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Study of the antibacterial properties and the physical chemistry of Moroccan and French honeys[1]

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Introduction

The international institutions (Codex Alimentarius, 1981 and European Pharmacopoeia, 2008)[2] agree to define honey as a natural sweet substance produced by honeybees. The raw materials are the nectar of plants or honeydew, a sweet sticky substance excreted by aphids or other sucking insects and often deposited on leaves and stems. The bees collect these materials, to transform them in honey by combining with their own specific substances. Finally the honey is dehydrated and stored in the honeycomb to ripen and mature. Honey is a hyper-osmotic solution, mainly composed of water (14 to 25%) and sugars, glucose and fructose being the main ones with a total sugar content of 95 to 99%. Many other compounds participate to the complex composition of honey: organic acids, amino acids and proteins, minerals, lipids, enzymes, pigments, flavors and flavonoids. Depending on composition, season and origin, honey displays various aspects considering both consistency and color.

Since the prehistoric times, man is interested in this product and the medicinal activities were recognized since ancient times thanks to the Dioscorides' treatise "De Materia Medica". Thus, we traditionally know honey as a topical agent and more specifically as a healer. The earliest record of its use in a wound treatment is an inscription on a fragment of a clay tablet dated to approximately 4500 years ago that described a recipe for an ointment. Tradition did not really change nowadays. Even if the place of honey has changed, we now mainly know

[1] "Les données issues du travail d'Audrey Crousilles sur les propriétés antibactériennes des miels sont la propriété du laboratoire de bactériologie de la faculté de pharmacie de Montpellier où ont été réalisées toutes les analyses".

[2] European Pharmacopoeia, 7th Edition, Honey, 2008, n°2051, p. 2163-2165.

it for its nutritional properties or as a sweetener, it is still perceived as a wound treatment. Scientific research was interested in the biological activities of honey in the seventies (Sedova et al., 1973[3]), and the studies are still ongoing to explain its effectiveness in therapeutics (Kwakman et al., 2012). Therefore, we are rediscovering honey to manage burn or infection wounds and published works present honey as an alternative for the treatment of these wounds (Molan, 2002).

Morocco is a great producer of honey, and this one occupies a particular place in the culture. Nourished by the sacred aspect in the Koran (which partially explains the effectiveness of it), honey is very used by the population as a first therapeutics. Traditional medicine is perennial because of this strong habits and knowledge of healers. Local populations primarily use plants and honey for therapeutics but also for economic reasons. Thus, honey is used to treat all the diseases touching all the biological spheres, and even as a complementary treatment to cancer.

In a general point of view, this work fits to the renewal of interest for natural resources. Indeed, we deal with the characterization of honey activities in order to bring new insights about a daily used natural product. The main objective of this study is focused on the antibacterial activity of honey. We proposed an *in vitro* analysis of honeys from south of Morocco in order to characterize their antimicrobial activity on a large spectrum of bacteria including both reference strains and natural isolates. Chestnut and oak honeys from Languedoc-Roussillon (France) have been previously studied (Ferrier, 2010) and displayed a particular antimicrobial activity. These honeys have been included in this study in order to compare their activity to that of honeys from south of Morocco. Owing to the main medical use of honey in wound dressing and healing, the experiments developed in this course have focused on pathogenic bacteria that can be found on wound infections and their complications. A chemical approach provides further

[3] Sedova N. N., Usmanov M. F. et al., The antimicrobial properties of several types of honey from Uzbekistan, Voprosy pitaniia, 1973, vol. 32, no 2, p. 84.

data about the physicochemical parameters that gave some clues about the biological activity of honey.

1 – Materials and Methods

1.1 Labs contexts

The work presented here was carried out under the leadership of bacteriology and analytical chemistry laboratories. Bacteriology laboratory is oriented to the pathogen-environment interface. This topic is therefore fully in this perspective through work on the antibacterial properties. Background works on antimicrobial activity of honey resumed mainly French honeys, particularly from the Languedoc Roussillon region (Ferrier, 2010)[4]. The Bromatology Laboratory is focused on the sanitary quality of food products. In this context, honey is now considered as a product subjected to high anthropogenic pressures (use of pesticides, standards, interest in natural product).

1.2 Honey samples and sugars standard solution

The honeys (without any traceability document) presented in the Table below (Table 1) were from a sampling campaign achieved in Morocco in June and July 2012; French honeys were from supermarkets or local markets.

For the microbiological experiments, we prepared aseptically a stock solution at 0.8 g/mL for each honey. Then these solutions and the honeys were stored at 5°C in the dark in order to use them extemporaneously.

[4] Ferrier J., Étude de l'effet antibactérien de différents miels pyrénéens, thesis, 2010.

Code	Plant origin, English name *(Scientific name)*	Arabian name	Geographic origin, Harvest year
Thy1	Thyme (*Thymus sp.*)	Tazukunnit	Igri, 2011
Thy2	Thyme (*Thymus sp.*)	Tazukunnit	Igri, 2004
Thy3	Thyme (*Thymus sp.*)	Tazukunnit	Imouzzer, 1987
Fle	Flowers	Ashdig	Igri, 2008-2009
Thu	Cedar (*Tetraclinis articulata*)	Azuka	Imouzzer, 2011
Ora	Orange tree (*Citrus sp.*)	Limon	Imouzzer, 2012
Carou	Carob (*Ceratonia siliqua*)	Carub	Tamri, 2011
Eu1	Spurge (*Euphorbia officinarum*)	Tikiut	Igri, 2009
Eu2	Spurge (*Euphorbia officinarum*)	Tikiut	Mesti, 2011
Char	Thistle (*Eryngium sp.*)	Tasenent	Tamri, 2010
Juju	Jujube (*Zizyphus sp.*)	Azugar	Imouzzer, 2008
Azu	Sugar syrup given to the bees as supply	Azucar	Souk of Agadir
Amse	*Ceratolimon feei* var. *grandiflorum*	Amslig	Mesti, 2010
Iki	Spurge (*Euphorbia officinarum*)	Ikiu	Aït-Oussa, 2011
Balsa	Balsamic spurge (*Euphorbia balsamifera*)	Talalt	Around Mesti, 2011
Rico	Flowers	-	Lozère, 2012 (48)
Chê	Oak (*Quercus sp.*)	-	Les Butineuses Catalanes (66)
ChaA	Chestnut (*Castanea sativa*)	-	Lluch, Saint Jean de Védas (34)
ChaB	Chestnut (*Castanea sativa*)	-	Miellerie Ardéchoise, Miel d'Ardèche (07)
ChaC	Chestnut (*Castanea sativa*)	-	Maître Rucher, Fuilla (66)

Table 1: Characteristics of the studied honeys

The control solution reflected the major sugars composition of honey (based on the mean analysis of sugars in bromatology laboratory). It was prepared aseptically by the mix of 1.75 g of glucose, 1.75 g of fructose and 0.5 g of sucrose in 2.5 mL sterile water. The solution was leave at room temperature for 24 to 48h to achieve the complete dissolution and also stored in the fridge.

1.3 Bacteriological study

1.3.1 Microbial strains and culture media

In order to study the MIC, bacterial (n=15) and fungal (n=4) strains have been used and were presented in table 2. Seven reference strains used for antimicrobial essays in pharmaceutical industry and control are included (Table 2). As mentioned in Table 2, all the clinical strains have been collected on wounds or injuries, and were sometimes involved for septic complications (see *Propionibacterium acnes*). All the strains have been pricked out on Tryptic-Soy Agar (TSA) except for *Propionibacterium acnes*, which required media enriched with 5% of sheep's blood (blood sheep agar). *Candida*

albicans strains have been isolated on Sabouraud medium. After incubation for 24h to 37°C, all the strains (bacterial and fungal) were prepared in Mueller-Hinton nutrient Broth (MHB) except for *P. acnes* which is sub- culturate in Brain Heart Infusion (BHI).

Species/Strain	Type	Origin	Antibiotics resistance phenotype
Staphylococcus aureus ATCC 6538	Cocci/Gram+	Reference strain for antimicrobial assays	Wild type, MSSA
Staphylococcus aureus MRSA 1508	Cocci/Gram+	Clinical, burn wound	Methicillin-resistant, MRSA
Staphylococcus aureus GISA T29-1	Cocci/Gram+	Clinical, nostril	Glycopeptide intermediate, GISA
Pseudomonas aeruginosa ATCC 9027	Bacilli/Gram-	Reference strain for antimicrobial assays	Wild type
Pseudomonas aeruginosa PAB04	Bacilli/Gram-	Clinical, burn wound	Multiresistant and resistant topical agents
Pseudomonas aeruginosa PAB13	Bacilli/Gram-	Clinic, burn wound	Wild type
Escherichia coli ATCC 8739	Bacilli/Gram-	Reference strain for antimicrobial assays	Wild type
Escherichia coli B2502	Bacilli/Gram-	Clinical	Extended-Spectrum Beta-Lactamase (multiresistance)
Escherichia coli B1525	Bacilli/Gram-	Clinical	TEM Beta-Lactamase
Enterococcus faecalis ATCC 29212	Cocci/Gram+	Reference strain for antimicrobial assays	ND
Enterococcus faecium B2477	Cocci/Gram+	Clinical scarpa post surgery infection	ND
Acinetobacter baumanii ATCC 13932	Coccobacilli/Gram-	Reference strain for antimicrobial assays	Wild type
Acinetobacter baumanii B2335	Coccobacilli/Gram-	Clinical, burn wound	Multiresistant
Aeromonas hydrophila BVH45	Bacilli/Gram-	Clinical, open wound abscess	Wild type
Propionibacterium acnes B1097	Bacilli/Gram+	Clinical, post surgical wound with sepsis	Wild type
Candida albicans ATCC 10231	Yeast	Reference strain for antimicrobial assays	ND
Candida albicans n°1	Yeast	Clinical	ND
Candida albicans n°14	Yeast	Clinical	ND
Aspergillus niger ATCC 16404	Filamentous fungus	Reference strain for antimicrobial assays	ND

Table 2 : Characteristics of the studied bacterial and fungal strains
(ND : Not Determined)

1.3.2 Minimal Inhibitory Concentration (MIC) determination

The MICs were determinated using microbroth dilution methods in polypropylene micro-plates (NUNC®) 96 wells with flat bottom. An example of MIC plate scheme was given in Supplementary figure 1. For each experiment, the stock solution of honey was diluted serially in water. Considering the previous dilution of the stock solution the serial dilutions of honey were 41%, 20%, 10%, 5%, 2.5%, 1.25%, 0.65%, 0.32% and 0.16% (w/v). Each MIC experiments were performed in triplicate, which corresponded to 3 plates for one strain and one honey.

The bacterial load is prepared into a trypton-salt solution from a stationary phase culture 24h adjusted to 0,5 McFarland, using a spectrophotometer (620 nm), in order to obtain a suspension corresponding to 10^8CFU/mL. This suspension was 100x diluted (100 µL of this suspension into a final volume of 10 mL of MHB or BHI for *P. acnes*) to obtain a final concentration of 10^6CFU/mL. One hundred microliters of this final suspension were loaded in each of the 96 wells of the plate previously filled with 100 µL of serially diluted honey. The plates were incubated at 37°C for 18 to 24h and the bacterial growth was evaluated visually by the observation of a turbidity higher than that observed in the negative control well inoculated with honey and MHB or BHI without bacteria.

1.3.3 Minimal Bactericidal Concentration (MBC) determination

Mueller-Hinton Agar plates (MHA) are inoculated by 1 µL of the suspension of each MICs well (see above) after incubation of 24h at 37°C. The inoculation has been achieved with a multiplier unit (Inoculator MIC-2000, Dynatech Product®), which deposits about 1 µL per drop. Bacterial survival was evaluated by the observation of a colony at the inoculation point after an overnight incubation at 37°C.

1.3.3 Time-kill kinetic study

The bacterial suspension was adjusted to 0.5 McFarland with

the spectrophotometer. 80 µl of the suspension was diluted into a final volume of 4 mL of MHB. Aliquots of 300 µL of this suspension were distributed into 1.5 mL microtubes (Eppendorf®) according to the filling scheme presented in the supplementary Figure 2. Microtubes were incubated in a shaking bain-marie (37°C) through all the time of the experimentation. We followed the kinetic of growth in the times t0, t1h, t4h, t6h, and t24h. At each time 11 µL of suspension of each tube were diluted into 99 µL of ultrapure water, and then make a 1/100 dilution. Finally, 100 µL of the latter suspension were inoculated on TSA dishes and incubated for 24h at 37°C. The colony counting was performed using a device Bioblock Scientific® Colony Counter.

1.4 Chemical study

1.4.1 Extraction Attempts

Several extractions had been tested to avoid the important content of sugars, thus the viscosity, and to allow the injection of the samples.

Liquid-Liquid extraction (L-L): We prepared several samples by the mix of 8 g of honey, with 8 mL of methanol, ethyl acetate or acetonitrile. This solution was heated and mixed. The extraction conditions were 2h, at 40°C and 300 rpm.Another protocol was asking fewer reagents by adding 1 to 2 mL of solvent (ethyl acetate, dichloromethane, hexane, xylene) to test different polarity on 1 g of honey. We shook by hand and waited for clarification.

SPE extraction: The columns Oasis Waters® HLB 1cc were used following the protocol provided. They needed a conditioning step (methanol), then a washing (water) and the samples have been treated (Posyniak at al., 2002)[5]. These last were prepared from 1 g of honey added with 2 ml of water or buffer following the perspectives considered.

[5] Posyniak A., Sniegocki T. et Zmudzki J., Solid phase extraction and liquid chromatography analysis of sulfonamide residues in honey, Bulletin-Veterinary institute in Pulawy, 2002, vol. 46, n°1, p. 111-118.

SPME Headspace extraction: Following the same principle than the L-L extraction, we heated the honey/water mix and introduced the retractable fiber (silica + polymers) into the space above the liquid level. The extraction lasted 3h, at 40°C and 300 rpm. Then, this device has been used directly as an injection system for GC-MS.

1.4.2 Physicochemical measurements

Four of the primary characteristics have been studied: pH, redox potential, H_2O_2 content and color of honey.

pH measurement : Initially, we prepared 2 g of honey into a test tube and added 400 µL of distilled water (DW). With as many tubes as necessary filled with 500 µl of DW, we made a series of 2-fold dilutions by transferring 500 µL from the previous tube (same distribution than the plate layout). pH measurement was performed using a calibrated electrode, and then reading the value after stabilization of the device.

Redox potential measurement: The same samples than the ones for the pH are used for the experiment, but into a glovebox and with nitrogen pressure. Then redox potential was performed using a calibrated electrode checked with Whitney buffers, and the values are read after stabilization of the device.

H_2O_2 measurement: The hydrogen peroxide is studied with the colorimetric method using specific strips (Peroxide test MERCK®).

Color study: Based on the accepted standards in the United States (USDA, 1985)[6], we used the Pfund® scale as a relative evaluation (no standardized conditions applied, e.g. sample thickness). Moreover, it exists a correspondence with the Lovibond® scale. It simply required placing the honey next to the color that best suited it.

[6] USDA Agricultural Marketing Service, 1985, United States Standards for Grades of Extracted Honey. 1985, USDA, Washington DC.

1.4.3 GC-MS Analyzes

The apparatus used for these experiments is a gas chromatograph TRACE GC Ultra ThermoScientific®, coupled with a mass spectrometer quadripole published by the same company. The apparatus and the resulting data are processed using Xcalibur software ThermoScientific®. The injection volume was 2 µL and was in split mode at 250°C. The carrier gas was helium with a flow rate of 1 mL/min. The oven temperature ranges from 50 to 300°C.

2. Results

2.1 Antimicrobial effect of honeys

2.1.1. Inhibition of microbial growth : bacteriostatic effect of honey

MIC is the lowest concentration of honey (highest dilution's value) that inhibits any visible bacterial growth. It then evaluates the bacteriostatic effect of honey.

Results were presented in Table 3. First of all, we observed that all the honeys inhibited microbial growth at 41% (as observed by Malika et al., 2004)[7] or below whereas the control sugar solution not always inhibited the growth and at the best the inhibition occurred in the 21% concentrated well. These results are consistent with those previously obtained in the laboratory. We showed that a high concentration of sugars could not effectively inhibit bacterial growth suggesting that honey inhibited microbial growth by another mechanism than hypertonicity. Although we saw variable results with a same honey and a same strain, inter- assays variations remained acceptable because differences did not exceed one dilution. We observed that the fungi were not sensitive to the honeys tested in this study. The MIC values observed are not higher than those observed for the sugar control solution.For the majority

[7] Malika N., Faid M. et El Adlouni C., Antimicrobial activities of natural honey from aromatic and medicinal plants on antibio-resistant strains of bacteria, Int. J. Agric. Biol, 2004, vol. 6, p. 289-293.

of honey samples the MIC values were between 41% and 20%. Referring to published studies, we considered that a MIC above 10% was notable.

On the other hand, the comparison between honeys reveals interesting elements. Globally, in the first series of honey, MIC values are 41% or 20% (ranges 2 to 9 in Table 3). Only two values reached 10% for Thy1 on *Pseudomonas aeruginosa* B13 and *Acinetobacter baumanii* ATCC. In general, the honeys of the first series do not stand out particularly from the sugar control. On the opposite, the second series is remarkable (ranges 10 to 17 in Table 3). We obtained for the thistle, jujube and sugars honeys MIC values equivalent to sugar control but other honeys of this second series displayed significant antimicrobial activities (bold in Table 3). The spurge, endemic of the country, and particularly Eu2, displayed MICs of 10% on clinical strains of *S. aureus*, *Aeromonas*, and *Acinetobacter*. MICs of Amse are quite low with 5% on the strains of *S. aureus*, *E. coli*, *Acinetobacter* and *Aeromonas*. Concerning Iki, it was most effective on *Acinetobacter baumanii* and *Aeromonas hydrophila* clinical species.

The third set includes all French honeys. We noted that these honeys have results beyond the average of other Moroccan honeys of the first series (the less effective one). In particular Chê, ChaA and ChaC honeys. These MICs are ranging from 10% to 5% on the reference and clinical strains of *S. aureus*, *E. coli*, *Acinetobacter* and *Aeromonas*.

The best results now. They concern the balsamic spurge honey (Balsa). First, we note that this honey inhibited the growth of all bacteria. The lowest MIC is 10%, for *Pseudomonas aeruginosa* and *P. acnes*. We observe very low MIC values 2.5% on *S. aureus*, *E. coli A. baumanii* and *A. hydrophila*. A MIC value of 1.25% was reached for Balsa on the strain GISA T29-1. This efficiency was particularly interesting considering the high level of resistance of this strain. The results presented herein showed that multi resistant strains such as *S. aureus* methicillin-resistant or GISA, *E. coli* producing BLSE, multiresistant *P. aeruginosa* were inhibited at the same MICs

or lower than their sensible counterparts.

P. aeruginosa, P. acnes and *Enterococcus* appeared as the less sensitive bacteria but again Balsa presented a significant activity on these species or genus. On the contrary, *S. aureus, E. coli, A. hydrophila, A. baumanii* displayed a global sensitivity with very low MIC values for some honeys. We could notice that Gram negative bacteria seemed more susceptible (Malika at al., 2004)[8]. Indeed, *P. aeruginosa* was inhibited by seven honeys, *E. coli* by five, *Acinetobacter* has remarkable MIC values for eight honeys and *Aeromonas* for nine of them while Gram positive bacteria inhibited less honeys but with MIC values lower for some particular strains such as *S. aureus* GISA T29-1.

Finally, we observed that the bacterial behavior facing honey activity varies according to bacteria and honey types

	Staphylococcus aureus ATCC 6538	*Staphylococcus aureus* 1508	*Staphylococcus aureus* GISA T29-1	*Pseudomonas aeruginosa* ATCC 9027	*Pseudomonas aeruginosa* B04	*Pseudomonas aeruginosa* B13	*Escherichia coli* ATCC 8739	*Escherichia coli* BLSE B2502	*Escherichia coli* NBLSE B1525	*Enterococcus faecalis* ATCC 29212	*Enterococcus faecium* B2477	*Acinetobacter baumanii* ATCC 13932	*Acinetobacter baumanii* B2335	*Aeromonas hydrophila* BVH 45	*Propionibacterium acnes* B1097	*Candida albicans* ATCC 10231	*Candida albicans* n°1	*Candida albicans* n°14	*Aspergillus niger* ATCC 16404
Sugars	x	x	x	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	x	x	41%	41%
Balsa	**2.5%**	**2.5%**	**1.25%**	**10%**	**10%**	**10%**	**2.5%**	**5%**	**2.5%**	**5%**	**5%**	**2.5%**	**2.5%**	**2.5%**	**10%**	41%	41%	41%	41%
Sugars	x	x	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	x	41%	x	x	41%
Thy1	20%	20%	20%	20%	20%	**10%**	20%	41%	41%	20%	20%	**10%**	20%	20%	**10%**	41%	41%	41%	41%
Thy2	20%	20%	20%	20%	20%	41%	20%	41%	41%	20%	20%	41%	41%	20%	41%	41%	x	41%	20%
Thy3	41%	41%	41%	20%	20%	41%	41%	41%	41%	41%	41%	41%	41%	20%	20%	41%	x	41%	20%
Fle	41%	20%	20%	20%	20%	20%	41%	41%	41%	41%	41%	41%	20%	20%	20%	41%	x	41%	41%
Thu	41%	20%	20%	20%	20%	20%	41%	41%	41%	41%	20%	20%	20%	20%	20%	41%	x	41%	x
Ora	41%	20%	20%	20%	20%	20%	41%	41%	41%	41%	41%	20%	41%	20%	x	41%	x	41%	41%
Carou	41%	41%	41%	41%	20%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%
Sugars	x	x	x	41%	41%	41%	41%	41%	41%	41%	41%	41%	41%	20%	41%	x	x	x	41%
Eu1	20%	20%	20%	20%	ND	**10%**	20%	41%	41%	20%	20%	20%	20%	**10%**	41%	41%	41%	41%	41%
Eu2	**10%**	**10%**	**10%**	**10%**	**10%**	20%	20%	20%	20%	41%	20%	**10%**	**10%**	**10%**	41%	41%	x	x	41%
Char	20%	41%	20%	41%	20%	20%	41%	41%	41%	41%	41%	20%	20%	20%	20%	41%	41%	41%	41%
Juju	41%	41%	41%	20%	20%	20%	41%	41%	41%	41%	41%	20%	20%	20%	20%	41%	x	x	41%
Azu	41%	41%	41%	41%	41%	20%	41%	41%	41%	41%	41%	41%	41%	20%	41%	x	x	x	41%
Amse	**5%**	**5%**	**5%**	**10%**	**10%**	**10%**	**10%**	**5%**	**5%**	20%	20%	**5%**	**5%**	**5%**	20%	41%	41%	41%	41%
Iki	41%	41%	41%	20%	**10%**	41%	20%	20%	20%	41%	20%	**10%**	**10%**	**10%**	41%	41%	41%	41%	41%
Sugars	x	x	x	20%	41%	41%	41%	41%	41%	41%	41%	41%	41%	20%	x	41%	x	x	41%
Rico	20%	**10%**	**10%**	20%	20%	20%	20%	20%	20%	41%	20%	20%	20%	**10%**	20%	41%	x	41%	41%
Chê	**10%**	**10%**	**10%**	20%	**10%**	20%	20%	**10%**	**10%**	20%	**10%**	**10%**	**10%**	**10%**	20%	41%	41%	41%	41%
ChaA	**5%**	**10%**	**10%**	20%	20%	20%	20%	**10%**	**10%**	41%	**10%**	**10%**	**5%**	**10%**	20%	41%	41%	41%	41%
ChaB	41%	41%	ND	20%	20%	20%	20%	41%	41%	41%	41%	20%	20%	20%	41%	41%	x	x	41%
ChaC	**10%**	**10%**	**5%**	20%	20%	20%	**10%**	**10%**	**10%**	20%	20%	**10%**	**10%**	**5%**	20%	41%	x	x	41%

Table 3 : MIC values for the 15 bacterial and 4 fungal strains on the 20 honeys
(ND : Not Determined)

	Staphylococcus aureus ATCC 6538		*Staphylococcus aureus* 1508		*Staphylococcus aureus* GISA T29-1		*Pseudomonas aeruginosa* ATCC 9027		*Pseudomonas aeruginosa* B04		*Pseudomonas aeruginosa* B13		*Escherichia coli* ATCC 8739		*Escherichia coli* BLSE B2502		*Escherichia coli* NBLSE B1525		*Enterococcus faecalis* ATCC 29212		*Enterococcus faecium* B2477		*Acinetobacter baumanii* ATCC 13932		*Acinetobacter baumanii* B2335		*Aeromonas hydrophila* BVH 45		*Propionibacterium acnes* B1097	
	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB
Sugars	x	x	x	x	x	x	41%	41%	41%	41%	41%	41%	41%	x	41%	x	41%	x	41%	x	41%	x	41%	x	41%	x	41%	41%	41%	x	41%	x
Balsa	2.5%	5%	2.5%	5%	2.5%	2.5%	10%	10%	10%	20%	10%	20%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	10%
Eu2	10%	10%	10%	10%	5%	5%	20%	20%	20%	20%	20%	20%	20%	20%	20%	NE	20%	20%	41%	x	20%	x	10%	10%	10%	10%	10%	10%	20%	41%
Amse	5%	5%	5%	5%	5%	5%	10%	10%	20%	20%	20%	20%	10%	10%	10%	10%	10%	10%	20%	x	20%	x	10%	10%	5%	5%	5%	5%	20%	20%
Iki	10%	10%	5%	5%	5%	5%	20%	41%	20%	20%	20%	20%	20%	20%	20%	20%	20%	20%	20%	x	20%	x	10%	10%	10%	10%	10%	10%	41%	41%
Chê	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	20%	10%	20%	20%	20%	20%	20%	20%	20%	10%	20%	10%	10%	10%	10%	10%	10%	20%	20%
ChaA	10%	10%	5%	5%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	20%	20%	10%	20%	5%	5%	5%	5%	5%	5%	20%	41%
ChaC	10%	10%	5%	5%	5%	5%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	20%	20%	20%	20%	10%	10%	10%	10%	5%	5%	20%	41%

Table 4 : MBC values for the 15 bacterial strains on the 7 most efficient honeys
The recurrence of the MIC values is explained because of the duplication of the experiments

bacteria, Int. J. Agric. Biol, 2004, Vol. 6, p. 289-293.

confirming that the antimicrobial activity of honeys corresponded to a specific mechanism rather than a generalist activity such as hypertonicity.

2.1.2. Bactericidal effect of honeys

The bactericidy is evaluated by the determination of MBC. A product will be considered as bactericide if it leaves less than 0.01% of survivors after a contact of 18h. In our experiments, this means less than 10^{-4} surviving bacteria in the initial medium after contact with honey solutions at the CMI. The MBC determination was focused on the seven honeys with the best outcomes in MIC experiments: Balsa, Eu2, Amse, Iki, Chê, ChaA and ChaC. Results were given in Table 4.

As well as in MIC determination, we found that MBC values for the sugar control are the highest (41% or 20%). Again, we can say that the only high osmolarity cannot explain the bactericidy of honey.

Generally, there was a certain homogeneity and reproducibility in the results. Not only between Tables 3 and 4, representing the same MIC determination experiments performed at different times, but also between MIC and MBC. The values are mostly identical or differed by only one dilution MBC values higher than MIC values as expected. The MBC/MIC ratio was less than or equal to 2. The definition of the bactericidal activity states that a product has a bactericidal activity if MBC/MIC ! 2. Therefore, we demonstrated herein that antimicrobial effect of honeys was mostly a bactericidal effect.

The Gram positive coccus, *Staphylococcus aureus*, was the more sensitive species whatever the origin of honeys, with MIC and MBC reaching sometimes 2.5% in particular for balsamic spurge honey. Clinical isolates resistant to antibiotics reacted significantly to the dilutions of honey. It is noteworthy that multiresistant strains such as MRSA and GISA were more sensitive than wild type strains (MSSA). The highest MBC

values observed in these experiments amounted to 10%, mainly for French honeys or Moroccan spurge honey and for MSSA strain.

In comparison with *S. aureus*, the other Gram positive cocci i.e. *Enterococcus faecalis* and *Enterococcus faecium*, were relatively unaffected by honey since they displayed the highest MBC values. Moreover, we noted that there was no effect at all for three Moroccan honeys (Eu2, Amse and Iki) at the concentration of 41%. Other MBC values for French honeys had an average of 10%. Again, balsamic spurge honey standed out with MBC at 2.5%. The differential effect of honeys on Gram positive cocci very related regarding their cell structure clearly suggested that honeys displayed a very specific mechanism of action.

For the species *P. aeruginosa*, which is a Gram negative bacillus, we note that the values are among the highest, i.e. the honey activity was the lowest. Even the most effective honey observed previously (balsamic spurge) gives a maximum of 10% for the MBC. Whether they are reference or clinical strains, wild type or multiresistant, they reacted similarly to the dilutions of honey. The MBC values are also high for *Escherichia coli*. However, we note again a remarkable susceptibility of the balsamic spurge honey.

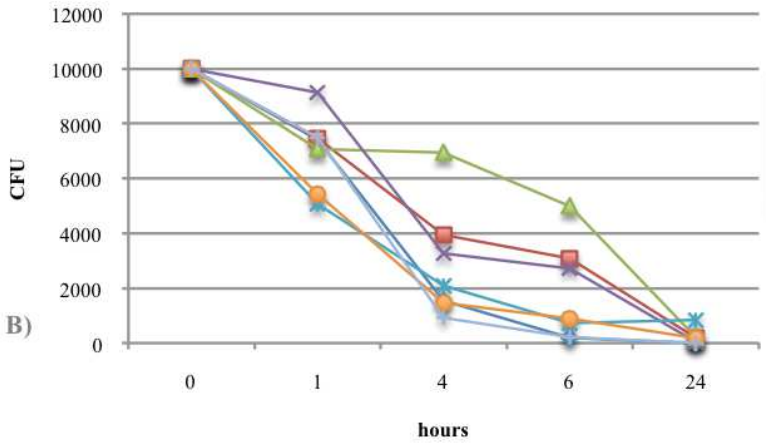
For both strains of *Acinetobacter baumanii*, we note - as most of the data previously mentioned - that the values are identical. MBC and MIC merge. Moreover, this bacterial species seems more susceptible to the effect of honey. Among the Gram negative bacteria tested in this study, they were those, which reacted to 5% Balsa, Amse and ChaA. The same 5% MBC value was observed for *Aeromonas hydrophila* in contact with, again, Balsa, Amse and ChaA, but also for ChaC.

Finally, the anaerobic species *Propionibacterium acnes* was poorly sensitive to most honeys (MCB oscillating between 10% and 20%) but better values were observed for the balsamic spurge honey with MBC at 5%.

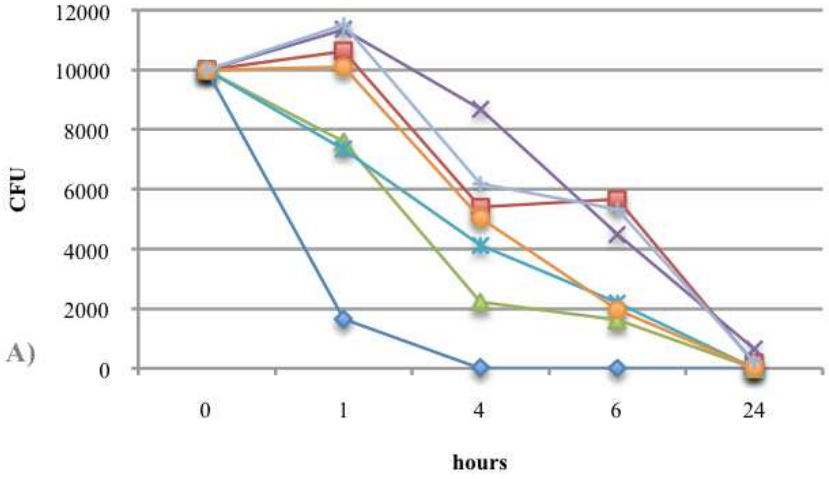
2.1.3. Bactericidal kinetics

After determining the bactericidal effect of honey, we investigated the bactericidal kinetics. Thus, as for the regular antibiotics found on the health market, two hypotheses are advanced. Is the effect, and then the kinetics, concentration-dependent or time-dependent? That's why we created a protocol able to answer these two questions at the same time. We finally investigated only *Staphylococcus aureus* strains because this approach is time-consuming and we underwent constraints in time and logistics.

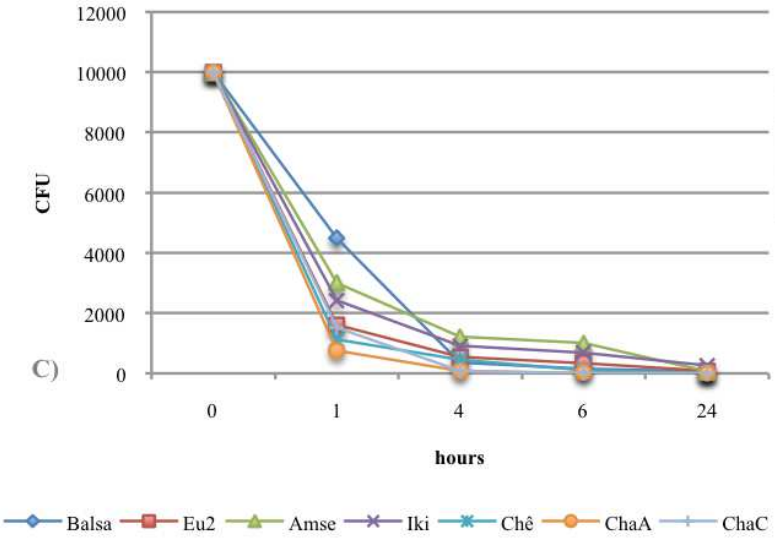
The results presented in Figure 1 are the mean curves for the honeys. Thus, we calculated these mean curves according to the tables of values collected for each experiment with one honey on one strain and on five to seven dilutions (see Supplementary figure 3).



	Balsa	Eu2	Amse	Iki	Chê	ChaA	ChaC
0	10000	10000	10000	10000	10000	10000	10000
1	7422	7454	7071	9131	5072	5426	7500
4	1551	3944	6945	3266	2083	1462	923
6	200	3074	4988	2729	730	896	213
24	0	192	209	46	835	180	1



	Balsa	Eu2	Amse	Iki	Chê	ChaA	ChaC
0	10000	10000	10000	10000	10000	10000	10000
1	1640	10619	7589	11353	7339	10089	11499
4	27	5402	2222	8678	4124	5036	6185
6	8	5659	1618	4486	2198	1955	5313
24	1	175	0	646	0	3	208



	Balsa	Eu2	Amse	Iki	Chê	ChaA	ChaC
0	10000	10000	10000	10000	10000	10000	10000
1	4493	1613	3012	2413	1119	758	1530
4	361	535	1206	906	457	81	83
6	160	344	1006	689	109	27	32
24	62	82	68	262	1	1	2

Figure 1 : Bactericidal kinetics on *Staphylococcus aureus* with the 7 most effective honeys
A) *Staphylococcus aureus* ATCC 6538
B) SAUR 1508
C) GISA T29-1

We showed different aspects for the bactericidal dynamics but globally, we observed a rapid and dramatic decrease of the

bacterial population, from 10^4 CFU to 0 CFU for some honeys and some strains (Balsa on MRSA 1508, Amse and Chê on MSSA ATCC 6538). Then surprisingly, honey seemed to be able to induce a bacterial decrease compatible with the bactericidal activity of antibiotics. Considering the more detailed results in Supplementary figure 3, we saw that the effect did not seem concentration-dependent but more likely time-dependent because the graphs for each honey concentration was similar. This result justified the calculation and the presentation in figure 1 of a mean graph for each honey. The graphs for each honey were pooled for a better visualization of the bactericidal effect on the three strains of *S. aureus*.

For MSSA ATCC 6538 first (Figure 1A), we observed a relatively slow decrease according time. We also noted a slight increase of the population after 1h for Eu2, Iki, ChaA and ChaC, whereas for the other honeys, the bacterial population decreased directly and throughout the day of experiment. Balsamic spurge honey had a particular effect that differed from other honeys. The decrease observed after 1h of contact is more marked and after 4h, the bacterial population was already as low as we could detect, i.e. less than 2700 CFU/mL. Iki honey gave the worst results for this strain; the decrease in bacterial load was slow. Indeed, the maximum effect observed in 1h with Balsa was observed in 24h with Iki. In between those two marked behaviours, we observed a nearly linear decrease for Amse and Chê whereas ChaA, Eu2 and ChaC presented more fluctuant effect.

For MRSA 1508 (Figure 1B) globally, all the curves show a complete decrease of bacterial load from 1h to 4h, the slope is more important than for MSSA strain. It is noteworthy that most honeys displayed a stronger effect on the antibiotic resistant MRSA strain than on the antibiotic susceptible MSSA. The sole exception was Amse that was clearly more effective on MSSA. From 4h to 24h, the slope was less important, and then the decrease in bacterial load was weaker. The bactericidal kinetics obtained for Balsa, Iki, Chê and ChaC were very encouraging. The other honeys are not as effective as the four

ones.

Considering *S. aureus* GISA T29-1 (Figure 1C), we observed the most spectacular results. The dramatical decrease in the first hour divided per three to six the bacterial load. After 1h, the slope is more flat, maybe because of the first strong decrease leaving a few bacteria to inhibit and also because the experimental limit of bacterial numbering was reached. However, Supplementary figure 3 gives more precisions about the real values for the decrease. We saw the majority of honeys bring down the burden to 0 or nearly 0 CFU Then, this strain showed a particular susceptibility to honeys whatever their geographical and botanical origins. In contrast to that observed for MSSA and MRSA, all the GISA curves displayed similar aspect for all honeys, suggesting another mechanism of action on this particular *S. aureus* strain.

2.2. Chemical Study

2.2.1. Physicochemical measurements

All the measurements exposed in Table 5 below are the chemical reflect of the bacterial process. This means that we took the values in the same conditions (with a 2-fold dilution) until the MIC observed and then 2 dilutions further.As a first point, we'll now see the pH. The acidity of honey is mainly due to organic acids, whether free or combined (Chakir et al., 2011)[9]. The pH values ranged from 3.65 (Balsa) to 6.03 (ChaA). The control solution is the highest value, with pH 6.48, thus nearly neutral; except it contains only sugars and therefore no organic acids. We can note that the French honeys, and especially the chestnut honeys are quite consistent. However, most of these honeys have a pH under 4, which definitely inhibit the bacterial growth. All the Moroccan honeys concentrate the values between 3.65 (Balsa) and 4.37 (Thy2). These values are consistent with the ones from other studies on

[9] Chakir A., Romane A., Marcazzan G. L. et al., Physicochemical properties of some honeys produced from different plants in Morocco, Arabian Journal of Chemistry, 2011.

the same honeys (Chakir et al., 2011 and Gulfraz et al., 2010)[10]. Moreover, the Moroccan honeys are mainly from herbal origin. Whereas the French honeys give higher values, from 4.62 to 6.03 and are from a forest environment. Then, we see a slight difference according to the vegetal origin, but this would definitely need further similar sampling to compare equivalent vegetal and geographical origin.

Honeys	pH						Redox potential (mV)						Color
	41%	20%	10%	5%	2.5%	1.25%	41%	20%	10%	5%	2.5%	1.25%	
Sugars control	6.48	6.60	6.65	-	-	-	175.2	166.2	157.2	-	-	-	ND
Balsa	3.65	3.75	3.90	4.05	4.25	4.64	146.0	145.1	138.4	135.2	130.8	123.7	white
Thy1	4.36	4.51	4.60	4.66	-	-	104.3	115.3	112.7	112.3	-	-	extra light amber
Thy2	4.37	4.46	4.54	-	-	-	145.2	135.1	129.1	-	-	-	amber
Thy3	3.73	3.79	3.93	-	-	-	118.9	127.4	138.0	-	-	-	amber
Fle	3.67	3.71	3.87	-	-	-	263.3	266.1	265.5	-	-	-	water white
Thu	3.79	3.80	3.93	-	-	-	179.4	177.3	197.2	-	-	-	extra light amber
Ora	3.77	3.79	3.90	-	-	-	201.1	217.7	228.1	-	-	-	ND
Carou	4,26	4,45	4,59	-	-	-	198.9	165.0	158.1	-	-	-	extra light amber
Eu1	3.90	4.02	4.09	4.16	-	-	131.7	129.4	125.7	128.2	-	-	light amber
Eu2	3.71	3.87	3.99	4.10	-	-	200.1	224.0	303.3	304.0	-	-	extra white
Char	4.01	4.10	4.17	4.24	-	-	178.1	161.8	157.2	150.4	-	-	extra light amber
Juju	3.88	4.03	4.13	4.23	-	-	161.0	154.5	163.8	162.3	-	-	white
Azu	3.91	3.95	4.08	-	-	-	158.4	200.6	209.4	-	-	-	ND
Amse	3.69	3.74	3.91	3.99	4.08	-	132.1	126.9	123.0	119.7	114.3	-	extra light amber
Iki	3.68	3.81	3.97	4.10	-	-	224.0	213.3	213.4	193.9	-	-	extra light amber
Rico	4.32	4.40	4.50	4.64	-	-	209.0	231.4	244.1	245.2	-	-	water white
Chê	4.62	4.71	4.82	4.96	-	-	182.9	198.1	256.2	254.1	-	-	white
ChaA	6.03	6.42	6.65	6.85	7.06	-	141.7	106.7	113.2	135.7	132.1	-	extra white
ChaB	4.95	5.07	5.19	-	-	-	197.2	188.0	190.4	-	-	-	extra white
ChaC	5.19	5.35	5.49	5.69	5.99	-	178.8	144.8	185.1	173.7	160.1	-	water white

Table 5 : pH, redox potential values and color for the 20 honeys and the sugars control solution
(ND : Not Determined)

Another characteristic is the redox potential. It measures the tendency of the solution to either gain or lose electrons when it is subject to change by introduction of a new species. The values oscillate from the lowest 104.3 mV (Thy1) to the highest 263.3 mV (Fle). We see that these values are very fluctuating. There is not consistence in these measurements as for the pH.

[10] Gulfraz M., Iftikhar F., Raja S. et al., Quality assessment and antimicrobial activity of various honey types of Pakistan, Afr J Biotech, 2010, vol. 9, p. 6902-6909.

Moroccan or French honeys have both low and high values. Moreover, the seven effective honeys (Balsa, Eu2, Amse, Iki, Chê, ChaA and ChaC) do not have particular values for this characteristic. The most effective one (Balsa) is part of the lower values but comparatively to the lowest values (such as Thy1, Thy3) it does not explain the effectiveness of honey.

Last point, the color. This one is explained because of the different vegetal origins. The nectar of each species collected by the bees is specific. And then, it contains several compounds giving his color to honey. We evaluated it with the Pfund® scale using about 1 g of honey into a test tube. Globally, the honeys available for this study fall into the category of light colors. French honeys are particularly light, with water white to white aspect. Some Moroccan honeys are a bit darker. Especially thyme honeys (Thy2 and Thy3), they are amber but on the lightest part of this category; whereas some other are too light to be determined with the scale (Ora and Azu).

2.2.2. H2O2 content

Following the MIC study, we investigated the H_2O_2 content at the values obtained for the seven most effective honeys. Plus some other honeys effective on several strains.

As we can see, the values are not equivalent and are ranging between 0 and 25 mg/L. H_2O_2 is proposed to be one of the antibacterial component of honey. And this peroxide is produced thanks to the glucose oxidase enzyme added to nectar by bees (Kwakman et al., 2012)[11].

But these results, and especially the values obtained for Balsa, Thy1 or Chê, are conversely. As Kwakman proposes it, with more H_2O_2, we could anticipate a better efficiency of honeys on bacterial strains. But several publications and the values obtained here are not consistent with this idea. Even if, the chestnut honey (ChaC) is very effective on bacteria, compared to the balsamic spurge one, the H_2O_2 content cannot explain

[11] Kwakman P. HS et Zaat S. AJ., Antibacterial components of honey. IUBMB life, 2012, vol. 64, no 1, p. 48-55.

thebacteriological observations. Considering the 3% hydrogen peroxide solution sold in pharmacy, representing ten O_2 volumes, it contains around 30 g/L of H_2O_2. We thus see, the relative dilution of this compound in honey.

Honey	Max dilution	H_2O_2 content (mg/l)
Balsa	1/64	0.5
Thy1	1/8	0
Eu1	1/8	2
Eu2	1/8	2
Amse	1/16	2
Iki	1/8	0.5
Rico	1/8	25
Chê	1/8	0.5
ChaA	1/16	2
ChaC	1/16	25

Table 6 Hydrogen peroxide content for the most effective honeys

2.2.3. Chemical aspects

Two honeys had been tested in GC-MS following the L-L extraction protocol, balsamic spurge (Balsa) and chestnut (ChaC) honeys. Consequently, for these two tests, four samples had been prepared (ethyl acetate, dichloromethane, hexane and xylene). In this stage, we wanted to investigate the honey composition. Exploring other compounds than sugars. Thereby, we will share an overview of the results obtained (Supplementary figure 4 for the chromatograms).

Considering Balsa, and the ethyl acetate sample, we find several components (Figure 2) with a major peak matching the 4-hydroxy-3,5-dimethoxybenzohydrazide (RT=32.41 min, $C_9H_{12}N_2O_4$). Following it several well defined peaks were forming a cluster between RT=44 et RT=54 min and composed of alkanes. We then find heptacosane (RT=47.18 min, $C_{27}H_{56}$), 17-pentatriacontene (RT=54.76 min, $C_{35}H_{70}$), ethyl iso-allocholate (RT=44.21 min, $C_{26}H_{44}O_5$).

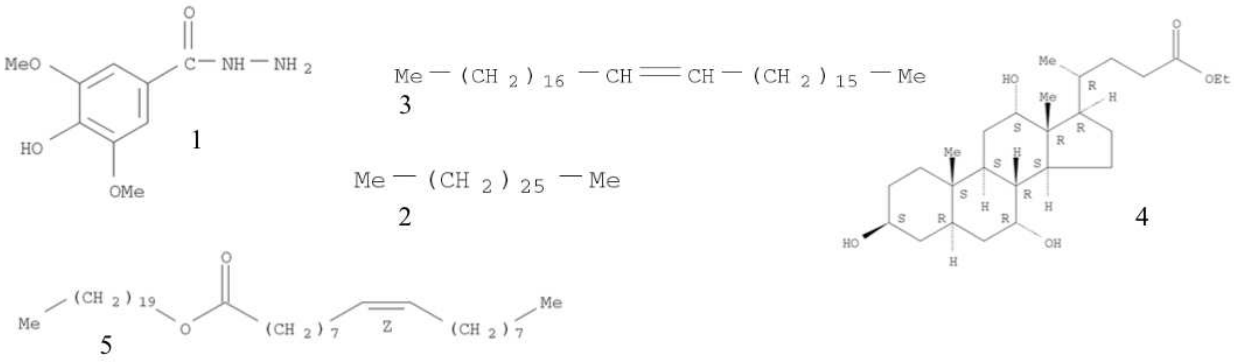


Figure 2 : Major components of the balsamic spurge honey (Balsa)
1) 4-hydroxy-3,5-dimethoxybenzohydrazide 2) heptacosane 3) 17-pentatriacontene
4) ethyl iso-allocholate 5) 9-Octadecenoic acid (9Z)-, eicosyl ester

The dichloromethane sample gives nearly the same compounds, even if we less find alkanes but more sterols between RT=54 and RT=58 min. However, we find again a peak with the 4-hydroxy-3,5-dimethoxybenzohydrazide (RT=32.61 min). But we find again also the ethyl iso allocholate (RT=54.73 min). An important peak represents the 9-octadecenoic acid (9Z)-, eicosyl ester (RT=55.53 min, $C_{38}H_{74}O_2$). But as mentioned above, the RT of this component is part of the sterols cluster, and comparing the MS spectrum, it is very close to the ethyl iso- allocholate, which is effectively a sterol.

The hexane sample is less rich in components compared to the more polar solvents. In the end we find again the major 4-hydroxy-3,5-dimethoxybenzohydrazide around 32 min. Followed by the heptacosane at 47 and 49 min ; and finally the sterol ethyl isoallocholate (RT=55.03 min). To finish, the most apolar solvent tested (xylene) does not give any peaks on the spectrum. We only observe a few peaks at the beginning picturing the solvent.

Considering ChaC now (Supplementary figure 5 for the spectra), the dichloromethane chromatogram is nearly the only one workable. It gave almost all the information presented in Figure 3. But on the first spectrum (ethyl acetate) we find again

this plateau starting at 54 min, representing the sterols with the most important one ethyl iso-allocholate. We find the same profile for the hexane sample, the sterols and nothing more. Regarding the xylene chromatogram, as mentioned for Balsa honey, we only see the peaks for the solvents.

And finally the dichloromethane sample gives the most of information. The major components are listed in figure x, then the first important one is tetradecanoic acid (RT=31.80 min, $C_{14}H_{28}O_2$). We then find hexadecanoic acid (RT=36.45 min, $C_{16}H_{32}O_2$) with several other fatty acids such as oleic acid (RT=39.69 min, $C_{18}H_{34}O_2$). More particularly, there is the 1,2-benzenedicarboxylic acid, mono(2-ethyl-6-hydroxyhexyl) ester (RT=45.12 min, $C_{16}H_{22}O_5$) which is a well documented phthalate (Nuti et al., 2005). The last components presented here are squalane (RT=46.40 min, $C_{30}H_{62}$) and myristic acid, hexadecyl ester (RT=55.99 min, $C_{30}H_{60}O_2$), mainly found in cosmetic preparations.

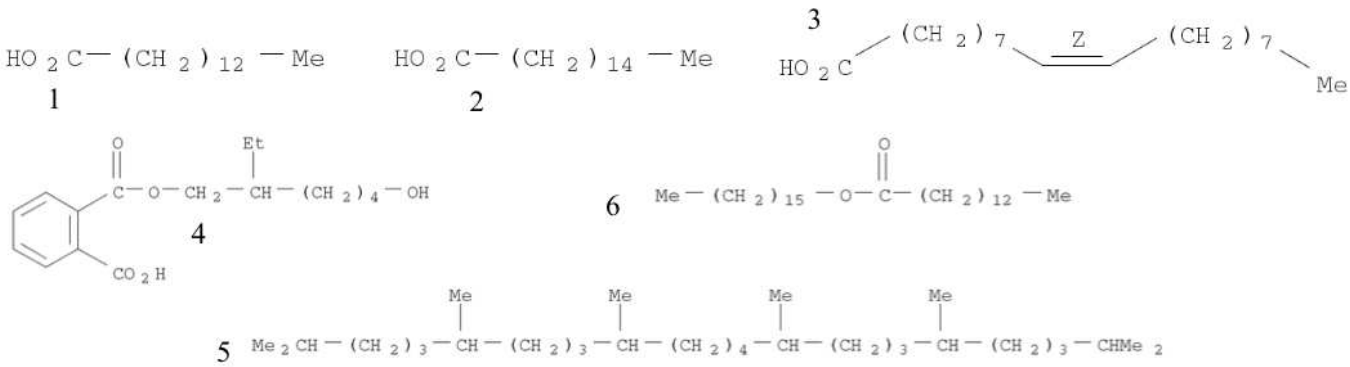


Figure 3 : Major chemical components from the chestnut honey (ChaC)

1) Tetradecanoic acid 2) Hexadecanoic acid 3) Oleic acid 4) 1,2-Benzenedicarboxylic acid, mono(2-ethyl-6-hydroxyhexyl) ester 5) Squalane 6) Myristic acid, hexadecyl ester

The other chemical aspect investigated here is the research about aminoglycoside molecules. We then investigated such molecules using the fluorescent properties of fluorescamine when bound to primary amino groups.

3. Discussion

All the strains used for this study are common in infectious diseases and are particularly involved in topical infections. As for *Propionibacterium acnes* (a bacteria of the cutaneous microbiota), which provoked a sepsis of the patient. Therefore, because of the increase of multiresistant bacterial strains, it is now important to find new ways to manage these diseases. And several studies have already showed the power of honey as a healer for burns, cutaneous infections or chirurgical wounds (Maghsoudi at al., 2011[12] and Molan, 2002[13]). Honey samples showed a potential activity against the growth of both Gram negative and Gram positive bacteria resistant to antibiotics. This would be a very interesting approach to control more dangerous species of microorganism in medical sciences.

In this study, we attempted to assess the value of honey from different botanical origins as an antimicrobial therapeutic agent. Moroccan honeys were particularly studied because of a strong culture around this product but also because of the existence of specific honeys due to endemic plants (e.g. *Euphorbia officinarum*) and rough climatic conditions. These conditions and the particular environment of South Morocco (no extensive cultivation) provide a high quality honey. And some of the samples presented here were never studied before.

These different types of honey showed different antimicrobial activities against clinically isolated strains (Tables 3 and 4). The antimicrobial effect was more pronounced for the endemic

[12] Maghsoudi H., Salehi F., Khosrowshahi M. K. et al., Comparison between topical honey and mafenide acetate in treatment of burn wounds. Annals of Burns and Fire Disasters, 2011, vol. 24, no 3, p. 132.
[13] Molan P. C., Re-introducing honey in the management of wounds and ulcers-theory and practice, 2002.

spurge (*Euphorbia officinarum,* highest value 10% and lowest value 1.25%) and even more particularly for the balsamic spurge (*Euphorbia balsamifera*, highest value 10% and lowest value 1,25%). The third remarkable Moroccan honey is from *Ceratolimon feei* var. *grandiflorum*, and produced a large effect on several strains including Gram negative and Gram positive. The mean value on *S. aureus*, *E. coli*, *A. baumanii* and *Aeromonas* was 5%. While the highest, obtained on *P. aeruginosa* was 10%. French honeys also gave attractive results. Honeys from oak or chestnut had both an action on *S. aureus*, *E. coli*, *Acinetobacter* and *Aeromonas*. The mean value observed was 10% corresponding at the value accepted as remarkable from other studies. From the first series tested, the mean value was 41% or eventually 20% on Gram negative strains. These results are equivalent with other studies (Voidarou et al., 2011[14] and Malika et al., 2004[15]). Honey was not as effective on yeast and fungus as on bacteria. However, the mean value observed was 41%, which is quite consistent with Moussa at al. (2012)[16]. They found an effectiveness of honey on *C. albicans* with MIC of 50% (even if we do not know the botanical origin of honeys).

But honey is not only bacteriostatic (as explained by the MICs); it is effectively bactericidal (through the MBCs observed). And that is even more interesting because the ratio MBC/MIC was always less than or equal to two, proving the bactericidal effect of honey. Indeed, this ratio is used to qualify a regular antibiotic and answering to this condition for a natural product indicates the importance of such product and then research.

As previously mentioned, the most surprising results were

[14] Voidarou C., Alexopoulos A., Plessas S. et al., Antibacterial activity of different honeys against pathogenic bacteria, Anaerobe, 2011, vol. 17, n°6, p. 375-379.
[15] Malika N., Faid M. et El Adlouni C., Antimicrobial activities of natural honey from aromatic and medicinal plants on antibio-resistant strains of bacteria, Int. J. Agric. Biol, 2004, vol. 6, p. 289-293.
[16] Moussa A., Noureddine D., Saad A. et al., Antifungal activity of four honeys of different types from Algeria against pathogenic yeast: Candida albicans and Rhodotorula sp, Asian Pacific journal of tropical biomedicine, 2012, vol. 2, no 7, p. 554-557.

obtained for the balsamic spurge honey. Considering *S. aureus*, the values between the lowest 1.25% on GISA T29-1 and the highest 2.5% on the other cocci are very interesting. Comparatively to the most studied and acclaimed honey: Manuka honey, the values observed in this study are more pronounced. Indeed, Manuka honey (which originates from the New Zealand Manuka tree, *Leptospermum scoparium*) is a well know honey all over the world for its strong activity on infectious bacteria. It is the major honey in medical use and is available as licensed wound dressing (Medihoney®). Sherlock et al. (2010)[17] investigated this honey on reference and clinical strains. The lowest MIC value obtained was 12.5%, compared to previous study where the lowest MIC was 6.25% for *S. aureus* (Patton et al., 2006)[18]. But they also investigated another Chile honey: Ulmo honey (which originates from the Chile Ulmo tree, *Eucryphia cordifolia*) giving better results than the Manuka ones. A lower MIC was observed for Ulmo honey ranging from 3.1% to 6.3%. As far as, Müller et al. (2013)[19] investigated raw Manuka honey and Medihoney®. Both honeys gave an MIC of 8% on *S. aureus* reference strain. Finally, we see that Balsamic spurge honey is well above Manuka honey. Moreover, we already know that Manuka honey has a very low H_2O_2 content and is qualified as a non-peroxide honey. This definition could also be applied for the balsamic spurge honey considering the low level measured with the test-strips (Table 6). Then this honey brings new elements, certainly a different composition, to explain this activity.

There are a few studies about time-kill kinetics of honey. We then fully studied it on three *S. aureus* strains to better

[17] Sherlock O., Dolan A., Athman R. et al., Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. BMC complementary and alternative medicine, 2010, vol. 10, no 1, p. 47.

[18] Patton T., Barrett J., Brennan J. et al. Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. Journal of microbiological methods, 2006, vol. 64, no 1, p. 84-95.

[19] Müller P., Alber D. G., Turnbull L. et al., Synergism between Medihoney and Rifampicin against Methicillin- Resistant Staphylococcus aureus (MRSA), PloS one, 2013, vol. 8, no 2, p. e57679.

understand how honey works. This coccus was the one which provided the best results and also a wide range of resistance. From reference (MSSA ATCC 6538) to clinical (MRSA 1508 and GISA T29-1) strains, the effect of honey was completely different. MSSA was the slowest strain to decrease. The bacterial decrease was very fluctuant according to the honeys tested. MSSA 1508 presented a more pronounced decrease after 4h of contact with honey. It is clear that honey is more effective on MRSA than on MSSA. At least, the results on GISA are very interesting. After only 1h of contact the dramatical decrease has divided the bacterial population three to six times. The resistance of this strain is an increase in the biosynthesis of the peptidoglycan, involving a thickened cell wall. We thus could think that the mechanism involved is due to the high osmolarity of honey.

Nevertheless, efficacy of honey still remains uncertainties. Several factors are put forward, several specific honey products, but we cannot restrict the effectiveness at a single element. The low pH and water content, high osmolarity, hydrogen peroxide, methylglyoxal, bee-defensin-1 peptide and additional components (Kwakman et al., 2012)[20] are the key elements advanced to explain the effectiveness of honey. However, some honeys (Manuka, Balsamic spurge) are very low for H_2O_2 and it thus cannot explain the activity observed. The pH of honey is always under four; and we already know that such acidity provides difficulties for the bacterial growth. Methylglyoxal and bee-defensin-1 are not fully investigated in all the studies about honey, it then remains unclear whether such compounds actually are effective as antimicrobial. But concerning our study, we mainly investigated the pH and hydrogen peroxide. Even if, we observed a slight increase of the pH diluting honey (which is explained by adding H_2O), the mean values are always under 4; a too acid environment for a well bacterial growth. Hydrogen peroxide was an argument advanced to explain on its own the antimicrobial effect. At

[20] Kwakman P. HS et Zaat S. AJ., Antibacterial components of honey. IUBMB life, 2012, vol. 64, no 1, p. 48-55.

least, publications (Kwakman et al., 2012[21] and non-peroxide honey) have already revealed a doubt about H_2O_2 involvement. Furthermore, the values obtained in this study, ranging from 25 mg/L (Chestnut honey) to 0,5 mg/L (Balsamic spurge honey) do not explain the effectiveness observed. MICs and MBCs values for *Euphorbia balsamifera* were the lowest. And compared to the commercial hydrogen peroxide, the content in honey is very relative. Finally, considering the redox potential, the findings were very fluctuant and no evidence appeared to explain the activity. Methylglyoxal, bee-defensin-1 or other components have not been investigated. It would be interesting to explore these parameters for further analyzes, and to compare with other studies. However, considering the traceability component of food product, pesticides exploration is needed to achieve such study. We planned this investigation at the ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail) laboratory, specialized in such work. Indeed, it is a tool to assess the contamination of honey by anthropic pressure on the hives. After this evaluation, honeys studied here would be certified even if the results already obtained proved a major activity against multiresistant bacteria. That is thus an important step arguing the real capacity of honey on its own as an antimicrobial agent. And all the publications are already showing the fabulous power of honey as a healer.

ABBREVIATIONS LIST

ATCC : American Type Culture Collection

BHI : Brain Heart Infusion□CFU : Colony Forming Unit□DW : Distilled Water

CFU : Colony Forming Unit□

DW : Distilled Water

[21] *Ibid*

ESBL : Extended-Spectrum Beta-Lactamases

GC-MS : Gas Chromatography-Mass Spectrometry

GISA : Glycopeptide Intermediate *Staphylococcus Aureus*

HPLC : High Performance Liquid Chromatography

L-L Extraction: Liquid-Liquid Extraction

MBC : Minimum Bactericidal Concentration

MHA : Mueller-Hinton Agar

MHB (CX2) : Mueller-Hinton Broth (Concentrated 2 times)

MIC : Minimum Inhibitory Concentration

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MSSA: Methicillin-Sensitive *Staphylococcus aureus*

NESBL : Non Extended-Spectrum Beta-Lactamases

ROW : Reverse Osmosis Water

SAUR : *Staphylococcus AUReus*

SPE : Solid Phase Extraction

SPME : Solid Phase Micro Extraction

TSA : Tryptic Soy Agar