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## The antibacterial activity of acacia, multifloral and oak honeydew honeys

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### Abstract

In the study were presented data for comparison of antibacterial activities between long time stored acacia (*Robinia pseudoacacia* L.), multifloral and oak (*Quercus* spp.) honeydew honeys towards *Staphylococcus aureus* (ATCC 9144). The results showed that the antibacterial activity of oak honeydew and multifloral honeys was much higher in comparison with acacia honeys with the lowest antibacterial activity. In the oak honeydew honey samples there was remaining long time stored antibacterial activity towards *Staphylococcus aureus* (ATCC 9144).

**Keywords:** Bee honey, antibacterial activity, *Staphylococcus aureus*

### Introduction

The antibacterial action of honey was reported for the first time in 1892 (Van Ketel, 1892) [1]. The different aspects of the antibacterial properties of honey have been recently extensively reviewed. The main honey substances are sugars, which by their osmotic effect exert an antibacterial action (Molan, 1992) [2]. There are two sorts of antibacterial agents or so called „inhibines“. One of them is heat- and light- sensitive and has its origin in the H<sub>2</sub>O<sub>2</sub>, produced by honey glucose oxidase (White *et al.*, 1963) [3]. Some workers believe that hydrogen peroxide is the main antibacterial agent (Dustmann, 1979 [4]; White *et al.*, 1963 [5]). Other authors find that the non-peroxide activity is the more important one (Radwan *et al.*, 1984) [6]. The non-peroxide antibacterial activity is insensitive to heat and light and remains intact after storage of honey for longer periods (Bogdanov, 1984) [7]. It was found that the dandelion, honeydew and rape honeys had a relatively higher non-peroxide antibacterial activity. These results indicate that a part of the antibacterial activity might have a plant origin, but the major part of the antibacterial activity of honeydew honey has a bee origin. It can thus be concluded that the honey acids exert the main antibacterial action, while honey pH could additionally act as an antibacterial factor (Bogdanov, 1997) [8].

Honey has been applied for medicinal purposes since ancient times. Its antibacterial effects have been established during the past few decades. Still, modern medical practitioners hesitate to apply honey for local treatment of wounds. This may be explained by the expected messiness of such local application. Moreover, secondary infectious disease may be caused by contamination of honey with microorganisms. Hence, if honey is to be applied for medicinal purposes, it has to meet certain criteria (Ahmed *et al.*, 2003) [9].

In this moment all over the world in medicine is used disc-diffusion method for evaluation of sensitivity of microorganisms to antibiotics. On the other hand it is observe increasing of cases of antibiotic resistance from usually found in wounds microorganisms from *Staphylococcus* spp.

Thus, the aim of the study was to compare antibacterial activity of long time stored Bulgarian's acacia (*Robinia pseudoacacia*), multifloral and oak (*Quercus* spp.) honeydew bee honeys to referent strain of *Staphylococcus aureus* (ATCC 9144).

### Materials and Methods

It was analyzed Bulgarian's acacia (n=10) and multifloral (n=10) honeys, collected near the towns Stara Zagora, and oak honeydew samples harvested near towns Madzharovo (Strandzha mountain), (n=10), all produced in 2006.

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With data from apiarists the samples were from bee families not treated with sugars or antibiotics. Additional analyses from samples were made with methods of the European Honey Commission and collected data were in compliance with regulations for honeydew honeys in Europe with conductivity more than  $0.8 \text{ mS}\cdot\text{cm}^{-1}$ . (Bogdanov *et al.*, 1997<sup>[10]</sup>; Oddo *et al.*, 2004<sup>[11]</sup>).

To determinate antibacterial activity of samples it was used the closes to usually used from determination of antibiotic sensitivity of honey disc-diffusion method, agar well diffusion method, adapted from the punch plate assay for inhibitory substances, described by Molan (Molan, 1992)<sup>[2]</sup>, with some differences, used for blossom and oak honeydew honeys. The antibacterial activity was evaluated by measuring the zones around the wells and expressed in phenol concentration possessing equivalent activity.

### Inoculum Preparation

A freeze-dried culture of referent strain of *Staphylococcus aureus* (ATCC 9144), was reconstituted in Trypticase Soy (TS) broth (Merck 1.18419) and incubated at  $37^\circ\text{C}$  for 18 hours. A loopful of the broth culture was subcultured onto nutrient agar plates (Merck 1.05450.0500) incubated for 24 hours at  $37^\circ\text{C}$ . Working cultures were obtained by placing one bead from the preserver ampoule stock into 10 ml of TS broth and incubating for 18 hours at  $37^\circ\text{C}$ . A further working culture was prepared by inoculating a 200  $\mu\text{l}$  volume of the prepared culture from the previous day, into another 10 ml TS broth. This was incubated for approximately 5 hours at  $37^\circ\text{C}$ . This culture was then adjusted to an absorbance of 0.5 measured at 540 nm using sterile TS broth as a blank and a diluent and a cuvette with a 1 cm pathway. A volume of 100  $\mu\text{l}$  of the culture adjusted to 0.5 absorbance was used to seed 150 ml nutrient agar to make the assay plates.

### Plate preparation

To prepare the assay plates 150 ml nutrient agar (23 g/l) was sterilised then held at  $50^\circ\text{C}$  for 30 minutes before seeding with 100  $\mu\text{l}$  of *Staph. aureus* (ATCC 9144) culture adjusted to 0.5 absorbance as above. The agar was swirled to mix thoroughly and poured into plates for microbiology which had been placed on a level surface. As soon as the agar was set the plates were placed upside-down at  $4^\circ\text{C}$  overnight before using the next day.

The agar must be removed from the autoclave as soon as possible then allowed to cool. One weeks supply can be autoclaved at one time then each day requirements steamed in a saucepan of boiling water for 30 minutes then cooled in a  $50^\circ\text{C}$  water bath for 30 minutes before seeding the agar and pouring the plates. It is essential that the agar is not left at high temperatures for a long time as this change the consistency of the agar.

### Catalase solution

The antibacterial activity of honeydew honeys was investigated after added solution of Catalase, Bovine Liver (CALBIOCHEM, 5MU, activity: 12523.0 U/mg, Cat No 219001), 2 mg/ml in distilled water was prepared fresh each day to sample solution to reduce possible peroxide activity.

### Sample preparation

A primary honey solution was prepared by adding 10 g of well mixed honey to 10 ml of distilled water in universals and placed at  $37^\circ\text{C}$  for 30 minutes to aid mixing. To prepare secondary solutions, 1 ml of the primary honey solution was

added to 1 ml of distilled water in a bijou for total activity testing and 1 ml of the primary honey solution was added to 1 ml of catalase solution for non peroxide activity testing. The density of honey, which is  $1.35\text{g/ml}$ , is allowed for in the final calculation.

### Sample and Standard Application

Each sample was tested in quadruplicate by adding 100  $\mu\text{l}$  to each of 4 wells with the same allocated number on the assay plate.

### Plate incubation

After application of samples and standards the plates were incubated on individual racks not stacked on top of one another, for 18 hours at  $37^\circ\text{C}$ .

### Zone measurement

The plates were placed back over the black quasi-Latin square to measure the diameter of the zones of inhibition with digital calipers using the points of the prongs used to measure inside diameters of tubes.

### Preparation of phenol standards

Standards of 2%, 3%, 4%, 5%, 6%, and 7%, were prepared from a 10% w/v solution by Phenol BDH A.R. (merck CgAA, Germany, Cat No 1.00206.0250). These solutions were kept at  $4^\circ\text{C}$  for one month before making fresh standards and brought to room temperature in the dark before use. Each standard was placed in two wells to test in duplicate.

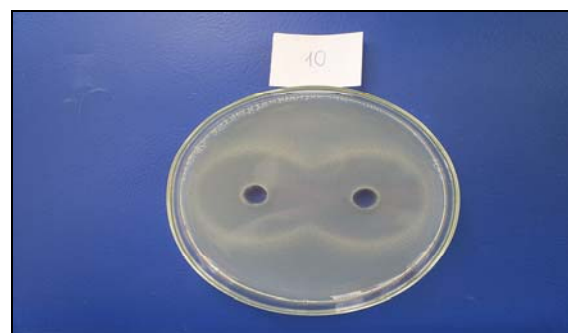
### Calculation of antibacterial activity of honey

The mean diameter of the clear zone around each phenol standard was calculated and squared. A standard graph was plotted of % phenol against the square of the mean diameter of the clear zone. To allow for the dilution and density of honey this figure was multiplied by a factor of 4.69 and the activity was then expressed as the equivalent phenol concentration (% w/v). The factor of 4.69 is based on a mean honey density of  $1.35\text{g/ml}$ .

Statistical analyses were tested by t-recr (Statmost<sup>TM</sup> for Windows). All analyses were done in 2010 in the Department of Hygiene, Technology and Control of Animal Foodstuffs, Veterinary Legislation and Management, faculty of Veterinary Medicine, Trakia university, Bulgaria.

### Results

Results from our experiments, shows that in all cases for all tested honeys without adding of catalase to sample solutions we found high zones with total inhibition (antibacterial activity) towards *Staph. aureus* (ATCC 9144), (Figure 1).



**Fig 1:** Antibacterial activity towards *Staph. aureus* (ATCC 9144) without adding of catalase solution to sample No 10 (oak honeydew honey, harvested near town Madzharovo).

In all cases before adding of catalase solution we found similar zones of antibacterial activity to referent strain of *Staphylococcus aureus* (ATCC 9144). The lowest value ( $29.45 \pm 1.01$  mm) of inhibition was found from acacia

honeys and the highest value from investigated oak honeydew ( $37.90 \pm 0.99$  mm) and multifloral ( $37.50 \pm 3.27$  mm), honeys (Table 1).

**Table 1:** Antibacterial activity (zones of inhibition) for acacia, multifloral and oak honeydew honeys, mm.

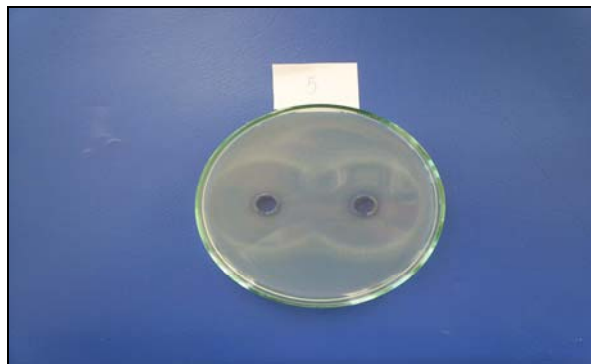
Types of honey and number of samples	Statistical parameters	Antibacterial activity	
		Without catalase	Catalase
Acacia ( <i>Robinia pseudoacacia</i> L.) honey, n=10	X ± SD Min Max	$29.45 \pm 1.01^a$ 28 31	n.f.
Multifloral honey, n=10	X ± SD Min Max	$37.50 \pm 3.27^{a,b}$ 32 42	n.f.
Oak ( <i>Quercus</i> spp.) honeydew honey (Madzharovo, Bulgaria), n=10	X ± SD Min Max	$37.9 \pm 0.99^{a,b}$ 37 40	$12 \pm 1$ (n=3) 11 13

Mean values (X); standard deviations ( $\pm$ SD); n.f. – not found antibacterial activity;

<sup>a</sup> - In most cases (acacia – blossom; acacia – honeydew; blossom – honeydew) - statistically significant differences between antibacterial activities ( $p < 0.0001$ ).

<sup>b</sup> - Comparison between antibacterial activity of oak honeydew and multifloral honeys - not found significant differences.

In all cases after adding of catalase solution by used agar well diffusion method we not found antibacterial activity to used strain of *Staphylococcus aureus* (ATCC 9144). But, in three from oak honeydew samples possesses zone of long time stored antibacterial activity against tested resistant bacterial strain, after adding of catalase solution (2 mg/ml), equivalent to 3% phenol standard (Table 1 and Figure 2).



**Fig 2:** The long time stored antibacterial activity after adding of catalase solution (2 mg/ml) to sample No 5 (oak honeydew honey, harvested near town Madzharovo).

### Discussion

Our results from antibacterial activity of honeys were similar to these of Portuguese scientists which point that all natural honeys possessed peroxide antibacterial activity (activity destroyed by catalase), (Henriques *et al.*, 2005) [12]. In most cases after adding of catalase solution by used agar well diffusion method we not found antibacterial activity to *Staphylococcus aureus*, but three from oak honeydew honeys have the long time stored antibacterial activity (Table 1 and Figure 2).

With compliance with results from other authors we could point that the antibacterial activity of blossom honeys was mainly from honey glucose peroxidase system (Molan, 1992 [2], Bogdanov, 1997 [8]). The greater the difference in the molar concentration of glucosoxidase and catalase in honeys

then the greater will be the potential to generate hydrogen peroxide and the greater will be the amount of catalase required to add to that sample for an antibacterial assay of “non-peroxide” activity.

We could point, that in the moment we know that all *Staphylococcus* spp. have enzyme catalase. But we don't know the quantity of this enzyme in different strains of this microorganism. We subscribe to opinion of other scientist's (Weston, 2000) [13], that it is conceivable that absence of catalase in different honeys might be the “unique factor” which differentiates “active” from “inactive” honeys. We make the hypothesis that the quantity or absence of glucosoxidase and catalase in some of oak honeydew honeys after long time storage and different quantity of catalase in *Staphylococcus* spp. could be in responsibility with sensitivity of this microorganism to this type of bee honey. To prove these hypotheses it is need additional surveys from content of catalase in different *Staphylococcus* spp. and also content of glucosoxidase and catalase in oak honeydew honeys after long time storage.

### Conclusion

In this study were presented from the first time data for comparisons between antibacterial activities from long time stored acacia, multifloral and oak honeydew honeys. It was found that some honeydew honeys from different geographical origin could be with long time stored antibacterial activity potential.

### Acknowledgement

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