other hand to chemical contamination, especially by pesticides (11)(12).

Honey has various therapeutic properties, the most important of which is its antimicrobial effect (antibacterial, antifungal, antiviral) which we will be developed in this work, but the other effects, namely the healing effect and the anticancer effect for the most important are not negligible either (13)(14)(15)(16). The antimicrobial effect is marked above all by the antibacterial activity, the antifungal effect of honey is observed especially on yeasts of medical interest, the antiviral effect has been tested with good results on viruses of the herpesviridae family (17)(18)(19)(20)(21).

The purpose of our study is to evaluate the microbiological quality of some natural honeys, and also the determination of their presumed antibacterial activity.

Methods

Honey samples

This is a prospective study involving 7 samples of honey from different floral origins (fig honey from Kahf El Ghar (Honey 1), arbutus honey from Taïnaste (Honey 2), bupleurum honey from Bouiblane (Honey 3), oak honey from Tazekka (Honey 4), jujube honey from Tazekka (Honey 5), eucalyptus honey from Sidi Slimane (Honey 6), jujube honey from Laioune sidi mellouk (Honey 7)).

Microbiological quality

We carried out a study of the microbiological quality by inoculating these honeys in their pure state and after decimal dilutions using physiological serum (to reduce the number of microorganisms per unit volume in order to facilitate microbiological examination) on different appropriate culture mediums (Mueller-Hinton agar Oxoid Ref: CM0337B, Columbia Blood Agar Base Oxoid Ref: CM0331B with blood, …), the protocol used was developed by analyzing several previous studies, in particular the study by Kamal and al. 2019 (22). The reading of the inoculated boxes will be carried out after 24 hours, 48 hours and 72 hours of incubation of the stock solution and the corresponding dilutions of each type of honey. The identification includes a macroscopic, microscopic examination, and enzymatic tests (catalase, oxidase), as well as a certainty identification based on the mass spectrometry (MALDI-TOF), the microorganisms sought are the bacteria of the total mesophilic aerobic flora (TMAF) which are capable of multiplying aerobically at temperatures between 20 and 45°C and fungal flora which includes yeasts and molds.

Antibacterial activity

The antibacterial activity of these honeys was studied against reference bacterial strains (ATCC = American Type Culture Collection) and clinical strains isolated from biological samples from our laboratory. The designed strains are: Escherichia Coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 29213, Klebsiella pneumoniae ATCC 27853, Streptococcus pyogenes (Clinical strain isolated on superficial pus, May 2023 by MALDITOF), Staphylococcus aureus (Clinical strain isolated on deep pus, July 2023 by MALDITOF), Staphylococcus hominis (Clinical strain isolated on ascitic fluid, August 2023 by MALDITOF), Staphylococcus epidermidis (Clinical strain isolated on superficial pus, October 2023 by MALDITOF) and Candida albicans (Clinical strain isolated on urine, November 2023 by MALDITOF).

To determine this antibacterial activity, we used two

methods (well diffusion method and liquid dilution method (MIC)).

Well diffusion method

Honey samples were prepared at different concentrations 100%, 75%, 50%, 25% by dissolving the honey with physiological water (0ml, 0.5ml, 1ml and 1.5ml) to obtain a 2ml preparation. 80µl of each honey at different concentrations (100%, 75%, 50% and 25%) was deposited in the wells after seeding the bacterial strains. For each strain to be tested, control experiments (wells without honey) were performed.
The Petri dishes were refrigerated (4°C) for 8 hours for pre-diffusion before incubation at 37°C for 24 hours. Reading after 24 hours and determination of the inhibition diameters if they exist (23).

Determination of the MIC (Dilution in liquid medium "microplates")

Incubation of a standardized inoculum of each bacterial strain with increasing concentrations (25%, 50%, 75%, 100%) of each honey and determination of the MIC which is the lowest concentration of honey able to inhibit all visible growth of the bacterial strain.

Results

Microbiological quality

This parameter makes it possible to assess the microbiological purity of these honeys (22). Indeed, we were able to notice the presence of total mesophilic aerobic bacteria in 3 samples of honey (jujube honey from Laioune sidi mellouk with a bacterial load greater than 105 CFU/ml, fig honey from Kehf El Ghar with 4.104 CFU/ml of bacterial load and Tazekka holm oak honey with 104 CFU/ml of bacterial load). However, the other honeys were free of these germs (Total Mesophilic Aerobic Flora).

Table 1. Different isolated germs from the honey samples studied

Samples	Total mesophilic aerobic flora CFU/ml	Yeasts CFU/ml	Molds CFU/ml
Honey 1	**4. 10^4**	-	-
Honey 2	-	-	-
Honey 3	-	-	-
Honey 4	**104**	**30**	-
Honey 5	-	-	-
Honey 6	-	-	-
Honey 7	**> 10^5**	-	-

Concerning the micromycetes, only Tazekka Holm Oak Honey contained a yeast (Cryptococcus neofromans) identified by mass spectrometry (MALDI-TOF: MALDI Biotyper® Sirius, BRUKER MBT Compass HT IVD, Version 5.2.320) with 2,36 as Confidence Score, However, all the other honeys contained neither yeasts nor molds. The confidence score is an index that allows us to decide on the accuracy of the germ identification. The higher this index is (>2), the more we are 99.99% sure that the identified germ is correct.
MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) is a robust, rapid, and cost-effective technique used for the accurate identification of bacteria and fungi. Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and the ratio of their mass-to-charge (m/z) can be measured. In MALDI-TOF mass spectrometry, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is a time-of-flight (TOF) analyzer (24).
To identify the bacterial strains that grew on the culture mediums inoculated with these honeys, we first carried out a macroscopic examination of the colonies, a microscopic examination after Gram staining, then we completed the identification with enzymatic testing (catalase, oxidase), in order to have an orientation, but the identification of certainty was carried out by the mass spectrometry (MALDI-TOF).
Honey 1 contained the following germs (*Staphylococcus hominis, S. epidermidis, and S. pettenkoferi*), Honey 4 contained Aerococcus viridans, while Honey 7 contained Bacillus subtilis.

Table 2. Identification of isolated bacterial species

Samples	Isolated bacterial species (MALDI-TOF)
Honey 1	*Staphylococcus hominis* (Confidence Score = 2,34)
	Staphylococcus epidermidis (Confidence Score = 2,28)
	Staphylococcus pettenkoferi (Confidence Score = 2,38)
Honey 4	*Aerococcus viridans* (Confidence Score = 2,18)
Honey 7	*Bacillus subtilis* (Confidence Score = 2,22)

Honey 1 : Fig honey ; Honey 4 : Oak honey ; Honey 7 : Jujube honey.

Antibacterial activity

Well diffusion method

Streptococcus pyogenes (CHU) was inhibited by all honeys tested at their different concentrations, with inhibition diameters ranging from 31mm to 41mm. *Staphylococcus aureus* ATCC 29213 was inhibited only by fig honey in its pure state (100%) with an inhibition diameter of 11mm, and to oak honey at its different concentrations 100%, 75%, 50% and 25% with respective inhibition diameters of 22mm, 18mm, 15mm, and 11mm. *Staphylococcus hominis* (CHU) had the same profile as *S. aureus* ATCC 29213 with an inhibition diameter of 10mm in response to pure fig honey (100%), and inhibition diameters of 18mm, 17mm , 15mm and 10mm in response to the respective concentrations of 100%, 75%, 50% and 25% of oak honey. The last two strains did not respond well to honeys, namely *Staphylococcus epidermidis* (CHU) which responded only to fig honey and oak honey in its pure state (100%) with respective inhibition diameters of 11mm and 14mm, while fig honey had no activity on *Escherichia coli* ATCC 25922 which responded to oak honey and eucalyptus honey in their pure state (100%) with the same inhibition diameter of 15mm.

Table 3. Inhibition diameters (mm) of the different bacterial strains studied produced by the 7 honey samples and their dilutions.

Bacterial strain	Honey concentration	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5	Honey 6	Honey 7
Streptococcus pyogenes (CHU)	100%	40 mm	41 mm	40 mm	40 mm	40 mm	39 mm	36 mm
	75%	38 mm	39 mm	39 mm	39 mm	38 mm	37 mm	34 mm
	50%	35 mm	35 mm	38 mm	37 mm	36 mm	35 mm	33 mm
	25%	33 mm	33 mm	32 mm	32 mm	33 mm	33 mm	31 mm
Staphylococcus aureus ATCC 25922	100%	11 mm	-	-	22 mm	-	-	-
	75%	-	-	-	18 mm	-	-	-
	50%	-	-	-	15 mm	-	-	-
	25%	-	-	-	11 mm	-	-	-
Staphylococcus hominis (CHU)	100%	10 mm	-	-	18 mm	-	-	-
	75%	-	-	-	17 mm	-	-	-
	50%	-	-	-	15 mm	-	-	-
	25%	-	-	-	10 mm	-	-	-
Staphylococcus epidermidis (CHU)	100%	11 mm	-	-	14 mm	-	-	-
	75%	-	-	-	-	-	-	-
	50%	-	-	-	-	-	-	-
	25%	-	-	-	-	-	-	-
Escherichia Coli ATCC 25922	100%	-	-	-	15 mm	-	15 mm	-
	75%	-	-	-	-	-	-	-
	50%	-	-	-	-	-	-	-
	25%	-	-	-	-	-	-	-

The other bacterial strains studied (Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC 27853, Staphylococcus aureus (CHU) and Candida albicans (CHU)) were completely insensitive to the 7 honey samples tested in our study.

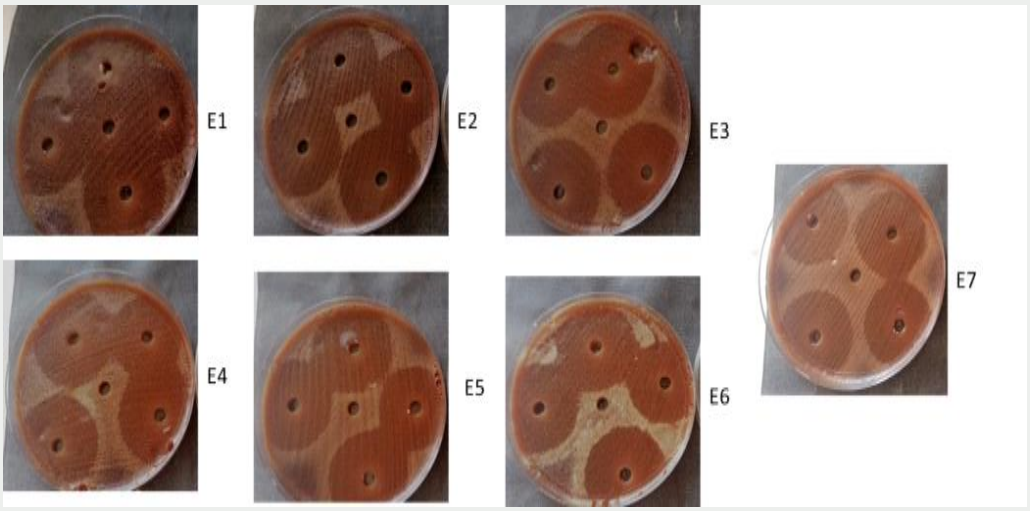

Figure 1. Inhibition diameters of Streptococcus pyogenes in response to the 7 honey samples and their dilutions.

E1: Sample 1 (Fig honey); E2: Sample 2 (Arbutus honey); E3: Sample 3 (Bupleurum honey); E4: Sample 4 (Oak honey); E5: Sample 5 (Jujube honey); E6: Sample 6 (Eucalyptus honey); E7: Sample 7 (Jujube honey).

Determination of the MIC (Dilution in liquid medium "microplates"):

Concerning Streptococcus pyogenes (CHU) the MICs were 25% for oak honey, 50% for fig and arbutus honeys and 100% for bupleurum, Tazekka jujube, eucalyptus and jujube tree from Laioune. For Staphylococcus aureus (ATCC) and Staphylococcus epidermidis (CHU) the MICs were 50% for fig honey and 25% for oak honey. For Staphylococcus hominis (CHU) the MICs were 100% for fig honey and 25% for oak honey. Finally, the MICs were 25% for oak honey and 100% for eucalyptus honey for Escherichia coli (ATCC).

Discussion

Honey is a natural product from the hive, it is generally unfavorable to the development of microorganisms thanks to its high sugar content, its acidic pH, its low water activity and its content of antimicrobial substances (10)(18). However, honey may be contaminated by microorganisms from unavoidable sources such as pollen, nectar, the digestive tract of bees, dust or even air and soil, but other sources of contamination are avoidable such as contamination at the time of harvest, handling or storage.

Table 4. Determination of the Minimum Inhibitory Concentrations (MIC) of the 7 honey samples.

Bacterial strain	Minimum Inhibitory Concentration (MIC)						
	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5	Honey 6	Honey 7
Streptococcus pyogenes (CHU)	50%	50%	100%	25%	100%	100%	100%
Staphylococcus aureus ATCC 25922	50%	-	-	25%	-	-	-
Staphylococcus hominis (CHU)	100%	-	-	25%	-	-	-
Staphylococcus epidermidis (CHU)	50%	-	-	25%	-	-	-
Escherichia Coli ATCC 25922	-	-	-	25%	-	100%	-

These contaminations caused by human acts can be avoided by adopting drastic hygiene measures during the harvest and handling of these honeys (11)(12). Indeed, in our study, some of these honeys were contaminated by mesophilic bacteria and yeasts.

Concerning the microbiological quality part of our study, we noted the presence of aerobic mesophilic bacteria in 3 samples of honey, namely jujube honey which contained Bacillus subtilis, fig honey which contained Staphylococcus hominis, S. epidermidis and S. pettenkoferi, as well as holm oak honey which contained Aerococcus viridans. Mycologically, only holm oak honey contained Cryptococcus neoformans which is an ubiquitous yeast, however, all other honeys tested were free of yeasts and molds. The bacteria and yeast isolated in our study are not strictly pathogenic bacteria which shows that the hygienic conditions were not systematically respected during the harvest of these honeys, also the honeys tested were free of coliforms which rules out the possibility of fecal contamination, other studies have found similar results with the isolation only of non-pathogenic and non-coliform bacteria, This shows that the honey harvest was carried out in relatively good conditions and that these honeys were free from any fecal contamination (25)(26), however, if the hygienic conditions during harvest, handling or the storage of honey were defective, pathogenic microorganisms or coliforms can reside there as was the case in certain studies which were able to isolate Salmonella spp, Shigella spp, Clostridium spp or other coliforms (27)(28)(29).

The second part of this study focuses on the antibacterial activity of the tested honeys, in fact, this activity showed variability depending on the kind of honey used, but also depending on the bacterial strains tested. Of the 7 honeys in the study, only holm oak honey (Honey 4) showed antibacterial activity on the 5 responding bacterial strains. While the other honey samples tested were more or less active on certain bacterial strains of the 5 responding strains but completely inactive on others, as was the case for example with arbutus honey (Honey 2) which was active on Streptococcus pyogenes (CHU), but completely inactive against Staphylococcus aureus (ATCC), Staphylococcus hominis (CHU), Staphylococcus epidermidis (CHU) and Escherichia coli (ATCC). This strong effectiveness of holm oak honey (Honey 4) is perhaps due to the floral origin of this honey, which pushes us to deepen the research on this honey in order to determine the different active ingredients that are present in this honey and which give it this marked antibacterial activity.

Concerning the bacterial strains tested, only Streptococcus pyogenes (CHU) showed a blatant sensitivity to all honeys studied at their different concentrations, which conforms with the use of honey in traditional medicine to treat bacterial tonsillitis which are for the most part due to the Streptococcus pyogenes germ (Group A Streptococcus according to the Lancefield classification (30)). The relief of symptoms caused by tonsillitis after using honey is probably due to the inhibition of the bacterial growth of this Streptococcus, this inhibition which will be more or less effective depending on the kind of honey used, because the MICs inhibiting this bacterium were variables depending on the honey tested with better effectiveness of holm oak honey (Honey 4) against this germ in our study. The sensitivity of the other responding bacterial strains was variable to these different honeys tested, with a noted effect of fig honey (Honey 1) and especially of holm oak honey (Honey 4). These observations push us to expand research into other kinds of honey to test their effects on bacterial strains that did not respond well to some or all of the honeys in our study.

Conclusion

The antibacterial activity of honey has been highlighted during this study, especially against Streptococcus pyogenes, and this activity was conditioned by the floral nature of honey. So, the traditional use of honey to treat certain conditions such as tonsillitis is fully explained by this study, and this use helps avoid the need for inappropriate administration of antibiotics. This study could also lead us towards a path of fundamental research with the objective of isolating active ingredients from different honeys in order to use them as antibacterial agents to overcome this problem of antibiotic resistance and thus limit the use of conventional antibiotics to only the documented cases.

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