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Comparison of methods to determine antibacterial activity of honeys against *Staphylococcus aureus*

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ABSTRACT

Nowadays, researching potentially functional properties of honey such as antimicrobial activity is interesting due to the overwhelming problem of bacteria strains resistant to antibiotics, and the expected higher value for honey that consumers constantly demand. In this research, we compared three different methods (agar dilution, broth dilution, as well as agar well diffusion), to analyse honey's antimicrobial activity against *Staphylococcus aureus*, using 56 unpasteurized honeys from different botanical origins. Agar well diffusion method showed to be a rapid and low cost screening method, using less medium and material, to distinguish the samples with and without antibacterial activity. Agar dilution and broth dilution procedures gave similar values. However, the latter proved to be faster and much more informative, providing with minimal antimicrobial and bactericidal concentrations. Higher values of antimicrobial activities were found in honeydew honeys, polyfloral honeys, *Calluna vulgaris* and *Erica* spp. honeys. Conversely, *Lavandula* spp. samples showed lower antimicrobial activity against *Staphylococcus aureus*.

1. Introduction

Honey has been used as a medicine since ancient times, mainly for the treatment of skin wounds, burns, ulcers, ocular infections, sore throat and digital dermatitis, among others [1–3]. The healing capacity of honey is strongly influenced by both physical and chemical properties of this food [4], which are also related to botanical source, honey bee's metabolism, as well as environmental, seasonal and climatic conditions. Apart from healing, honey has been also employed as an excellent preservative for other food commodities, due to its antimicrobial activity [5,6].

The inappropriate use of antibiotics has led to many forms of bacterial resistance, thereby limiting the use of these agents in strains of microorganisms resistant to antibiotics [7]. Research on potentially antibacterial products, such as honey, is of great interest because they could be successfully used against certain microorganisms' strains.

The antibacterial activity of honey has been extensively studied throughout the last years. Nevertheless, most research was carried out on Australian and New Zealand honeys [8–10], existing few studies on European honeys in general [11,12], and on Spanish samples in particular [13,14]. The antimicrobial activity of honeys has been attributed to osmolarity, pH, hydrogen peroxide production, flavonoids, phenolic compounds and the presence of other phytochemical components, such as methylglyoxal, leptosin, melanoidins, bee defensing, jelleins, and hydroxyl radicals [15–21]. Different processing or storage conditions are able to change the composition of honey, modifying its antimicrobial activity. For example, Chen and colleagues [22] observed a decrease in antibacterial and antifungal activity in processed honey (heat to 45 °C for 8 h), while Elbanna and colleages [23] shown a decrease in antibacterial activity for honeys stored at room temperature for 12 and 24 months and also in autoclaved honeys. The great variability of information about the antimicrobial honey compounds may be attributed to differences in both botanical and geographical origins, and consequently to the chemical composition [24,25]. In addition, many factors (climate, genetic composition of plants and bee species), are known to affect honey composition, and thereby such properties as antibacterial activity.

Staphylococcus aureus is a Gram-positive bacterium widely distributed throughout the world. Nowadays, this microorganism is one of the main causes of infections related to hospital care [26]. This is favored by the fact that this species is found in both the skin and mucous membranes of humans, which allows its penetration into the patient's bloodstream through surgical wounds, as well as through direct or indirect contact with medical personnel

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with a contaminated object, or with another patient [27]. Furthermore, this microorganism is also an important foodborne pathogen responsible of several outbreaks [28]. *Staphylococcus aureus* is the bacterium most commonly chosen by researchers to assay antimicrobial activity of honeys. This is because *Staphylococcus aureus* can tolerate honey's high sugar contents and acidity levels, while being sensitive to the antimicrobial action of hydrogen peroxide and the non-peroxide inhibitory action of honey [29,30].

Different protocols have been used to assess honey's antimicrobial activity with very different results. Procedures can be classified as agar diffusion methods, such as agar well diffusion and paper disc diffusion; dilution methods, such as agar dilution and broth dilution; as well as gradient plates, such as wedge system and spiral plating [11,12,19,31]. The use of a great number of non-standardized methods in the determination of honey's antimicrobial activity is a hurdle when assessing and interpreting results. Patton and colleagues [10] compared several antimicrobial activity assays but using only one honey sample. Allen and colleagues [8] evaluated a huge amount of samples, but using only one method (agar well diffusion). With this background, it follows that it would be desirable, for the drawing of reliable conclusions, to compare the most used methods to determine antibacterial activity of honeys using a representative number of samples. Hence, the purpose of this study has been to compare, on more than fifty samples, three different methods (agar well diffusion, agar dilution and broth dilution), widely used to assess honeys' antibacterial activity against *Staphylococcus aureus* in order to propose the most suitable procedure. An additional purpose has been to research possible differences among minimum inhibitory concentrations against *Staphylococcus aureus* of honeys from different botanical origins.

2. Materials and methods

2.1. Samples

This work has been carried out on 56 representative, unpasteurized Spanish honeys, harvested in 2011 in "Castilla y León". The sampling area was larger than 94,200 square kilometers with different landscapes (brushwood, steppe grasslands, prairies and mountains). Botanical origins were determined by melissopalynology [32–34], sensory analysis and such physicochemical parameters as pH, conductivity, color and sugars profile. Most samples were polyfloral honeys. According to their botanical origins, honeys were classified in five groups coded as: A) Polyfloral, B) Honeydew and honeydew-blends (commonly known as "forest honeys"), C) Heather (*Calluna vulgaris* L. (Hull), *Erica* spp. and *Erica* spp./*Calluna vulgaris*), D) *Lavandula* spp. and E) Leguminosae.

The samples were stored in darkness at 4 °C until analysis.

Minimum inhibitory concentration (MIC) assay was developed using fresh-daily serial honey dilutions (75%, 37.50%, 18.75%, 9.38%, and 4.69% [w/v]), aseptically prepared in nutrient broth (Oxoid, Basingstoke, United Kingdom). Agar well diffusion method (AWD) was carried out with 75% (w/v) honey dilution.

2.2. Bacterial strain

Honeys' antibacterial activities were tested against *Staphylococcus aureus subsp. aureus* CECT 976, using decimal dilutions in RINGER (OXOID).

2.3. Antimicrobial activity

2.3.1. Agar well diffusion

Tubes of 20 ml sterile liquid nutrient agar (OXOID) at 50 °C were inoculated with 500 μl of 7 log CFU/ml *Staphylococcus aureus* (overnight cultures grown at 37 °C on nutrient broth, and plated out in nutrient agar media to take colony count), mixed thoroughly, poured into sterile empty plates and left 1 hour at room temperature until solidification. Then, 8 mm diameter wells were cut into the surface of the agar using the back of a sterile blue tip and 150 μl of 75% (w/v) honey were added to each well. After 24-hours incubation at 37 °C, zones of inhibition were measured using a Vernier caliper. The diameter of zones, including the diameter of the well, was recorded. Each assay was carried out in triplicate.

2.3.2. Determination of minimum inhibitory concentration (MIC)

MIC is generally defined as the lowest concentration of a given antimicrobial that prevents growth of a microorganism after a specified incubation period [35]. In this study MIC was calculated using agar dilution and broth dilution methods. MIC was determined in this work as the minimal honey concentration where *St. aureus* growth was not visually observed.

Agar dilution method. Four tubes containing 10 ml sterile nutrient broth (OXOID) were used for each honey in order to perform successive serial half-dilutions to obtain final concentrations from 37.50% to 4.69% (w/v). Ten milliliters of each honey dilution (from 75% to 4.69% [w/v]) was added to 10 ml sterile liquid nutrient agar at 50 °C (final concentration from 37.50% to 2.35% [w/v]). This mixture was homogenized in a vortex mixer, poured into plates and then the agar was allowed to solidify. *Staphylococcus aureus* was added at 5 log CFU/ml to each plate in 5 μl spots. A control plate with no added antimicrobial was prepared and inoculated, ensuring adequate growth of *Staphylococcus aureus*. Plates were incubated at 37 °C for 24 h. The MIC was the lowest concentration that completely inhibited growth.

Broth dilution method. Sterile 96 well round bottomed polystyrene microtitre plates (Brand, Wertheim, Germany) were used. A hundred microliters of 5 log CFU/ml *Staphylococcus aureus* was added to 100 μl of test honey, at different concentrations (from 37.5% to 0.6% [w/v]) in each well (three replicates per dilution, seven dilutions tested). Control wells from each honey were performed adding to each honey dilution 100 μl of RINGER instead culture, in order to observe contamination. Also control wells contained only broth (negative control) or only bacteria and broth (positive control) were made. Plates were incubated at 37 °C during 24 h. Both visible growth and MIC were recorded. Then, 10 μl of each well in which bacterial growth had been inhibited, were plated on to nutrient agar (Oxoid) and incubated overnight at 37 °C in order to research if the antibacterial activity of the honey samples was bacteriostatic or bactericidal. Minimum bactericidal concentration (MBC) was determined as the minimal honey concentration where *St. aureus* didn't grow in agar plates after 24 h at 37 °C. Plates with visible colony growth were considered to exhibit bacteriostatic activities, while those with no growth were recorded as exhibiting bactericidal activities.

2.4. Statistical analysis

Antimicrobial data were statistically analyzed using principal components analysis (PCA) and ANOVA test, where appropriate means were ranked using the least significance difference test (LSD 0.05). Data analyses were conducted using the statistical software package Statgraphics Centurion XVI (2010).

3. Results and discussion

Table 1 shows the results obtained after applying the three different methods to determine honeys' antibacterial activity against *Staphylococcus aureus.* Most of the samples (78.5%) have exhibited antibacterial activity in concentrations lower than 10% (4.69% and 9.38% [w/v]) by both agar dilution and broth dilution methods.