EFFECTS OF EXTRACTION, STORAGE CONDITIONS AND HEATING TREATMENT ON ANTIBACTERIAL ACTIVITY OF *Zanthoxylum fagara* HONEY FROM COJEDES, VENEZUELA

Efectos de las Condiciones de Extracción, Almacenamiento y Tratamiento Térmico en la Actividad Antibacteriana de Mieles de *Zanthoxylum fagara*, Cojedes, Venezuela

***Agustina Manuela Ríos*[1], *María Luisa Novoa*[1] *y Patricia Vit*[2]**

[1]*Departamento de Microbiología de Alimentos, Instituto Nacional de Higiene "Rafael Rangel", Ciudad Universitaria. Caracas, Venezuela.*
[2]*Apiterapia y Vigilancia Ambiental, Departamento Ciencia de los Alimentos, Facultad de Farmacia, Universidad de Los Andes. Mérida, Venezuela.*

ABSTRACT

In this study, the influence of extraction, storage conditions and heating treatment on the antibacterial activity of *Zanthoxylum fagara* honey produced by *Apis mellifera* was researched. For that purpose, scraped and centrifuged honey samples from the Guilan apiaries (Cojedes state, Venezuela) were stored in darkness or sunlight for four months and exposed to different heat treatments (40°C for 30 days, and 78°C for 5, 10 and 15 min). The antibacterial activity was studied for 5%, 10%, 15%, 20% and 25% of bees honey in nutrient agar and the inhibitory grade was ranked from 0 to 5. The selected bacterial strains for this study were *Bacillus subtilis, Escherichia coli, Listeria* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Storage in darkness or sunlight for four months, as well as mild heat treatment (40°C for 30 days) did not affect the antibacterial properties. Antibacterial activity was higher in scraped honey, but it was more vulnerable to heat treatment than centrifuged honey. Exposures of scraped honeys at 78°C for 15 minutes significantly decreased the antibacterial activity, with partial losses of 67,4% for *E. coli* and 54% for *S. aureus. Listeria* spp. was the most sensitive bacteria. *E. coli was* the most resistant bacteria for heated honey and *B. subtilis* the most resistant for fresh honey.

Key words: Antibacterial activity, honey, heat treatment, storage.

RESUMEN

En este estudio se investigó el efecto de las condiciones de extracción, almacenamiento y tratamiento térmico sobre la actividad antibacteriana de mieles de *Zanthoxylum fagara* producidas por *Apis mellifera*. Para ello se extrajeron mieles por raspado y por centrifugación en los apiarios Guilan (Cojedes, Venezuela). El almacenamiento se realizó en condiciones de oscuridad o exposición a la luz solar durante cuatro meses. Se seleccionaron cuatro tratamientos térmicos (40°C por 30 días, y 78°C por 5, 10 y 15 min). La actividad antibacteriana se midió en concentraciones crecientes de 5%, 10%, 15%, 20% y 25% de miel de abejas en agar para medir el grado de inhibición en un rango de 0-5. Las cepas bacterianas seleccionadas fueron *Bacillus subtilis, Escherichia coli, Listeria* sp., *Pseudomonas aeruginosa* y *Staphylococcus aureus.* El almacenamiento en la oscuridad y bajo exposición a la luz solar por cuatro meses, y el tratamiento térmico de 40°C por 30 días, no alteró las propiedades antibacterianas. Si bien las mieles raspadas presentaron mayor actividad antibacteriana, también fueron más vulnerables al tratamiento térmico. La exposición a 78°C por 15 min de mieles raspadas disminuyó significativamente la actividad antibacteriana, con pérdidas parciales de 67,4% para *E. coli* y de 54% para *S. aureus. Listeria* spp. fue la bacteria más sensible. *E. coli* fue la bacteria más resistente en miel recalentada y *B. subtilis* la más resistente en miel recién cosechada.

Palabras clave: Actividad antibacteriana, miel, tratamiento térmico, almacenamiento.

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INTRODUCTION

The therapeutical use of honey has proved to be beneficial in the treatment of burns, wounds and skin ulcers because of its antibacterial properties [23]. The nature and extent of its antibacterial activity has been attributed to several factors, but

is not yet completely understood. Beeswax, flower volatiles, nectar, pollen, propolis and honey are collected or manufactured by bees to construct their hive and feed the colony [25]. These materials and some of its components may explain the antibacterial behaviour of honey. The low pH and the high sugar content of honey are sufficient to prevent the growth of many species of bacteria or fungi. However, both factors may be neutralized if honey is diluted. The presence of hydrogen peroxide generated by enzymatic activity of glucose oxidase in diluted honey is considered as the major antibacterial factor [27]. The findings of antibacterial activity that is stable to heating [3, 4, 9, 14, 19, 24] and resistant to catalase treatment [1, 2, 14, 15] are evidence for the existence of non-peroxide antibacterial factors, such as pinocembrin [3], syringic acid, methyl syringate and other phenolic acids [20], terpenes and benzyl alcohol [24].

The antibacterial activity of honey from the same floral source can vary from lot to lot and can be easily lost by inappropriate handling and storage. Hydrogen peroxide is destroyed by components of honey and declines with time [27]. Honeys with high level of catalase have low level of hydrogen peroxide [8]. As hydrogen peroxide decomposes, it generates highly reactive free radicals which react and kill bacteria [16]. Thermal stability and denaturation of glucose oxidase by light vary with floral sources [26, 28, 29]. In one study on thermal stability of antibacterial properties of honey, an almost complete loss of these properties was found after heating at 100°C for 10 min [29], while in another, the activity was not completely lost after exposure to 100°C for 15 min [5]. Lower degrees of heating caused partial loss or even increase of the antibacterial activity. The minimum inhibitory concentration of honey was found to increase from 4% to 8% after exposure of honey to 46°C for 8 h, and increase to 12% after exposure to 55°C for 8 h [31]. Allen *et al.* [2] found no correlation between the antibacterial activity and the age of honey. Little or no loss of activity was found in honeys stored for one year at room temperature in closed containers, but loss was observed in samples frequently opened [12].

Being an edible natural product with medicinal properties, honey antibacterial activity should be required in batches for therapeutical use [17, 18]. The present study was done to observe the effect of extraction, storage and heating conditions on the antibacterial activity of *Zanthoxylum fagara* (Rutaceae) honey, measured with the conventional *B. subtilis*, but also with *E. coli, Listeria* spp., *P. aeruginosa* and *S. aureus* because they are resistant and frequent bacteria in ulcers and wounds. Scraped and centrifuged honeys were tested to detect what extraction method would be more desirable to achieve a higher antibacterial activity of honey. Storage in the darkness was compared with storage under sunlight exposure. Heating treatments were considered, due to the belief that only liquid honey is pure honey and heating is generally applied without control to melt natural crystals of Venezuelan commercial honey.

MATERIALS AND METHODS

Samples

Ten *Apis mellifera* honey samples (750 to 1000 g each) were collected from Guilan apiaries in Taguanes, Cojedes state, Venezuela. This area has adequate characteristics for beekeeping and honey production on a commercial scale. Pollen analysis to estimate the botanical origin of the honeys revealed a monofloral condition for *Zanthoxylum fagara*, a Rutaceae locally known as "arañagato" (López-Palacios, 1986) with presence of *Protium heptapyllum, Psidium guajaba, Roupala montana, Hyptis suaveolens, Verbesina turbacensis, Tridax procumbens, Cocos nucifera, Mimosa pudica* and *Mimosa albida*. The pollen density of the samples fell into Maurizio class II (2000 to 10000 pollen grains/g honey). To ensure homogeneity, all the samples were mixed inside a 1000 mL jar by upside down inversion every 2 min during 1 h, followed by 25 vertical and spinning movements with a large spoon. The homogenized honey samples were distributed in 50 g portions in 2 cm diameter glass test tube and capped.

Methods of honey extraction

Five of the samples were harvested from totally operculated combs, scraped and sieved in the laboratory; the other five samples were extracted by centrifugation.

Storage conditions and heating treatments

Storage conditions and heating treatments were: control at harvest time (1), stored in darkness (2) and exposed to daily sunlight (3) during four months, incubated at 40°C during 30 days (4), heated in a water bath at 78°C during 5 min (5), 10 min (6), 15 min (7).

Analysis

The antibacterial activity of the honeys was tested incorporating (5%, 10%, 15%, 20% and 25% w/v) honey in nutrient agar plate following the technique published in the *Methodes Officielles d'Analise du Miel* [13]. The plates were inoculated immediately after solidification, with 0.1 mL of a suspension from a 24h culture at 32°C, previously adjusted to 10^6 cfu/mL of *Bacillus subtilis* (ATCC 6633), *Listeria spp.* (Bureau of Microbial Hazard, Health Protection Branch, Canada), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 14502). The plates were incubated for 24 hours at 35°C. The inhibition grade was ranked from 0 (without inhibition by 25% honey) to 5 (without inhibition by 5% honey), according to the honey dilution required to observe growth inhibiton. Fractions of 0.25, 0.50 and 0.75 indicated inhibition of 3/4, 1/2 and 1/4 of the plate. The minimum inhibitory concentration was determined by the method of serial dilution tubes. Statistical differences were considered significant at $p<0.05$ with *t*-test.

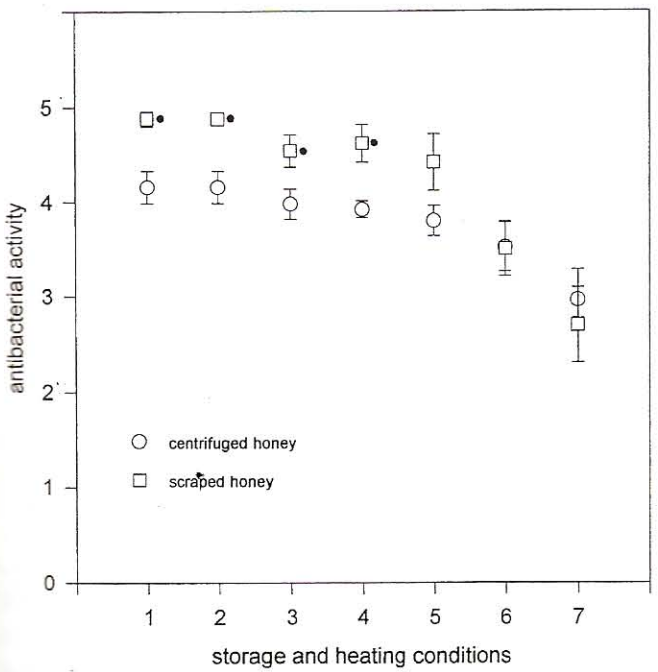

FIGURE 1. VARIATION OF ANTIBACTERIAL ACTIVITY IN CENTRIFUGED AND SCRAPED HONEYS AT STORAGE AND HEATING CONDITIONS 1-7 (SEE MATERIALS AND METHODS). VALUES ARE MEAN ± S.E.M. FOR THE FIVE TESTED BACTERIA STRAINS.

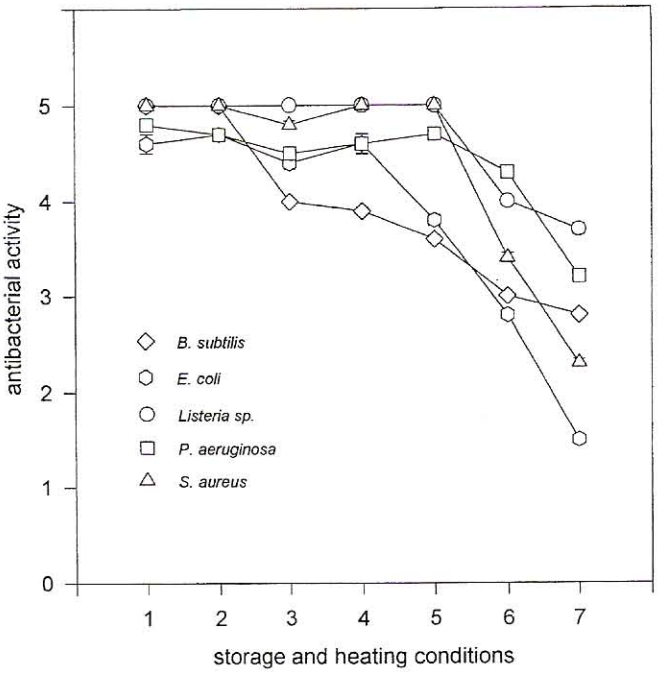

FIGURE 2. EFFECT OF BACTERIA TYPE ON ANTIBACTERIAL ACTIVITY MEASUREMENTS IN SCRAPED HONEYS AT STORAGE AND HEATING CONDITIONS 1-7 (SEE MATERIALS AND METHODS). VALUES ARE MEAN ± S.E.M., n = 5.

RESULTS

FIG. 1 shows the average antibacterial activity for the five selected bacterial strains measured for centrifuged and scraped honeys, after, storage conditions and heating treatments (1-7) were applied. Compared with the control (1), antibacterial properties of honey were not affected after storage in the dark (2), sunlight (3), heat treatment at 40°C for 30 days (4) and 78°C for 5 min (5), but a decline was observed after heating at 78°C for 10 min (6) and 15 min (7). Differences between scraped and centrifuged honeys were significant ($p<0.05$) for the control (1), storage in the dark (2), sunlight (3) and at 40°C (4). Heating at 78°C increased the variation in response of the different bacteria and gave non-significant difference between the two extraction methods.

The behaviour of individual strains after different treatments is presented in FIG. 2 for scraped honey and FIG. 3 for centrifuged honey. In all strains, both the antibacterial activity values and the range of variation at harvest time were higher in scraped honey than in centrifuged samples. Reduction of activity due to sunlight exposure was well detected by *B. subtilis* in scraped honey, while in centrifuged honey minor losses were detected by all strains except *S. aureus*. For both extractive methods, major decreases of antibacterial activity were caused by heating at 78°C during 10 and 15 minutes, as observed in treatments 6 and 7.

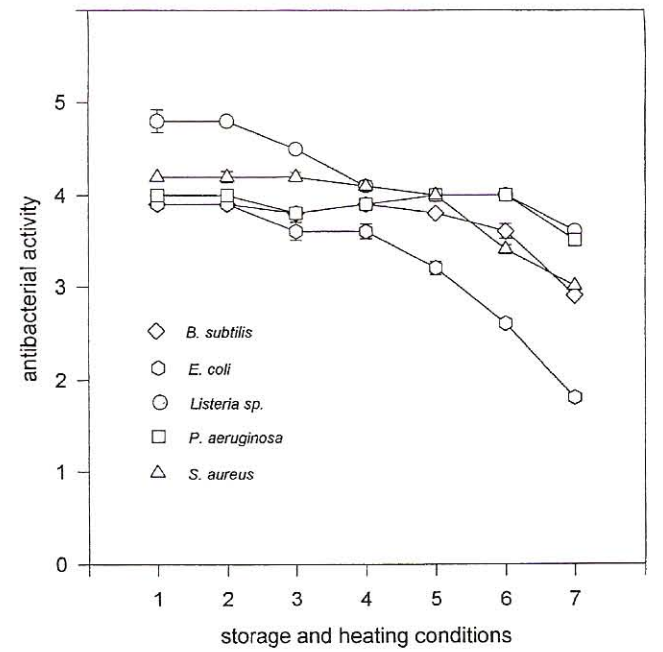

FIGURE 3. EFFECT OF BACTERIA TYPE ON ANTIBACTERIAL ACTIVITY MEASUREMENTS IN CENTRIFUGED HONEYS AT STORAGE AND HEATING CONDITIONS 1-7 (SEE MATERIALS AND METHODS). VALUES ARE MEAN ± S.E.M., n = 5.

TABLE I
PERCENTAGE OF LOSS OF ANTIBACTERIAL ACTIVITY IN SCRAPED AND CENTRIFUGED HONEYS HEATED AT 78°C DURING 5 min (5), 10 min (6) AND 15 min (7)

Honey treatments		Percentage of loss of antibacterial activity				
		Bacillus subtilis	*Escherichia coli*	*Listeria* spp.	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Scraped	5	28.0	17.4	26.0	2.1	0.0
	6	40.0	39.1	20.0	10.4	32.0
	7	44.0	67.4	26.0	33.3	54.0
Centrifuged	5	3.0	17.9	16.7	0.0	4.8
	6	28.0	33.3	16.7	0.0	19.0
	7	42.0	53.8	25.0	12.5	28.6

The percentage of loss of antibacterial activity shown in TABLE I was calculated for heated honeys (treatments 5, 6 and 7), referred to values at harvesting time. In all treatments, antibacterial activity losses were higher in scraped honey, except for the 5 min heating measured with *E. coli* and *S. aureus*. The gradient of antibacterial activity loss according to the heating time was variable for the different strains. Heating of centrifuged honey for 5 and 10 min at 78°C, maintained its antibacterial activity against *P. aeruginosa*, but 12.5% of this activity was lost after 15 min heating at the same temperature. For both scraped and centrifuged honey the major losses of antibacterial activity against *E. coli* were present at 15 min heating at 78°C. *B. subtilis* and *Listeria* spp. had similar values for both extractive methods after 15 min heating.

The minimum inhibitory concentration of honey at the harvesting time and the extreme heat treatment in this study (78°C for 15 minutes) for the tested strains is presented in TABLE II. Averages of 7.9 and 12.6 g honey/mL were found for the two selected treatments.

TABLE II
MINIMUM INHIBITORY CONCENTRATION OF HONEY AT HARVEST TIME AND AFTER HEAT TREATMENT

Bacterial strain	Minimum inhibitory concentration of honey (g honey/ mL nutrient agar plate)	
	Harvest Time	78°C x 15 min
B. subtilis	10.0	14.0
E. coli	8.0	17.0
Listeria sp.	6.0	10.0
P. aeruginosa	8.0	10.0
S. aureus	7.5	12.0
Average	7.9	12.6
S.E.M.	0.64	1.33

DISCUSSION

When honey is squeezed from the comb, a small amount of propolis is integrated into the honey. Since propolis contain flavonoids with antimicrobial properties [10], the higher average antibacterial activity observed in scraped honeys, FIG. 1, can be explained by this fact. With all strains, the antibacterial activity values at harvest time were higher in scraped honeys, FIG. 2, than in centrifuged samples, FIG. 3.

Hydrogen peroxide is destroyed by honey catalase [21, 27]. In honeys of the same floral source, levels of hydrogen peroxide accumulated from enzymatic action of glucose oxidase were seen to decline with time and *S. aureus* has been observed more sensitive than *B. subtilis* to hydrogen peroxide [17]. This observation agrees with our results in scraped honeys after four months of storage in sunlight and one month at 40°C, because having the same initial antibacterial activity, *B. subtilis* became more resistant than *S. aureus* with time.

There are many reports with large differences in the stability of antibacterial activity of honey at mild heat treatments. Honey stored at 20°C for several months without deterioration of its antibacterial activity has been reported by Gonnet and Lavie [9], but losses of 15-16% for three months and 24-27% for six months were found by Radwan *et al.* [19]. Generally, it is accepted that honey is stable at room temperature below 40°C [5]. This is expected after considering that 34°C is an average temperature in the bee-hive, where honey can spend long time [17]. In the present study there were no differences between the antibacterial activity at harvesting time and after one month at 40°C.

Although the antibacterial activity was higher in scraped honey at different storage conditions, the heating at 78°C caused higher losses than in centrifuged honey, TABLE I. These results show that both heat-sensitive and heat-stable antibacterial substances are present in *Mimosa pudica* honeys. Volatile and thermostable substances of bee origin, non-volatile and thermostable derivatives of benzoic acid [20] and volatile fractions of different honeys [24] have been considered to explain this double nature of antibacterial substances. The floral source of honey assayed in this study has not yet been attributed antibacterial properties. The decline of antibacterial

activity in scraped honeys after heating for 15 min was higher than in centrifuged honeys, when measured with *E. coli*, *P. aeruginosa* and *S. aureus*, FIGS. 2 and 3. This fact suggests the thermosensitive nature of the antibacterial agent present in scraped honey.

Earlier studies found that direct sunlight decreased the antibacterial properties of honey, specially if it is exposed in thin films and transparent glass, while jars with transmission lower than 400 nm and diffuse sunlight did not affect it. Dustmann [8] found that in honeys from some floral sources, non-identified substances, made glucose oxidase more sensitive to denaturation by light. White and Subers [29] suggested the presence of a photosensitizer for the photo-oxidation of the enzyme. Glucose oxidase being sensitive to light, the loss of activity is affected by the type of container and the intensity of light; gradual loss occurs when exposed to direct sunlight, but not with diffuse daylight [29]. Dark honey was found to be more light stable than light honey, due to lower light penetration into the bulk of honey. In this study the variables for the sunlight test were bacterial strain and extraction method. Scraped honeys could have had a higher presence of a photosensitizer required to oxidize the antibacterial substance to which *B. subtilis* was more sensitive, therefore only this strain detected the variation caused by exposure to sunlight for four months. However these changes were not detected with *Listeria* spp. In centrifuged honeys, inhibition of growth did not vary for *S. aureus*, but losses of antibacterial activity were detected with all other strains.

In a previous study of non-specified age of commercial honeys from Caracas, the minimum inhibitory concentration of honey was 15% against *B. subtilis* whereas lower values of 10.3% for *Apis mellifera* and 11.0% for stingless bee honeys were found in Brazil [7]. In TABLE II, the average inhibitory concentration of honey varied from 7.9% at harvest time to 12.6% for honey heated at 78ºC during 15 minutes, showing that honey treatment is controlling the antibacterial activity to a greater extent than the entomological origin of honey.

CONCLUSIONS

Scraped honey had higher values of antibacterial activity than centrifuged honey, but it was more vulnerable to heat treatments than centrifuged honey. Main losses of antibacterial activity under sunlight exposure were measured against *B. subtilis* in scraped honey and *Listeria* spp. in centrifuged honey.

Listeria spp. also was the least resistant bacteria, with 6% minimum inhibitory concentration of fresh honey and *E. coli* the most resistant with 17% minimum inhibitory concentration of honey after heating at 78°C for 15 min. *B. subtilis* is the most resistant at harvest time, confirming the international preference to test the antibacterial properties of traded honey [13] which is not intended to undergo any overheating process.

RECOMMENDATIONS

Allen *et al.* [2] found that differences in floral origin were more significant than differences in storage conditions, to explain losses in antibacterial activity. Plant-derived substances influenced the stability of the glucose oxidase, and consequently the antibacterial activity. A detailed profile of chemical groups, such as the flavonoids proved the presence of common and distinctive species according to the botanical origin of honeys [22]. A future study of these components in *Zanthoxylum fagara* honey will provide a complementary information for the botanical origin determined by pollen content and a basis to observe the antibacterial effects of individual compositional factors.

A great contribution for Venezuelan apiculture would be the study of antibacterial properties of other monofloral honey. Some medicinal honeys may be described under such approach and will provide evidence to improve the local regulations of honey, with a comprehensive review like A Century of Federal Honey Research [30]. Venezuelan honey standards [6] show the lack of microbiological control and antibacterial tests. A database is under construction to support the addition of standards on antibacterial properties of honey.

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