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Antibacterial Comparative Study Between Extracts of Mexican Propolis and of Three Plants Which Use *Apis mellifera* for its Production

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Abstract: The aim of this study was to evaluate the antibacterial properties of extracts obtained from three Mexican plants (*Callistemon citrinus* Stapf, *Eucalyptus camaldulensis* Dehnhardt and *Ricinus communis* L) and of extracts of propolis produced by *Apis mellifera* in the same region comparing them by diffusion and plate dilution tests. Hexane and ethyl and methanol acetate extracts from each plant as well as propolis hexane and ethanol extracts were tested on 2 gram-positive and 8 gram-negative bacterial strains: *Staphylococcus aureus, S. epidermidis, Shigella dysenteriae, Salmonella tiphy, Yersinia enterocolitica, Enterobacter aerogenes, Enterobacter agglomerans* (*Pantoea agglomerans*), *Escherichia coli, Vibrio cholerae* Nr. 01 and *V. cholerae* (clinical case). The propolis extracts was analyzed by HPLC chromatography. Propolis extracts were active against *Staphylococcus aureus, S. epidermidis, Vibrio cholerae* Nr. 01 and *V. cholerae* (clinical case). *Callistemon citrinus* Stapf extracts were effective against *Staphylococcus aureus, S. epidermidis, Vibrio cholerae* Nr. *01* and *V. cholerae* (clinical case), *Eucalyptus camaldulensis* Dehnhardt extracts acted against *Staphylococcus aureus, S. epidermidis* and *Vibrio cholerae* Nr. 01 and *Ricinus communis* L extracts were effective against *V. cholerae* (clinical case), *Shigella dysenteriae* and *E. agglomerans*. In general, antimicrobial activity of propolis coincided with plant extract activity against *Staphylococcus aureus, S. epidermidis, Vibrio cholerae* Nr. 01 and *V. cholerae* (clinical case). The major compounds were the flavones, cinnamic acid derivative and the caffeic acid derivative. These data corroborate the close relationship between propolis composition and the constitution of plants serving as source for some of its components. Thus, the antibacterial spectrum of propolis will vary depending on the geographical situation and the types of vegetation of a given region. On the other hand, plants used by *Apis mellifera* to produce propolis are being considered as source of secondary metabolites with antimicrobial action.

Key words: Propolis, *Apis* mellifera, mexican plant extracts, antibacterial properties, beehives, floral extracts

INTRODUCTION

Propolis is a multifunctional material used by bees to build and maintain beehives. It is a resinous, balsamic, rubbery substance of viscous consistency. Its color can be brownish green, chestnut or even black, it tastes bitter but has a sweet and pleasant odor. Color and composition largely depend on its botanical origin and the type of bee that produced it (Kujumgiev *et al*., 1999). Propolis samples show significant chemical differences related to its origin and have therefore become a matter of interest for chemists and biologists and have recently been used as source of new biologically active compounds (Bankova *et al*., 2002).

It has been widely reported that propolis is effective against bacteria, fungi, parasites and viruses, it shows antitumor, antioxidant, scar-forming and tissue-regenerating properties, as well as low toxicity in humans, among other properties (Kartal *et al*., 2003; Kujumgiev *et al*., 1999; Prytzyk *et al*., 2003). Propolis is

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made by bees with substances collected from the leaf buds and stems of various plants, then mixed with wax and salivary secretions at the beehive. The complex chemical composition of this blend contains >160 active components. Its biological activity depends on various factors among them the type of plants used to collect the ingredients similarly to honey in which differences have been established according with the flowering features and type of nectariferous plants used by the bees to prepare it (Bankova *et al*., 2002; Sforcin *et al*., 2000). Some researchers consider that sesquiterpenes, in particular bisabolol are some of the main components responsible for the biological activity of propolis as well as flavonoids, phenolic acids and their esters (Martins *et al*., 2002).

Along the last 3 decades, the frequency, etiology and epidemiology of systemic infections has changed as medical attention has evolved, particularly among intensive care patients, who require hospitalization. Many Gram positive cocci associated to nosocomial systemic infections are now resistant to commonly used antibiotics. At present, the three most common causes of nosocomial systemic infections in the United States are coagulase negative staphylococci, *Staphylococcus aureus* and enterococci (Karchmer, 2000).

Resistance to antibiotics of frequently isolated pathogens has jeopardized the clinical usefulness of several types of important antimicrobial compounds, including beta-lactamic antibiotics, macrolids, aminoglycosides, glycopeptides and fluoroquinolones. Critical Gram-positive pathogens such as *staphylococci, beta-hemolytic streptococci, enterococci and Gram-negative bacilli* such as enterobacteria or *Pseudomonas* sp. have developed resistance to fluoroquinolones by prolonged exposure (Jones *et al*., 2008). This has rendered the search for new antibacterial agents critical.

Medicinal plants are natural resources providing valuable herbal products, they have their roots in antiquity (Chandrasekaran and Venkatesalu, 2004) and are presently still used in routine treatment against certain diseases among them, those caused by fungi (Rios and Recios, 2005).

However, even though propolis has been used for many years few studies have been performed in Mexico regarding its antibiotic activity. The aim of the present study was to evaluate and compare the antibacterial activity of propolis with that of three plants from which they collect the components to prepare it and other bee products.

MATERIALS AND METHODS

Plants and propolis: Samples from plants visited by bees and the samples of propolis used in the present study were collected at the Faculty of Higer Studies Cuautitlan facilities (FES-C), Campus 4 of the National Autonomous University of Mexico located in the municipality of Cuautitlán Izcalli, State of México, which is located in central México located geographically between parallels 18°21' and 20°17' North latitude and 98°36' and 100°36'W, at 2,683 m above sea level in its highest plain.

Samples of three plants from which bees take the components to make propolis and other bee products were collected. Aerial parts of the botanical material were identified by Ma. Edith Lopez Villafranco. Voucher specimens were deposited at the National Herbarium of Mexico (MEXU) at the National Autonomous University of México and at the herbarium IZTA at the at the Facuty of Higer Studies Iztacala (FES-I) and a sample of each was saved for the ethnobotanic collection of this institution. Specimens were identified as *Callistemon citrinus* Stapf (IZTA 42143), *Eucalyptus camaldulensis* Dehnhardt (IZTA 42144) and *Ricinus communis* L (IZTA 42146).

Preparation of propolis extracts: Propolis of *Apis mellifera* bees was macerated in 70% ethanol. It was left to rest at room temperature protected from the light for 2 weeks. Then, it was filtered and its fractions obtained by partition with hexane and ethanol. Both extracts were concentrated until dry and stored in a fresh and dry place, protected from light.

Preparation of herbal extracts: Flowers of the plant species visited by bees were collected at the FES-C premises, dried, ground and stored in a dry place. Extracts of this material were obtained by adding solvents in order of increasing polarity (hexane, ethyl acetate, methanol). Extracts were filtered and concentrated until dry by low-pressure distillation. Solvent remnants were eliminated by aeration.

Microorganisms: The strains used in this study were *Staphylococcus aureus* ATCC 12398, *Salmonella tiphy* ATCC 19430, *Enterobacter agglomerans* (*Pantoea agglomerans*) ATCC 27155, *Escherichia coli* ATCC25922, *Staphylococcus epidermidis, Enterobacter aerogenes, Shigella dysenteriae, Vibrio cholerae* serotype 01 and *V. cholera* (a clinical isolate corresponding with group 01, producing enterotoxin, serotype Inaba, biotype El Tor), which were obtained at the Phytochemistry Laboratory (FES-I) and *Yersinia enterocolytica* (donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala).

Qualitative sensitivity tests (Agar diffusion test): Bacteria were sown in Müller-Hinton growth medium (Bioxon,

Mexico) and incubated at 35°C for 24 h. Inoculum density was adjusted to tube 0.5 of the Mc Farland nephelometer (1-5×10^8 UFC mL^{-1}). Whatman study discs (Nr. 5) of 5 mm diameter were impregnated with 10 μL per disc of a 2 mg10 μL^{-1} solution of each crude extract and left to dry at room temperature. A sample of the adjusted inoculum was massively sown on Müller-Hinton agar (DIBICO) and the crude-extract impregnated discs were placed on it. They were incubated for 24 h subsequently, the inhibition halo diameters were measured. Discs impregnated with 10 μL of each solvent were used as negative controls. Positive controls were discs impregnated with 25 μg chloramphenicol. Each experiment was repeated at least three times (Berghe and Vlietinck, 1997).

Quantitative sensitivity tests and statistical analysis Agar dilution test: This test was performed based on document CLSI/NCCLS M44A with bacteria showing sensitivity to qualitative tests with the corresponding extracts. Petri dishes (60×15) containing 6 mL Müller-Hinton agar were added with extracts at different concentrations: 3.0, 2.5, 2.0, 1.5, 1.0, 0.75, 0.50, 0.25, 0.125 mg mL^{-1} starting from a standard DMSO solution. Bacteria (1-5×10^8 UFC mL^{-1}) were dotted onto the extract-containing growth media at three different places and Petri dishes were incubated at 35°C for 24 h. Controls were bacteria grown on media without extract. The Minimum Inhibitory Concentration (MIC) for each bacterial strain was determined. Each experiment was repeated at least three times. The results were analyzed by two-way ANOVA.

Chemical analyses of propolis: The propolis extracts obtained was analyzed by HPLC chromatography.

HPLC chromatography: The equipment used was Hewlett-Packard HP model 1100 series (Hewlett-Packard, Wilmington, DE, USA), equipped with a Detector Array of Diodes (DAD) 1100 operated with mobile ChemStation AO903 phase methanol: acetonitrile: water (25: 25: 50); column Allsphere ODS 1 (250x4.6 mm) 5 mm inside diameter, flow 1 mL min^{-1}, detector array of diodes with detector setting at 260 nm and sweeps 200-700 nm.

Identification of the components: The identification of the constituents was assigned on the basis of comparison of their retention indices with those given in the literature (Harborne, 1994).

RESULTS AND DISCUSSION

Studies that relate floral compounds with bee products are predominantly focused on *Apis mellifera* pollen and its human use as food as a remedy for diseases or as a nutritional supplement. This research compares the antibacterial activity of propolis prepared by *Apis mellifera* with that of floral extracts from the plants visited by these bees. Table 1 shows antibacterial activity results for each extract.

Both, the propolis and the floral extracts showed antibacterial activity mainly against Gram-positive strains, as shown by their effects on the two species (*S. aureus* and *S. epidermidis*) tested here. Growth was inhibited by propolis extract in both, its hexane and ethanol partitions and by the hexane and methanol extracts of *R. communis*, the methanol extract of *E. camaldulensis* and the hexane, ethyl acetate and methanol extracts of *C. citrinus*.

The *V. cholerae* strains, *V. cholerae* Nr. 01 and the *V. cholerae* (clinical case) were both inhibited by the two propolis extracts and by the *C. citrinus* ethyl acetate and methanol extracts. Additionally, *V. cholerae* Nr. 01 was inhibited by the *E. camaldulensis* methanol extract and the *V. cholerae* (clinical case), by the *R. communis* ethyl acetate extract.

The highest sensitivity level was shown by *S. aureus*, *S. epidermidis* and the two *V. cholerae* strains treated with the propolis ethanol extract and the *C. citrinus* ethyl acetate extract, which gave MIC values <0.125 mg mL^{-1}.

The strains *S. tiphy*, *Y. enterocolitica*, *E. agglomerans* (*P. agglomerans*) and *E. coli* were not inhibited by any of the extracts tested herein.

Two-factor ANOVA statistical analysis found that the extracts activity depend on the species of bacteria or the sensitivity of bacteria depends on the extract, the above is the interaction between bacterial strain and extracts ($p<0.01$).

Table 2 shows the composition of propolis extracts and its fractions. The major compounds in ethanol fraction were the flavones and cinnamic acid derivative and in the hexane fraction were cinnamic acid derivative and the caffeic acid derivative.

Propolis biological activity has been related to several factors including bee species, geographical region, type of vegetation, collection period and solvent used for extraction purposes. Besides, a combination of different compounds is known to be required for propolis to be biologically active (Bankova *et al*., 2002; Kujumgiev *et al*., 1999; Sforcin *et al*., 2000).

About the species of plants used in this research, we found that *C. citrinus* is only used for ornamental purposes and there are few reports of its antibacterial activity (Cock, 2008; Melendez and Capriles, 2008), *E. camaldulensis* has been reported elsewhere to possess

Table 1: Antibacterial prperties of propolis, *R. Communis*, *E. Camaldulensis*, *C. Citrinus* extracts (Inhibition halo diameter mm, MIC mg mL^{-1})

Extract	*S. aureus*	*S. epidermidis*	*Sh. dysenteriae*	*E. aerogenes*	*V. cholerae 01*	*V. cholerae* (clinical isolate)
Propolis						
Hexane						
Halo diameter	9.7	9.0	NA	NA	9.0	8.7
MIC	0.125	0.125	-	-	0.125	0.125
Ethanol						
Halo diameter	11.3	11.0	NA	NA	9.7	11.0
MIC	<0.125	<0.125	-	-	<0.125	<0.125
R.*communis*						
Hexane						
Halo diameter	13.6	14.0	NA	NA	NA	NA
MIC	>3.00	>3.00	-	-	-	-
Ethyl Acetate						
Halo diameter	9.3	NA	NA	NA	NA	NA
MIC	2.50	2.50	-	-	-	>3.00
Methanol						
Halo diameter	10.0	11.7	8.7	10.0	NA	NA
MIC	0.70	0.25	>3.00	>3.00	-	-
E.*camal dulensis*						
Hexane						
Halo diameter	NA	NA	NA	NA	NA	NA
MIC	-	-	-	-	-	-
Ethyl Acetate						
Halo diameter	NA	NA	NA	NA	NA	NA
MIC	-	-	-	-	-	-
Methanol						
Halo diameter	11.0	11.3	NA	NA	9.7	NA
MIC	2.00	0.75	-	-	>3.00	-
C.*citrinus*						
Hexane						
Halo diameter	9.3	9.0	NA	NA	NA	NA
MIC	2.50	>3.00	-	-	-	-
Etile Acetate						
Halo diameter	19.3	19.6	NA	NA	18.0	NA
MIC	<0.125	<0.125	-	-	<0.125	<0.125
Methanol						
Halo diameter	15.0	15.0	NA	NA	10.0	NA
MIC	0.125	0.125	-	-	0.50	1.00

Two-factor ANOVA statistical analysis found that there are significant differences between extracts ($p<0.01$), NA: No Activity

Table 2: Constituents of propolis fraction

Fraction	Retention time (min)	Max. (nm)	Compound assignment
Propolis-Ethanol	3.5	236, 260, 294	Isoflavone
	4.1	238, 276, 324	Ilavone
	4.6	250, 322	Quercetin
	4.8	242, 296, 324	Flavone
	5.9	236, 288	Flavone
	9.7	236, 293, 328	Flavone
	11.9	238, 292, 328	Flavone
	14.1	282	Cinnamic acid derivatve
	16.1	236, 290	Cinnamic acid derivatve
	19.3	290	Cinnamic acid derivatve
Porpolis-Hexane	7.0	314	The caffeic acid derivative
	13.1	286	Cinnamic acid derivatve
	16.2	236, 290	Cinnamic acid derivatve
	16.9	236, 290	Cinnamic acid derivatve

antibacterial activity (Martos *et al*., 2000) and the resin of *R. communis* has been traditionally used with due consideration of its high toxicity having shown that it has bactericide, insecticide and vermifuge activity among others (Challoner and McCarron, 1990).

CONCLUSION

Propolis extracts and extracts from the flowers of *C. citrinus*, *E. camaldulensis* and *R. communis* were found to possess antibacterial activity against *S. aureus*, *S. epidermidis*, *V. cholerae* 01 and *V. cholerae* (clinical case). These data underline the relationship between the antimicrobial activity of propolis and the plants from which bees take the components to make propolis. Thus, the antibacterial spectrum of propolis may vary depending on the geographical location and the surrounding vegetation. In Mexico, there is no much information that establishes this relation. Propolis extracts and extracts from the flowers of *C. citrinus*, *E. camaldulensis*

and *R. communis* could be used to prepare medicaments or directly can be probed as treatments. Further investigation is needed to establish the reach of these findings.

REFERENCES

Bankova, V., M. Popova, S. Bogdanov and A.G. Sabatini, 2002. Chemical composition of European propolis: Expected and unexcepted results. Zeitschrift Naturforschung C. A. J. Biosci., 57: 530-533.

Berghe, V.D.A. and A.J. Vlietinck, 1997. Screening Methods for Antibacterial Agents from Higher Plants. In: Methods in plant Biochemistry, Assay for Bioactivity, Dey, P.M., J.B. Harborne and K. Hostettman (Eds.). Vol. 6, Academic Press, London, ISBN: 0-12-214674-3(HB), pp: 47-69.

Challoner, K.R. and M.M. McCarron, 1990. Castor bean intoxication. Ann. Emerg. Med., 19: 1177-1183.

Chandrasekaran, M. and V. Venkatesalu, 2004. Antibacterial and antifungal activity of *Syzgium jambolanum* seeds. J. Ethanopharmacol., 91: 105-108.

Cock, I., 2008. Antibacterial activity of selected Australian native plant extracts. Internet J. Microb., 4: 1-12.

Harborne, J.B., 1994. The Flavonoids: Advances in Research Since 1986. 1st Edn., Chapman and Hall, England, ISBN: 0412480700, pp: 117-122.

Jones, R.N., T.R. Fritsche and H.S. Sader, 2008. Antimicrobial activity of DC-159a, a new *Fluoroquinolone*, against 1,149 recently collected clinical isolates. Antimicrob. Agents Chemother., 52: 3763-3775.

Karchmer, A.W., 2000. *Nosocomial* bloodstream infections: Organisms, risk factors and implications. Clin. Infect. Dis., 31: 139-143.

Kartal, M., S. Yildiz, S. Kaya, S. Kurucu and G. Topçu, 2003. Antimicrobial activity of propolis samples from two different regions of Anatolia. J. Ethnopharmacol., 86: 69-73.

Kujumgiev, A., I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Christov and S. Popov, 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol., 64: 235-240.

Martins, R.S., E.S.J. Pereira, S.M. Lima, M.I. Senna, R.A. Mesquita and V.R. Santos, 2002. Effect of commercial ethanol propolis extract on the *in vitro* growth of Candida albicans collected from HIV-seropositive and HIV-seronegative Brazilian patients with oral candidiasis. J. Oral Sci., 44: 41-48.

Martos, I., F. Ferreres, L. Yao, B. Arcy, N. Caffin and F.A. Tomás-Barberán, 2000. Flavonoids in monospecific eucalyptus honeys from Australia. J. Agric. Food Chem., 48: 4744-4748.

Melendez, P. and V. Capriles, 2008. Antibacterial properties of tropical plants from Puerto Rico. J. Phymed., 13: 272-276.

Prytzyk, E., AP. Dantas, K. Salomao, A.S. Pereira, V.S. Bankova, S.L. De Castro and N.FR. Aquino, 2003. Flavonoids and trypanocidal activity of *Bulgarian propolis*. J. Ethnopharmacol., 88: 189-193.

Rios, R.L. and M.C. Recios, 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol., 100: 80-84.

Sforcin, J.M., A. Fernandes Jr., C.A.M. Lopes, V. Bankova and S.R.C. Funari, 2000. Seasonal effect on Brazil propolis antibacterial activity. J. Ethnopharmacol., 73: 243-249.