The Science Behind The Active Honey Patagonia Factor

Certified antibacterial honey from Chilean Patagonia

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The research team, led by Gloria Montenegro, carried out different science, research, and technology and development projects over more than three decades.

These generated results provisionally called "analysis of the biological activity of various native/endemic Chilean honeys concerning its biocidal potential". (Lusby et al. 2005;Muñoz et al 2007; Lee et al. 2008; Montenegro & Ortega 2011; Schencke et al. 2011;Montenegro et al. 2013a; Montenegro et al. 2013b; Calderon et al. 2015; Schencke et al.2015; Bridi et al. 2016; Montenegro & Velasquez 2017, Bridi & Montenegro 2017;Giordano et al. 2018)

According to the Codex Alimentarius, honey is a natural sweet substance produced by bees (Apis mellifera) from flower nectar or secretions of living parts of the flowers. The nectar is collected and transformed into honey by enzymatic actions and dehydration, reaching water content of approximately 18% (Montenegro and Mejias, 2008). Honey contains proteins, organic acids, vitamins (especially vitamin B6, thiamine, niacin, riboflavin and pantothenic acid), minerals (including calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), pigments solid particles derived from bee pecoreo. Besides a great variety of volatile compounds and secondary compounds derived from the plant species from which it originates, such as phenolic compounds and terpenes. Chemical, sensory, physical and microbiological characteristics are the main determinants of honey quality (Alvarez-Suarez et al., 2010, Montenegro et al. 2013).

One of the main reasons for the antibacterial activity of honey is its high sugar content (close to 80%) and acid pH (between 3 and 4.5) due to gluconic acid, derived from glucose catalysis (Araya, 2004; Ordoñez, 2015, Ramírez, 2013; Cortez et al. 2011, Ulloa, 2010). Also, when bees visit the flowers, they introduce an enzyme to convert the nectar into honey. This enzyme, called hydrogen peroxide activity (HPA), gives it antibacterial properties (Garcia, 2018; Ramirez, 2013; Ordonez, 2015). A study published in 1937, was the first to examine in detail the honey's antimicrobial effect and its authors called "inhibin", which was initially responsible for this effect, before identifying it as hydrogen peroxide. It is important to note that most varieties of honey contain hydrogen peroxide when freshly harvested from the hive. However, this enzyme is thermolabile and photosensitive, so if it is excessively heated or exposed to direct light for a long time in its packaging or storage, it loses its antiseptic properties (Moore, 2011).

In bee honey, there are also non-peroxide type compounds that generate antibacterial activity; these vary according to the plant species from which the bees take the nectar (García, 2018). We identified the defensin-1 peptide, attributing it antimicrobial properties after studies carried out with several microorganisms (Fiorilli, 2015).

As the honey inherits properties, according to its botanical origin, one would expect to find significant differences in its biological activity. Worldwide, the honey of Eucalyptus marginata, Kunzea ericoides, Leptospermum scoparium and Knightea excelsa have high HPA levels and therefore used in many cures (Alqarni, 2012, Stephens et al., 2010).

In Chile, the botanical origin of honey has been studied, allowing its export with a certificate of botanical and geographical origin, while demonstrating, at the same time, the variety of native flora used by bees (Ramirez and Montenegro, 2004, 2000; Montenegro et al., 2003, 1992; Avila et al., 1993; Montenegro and Avila, 1995). Thus, among Chilean varieties of honey, the botanical origin of endemic species such as "Corontillo" Escallonia pulverulenta and "Quillay" Quillaja saponaria in the central and north-central zone of the country and native species such as "Ulmo" Eucryphya cordifolia, in the southern region, stands out (Montenegro, 2002; Montenegro et al., 2003; Montenegro et al., 2008; Montenegro et al., 2010a; Montenegro et al., 2010b). Studies conducted by the Pontificia Universidad Católica de Chile indicate that the different native honey of Chile show significant differences in both antioxidant activity and activity against pathogens, which depends on the botanical and geographical origin and may be associated with the content of polyphenols (Montenegro et al. 2012).

Different studies state that there is a close relationship between the floral origin vs. color vs. composition vs. antioxidant capacity vs. antimicrobial capacity of the bee honey, where the different darkest honey are the ones that reflect a higher content of bioactive compounds and therefore a higher biological activity. In a 2010 study, I observed significant antimicrobial activity for ulmo and Manuka UMF 25+. The scientists were amazed by the ulmo's activity and suggested to study it in depth. That is why ulmo single-flower honeys have been used for research in wound healing based on their proven antibacterial properties. Sherlock et al., (2010) tested the antimicrobial activity of Chilean honey produced from ulmo against a group of bacteria, showing this honey has a higher antibacterial effect for Staphylococcus aureus, Escherichia coli and Pseudomonas aeroginosa compared to Manuka honey (Schencke, 2013).

It is worth mentioning that recent studies indicated that dilutions of native Chilean ulmo honeys had an inhibitory action at different concentrations against bacteria such as Pseudomona aeruginosa and Escherichia coli. However, this did not occur in all ulmo honey, which could be because the antimicrobial characteristics of diverse kind of honey are affected, among other things, by the accompanying species, which vary according to the year and area, which would explain the differences between samples of the same botanical origin (Kucuk et al., 2007; Molan, 1992).

Several works by Montenegro and collaborators have proven the bactericidal properties of Chilean honeys that originates from native or endemic species of the temperate forest of southern Chile, the scrublands of central Chile and the shrubby steppes of the northern zone (Muñoz et al. 2007; Montenegro & Ortega 2011; Montenegro et al. 2013a; Montenegro et al. 2013b; Bridi et al. 2016; Montenegro & Velasquez 2017, Bridi & Montenegro 2017; Giordano et al. 2018;).

Chile has a great diversity of endemic and native plant species that can give rise to a range of honey produced by Apis mellifera. Continental Chile has an area of nearly 75 million hectares located in the southwest of South America. It has a length of approximately 4,300 kilometers from north to south and an average width of 180 kilometers. The presence of two mountain ranges, such as the Andes and the Coastal Range, generate very diverse geomorphology with very different valleys and microclimates, besides a diverse community of plants capable of producing nectar for Apis mellifera. Also, the presence of the Atacama Desert to the north and the Pacific Ocean to the west determines geographical isolation evolving towards biodiversity characterized by high endemism of species and ecosystems. Chile's vascular flora has just over 5,500 species, not including subspecies and varieties (Marticorena, 1990, Montenegro 2000; Montenegro et al 2002). Although the number of species compared to other Latin American countries is low, the most prominent feature is that almost half of the flora is endemic, giving a unique character to the products that arise from these species, such as "bee honey" which also result in unique biological properties. (Montenegro y Mejias 2013; Montenegro & Ortega 2011 WO/2011/057421;

Montenegro y Mejias 2013; Silva et al 2016; Bridi et al. 2016; Montenegro & Velázquez 2017; Bridi & Montenegro 2017; Giordano et al. 2018). Central Chile is a biodiversity hotspot where endemic plants account for 0.5% of the 300,000 plant species described worldwide. This means that 46.8% of the plants described in Chile are endemic (Myers et al., 2000).

The production of single-flower honeys of endemic or native floral origin arises in two large geographical regions of the country. The first corresponds to the Central Zone of Chile, which has a semi-arid Mediterranean climate, where the Evergreen Sclerophyll Scrub is the dominant vegetation. The second, towards the south, corresponds to the humid Mediterranean region where the Temperate Broadleaf Forest, also locally called Bosque Valdiviano, predominates. The characteristic of the central zone is the production of endemic single-flower honeys (Montenegro et al. 2008, Muñoz et al. 2007; Montenegro & Ortega 2011; Montenegro et al. 2013a; Montenegro et al. 2013b; Bridi et al. 2016; Montenegro & Velasquez 2017, Bridi & Montenegro 2017; Giordano et al. 2018) such as quillay (Quillaja saponaria), corontillo (Escallonia pulverulenta) corcolén (Azara petiolaris) and Tevo (Retanilla trinervia). While towards the south we find production of native single-flower honey of hazelnut (Genuina avellana), ulmo (Eucryphia cordifolia), and tineo (Weinmannia trichosperma) and tiaca (Caldcluvia paniculata). Besides, there is a production of honeys from the nectar of introduced species (Muñoz et al. 2007; Montenegro et al. 2009a; Montenegro et al. 2009b; Montenegro et al. 2013). The pollen profile in the honey reflects the vegetation surrounding the apiary, the seasonal floral diversity, and the species composition of the plants the bees have foraged. The relative frequency of pollen grains present in honey is determined through the melissopalynological method internationally accepted as an indicator of botanical/geographical origin, a tool of great importance for marketing honey with higher added value (Montenegro et al. 2010; Corvucci et al. 2015). In Chile, the official standard (NCh2981.Of2005, Montenegro, et al. 2008) establishes that the melissopalynological analysis must be used to differentiate the botanical origin of the diverse kind of honey produced in this country, which can classify according to three different types of floral or botanical sources: mono-flower, bi-flower, or poly-flower. Single-flower or uni-flower kinds of honey are those where at least 45% of pollen or more belongs to a particular species. Bi-flower honey is that where the pollen of two species is dominant, covering a total between both of more than 50%. There is not a difference higher than 5% between both. Finally, poly-flower honey is the one where no species covers the previous requirements (Montenegro et al. 2008).

Antimicrobial Analysis

Several studies have proven the antibacterial activity of honeys (Lusby et al. .2005Muñoz et al. 2007; Lee et al. 2008; Montenegro & Ortega 2011; Schencke et al. 2011; Montenegro et al. 2013a; Montenegro et al. 2013b; Calderon et al. 2015; Schencke et al. 2015; Bridi et al. 2016; Montenegro & Velasquez 2017, Bridi & Montenegro 2017; Giordano et al. 2018) describing their bacteriostatic or bactericidal activity. The antimicrobial nature of honey depends on different factors that act individually or synergistically, being one of the most significant, the presence of phenolic compounds of hydrogen peroxide, of the pH of honey among the most important. The antibacterial capacity of ulmo honey (Eucryphya cordifolia) has been previously described and patented (Montenegro and Ortega 2011, Montenegro et al. 2013, Sherlock et al. 2010). Moreover, the methanolic extract obtained using Amberlite XAD-2 columns indicates that phenolic compounds play an essential role in this capacity. In vitro trials showed that these extracts were able to inhibit the growth of Escherichia coli, Pseudomona aeruginosa, Staphylococcus aureus, and Streptococcus pyogenes for which we also determined the minimum inhibitory concentration (Montenegro and Mejias 2013, Montenegro et al. 2013).

Manuka honey derived from the tree or shrub species Leptospermum that grows in New Zealand and eastern Australia has been described as capable of inhibiting about 60 species of bacteria including aerobic and anaerobic, both highly negative and positive. The antimicrobial activity of this honey against Staphylococcus aureus bacteria makes it an essential functional ingredient for wound healing. The potential of this honey to heal wounds has been repeatedly demonstrated not only by its growth-controlling properties of this bacteria but also by maintaining moisture in the wound area and producing a viscosity that acts as a barrier and helps to protect and prevent infection (Lusby et al. 2005). Concerning quillay honeys (Quillaja saponaria), we analyzed their antibacterial and antifungal activity and showed a significant bacyeriotatic activity against the four bacterias: Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, and Pseudomona aeruginosa. Concerning quillay honeys (Quillaja saponaria), we analyzed its antibacterial and antifungal activity and proved a significant bacteriostatic action against four bacteria: Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, and Pseudomona aeruginosa. The antibacterial effect could be due to the phenolic compounds present in the phenolic extract, such as caffeic, coumaric, and salicylic acids, and to the flavonone naringenine and flavonol kaempferol detected by the High-Performance Liquid Chromatography technique.

APF FACTOR DEVELOPMENT

This seal called: "Active Patagonia Factor," whose abbreviation will be APF indicates different levels of biological activity — controlling the growth of 3 pathogenic bacteria (Escherichia coli, Staphylococcus aureus, Salmonella enterica) of Chilean Honeys from the Native Forest and the extreme south of the country.

The seal was created based on the results of scientific experimentation with more than 500 Chilean honeys of single-flower botanical and known origin coming from species of natural plant communities of Central Chile (evergreen sclerophyllous scrub) and plant communities of southern Chile (Rainforest)

In each of the honeys collected from apiaries located in these wild areas, we determined the total growth controlling activity of 3 pathogenic bacteria: Escherichia coli, Staphylococcus aureus, Salmonella enterica following the methodology described below.

Determination of the honey’s antimicrobial activity

The antibiotic activity of honey means the ability to eliminate or inhibit the development and growth of some microorganisms, commonly bacteria or fungi.

Evaluating the potential effectiveness of honey requires having a culture isolated from the pathogens, pathogenicity testing, and testing the efficacy of honey control on pathogen development.

To determine the honeys antibiotic capacity (which allowed to design the graduation and to express it in a seal), we used the Agar Diffusion method (Well Diffusion Agar, WDA)

We performed the WDA test as follows: We used the following bacteria: Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC25923), and enteric Salmonella from the Institute of Public Health of Chile. Initially, we performed an antimicrobial capacity analysis with DM, for which we used Petri dishes with 25 mL of soy agar (BBL TM Trypticase TM soy agar BD [Sparks, MD USA]), as culture medium, which we seeded with sterile swabs immersed in a bacterial solution. For the bacterial solution, we diluted each bacterial species in saline to a concentration of 106 cfu/mL. We made three holes of 6 mm diameter in each plate, where we placed 100 µL of the extract. Each analysis performed in triplicate. We incubated the plates at 37 °C for 24 hours, and we observed if there was inhibition in the growth of the bacteria, which we measured as millimeters of diameter of bacterial growth inhibition.

Besides, we performed the double micro-dilution test in series on ELISA plates (Pontino et al., 2006) to estimate the minimum bactericide concentration. For this purpose, we used 150 µL of soybean broth, 50 µL of bacterial solution (5 x 103 cfu/mL), and 150 µL of honey extract per hole. We incubated the plates at 37°C for 24 hours. Finally, we performed a subculture of the ELISA plate solutions in Petri plates with 25 mL of soya agar.

We expressed the minimum bactericide concentration (MBC) in g of honey/mL of distilled water, which is necessary to cause bacterial death.

Statistical analysis

For the analysis, we used the statistical software Infostat (Di Rienzo et al., 2012), with which we performed variance analysis to determine the existence of significant differences between honeys analyzed for each parameter evaluated. We determined the differences using Tukey's test (p < 0.05), and we calculated correlations between data using Pearson's correlation coefficient.

After these steps, we will select the honeys that show some degree of activity against the bacteria in question.

Factor Determination

The average level of antibacterial activity against these bacteria results in a number added to the factor, thus determining the levels elaborated according to the average diameter of the three bacteria Escherichia coli, Staphylococcus aureus, Salmonella enterica. To determine the factor, we considered working based on all the honeys that have shown some degree of activity against all the bacteria analyzed, specifying a minimum average diameter of 3 bacteria of 13 mm to consider it as honey with honey with anti-bacterial activity. Based on the results and the antibacterial capacities of the honeys with these characteristics, these honeys were divided into 4 groups:

HONEY WITH LOW ANTI-BACTERIAL ACTIVITY
HONEY WITH MEDIUM ANTI-BACTERIAL ACTIVITY
HONEY WITH HIGH ANTI-BACTERIAL ACTIVITY
HONEY WITH VERY HIGH ANTI-BACTERIAL ACTIVITY

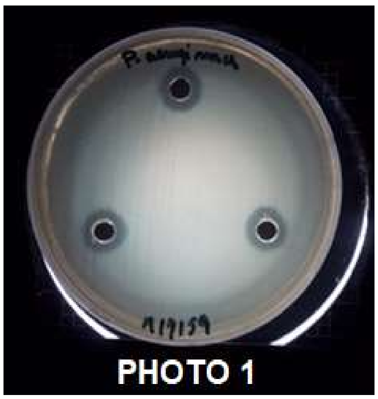
The distribution of % of these honeys under the classification made was:

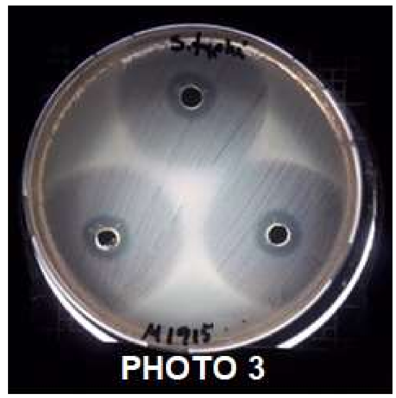
HONEY WITH LOW ANTI-BACTERIAL ACTIVITY	44%
HONEY WITH MEDIUM ANTI-BACTERIAL ACTIVITY	31%
HONEY WITH HIGH ANTI-BACTERIAL ACTIVITY	18%
HONEY WITH VERY HIGH ANTI-BACTERIAL ACTIVITY	7%

For the determination and equivalence of these results to a level of the APF factor, we decided to exclude all honeys with low anti-bacterial activity, so the factor will be determined with three levels, which we named as follows: APF 100+, APF 150+ and APF 200+, directly associated to their anti-bactericidal capacity.

HONEY WITH MEDIUM ANTI-BACTERIAL ACTIVITY	APF 100+
HONEY WITH HIGH ANTI-BACTERIAL ACTIVITY	APF 150+
HONEY WITH VERY HIGH ANTI-BACTERIAL ACTIVITY	APF 200+

PHOTOS OF RESULTS APF ANALYZIS:





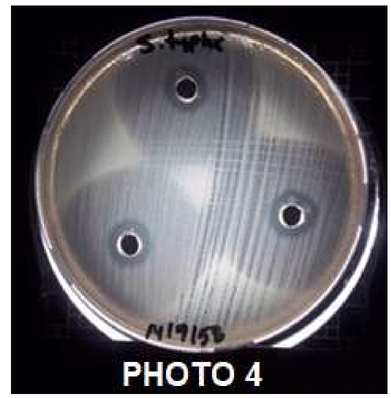


Photo 1: No Anti-bacterial activity level in the honey against bacteria. Result: **NO APF.**
Photo 2: Medium Anti-bacterial activity level in the honey against bacteria. Result: **APF 100+**
Photo 3: High Anti-bacterial activity level in the honey against bacteria. Result: **APF 150+**
Photo 4: Very high Anti-bacterial activity level in the honey against bacteria. Result: **APF 200+**