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Study of the *in vitro* sensitivity to honey bee propolis of *Staphylococcus aureus* strains characterized by different sensitivity to antibiotics

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Abstract - The sensitivity level to propolis in 44 *Staphylococcus aureus* strains, 42 isolated from patients of hospital environment and 2 ATCC reference strains, has been studied. The strains considered showed different behaviour towards antibiotics typically used in the hospitals in order to contain both Gram positive and Gram negative bacteria. On the contrary all the *S. aureus* isolates studied showed sensitivity towards 2 propolis samples collected from two different areas of Piedmont (Italy). In particular it is important to underline that 11 isolates resistant to methicillin showed sensitivity to propolis. The analysis of variance calculated on values of the minimum concentration of propolis inhibiting microorganism growth did not show any significant differences to the sensitivity level to the product among the 44 isolates studied. The results obtained from the research appear to confirm the antimicrobial property of propolis and contribute to increase interest in the power of this natural product in medical field.

Key words: propolis, *Staphylococcus aureus*, antibiotics, antimicrobial property.

INTRODUCTION

Propolis is a natural resinous substance which is collected by honey bees (*Apis mellifera* L.) from plant bud and bark exudates and mixed with hypopharyngeal gland secretions, beeswax and pollen (Serra Bonvehì and Ventura Coll, 1994; Cheng and Wong, 1996; Kujumgiev *et al*., 1999). The chemical composition of this bee-hive product is very dependant on the botanical origin of the exudates (Greenaway *et al.*, 1990, Bankova *et al.*, 1992; Marcucci, 1995), and very complex in that it contains at least 140 different known compounds (Greenaway *et al.*, 1990; Koo *et al.*, 2002). In Europe the main sources for propolis constituents include poplars (*Populus* spp.), birches (*Betula* spp.), oaks (*Quercus* spp.), alders (*Alnus*

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spp.), willows (*Salix* spp.), horse chestnuts (*Aesculus* spp.), hazels (*Corylus* spp.) and pine trees (*Pinus* spp.) (König, 1985; Cheng and Wong, 1996).

The potential of this bee-hive product as a natural antibiotic has long been recognised, and propolis has recently received increased attention in various formulations for dermatology, otorhinolaringology, ginecology, stomatology, odontology and veterinary medicine (Bankova *et al.*, 1992; Marcucci, 1995; Cheng and Wong, 1996). Propolis can be particularly effective against several human pathogenic fungi, viruses and Gram-positive bacteria (Pepeljnjak *et al*., 1982; Al-Masaudi, 1991; Detoma and Ozino, 1991; Amoros *et al.*, 1992; Serkedjieva and Manolova, 1992; Cheng and Wong, 1996; Ozino *et al*., 1996; Park *et al*., 1998).

The major bio-active components of propolis are aromatic acids, esters and the flavanoids galangin, quercetin, kaempferol, acacetin, pinocembrin and pinostobin (Bankova *et al*., 1992; Dimov *et al*., 1992; Serra Bonvehì *et al.*, 1994; Cheng and Wong, 1996).

The complexity of the chemical composition of propolis, together with the potential synergy between components, has made critical examination of possible modes of action of components (Cheng and Wong, 1996). A water soluble, UV absorbing component of propolis, possibly having an O-diphenolic functional group, has been shown to inhibit bacterial DNA-dependant RNA polymerase (Šimuth *et al.*, 1986), but the mode of action of the flavanoid components is largely unknown.

This bee-hive product has been successfully used as a natural remedy in folk medicine for many generations, and while the mode of action of propolis is not fully elucidated, the potential for microbial populations to develop resistance to this compound cannot be discounted.

The Gram-positive bacterium *Staphylococcus aureus* is one of the bacterial species that appears to be naturally susceptible to propolis (Brumfitt *et al*., 1990; Detoma and Ozino, 1991, Grange and Davey, 1990; Krol *et al.*, 1993). *S. aureus* is an important pathogen that causes a wide variety of suppurative diseases and toxinoses (Lyon and Skurray, 1987). There has recently been a dramatic increase in the incidence of nosocomial infections caused by strains of *S. aureus* that are resistant to as many as 20 antimicrobial compounds, including antibiotics, antiseptics and disinfectants (Al-Masaudi, 1991; Lyon and Skurray, 1987; Russel, 1997).

The aim of the present research was to check the sensitivity level of propolis in *S. aureus* strains that showed different behaviour towards antibiotics usually used in the hospitals in order to contain both Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains. Forty-four strains were included in the study (Table 1). They comprised 42 clinical isolates from three different hospitals of the Piedmont region (Italy), and 2 ATCC reference strains, including ATCC 25923 (recommended by NCCLS for antimicrobial disc susceptibility testing).

The strains were isolated, except for the ATCC strains, on Blood Agar Base or Mannitol Salt Agar (Oxoid, Unipath ltd., Hampshire, England) and stored at +4 °C on Mueller Hinton Agar (Oxoid). Confirmation of identification was undertaken by standard physiological and biochemical techniques (Kloos and Jorgenses, 1985).

TABLE 1 – The *Staphylococcus aureus* strains used in the study

Number	Strain	Clinical specimen	Origin*
1	76R OIRM2	pharyngeal swab	DSPM
2	12ESC OIRM3	expectoratum	DSPM
3	14ESC OIRM4	expectoratum	DSPM
4	153R OIRM5	pharyngeal swab	DSPM
5	23ESC OIRM6	expectoratum	DSPM
6	25ESC OIRM7	expectoratum	DSPM
7	26ESC OIRM8	expectoratum	DSPM
8	48ES OIRM10	expectoratum	DSPM
9	47ES OIRM11	expectoratum	DSPM
10	51ES OIRM12	expectoratum	DSPM
11	350R OIRM13	pharyngeal swab	DSPM
12	343R OIRM14	pharyngeal swab	DSPM
13	67ESC OIRM15	expectoratum	DSPM
14	65BR OIRM16	broncus-aspirate	DSPM
15	66N	expectoratum	DSPM
16	ATCC 2921		
17	ATCC 25923		
18	10STA	expectoratum	DSCB
19	22STA	expectoratum	DSCB
20	18STA	expectoratum	DSCB
21	19STA	expectoratum	DSCB
22	20STA	expectoratum	DSCB
23	21STA	expectoratum	DSCB
24	VII STA	expectoratum	DSCB
25	MSA (STAPH14)	urinoculture	DSCB
26	1ST	peritoneal catheter infection	ASL
27	2ST	foot wound	ASL
28	3ST	exudate	ASL
29	4ST	exudate	ASL
30	5ST	expectoratum	ASL
31	6ST	sacral decubitus ulcer	ASL
32	7ST	broncus-aspirate	ASL
33	8ST	peritoneal catheter infection	ASL
34	9ST	leg ulcer	ASL
35	10ST	leg ulcer	ASL
36	11ST	expectoratum	ASL
37	12ST	aerobic hemocolture	ASL
38	13ST	aerobic hemocolture	ASL
39	14ST	expectoratum	ASL
40	15ST	expectoratum	ASL
41	16ST	auricular pus	ASL
42	17ST	anaerobic hemocolture	ASL
43	18ST	ulcer	ASL
44	19ST	expectoratum	ASL

* DSPM: Università di Torino, Dipartimento di Sanità Pubblica e di Microbiologia.
DSCB: Università di Torino, Dipartimento di Scienze Cliniche e Biologiche, Ospedale San Luigi.
ASL: Azienda Sanitaria Lovale n. 12 (Biella), Laboratorio di analisi chimico-cliniche e microbiologia.

Antibiotic sensitivity testing. Sensitivity to methicillin (Oxoid), ampicillin, cephalothin, clyndamycin, co-trimoxazole, erythromycin, gentamicin, oxacillin and tetracycline (Biolife Italiana S.r.l., Milan, Italy) was tested using the Kirby-Bauer disc susceptibility method (Bauer *et al.*, 1966), according to the NCCLS (National Committee for Clinical Laboratory Standards, 1990) Document M2-A4 (Approved Standard) in triplicate for each strain.

Susceptibility was tested on Mueller Hinton Agar, inoculated with a 0.5-McFarland unit suspension of each strain. Plates were incubated at 35 °C for 18 h and the strains were defined as resistant, intermediate or susceptible to each antibiotic by determination of the inhibition zone sizes, according to the NCCLS standards.

Propolis samples and preparation of the ethanolic extract of propolis. Two samples of raw propolis from bee-hives located in two different areas of the Piedmont region were used. The two sample came from a hilly zone (sample A) and a mountain valley (sample B). The source of propolis in the hilly zone was largely from natural species and hybrids of poplar, while in the mountain area (>800m altitude), bees produced propolis from the resinous exudates of various plants, including alders, poplars, hazels and pine trees.

Extracts of the propolis samples were prepared freezing samples at –20 °C and grinding the frozen material in a pre-cooled mortar and pestle. The ground material was mixed with 99.8% (v/v) ethanol, in hermetically-sealed glass vessels at a ratio of 1g of propolis powder to 3 ml of ethanol. Vessels were then incubated for one week at room temperature in darkness with constant agitation. The resulting ethanol solutions were clarified by centrifugation at 7000 × *g* for 60 s and the supernatants collected and filtered through filter paper (Whatman #4). Ethanol soluble components were then collected by evaporation to dryness under vacuum. Final extracts were redissolved in pure ethanol to achieve 15% (w/v) solutions. Final solutions were stored in hermetically-sealed brown-glass bottles at room temperature (Detoma and Ozino, 1991).

Determination of the propolis MIC. Determination of the MIC for each propolis sample was performed by making different dilutions of the ethanolic extracts of propolis in Mueller Hinton Agar: different volumes of propolis extracts, between 20 µl and 400 µl, were added to 100 ml of molten medium at 45 °C, mixed and poured into Petri dishes. Concentrations of solid material were between 0.03 and 0.6 mg of propolis per ml of medium. Bacterial suspensions were obtained from 48 h cultures on Mueller Hinton Broth (Oxoid) incubated at 35 °C. Cultures were diluted with sterile Ringer solution to a concentration of 0.5 on the McFarland scale. Bacterial suspensions were inoculated at 50 µl per plate, corresponding to 1.5×10^6 CFU. All MICs were determined in triplicate at all dilutions. Growth and solvent inhibition controls were unammended Mueller Hinton Agar, and Mueller Hinton Agar with 10 µl of ethanol per ml respectively. All plates were incubated at 35 °C for 48 h. The MICs of each strain were expressed as the lowest propolis concentration which inhibited any visible microbial growth.

RESULTS

Table 2 shows the results concerning the antibiotic sensitivity of *S. aureus* strains studied. Four groups were created due to the different behaviour of the 44 strains considered. The first group contains 11 strains resistant to methicillin; 7 strains, sensitive to all tested antibiotics, belong to the second group; the third one contains 9 strains resistant only to ampicillin; the fourth group contains 17 strains sensitive to both methicilln and resistant to more than 1 of the tested antibiotics.

On the basis of the diameter values of the inhibition zone caused by the different antibiotics and according to the NCCLS standards, the strains were defined as resistant, intermediate or susceptible to each antibiotics; the total absence of inhibition has been indicated with R* symbol.

Table 3 shows the values of the minimum concentration of propolis inhibiting

TABLE 2 – Antibiotic sensitivity patterns of the strains used in the study

Groups	Antibiotics								
Group 1 Strains resistant to methicillin	MET 5 μg	AMP 10 μg	KF 30 μg	DA 2 μg	SXT 25 μg	E 15 μg	CN 10 μg	OX 1 μg	TE 30 μg
3	R	R	S	S	S	I	R	R	S
14	R*	R	S	S	S	R	R*	R*	S
20	R*	R*	R*	S	S	R	R*	R*	I
23	R*	R	R*	S	S	R	R*	R*	S
28	R*	R*	R*	R*	S	R*	R*	R*	S
29	R*	R	R	R*	S	R*	R*	R*	S
30	R*	R	R*	R*	S	R*	R*	R*	S
32	R*	R*	R*	R*	S	R*	R*	R*	S
36	R*	R*	R*	R*	S	R*	R*	R*	R
40	R*	R*	R*	R*	S	R*	R*	R*	R
44	R*	R	R*	R*	S	R*	R*	R*	S
Group 2 Strains sensitive to all antibiotics	MET 5 μg	AMP 10 μg	KF 30 μg	DA 2 μg	SXT 25 μg	E 15 μg	CN 10 μg	OX 1 μg	TE 30 μg
15	S	S	S	S	S	S	S	S	S
18	S	S	S	S	S	S	S	S	S
34	S	S	S	S	S	S	S	S	S
37	S	S	S	S	S	S	S	S	S
38	S	S	S	S	S	S	S	S	S
42	S	S	S	S	S	S	S	S	S
43	S	S	S	S	S	S	S	S	S

(*continued*)

TABLE 2 – Antibiotic sensitivity patterns of the strains used in the study (*follow*)

Groups	Antibiotics								
Group 3 Strains resistant to amphicillin	MET 5 μg	AMP 10 μg	KF 30 μg	DA 2 μg	SXT 25 μg	E 15 μg	CN 10 μg	OX 1 μg	TE 30 μg
1	S	R	S	S	S	S	S	S	S
2	S	R	S	S	S	S	S	S	S
6	S	R	S	S	S	S	S	S	S
12	S	R	S	S	S	S	S	S	S
17	S	R	S	S	S	S	S	S	S
25	S	R	S	S	S	S	S	S	S
26	S	R*	S	S	S	S	S	S	S
33	S	R*	S	S	S	S	S	S	S
35	S	R*	S	S	S	S	S	S	S
Group 4 Strains resistant to more than 1 antibiotic	MET 5 μg	AMP 10 μg	KF 30 μg	DA 2 μg	SXT 25 μg	E 15 μg	CN 10 μg	OX 1 μg	TE 30 μg
4	S	R	S	S	S	R	S	S	R
5	S	R	S	S	S	R	S	S	R
7	S	R	S	S	S	S	S	S	R
8	S	R	S	S	S	R*	S	S	S
9	S	R	S	I	S	R*	S	S	I
10	S	R	S	I	S	S	R*	S	S
11	S	R*	S	S	S	R	R*	S	S
13	S	R	S	S	S	I	S	S	S
16	S	R	S	I	S	S	S	S	S
19	S	R*	S	S	S	I	S	S	S
21	S	R*	S	S	S	I	S	S	R
22	S	R	S	S	S	I	S	S	R
24	S	R	S	I	S	I	S	S	S
27	S	R*	S	I	S	R*	S	S	S
31	S	R*	S	S	S	S	S	S	I
39	S	R	S	S	S	R*	S	S	S
41	S	R	S	I	S	S	S	S	S

R: resistant; I: intermediate; S: sensitive; R*: inhibition zone absent.
MET: methicillin R< 9 mm; I =10-13 mm; S> 14 mm; AMP: ampicillin R< 28 mm; S>29 mm; KF: cephalothin R< 14 mm; I =15-17 mm; S> 18 mm; DA: clyndamycin R< 14 mm; I =15-20 mm; S>21 mm; SXT: co-trimoxazole R< 10 mm; I = 11-15 mm; S>16 mm; E: erythromycin R< 13 mm; I = 14-22 mm; S> 23 mm; CN: gentamicin R< 12 mm; I =13-14mm; S>15 mm; OX: oxacillin R< 10 mm; I =11-12 mm; S> 13 mm; TE: tetracycline R< 14 mm; I = 15-18 mm; S> 19 mm.

TABLE 3 – Minimal concentration of propolis (sample A and sample B) inhibiting the growth of the *Staphylococcus aureus* strains

Groups	MIC*	
Group 1 - Strains resistant to methicillin	Sample A	Sample B
3	0.13	0.16
14	0.15	0.21
20	0.15	0.16
23	0.12	0.12
28	0.15	0.15
29	0.15	0.18
30	0.15	0.19
32	0.13	0.15
36	0.16	0.19
40	0.15	0.18
44	0.13	0.15
Group 2 - Strains sensitive to all antibiotic		
15	0.12	0.12
18	0.12	0.12
34	0.18	0.21
37	0.18	0.18
38	0.16	0.19
42	0.16	0.19
43	0.25	0.25
Group 3 - Strains resistant to amphicillin		
1	0.10	0.12
2	0.15	0.16
6	0.13	0.13
12	0.13	0.13
17	0.09	0.12
25	0.13	0.13
26	0.13	0.16
33	0.16	0.19
35	0.15	0.18
Group 4 - Strains resistant to more than 1 antibiotic		
4	0.16	0.16
5	0.13	0.16
7	0.13	0.13
8	0.13	0.13
9	0.15	0.15
10	0.13	0.13
11	0.16	0.16
13	0.13	0.13
16	0.09	0.13
19	0.12	0.12
21	0.13	0.16
22	0.16	0.16
24	0.13	0.13
27	0.12	0.12
31	0.18	0.18
39	0.18	0.21
41	0.24	0.27

* MIC expressed as mg of propolis per ml of culture medium.

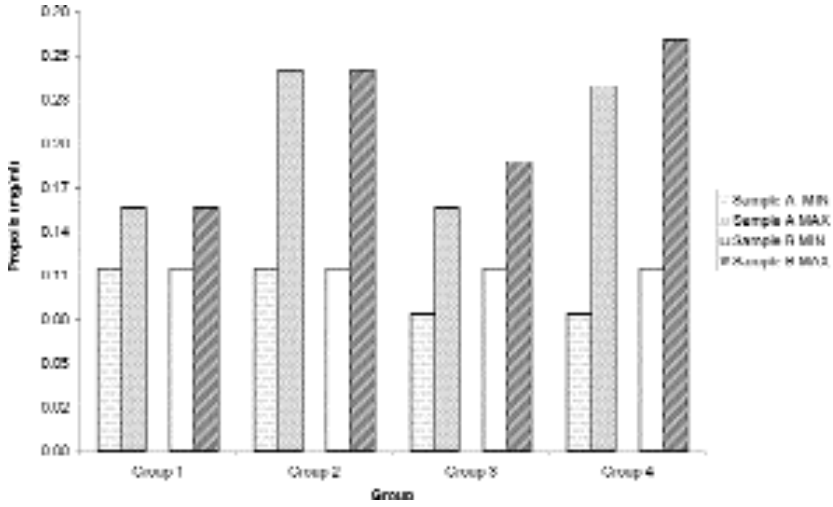

FIG. 1 – Minimal and maximal values of MIC of 2 propolis samples towards *Staphylococcus aureus* strains

(MIC) the growth of the 44 *S. aureus* strains distributed in the 4 groups. The MICs (expressed as mg propolis/ml cultural medium) of both propolis samples used in the research are reported for each strain.

All strains studied showed sensitivity to both propolis samples with amounts of product comprised between 0.09 mg/ml and 0.27 mg/ml. In particular MIC values for 42 of 44 isolates were comprised between 0.12 and 0.21 mg/ml of propolis A sample and between 0.09 and 0.18 mg/ml of propolis B sample. In order to inhibit the growth of the isolates 41 and 43 greater propolis amounts were needed, respectively 0.24 and 0.25 mg/ml of propolis A and 0.27 and 0.25 mg/ml of propolis B.

The statistical analysis of variance calculated on MIC values of the 2 propolis samples according to the 4 different groups, did not show any significant differences among the isolates.

Figure 1 shows minimum and maximum MIC values of the 2 propolis samples according to *S. aureus* strains belonging to the 4 groups.

MIC values are similar in the sphere of the 4 groups including *S. aureus* isolates characterized by a different level of sensitivity according to the 9 antibiotics considered.

CONCLUSIONS

The study of the behaviour of *S. aureus* strains according to propolis samples of piedmontese origin showed that all the considered isolates presented sensitivity to the natural product.

The analysis of variance calculated on values of the minimum concentration of propolis inhibiting microorganism growth did not show any significant differences, among the 44 isolates studied, about sensitivity level to the product.

On the contrary the strains showed a different behaviour according to antibiotics employed in the hospital routine to the control of Gram-positive and Gram-negative bacteria. It appears possible to assert that *S. aureus* strains's answer to propolis treatment was independent from their behaviour according to the antibiotics considered.

It's important to underline that propolis was effective according to 11 strains resistant to methicillin; in the medical field there is growing concern for the increasing frequency of resistance phenomenons in bacterial strains to antibiotics, in particular to methicillin, effective in the control of the most of nosocomial infections by *S. aureus*.

The results obtained from the research appear to confirm antimicrobical property of propolis and contribute to increase the interest in the power of this natural product in the medical field.

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