Relationship among antibiotic residues and antibacterial activity of the endemic spurge honey
(*Euphorbia Resinifera* o. Berg) from morocco

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ABSTRACT

Antibiotic-resistant bacteria continue to be of major health concern worldwide. In recent years, several reports and scientific articles claim the contamination of honey by antibiotics, detectable concentrations of antibiotic residues in honey are illegal. They, may cause hypersensitivity or resistance to drug therapy in humans, and are perceived by consumers as undesirable. In this sense, the purpose of this work was to examine the antibacterial activity of the *Euphorbia resinifera* (*E. resinifera*) honey against *Escherichia coli* and *Staphylococcus aureus* in vitro using the well-agar diffusion assay followed by dilution range to obtain more precise minimum inhibitory concentration values. The second aim is to evaluate the presence of antibiotics in honey using a screening test: Evidence Investigator™, an immuno-enzymatic method for detection of 27 antibiotic residues followed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) for confirmation of suspect samples; in order to assess the relationship between the presence of antibiotic residues and the antibacterial activity of honey. In this study, a total of 37 *E. resinifera* honey samples were analyzed. The results show that all samples of honey inhibited the growth of bacteria at the dilutions at 50% (v/v); the highest inhibition zone (25.98 ± 0.11 mm) was recorded from sample 5 for *Staphylococcus aureus* and (13.84 ± 1.10 mm) in sample 17 for *Escherichia coli* and that 50% (v/v) dilutions showed significant antibacterial effect compared to other dilutions (6.25, 12.5, 25% (v/v)). In all samples, there were no antibiotic residues detected except for one showing the detection of Trimethoprim at 6.48 µg kg⁻¹. Our research is one of the first studies that relate the relationship between the presence of antibiotic residues and the antibacterial activity of *Euphorbia resinifera* honey and showed that the antibacterial activity of honey might be due to the high osmotic nature, a low pH, its content of phenolic compounds and hydrogen peroxide and also to its content of methylglyoxal.

Keywords: *Euphorbia resinifera* honey; antibacterial activity; antibiotic residues; multi-array; screening; LC-MS/MS

INTRODUCTION

In Morocco, the *E. resinifera* unifloral honey, called “Zakkoum” honey in Arabic, is very much appreciated it represents an important medicinal and ethnopharmacological resource (Ihitassen, 2019). This type of honey is produced exclusively in a principal and unique area located in the Middle Atlas (Tadla-Azilal region). The plant *E. resinifera* O. Berg, a large perennial leafless cactus-like, is an endemic species of Morocco (Chakir et al., 2016). The annual flowering of this *Euphorbiaceae* is very limited between three and four weeks beginning at the end of July.

The very distinct quality of this unique honey is obtained through the leafy *E. resinifera* vegetation covering exclusively the mountains of the Tadla-Azilal region. This specific honey is well-known for its tasting and medicinal qualities that differentiate it from other types of Moroccan honeys. This typicity is sought by the majority of the beekeepers of Morocco who settle in the region during the flowering period of the *E. resinifera*. In fact, *E. resinifera* honey from
Detection of antibiotic residues in honey could provide interesting evidence of the close relationship between the presence of antibiotics and the antibacterial activity of honey. Therefore, there is a need to ensure that the antibacterial action of *E. resinifera* honey stems partly from a phytochemical component, and not from the presence of antibiotic residues used by beekeepers.

To our knowledge, no study has examined the relationship between the presence of antibiotic residues and the antibacterial activity of honey. The objective of this study is two-fold: First to determine in vitro antibacterial activity of *E. resinifera* honey against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), and to second to ensure that *E. resinifera* honey is free from antibiotic residues using screening test: “Evidence Investigator™,” is an immuno-enzymatic method for detection of 27 antibiotic residues in 37 *E. resinifera* honey samples. In the second level, LC MS/MS was used for confirmation of suspect samples.

**MATERIALS AND METHODS**

**Materials**

**Chemicals and reagents**

Antimicrobial Array I Ultra Kit (AM I, EV3843), Antimicrobial Array II Plus Kit (AM II, EV 4169 A/B), Antimicrobial Array III Kit (AM III, EV3695), Antimicrobial Array V (AM V, EV4027) were purchased from Randox Laboratories Ltd. 55 Diamond Road, Crumlin, County Antrim, United Kingdom. All chemicals and solvents used were of analytical grade and suitable for LC/MSMS. All references standards were from Sigma-Aldrich (Seelze, Germany). Pharmaceutical standards (chloramphenicol, semicarbazide, -1-aminoxydantoin,3-amino-2-oxazolidone, -5-morpholinométhyl-3-amino-2-oxazolidone, oxytetracycline, epoxycycline, tetracycline, epoxycycline, chlorotetracycline, epichloretetracycline, trémethoprine, marbofloxacine, norfloxacine, ciprofloxacine, danofloxacin, enrofloxacine, sarafloxacine, difloxacine, nalidixic acid, oxolinic acid and flumequine) were purchased from Sigma-Aldrich (Seelze, Germany). The commercial antibiotics discs were from Sanofi Diagnostics Pasteur, France. Mueller Hinton agar, nutrient agar was purchased from Bio-card France and tryppticase soy broth from TSB, BBL Microbiology Systems, USA.

**Honey samples**

Thirty-seven (37) samples of *E. resinifera* honey, produced by Union of beekeeping cooperatives of the Tadla-Azilal region in 2017, were purchased from the sales area in Afourer city and from the stand of this cooperative at the market of International Exhibition of Agriculture of the Tadla-Azilal region is the first officially honey labeled, by the Union of Beekeepers Cooperatives of Tadla-Azilal, as having protected geographical indication -PGI- in Morocco (Ministry of Agriculture and Fisheries of Morocco, 2012).

This type of honey has been the subject of several publications concerning its physicochemical composition and its color (Moujanni et al., 2018), its bacteriological quality (Moujanni et al., 2017a), antibacterial activity (Noaman et al., 2004), its anti-inflammatory capacity (Khiati et al., 2012) and identification of pesticides residues and heavy metals (Moujanni et al., 2017b).

The antimicrobial properties of honey have been investigated by a number of researchers worldwide, and it has been shown that the inhibitory activity has been attributed to osmolarity due to its high sugar content (Cooper et al., 1999), naturally low pH (Bang et al., 2003), production of hydrogen peroxide present in honey due to the action of glucose oxidase enzyme (Olaitan et al., 2007) and also the presence of phenolic acids (Estevinho et al., 2008; Biluca et al., 2016).

Even though it is strictly prohibited and no antibiotic has a marketing authorization for the treatment of bees, the antibiotics are used illegally in beekeeping, mainly tetracyclines, streptomycin, sulfonamides and chloramphenicol (Gaudin et al., 2014) for the treatment and prevention of diseases such as American and European foulbrood (Bogdanov, 2006).

The presence of antibiotic residues in honey present a risk to the health of consumers, because they could be a source of allergic reactions (Toldra and Reig, 2006) and can lead to obtaining bacterial resistant strains to antibiotics after consumption of honey (Bargańska et al., 2011). That is why, in recent years, several publications have focused on the determination of antimicrobial contaminants in beekeeping products especially honey (Kumar et al., 2020; Savarino et al., 2020).

Screening methods are the first step in controlling antibiotic residues in food (Gaudin, 2017; ANSES, 2019; AFNOR, 2014). They can detect the presence of an antibiotic or group of antibiotics at the level of interest, and usually provide qualitative results (Jakšić et al., 2018). Then, in a second step the residues of the positively tested samples are quantified mostly by quantitative confirmation methods such as using an analytical method based on high performance liquid chromatography associated with a mass detector (HPLC-MS/MS) (Gaudin, 2017; Jakšić et al., 2018; Laurentie et al., 2002; Kaufmann et al., 2002). For that reason, none of these factors taken individually seen to be enough to explain the antibacterial activity.
Meknes (Morocco). The samples have not been heated or pasteurized. All samples were stored at 4°C until assay, because levels of sulfonamides in honey are known to decrease over time when the honey is stored at room temperature (Sheth et al., 1990).

Microorganisms
Microorganisms were supplied by Microbiology Laboratory of the Pharmaceutical and Veterinary Products Division of National Office of Food Safety (ONSSA) Rabat-Morocco. All bacteria were of standard strains (ATCC, US) including one gram-positive bacteria (S. aureus (ATCC 6538)) and one gram-negative bacteria (E. coli (ATCC 10536)).

Preparation of honey solutions
Solutions of honey were handled aseptically and protected from bright light to prevent from photodegradation of the glucose oxidase that gives rise to hydrogen peroxide in honey (Nair and Chanda, 2006). Dilutions (v/v) of each honey sample were made in sterile distilled water to obtain final concentrations of 6.25, 12.5, 25 and 50%.

Antibacterial activity of honey
Preparation of bacterial suspensions
The isolates were identified based on standard microbiological techniques and sub-cultured in nutrient agar slopes at 37°C for 24 h. Colonies of fresh cultures of the different microorganisms from overnight growth were picked with sterile inoculating loop and suspended in 3 ml nutrient broth contained in sterile test tubes and incubated for 3 h at 37°C. This was diluted with distilled water to set inoculum density used in this study (Patton et al., 2006).

Susceptibility testing of honey
Well diffusion and Spectrophotometric assay by Chaibi et al., (1996). The antibacterial activity of the honey was tested against the gram-negative bacteria E. coli (ATCC 10536) and gram-positive S. aureus (ATCC 6538). The choice of E. coli and S. aureus strains is based on their parietal differences (Gram + and Gram -), the problems that they cause in a clinical setting as well as the challenge they to face with a modern anti-biotherapy especially the treatment of wounds.

The agar well diffusion technique was used to screen for antibacterial activity of honey. The well diffusion method was employed. Fresh culture suspension of the test microorganisms (100 µl) was spread on Mueller Hinton agar plates.

The concentration of cultures was 1×10⁷ CFU ml⁻¹.

The honey samples were first inoculated separately on standard nutrient media with no test organisms to evaluate their possible contamination. Thereafter, solidified nutrient agar plates were separately flooded with the liquid inoculums of the different test organisms using the pour plate method. The plates were drained and allowed to dry at 37°C for 30 min after which four equidistant wells of 5 mm in diameter were punched using a sterile cork borer at different sites on the plates. 10 µl of the different concentrations (6.25, 12.5, 25 and 50% (v/v)) of the honey samples were separately placed in the different punched wells with 1 ml sterile syringe. The plates were allowed to stay for 15 min for pre-diffusion to take place followed by an overnight incubation that lasted for 24 h at 37°C. The diameter of the well were recorded. Each assay was carried out in triplicate. Nutrient agar plate without honey was similarly inoculated as a control. All tests were performed in triplicate and the inhibition zones of honeys were compared with those of antibiotics used (Patton et al., 2006).

Determination of Minimum Inhibitory Concentration (MIC)
The MIC of honey was determined according to the method adopted by (Chaibi et al., 1996). The tubes containing 10 ml of trypticase soy broth (TSB, BBL Microbiology Systems, USA) were filled by different concentrations of the honey to be tested. These tubes were aseptically inoculated with the strain to be tested at the final concentration of 3×10⁶ CFU ml⁻¹ and then incubated at 35°C for 24 h. The optical density (OD) was determined initially in a spectrophotometer at 620 nm and after 24 h of incubation. The inhibition is expressed by the inhibition index (II) calculated according to the following formula:

\[ II = 1 - \frac{OD_1}{OD_2} \]

where, OD1: difference between the absorbance after 24 h of incubation and the absorbance at the starting time of incubation with the honey sample; OD2: difference between the absorbance after 24 h of incubation and the absorbance at the starting time of incubation without the honey sample. An II = 0 indicates that there is no inhibition, II > 1 shows total inhibition, II > 1 results in cell lysis and II < 0 would indicate that there is growth stimulation (Chaibi et al., 1996). The readings were repeated 3 times for each concentration of honey and for the three strains tested.

Antibiotic susceptibility test
Antibiotic susceptibility for the pathogens and their reference strains were detected using the disk diffusion method, according to the standards set by the Clinical and Laboratory Standards Institute (CLSI). An aliquot of 100 µl of an overnight culture was diluted in saline solution to about 1.5×10⁸ CFU ml⁻¹ (0.5 Units of McFarland turbidity standard).
Mueller Hinton agar plates were flooded with this suspension to give confluent colonies. The plates were then incubated at 37°C for 24 h to 48 h and the diameters of the clear zones around each disk were measured after incubation. The tested antibiotics were as follows: Erythromycin (15 μg), Ciprofloxacin (5 μg), Doxycycline (30 μg), Cephalothin (30 μg) and Ampicillin (2 μg).

**Evidence Investigator™ system**

**Antimicrobial Array kits used**

AMI is used in detecting multiple groups of antibiotics which are specifically found in honey. It, simultaneously, detects on a single honey sample: Sulfadiazine (SZ), sulphadimethoxine (SDM), sulphamethazine (SMT), sulfathiazol (ST), sulphisoxazole (SS), sulphamonemethoxine (SMM), sulphapyridine (SP), sulphamethoxypyridazine (SMP), sulphachlorpyridazine (SCP), sulphadoxine (SD), dapsone (DAPS), sulphaquinixaline (SQ), sulphamerazine (SM) and trimethoprim (TMP). AM II test is used for quinolones (QNL), ceftiofur (LSF), streptomycin (STR), tylosin (TYL) and tetracyclines (TC). AM III (EV3695) is used to detect the nitrofuran family: 3-amino-2-oxazolidinone (AOZ), 5- methylmorpholino-3-amino-2-oxazolidinone (AMOZ), 1-aminohydantoinhydrochloride (AHD) and semicarbazide (SEM) and AM V is used for the detection of chloramphenicol (CAP).

**Sample preparation**

AM I (EV3843), and AM II (EV 4169 A/B): A total of 1 g of honey sample is weighted out. Then 9 ml of diluted wash buffer warmed to 37°C are added. The tubes are placed on a roller for 10 min. The sample is now ready for application to the biochip.

AM III (EV3695): 1 g of honey was weighed and 4 mL of double denoised water warmed to 37°C. 0.5 ml of 1 M HCl and 145 μl of 10 mM 4’Nitrobenzaldehyde were added, vortexed for 1 min and centrifuged for 10 min at 4000 tr min⁻¹ at 25°C. 3 ml of the supper ethyl acetate layer was transferred to a clean glass test tube and dried down at 50°C. The samples were resuspended in 1 ml of hexane and 1 ml of diluted wash buffer, vortexed for 2 minutes and centrifuged at 4000 tr min⁻¹ for 10 min. 50 μl of the lower aqueous layer was used for the test of the biochips.

AM V (EV4027): 2 g of honey was weighed. 4 ml of diluted wash buffer warmed to 37°C are added. The tubes are placed on a roller for 10 min or until dissolved. Then 8 ml of acetonitrile and 1.5 g of sodium chloride are added, vortexed for 2 min and centrifuged for 10 min at 4000 tr min⁻¹ at 25°C. 2 ml of the top layer was removed and dried down at 50°C, sample was reconstituted with 500 μl of hexane, vortexed for 2 min and centrifuged at 4000 tr min⁻¹ for 10 min. 100 μl of the lower aqueous layer was used for the test of the biochips.

**Biochip analysis**

The Evidence Investigator™ Biochip Array technology is used to perform simultaneous quantitative detection of multiple analyses from a single sample. The core technology, the Randox Biochip, is supplied pre-fabricated with a panel of discrete test regions (DTRs) containing immobilized antibodies specific to different antibiotics. The biochip array assay here employs a competitive format; antibodies selective for the analyses of interest are immobilized at the DTRs. Increased levels of antibiotics in a specimen will lead to decreased binding of antibiotics labelled with horseradish peroxidase (HRP) and thus, a decrease in chemiluminescence being emitted. Detection is accomplished via imaging of a chemiluminescent signal with a CCD (charge-coupled device) camera. Each biochip contains 23 distinct test regions and unlike most current conventional immunoassay analyzers, allows multiple assays to be performed simultaneously on a single sample. The biochip assays methodology is based on standard immunoassay techniques. In most test panels, antibodies are attached to the surface of the biochip and analytes in the sample bind to them; competitive and sandwich immunoassays are used for the biochip assay and the methodology adopted is panel specific and dependent on the molecular weight of the target analytes. The concentration of analyte present in the sample was plotted and calculated from the calibration curve.

All analyses were performed according to the manufacturer’s instructions. The solutions required for the test has been prepared in accordance with the specifications of the producing company and all materials were brought to room temperature. The samples were analyzed by an Evidence Investigator AMI, AM II, AM III and AM V. 200 μl, 200 μl, 150 μl and 100 μl of assay diluted for AM I, AM II, AM III and AM V respectively were pipetted into the wells. Next, a calibrator or sample was pipetted into the wells. To mix the reagents, all sides of the plate was tapped and the holding plate was fixed onto the bottom plate of the thermo shaker and incubated for 30 min at 25°C and 370 rpm. 50 μl, 50 μl, 100 μl and 100 μl of conjugate for AM I, AM II, AM III and AM V respectively per well was pipetted. It was incubated in the thermo shaker for 60 min at 25°C and 370 rpm.

The reagents were removed by sharply moving the process plate. Two rapid washing processes were immediately performed with diluted washing solution per well. The washing cycle was performed four more times. For each cycle, all sides of the process plate were tapped for about 2 min. After the final wash 250 μl of signal reagent was
pipetted into the wells and incubated for 2 min in darkness and analyzed.

The imaging process was conducted within 30 min. The results were automatically assessed in the Randox Evidence Investigator software. Evidence Investigator (Randox Laboratories Ltd., Crumlin, County Antrim, UK) identifies images by using Relative Light Units (RLU) which conducts the reading process via Charge Coupled Device (CCD) camera at a temperature of –40°C. Antimicrobial Array kits have been validated by the manufacturer as a result of validation studies with reference samples.

**Sensitivity**
The limits of detection (LOD) for the Evidence™ analytes for the honey matrix is shown in Table 1.

**LC-MS/MS confirmation**
The validation method was performed with a high-performance liquid chromatography apparatus (HPLC) type FLEXAR (PerkinElmer, Inc. USA) coupled to triple quadrupole type mass spectrometer: AB SCIEX QTRAP 5500 with turbo ion spray interface and Analyst software according to (Bohm et al., 2012). This internal method of the national office of food safety (ONSSA) antibiotic residues laboratory complies with the requirements of Decision 2002/657/EC concerning the performance of methods for the determination and confirmation of antibiotics residues in honey samples (European Commission, 2002) and guidance paper of Community Reference Laboratories (CRLs) (Community Reference Laboratories, 2007). All chemicals and solvents used were of analytical grade and suitable for LC/MSMS. Chromatographic conditions, nebulizer current and other conditions according to the type of antibiotic are shown in Table 2.

Stock standard solutions were prepared individually by dissolving each compound in water or methanol at concentrations in accordance with their dissolution properties (Sigma Aldrich). Thus, all QNL analytes were solubilized in water at a concentration of 1 mg ml\(^{-1}\). Whereas NF, CAP, TC and SA/TMP analytes were solubilized in methanol at concentration of 0.5 mg ml\(^{-1}\). These stock solutions were then stored at –20 °C in darkness until use. A 1 µg ml\(^{-1}\) composite standard solution was obtained by further dilution of the stock solutions with methanol. This solution was employed to build the different calibration curves and to provide quality control samples after adequate spiking experiments. Before being applied for LC analyses, all solutions were filtered by micro-filter (4.5 µm).

**Statistical analysis**
Statistical differences between the different dilutions for each bacteria and the antibacterial effect for 50% dilution were determined by one-way ANOVA using Excel spreadsheets on Microsoft Office 2016. Differences were considered significant at p<0.05.

**Table 1: Results of antibiotic residues (µg/kg) in E. resinifera honey from Morocco (n=37)**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>LOD*</th>
<th>Positives*</th>
<th>%</th>
<th>High level*screened</th>
<th>LC/MSMS Confirmation</th>
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<tr>
<td>CAP</td>
<td>0.1</td>
<td>03</td>
<td>8</td>
<td>0.32</td>
<td>**</td>
</tr>
<tr>
<td>QNL</td>
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<td>03</td>
<td>8</td>
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<td>-</td>
</tr>
<tr>
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<td>2</td>
<td>0</td>
<td>-</td>
<td>3.44</td>
<td>-</td>
</tr>
<tr>
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<td>5</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
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<td>TYL</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
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<tr>
<td>TC</td>
<td>5</td>
<td>05</td>
<td>13</td>
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<tr>
<td>ST</td>
<td>2.09</td>
<td>0</td>
<td>-</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
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</tr>
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<td>-</td>
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<tr>
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<td>5.43</td>
<td>-</td>
</tr>
<tr>
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<td>5</td>
<td>0</td>
<td>-</td>
<td>1.11</td>
<td>-</td>
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<td>1.71</td>
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<td>SEM</td>
<td>0.5</td>
<td>04</td>
<td>10.81</td>
<td>&gt; 22</td>
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<td>0.3</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Randox kit, **<LOD
RESULTS AND DISCUSSION

Antibacterial effect of *E. resinifera* honey

Antibacterial effect of various types of 37 *E. resinifera* honey samples at different concentrations (6.25, 12.5, 25 and 50 % (v/v)) against *E. coli* and *S. aureus* using agar well diffusion method was investigated. As shown in Table 3. 50 % concentration showed significant antibacterial effect compared to other concentrations, 6.25, 12.5 and 25 %. In addition, the antibacterial effect for 50 % concentration in *E. coli* and *S. aureus* was statistically analyzed. *S. aureus* showed significant antibacterial effect (p <0.05) compared to *E. coli* for all samples studied, except sample 13 (p >0.05). In fact, highest inhibition zone of 13.84 mm was recorded for sample 17 against *E. coli* and 25.98 mm was recorded for sample 5 against *S. aureus* (Fig. 1). In Saudi Arabia honey, *Ziziphus spina-christi* honey showed an inhibition zone of 20.33 mm at concentration of 80 % against *S. aureus*, while, an inhibition zone of 18.34 mm was found for Lavandula dentata honey at concentration of 50 % against *Proteus mirabilis*. However, in Argentina, no antibacterial effect was found of various honey samples provided by apiarists against both *E. coli* and *S. aureus* (Basualdo, 2007) Similarly, no antibacterial effect was observed at concentration of 20 % for Malaysian Melaleuca honey against *S. aureus*, while, concentrations of 60 and 80 % showed an antibacterial effect against *S. aureus* with an inhibition zone of 8.1 and 13.7 mm, respectively (Ng and Lim, 2015). In our study, the inhibition zone was found to be positively linearly correlated with increasing honey concentrations, in addition, increasing honey concentrations showed a corresponding increase in the inhibitory effectiveness.

Regarding MIC, as shown in Table 4, the maximum microbial growth was observed for the concentrations 50 and 25%. However, at 12.5 and 6.25%, recorded inhibitions were very minimal for both *E. coli* and *S. aureus*, except for samples 11, 14, 18 and 19, which showed total inhibition on *S. aureus* at the concentration 6.25% with an inhibition index of 0.96, 1.10, 1.07 and 1.00 respectively. In general, the results of the inhibition index show that the honey concentrations affect the growth of *E. coli* and *S. aureus* differently. For 50 and 25% concentrations, the different honey samples showed a clear inhibition with an inhibition index of 0.95 in most cases as it is presented on Table 4. The MIC for each honey is presented in Table 5. The lower MIC was recorded in sample 11, 14 and 19 against *S. aureus* (6.25%) and these results are in agreement with (Dżugan et al., 2020) who found the MIC of the honeys against *S. aureus* ranges from 6.25 to 25%. (Anthimidou and Mossialos, 2013) have reported that the MIC of manuka honey was determined at 6.25% against *S. aureus* and Four Greek and Cypriot honeys demonstrated a MIC at 3.125 %.

![Fig 1. Inhibitory effect of honey sample 5 on *E. coli* and *S. aureus* at 50% concentration.](image-url)
Table 3: Antibacterial activity of Moroccan *E. resindifera* honey samples against *S. aureus* and *E. Coli* for different dilutions

<table>
<thead>
<tr>
<th>S</th>
<th>Mean diameter of inhibition zone (mm) (n=3)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>S. aureus</strong> (G+)</td>
<td><strong>E. Coli</strong> (G-)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>01</td>
<td>17.27±0.30±</td>
<td>8.40±3.50</td>
</tr>
<tr>
<td>02</td>
<td>16.73±0.45±</td>
<td>10.95±2.18</td>
</tr>
<tr>
<td>03</td>
<td>10.79±2.88±</td>
<td>0</td>
</tr>
<tr>
<td>04</td>
<td>16.79±1.50±</td>
<td>9.18±3.50</td>
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<tr>
<td>05</td>
<td>25.98±0.11±</td>
<td>18.83±0.23</td>
</tr>
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<td>06</td>
<td>25.37±0.45±</td>
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Inhibition of control antibiotics

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</table>

Results are expressed as mean ± standard deviation, n = 3, different letters in the table denote that the antibacterial activity studied (inhibition diameters) is influenced by the degree of dilution of the honey (1/2, 1/4, 1/8 and 1/16; *p < 0.05*) (letter a) and the bacterial strain (*E. coli* and *S. aureus*; *p < 0.05*) (letter b).

The sensitivity of *S. aureus* and *E. coli* to erythromycin, ciprofloxacin, doxycycline, cephalothin, and ampicillin has been tested. Both strains were sensitive to antibiotics except for AMP to which *E. coli* was resistant Table 3. The antibacterial effect of the honey observed, in comparison with that of the antibiotics tested, shows an equivalent effect to ampicillin for *S. aureus* (14.46 mm) and to cephalothin for *E. coli* (9.16 mm). Our results show, moreover, that the honey samples, diluted at 50%, show an interesting inhibitory effect against *E. Coli* which is resistant to ampicillin. Consiglio et al., (2013) and Roby et al., (2020) reported that the activity of honeys can vary considerably according to the different types of flowers. Moreover, the results of our study revealed that the antibacterial activity of honeys sharing the same
floral origin could differ considerably depending, on storage conditions processing and handling. Also, the gram-positive bacterial strains were the most susceptible to the effect of honey whereas the gram-negative microbes were less sensitive to all honey samples, which is in accordance with previous observations of Malanovic et al., (2016), Nair and Chanda, (2006) and Khan et al., (2009). The difference in sensitivity to honey and other antibacterial agents between gram-positive and gram-negative bacteria may be due to the outer membrane of the gram-negative bacterial cell which prevents some active substances from entering the cell. Gram-positive bacteria do not have an outer membrane protecting the peptidoglycan which facilitates the penetration of antimicrobial agents and causes damage (Malanovic and Lohner, 2016).

<table>
<thead>
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<th>Sample</th>
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The Evidence Investigator™ system is an adequate analytical method for screening analyses for detection of antibiotic residues in honey. It demonstrates an excellent specificity and ensures reliable results (O’Mahony et al., 2011; Popa et al., 2012; Gaudin et al., 2013, 2014, 2015).

Antibiotic residues
None of 37 E. resinifera honey samples were detected with LSF, STR, TYL, AMOZ, AOZ, ST, SS, SP, SMM, SMP, SCP, DAPS, SD, SZ, SDM, SQ, SMT and SM residues. CAP and QNL were found in 3 samples (8 %), SEM and AHD were detected in 4 samples (10.81 %), TC was found in 5 samples (13%) and TMPs and AOZ residues were found in 1 sample (2.7 %). The results obtained are shown on Table 1.
The samples that contained antimicrobial residues were analyzed by Confirmatory analysis, here was performed via LC-MS/MS. In all samples, there were no antibiotic residues detected except for one showing the detection of TMPs at 6.48 µg kg⁻¹ (Fig. 2). There is no fixed limit for TMPs residues neither in Morocco nor internationally (EU, Codex, FDA, etc.). Only Belgium sets a proposed recommended target concentration (PRTC) of 20 µg kg⁻¹. The level of TMPs values detected in our study are much lower than this target. The presence of TMPs at a very low level can be explained by the contamination of honey by wax.

Indeed, since honey is produced according to a specific reference prohibiting the use of antibiotics, it is possible to emit the hypothesis that the TMPs come from an old contamination of the wax following its recycling. Their possible accumulation in wax is related to the liposolubility of TMPs.

An analysis of many bibliographic studies shows that there are antibiotic specialties for bee species in several countries of the world including the United States, Australia and Canada (Baggio et al., 2009; Barbançon et al., 2013; Codex Alimentarius Commission, 2018; Community Reference Laboratories, 2007; Food and Drug Administration, 2003; Gaudin et al., 2013, 2015; Wang, 2004; Wang et al., 2014; FAO/WHO, 2018; Zhou et al., 2009). Among the EU Member States, only Slovakia has an antibiotic with a marketing authorization for the fight against European foulbrood (Oxyoharm® containing Ox-TC) (Vidal-Naquet, 2015). In France, only the use of TCs is regulated by the DGAL memo of 26 April 2005. These antibiotics are listed as authorized substances of EU commission regulation N° 37/2010 (European Commission, 2010).

In Morocco, on a positive list of authorized veterinary medicines, of more than 1800 specialties, only six products are authorized for honey bees with only one antibacterial substance (Bicyclohexyl-Ammonium Fumagillin) that was approved for control of Nosema disease (National office of Food Safety, 2019).

This product has not been marketed since 2000 because of its proven genotoxicity in humans and the lack of setting of its MRL (Barbançon et al., 2013). In a study on honey, Barrasso et al., (2019) reported that 66 samples of Apulian honey tested by Evidence Investigator (AM II); TYL was detected in 38 honey samples, TCN in 36 honey samples, QNL in 9 honey samples, TAF (Thiamphenicol) in 21, LSF in 19 samples and STR was detected in 1 sample. In another study conducted by Aksem, (2019) examining a total of 45 honey samples including 5 brands of honey and 40 local honey (honey produced in the region by beekeepers) by (AM IV) test Kit; Erythromycin was detected in 41 samples (91.1%), 10 samples (22.2%) had STR, 8 (17.8%) had Amikacin, 6 (13.3%) had Lincosamides, whereas no samples were detected in spiramycin, apramycin, bacitracin, tobramycin, spectinomycin or virginiamycin antibiotic residues. On the other hand, Korkmaz et al., (2017) determined a sulfonamide and tetracycline group antibiotics in 59 natural pine honey samples collected from Aegean Region of Turkey by competitive enzyme-linked immunoassay method y (ELISA); tetracycline group antibiotics were found in 35 honey samples (52.5%) between 6 and 42 ppb while The highest amount was 42 and 38 ppb, sulfamethazine antibiotic was found in 31 honey samples (59.3%) between 3 and 32 ppb whose the highest amount was 32 and 26 ppb.

Regarding the determination of honey antibiotics by confirmatory methods (Loupips et al., 2017) determined Thirty-six different antibiotics and residues from four different families (sulfonamides, tetracyclines, amphenicols, fluoroquinolones) and some individual antibiotics (penicillin, trimethoprim, and tiamulin) were tested in 20 commercial honey samples originating from Cyprus and Greece of different types (thyme, multifloral, pine, and orange blossom) by LC-MS/MS, it was reported that Oxolonic acid was determined (2.0 µg kg⁻¹) in one of the analyzed Greek flower honeys, sulfathiazole (11.2 µg kg⁻¹) in one Cypriot thyme honey, and sulfadimethoxine (17 µg kg⁻¹) in one Cypriot pine honey.

It can be seen that the differences between the results of the researchers depend on the method used in the detection of antimicrobials. Our research is one of the first studies that relate the impact of the presence of antibiotic residues on the antibacterial activity. According to our negative research regarding the results for antibiotic residues in E. resinifera honey, we can conclude that the antibacterial activity of this honey might be attributed to a high osmotic nature, a low pH (Olaitan et al., 2007) its content of phenolic compounds (Velásquez et al., 2020) and hydrogen peroxide (H₂O₂) (Liang et al., 2020) and also to its content of methylglyoxal which is found in high concentration in Manuka honey (Atrott and Henle, 2009). Consequently, faced with these unharmonized global rules for antibiotic use in beekeeping, the Codex and consequently most countries have not laid down MRLs for antibiotic residues in honey. In addition, harmonized rules do not exist with regard to acceptable control methods, LOD or sampling methods. In some countries (e.g. Australia, Canada, India, Korea), MRLs have been set for each class of antibiotics (Community Reference Laboratories, 2007). In other countries, it was decided to establish different residues limits like action limits, recommended target concentrations, minimum required performance limit, and recommended concentration for screening and
Fig 2. LC-MS/MS Chromatograms obtained for TPMs of honey sample; (A) blank sample, (B) standard and (C) suspect sample.
non-conformity or tolerance levels (Reybroeck, 2003). Regarding the biochemistry of antibiotics in foods, they are stable in honey as parent molecules or metabolites after degradation, hence the need to look for them.

The greatest danger, in terms of human health, concerns prohibited substances, namely CAPs and NFs and, to a lesser extent, SAs. In addition, we must emphasize that, to our knowledge, this is the first published work, which deals with the important issue of antibiotic residues in Moroccan honeys.

This type of honey may be suggested for use as a natural adjunct to many diseases because of its positive health effects. Therefore, to protect the image of this kind of honey and all honey types of Moroccan origin as a healthy natural product, researchers should highlight a research program targeting Moroccan honeys labeled for their quality and benefits.

CONCLUSION

Our study demonstrates the relationship between the presence of antibiotic residues and the antibacterial activity of *E. resinifera* honey. According to our negative research regarding the results for antibiotic residues in *E. resinifera* honey, it could be concluded that the antibacterial activity of honey might be due to the honey’s phytochemical characteristics, pH, viscosity, and content of H$_2$O$_2$. The present study concluded that there is an opportunity that *E. resinifera* honey may be suggested according to its positive health effects, for use as a natural adjunct to many diseases whose pathogen is *E. coli* and *S. aureus*. However, further clinical studies are necessary to elucidate this hypothesis.

From a methodological point of view and through our results, we recommend that studies of the antimicrobial effects of honey can only be done after validation, of the samples studied, of the absence of antibiotic residues. It may be assessed by a rapid, simple screening method offering the detection of multiple analyts.

**Author contributions**

**Study conception and design:** A. Essamadi, A. Moujanni & A. Terrab; **Experimental work and data acquisition:** R. Benjamaa, N. Zyate, A. Talmi & S. Darkaoui; **Analysis of results and bibliographic research:** R. Benjamaa, A. Moujanni, & A. Essamadi; **Data interpretation:** R. Benjamaa, A. Moujanni, & A. Essamadi; **Manuscript writing:** R. Benjamaa & A. Moujanni; **Critical review of the manuscript:** A. Essamadi, A. Moujanni, B. Nasser, R. Eddoha, M. Benbachir & A. Moujahid

**REFERENCES**


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