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EFFECTS OF PROPOLIS FROM BRAZIL AND BULGARIA ON *Salmonella* SEROVARS

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ABSTRACT: Propolis shows biological properties such as antibacterial action. This bee product has a complex chemical composition, which depends on the local flora where it is produced. *Salmonella* serovars are responsible for human diseases that range from localized gastroenteritis to systemic infections. The aim of the present study was to investigate the susceptibility of *Salmonella* strains, isolated from food and infectious processes, to the antibacterial action of Brazilian and Bulgarian propolis, as well as to determine the behavior of these bacteria, according to the incubation period, in medium plus propolis. Dilution of ethanolic extract of propolis in agar was the used method. Brazilian and Bulgarian propolis showed an antibacterial action against all *Salmonella* serovars. The minimal inhibitory concentrations (MIC) of propolis were similar, although they were collected in different geographic regions. *Salmonella typhimurium*, isolated from human infection, was more resistant to propolis than *Salmonella enteritidis*.

KEY WORDS: propolis, susceptibility profile, *Salmonella*, food, infection.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Because of its several biological properties, propolis has attracted much attention in recent years, although it has been used in folk medicine since ancient times (4).

Propolis antibacterial activity has been widely investigated (8-10, 14-16, 18). Its complex chemical composition shows more than 300 constituents identified to date (7).

Salmonellae are Gram-negative, facultative anaerobic, non-endospore forming, usually motile rods that cause gastroenteritis and enteric fever, constituting a significant ongoing threat to worldwide public health. Its virulence requires the expression of complex arrays of virulence factors that allow the bacterium to evade the host's immune system (13).

Therefore, the aim of the present work was to investigate the behavior of *Salmonella typhimurium* (isolated from human infections) and *Salmonella enteritidis* (isolated from contaminated poultry) subjected to increasing propolis concentrations in order to determine the MIC for microbial growth. The survival curve was also analyzed according to the incubation period in culture medium and propolis. The effects of Brazilian propolis were also compared with those produced by a propolis sample from Bulgaria in the same assays.

MATERIALS AND METHODS

Propolis Samples

Brazilian propolis was collected in the Beekeeping Section of the School of Veterinary Medicine and Animal Husbandry, UNESP, Botucatu, São Paulo State, Brazil. Bulgarian propolis was supplied by Dr. Bankova from the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Propolis samples were ground and extracted (30g of propolis; volume was completed to 100 ml with 70% ethanol) in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and final concentrations were calculated to obtain the dry weight (Brazilian propolis: 133 mg/ml; Bulgarian propolis: 170mg/ml). Specific dilutions of these solutions were prepared for each assay in appropriate media.

Salmonella Serovars

Strains of the different *Salmonella* serovars used in this study were obtained as follows: Serovar-*typhimurium* clinical isolates were obtained from patients of the University Hospital of the Botucatu Medical School, UNESP. Serovar *enteritidis* was isolated from poultry, according to Andrews *et al*. (1). These strains were identified at the Adolpho Lutz Institute, São Paulo, Brazil. Standard strains of *Salmonella typhi* (00238) and *Salmonella typhimurium* (13311) were supplied by Oswaldo Cruz Foundation, Rio de Janeiro, Brazil.

All *Salmonella* strains were maintained in trypticase soy agar (TSA, Oxoid) at room temperature.

Susceptibility Test

Minimal inhibitory concentrations (MIC) were determined by the agar dilution method, following the National Committee of Clinical Laboratory Standards Guidelines (12).

Bacterial strains were grown in Brain Heart Infusion Agar (BHI, Oxoid) at 37ºC/24h. After incubation, five colonies of each strain were suspended in 5ml of sterile saline and diluted to yield a final inoculum of approximately $1X10^6$ colony forming units (CFU/ml).

Serial concentrations (% v/v) of propolis from Brazil and Bulgaria were obtained in plates containing Mueller Hinton Agar, ranging from 1% to 14%. Each antimicrobial test also included plates containing the culture medium plus ethanol, in order to obtain a control of the solvent antimicrobial effect.

After the inoculation procedures using a multiloop replicator, plates were incubated at 37ºC/24h and MIC endpoints were read as the lowest concentration of propolis that resulted in no visible growth or haze on the surface of the culture medium. Populational analyses of data were carried out by calculating the MIC for 90% of the strains of each group of microorganism (17).

Survival Curve

The survival curve of *Salmonella* strains was obtained in order to observe the incubation period responsible for propolis antibacterial activity. Thus, $1X10^6$CFU/ml were inoculated into BHI plus propolis at the corresponding $MIC_{90\%}$ previously obtained for each strain.

After 1.5, 3, 6, 9 and 24 hours of incubation (37ºC), aliquots of each culture were recovered and plated on Mueller Hinton Agar by the pour plate method. Plate counts (CFU/ml) were carried out after 24h incubation and the survival percentage was calculated (17).

Statistical Analysis

Analyses of variance were used to verify the treatment effects on the survival curve, according to the incubation period in medium plus propolis from Brazil and Bulgaria. The probability of 0.001 was chosen as the significant level (20).

RESULTS

Salmonella enteritidis (isolated from food) was more susceptible to Brazilian and Bulgarian propolis ($MIC_{90\%}$=8.70% and $MIC_{90\%}$=8.54%, respectively) in comparison with *Salmonella typhimurium*, isolated from human infection ($MIC_{90\%}$=9.90% and $MIC_{90\%}$=10.0%, respectively). Standard strains showed a MIC value identical to that of infection-isolated strains (Table 1). With regards to ethanol, which was used as a solvent for propolis in this assay, its inhibitory action was observed only at the concentration of 12.6%.

There was a reduction in the CFU of *Salmonella enteritidis* within 24h in medium plus Bulgarian propolis (Table 2), indicating a remarkable bactericidal effect on this strain after 1.5h ($p<0.001$). Brazilian propolis showed only a non-significant bacteriostatic effect.

With respect to *Salmonella typhimurium*, Brazilian and Bulgarian propolis showed an efficient inhibitory action (Table 3), with a bactericidal effect ($p<0.001$) after 24h.

Bulgarian propolis reduced the CFU number of standard strains (Tables 4 and 5), showing a bactericidal effect after 1.5h. On the other hand, these strains were also susceptible to Brazilian propolis, which had a bactericidal effect on *Salmonella typhimurium* (13311) (Table 4) and a bacteriostatic action on *Salmonella typhi* (00238) (Table 5). Ethanol 70% showed only a bacteriostatic effect for all strains.

Table 1. Antibacterial activity (Minimal Inhibitory Concentration – $MIC_{90\%}$) of ethanolic extract of propolis from Brazil and Bulgaria against *Salmonella* serovars.

Microrganisms		**Brazilian Propolis**		**Bulgarian Propolis**	
		%v/v	**mg/ml**	**%v/v**	**mg/ml**
S. enteritidis (food) (n=13)	MIC 90%	8.70	217.5	8.54	221.0
S. typhimurium (infection) (n=14)	MIC 90%	9.90	247.5	10.00	260.0
S. typhimurium (13311)	MIC	9.90	247.5	10.00	260.0
S. typhi (00238)	MIC	9.90	247.5	10.00	260.0

Table 2. Viable count (CFU/ml - log10) and survival percentage (%) of *Salmonella enteritidis* according to its susceptibility to ethanolic extract of propolis from Brazil and Bulgaria ($MIC_{90\%}$=8.70% and $MIC_{90\%}$=8.54%, respectively) and ethanol 70% ($MIC_{90\%}$=12.60%).

TIME (hours)	**Control (A*)**	**Ethanol 70% (12.6%) (B*)**		**Brazil (8.70%) (B*)**		**Bulgaria (8.54%) (C*)**	
	log-CFU	**log-CFU**	**%**	**log-CFU**	**%**	**log-CFU**	**%**
0	6.0	5.9	100	6.0	100	5.9	100
1.5	6.4	5.7	70.5	5.7	59.8	3.5	0.3
3	7.2	5.7	68.0	5.6	44.3	3.5	0.2
6	9.0	5.6	57.7	5.4	23.8	3.7	0.5
9	9.7	5.5	41.0	5.3	20.6	3.0	0.1
24	8.9	4.9	9.7	4.4	2.5	0	0

* Different letters indicate statistical difference between groups

Table 3. Viable count (CFU/ml - log10) and survival percentage (%) of *Salmonella typhimurium* according to its susceptibility to ethanolic extract of propolis from Brazil and Bulgaria ($MIC_{90\%}$=9.90% and $MIC_{90\%}$=10.00%, respectively) and ethanol 70% ($MIC_{90\%}$=12.60%).

TIME (hours)	**Control (A*)**	**Ethanol 70% (12.6%) (B*)**		**Brazil (9.90%) (B*)**		**Bulgaria (10.00%) (C*)**	
	log-CFU	**log-CFU**	**%**	**log-CFU**	**%**	**log-CFU**	**%**
0	6.0	5.9	100	5.9	100	6.0	100
1.5	6.3	5.8	75.5	5.1	40.0	3.3	0.9
3	7.3	5.8	68.0	4.3	7.3	3.6	1.7
6	9.0	5.7	61.7	4.1	4.0	3.7	2.2
9	9.4	5.6	44.7	3.5	1.0	3.5	1.3
24	8.3	4.9	8.2	0	0	0	0

* Different letters indicate statistical difference between groups

Table 4. Viable count (CFU/ml - log10) and survival percentage (%) of *Salmonella typhimurium* (13311) according to its susceptibility to ethanolic extract of propolis from Brazil and Bulgaria ($MIC_{90\%}$=9.90% and $MIC_{90\%}$=10.00%, respectively) and ethanol 70% ($MIC_{90\%}$=12.60%).

TIME (hours)	**Control (A*)**	**Ethanol 70% (12.6%) (B*)**		**Brazil (9.90%) (B*)**		**Bulgaria (10.00%) (C*)**	
	log-CFU	**log-CFU**	**%**	**log-CFU**	**%**	**log-CFU**	**%**
0	5.9	5.8	100	5.7	100	5.97	100
1.5	6.5	5.2	26.6	3.0	0.2	0	0
3	7.4	5.0	17.2	0	0	0	0
6	8.8	4.7	8.7	0	0	0	0
9	9.6	4.6	6.9	0	0	0	0
24	10.5	3.8	0.9	0	0	0	0

* Different letters indicate statistical difference between groups

Table 5. Viable count (CFU/ml - log10) and survival percentage (%) of *Salmonella typhi* (00238) according to its susceptibility to ethanolic extract of propolis from Brazil and Bulgaria ($MIC_{90\%}$=9.90% and $MIC_{90\%}$=10.00%, respectively) and ethanol 70% ($MIC_{90\%}$=12.60%).

TIME (hours)	Control (A*)	Ethanol 70% (12.6%) (B*)		Brazil (9.90%) (B*)		Bulgaria (10.00%) (C*)	
	log-CFU	log-CFU	%	log-CFU	%	log-CFU	%
0	5.95	5.96	100	6.0	100	5.99	100
1.5	6.4	5.5	41.1	4.6	31.5	0	0
3	7.5	5.4	35.6	4.5	25.4	0	0
6	8.6	5.2	21.5	4.4	22.3	0	0
9	9.3	5.1	20.1	3.8	5.4	0	0
24	10.8	4.7	7.8	3.0	0.8	0	0

* Different letters indicate statistical difference between groups

DISCUSSION

Interactions between *Salmonella* and human hosts, as well as the adaptive mechanisms to guarantee survival, are important to bacteria virulence and capability to resist and evade the host defense (19). In the present work, *Salmonella enteritidis* (isolated from food) was more susceptible to Brazilian and Bulgarian propolis in comparison with *Salmonella typhimurium* and standard strains.

Thus, the higher resistance of patients-isolated *Salmonella* may be due to survival strategies developed by this intracellular pathogen during its permanence in the host's body (6), also showing higher resistance to the propolis action.

In recent decades, a great interest has arisen in propolis composition, therapeutic properties, botanical origin and geographic region of production (7).

Obviously, in different propolis samples, different substance combinations are essential for its biological activity (10). In the temperate zone of the Northern Hemisphere, bees produce propolis from late spring until early autumn, collecting the material mainly from the bud exudates of polar trees (2). In Brazil, propolis production occurs throughout the entire year and seasonal variations in its chemical composition are not significant and are predominantly quantitative (2, 5). *Baccharis dracunculifolia*

DC. has shown to be the main propolis source in Botucatu, Brazil, followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (3).

Brazilian propolis sample has been analyzed by gas chromatography (GC), gas chromatography mass spectrometry (GC-MS) and thin-layer chromatography (TLC). It presents small quantities of flavonoids: kaempferol, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether; one prenylated *p*-coumaric acid and two benzopyrenes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyrenes; essential oils: spathulenol, (2Z,6E)-farnesol, benzyl benzoate, and prenylated acetophenones; aromatic acids: dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid (which are common for poplar propolis), 3,5-diprenyl-*p*-coumaric acid, and 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran; di and triterpenes; among others. On the other hand, Bulgarian propolis sample contains predominantly phenolic compounds, including several flavonoids, aromatic acids and their esters (2, 3, 5).

Much research has also been carried out on isolated compounds. Mirzoeva *et al*. (11) reported that propolis and some of its cinnamic and flavonoid components were found to uncouple the energy-transducing cell membrane and to inhibit bacterial motility. Santos *et al*. (16) evaluated the antibacterial activity of Brazilian propolis and its fractions against oral anaerobic bacteria. None of the assayed fractions was more active than propolis aqueous-ethanolic extract, suggesting that the antibacterial action is probably caused by the synergistic effects of different compounds.

In the present study, Brazilian and Bulgarian samples had similar effects considering their MIC, although they were produced in widely separated geographic regions and had different actions on the survival curve.

As propolis has been used since early times and as there is increasing scientific and commercial interests, a better understanding of its action will provide a scientific basis for its better therapeutic application in human or veterinary medicine whether it is associated or not with conventional treatments.

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