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Multifactorial aspects of antimicrobial activity of propolis

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Summary

We investigated the antibacterial activity of sub-inhibitory concentrations of ethanolic extract of propolis (EEP), and its effect on the antibacterial activity of some antibiotics. Some clinically isolated Gram-positive strains were used.

Moreover, sub-inhibitory concentrations of EEP were used to value its action on some important virulence factors like lipase and coagulase enzymes, and on biofilm formation in *Staphylococcus aureus*.

Our results indicated that EEP had a significant antimicrobial activity towards all tested clinical strains.

Adding EEP to antibacterial tested drugs, it drastically increased the antimicrobial effect of ampicillin, gentamycin and streptomycin, moderately the one of chloramphenicol, ceftriaxon and vancomycin, while there was no effect with erithromycin.

Moreover, our results pointed out an inhibitory action of EEP on lipase activity of 18 *Staphylococcus* spp. strains and an inhibitory effect on coagulase of 11 *S. aureus* tested strains.

The same EEP concentrations showed a negative interaction with adhesion and consequent biofilm formation in *S. aureus* ATCC 6538P.

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Introduction

Propolis (bee glue) is a natural resinous hive product, collected from various plant sources, manipulated by honeybees and extensively used in folk medicine.

Recently it has attracted much attention as a useful substance applied in medicine and cosmetics due to its antibacterial and antifungal activities (Burdock, 1998).

In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris depending on the place and time of collection (Nieva Moreno et al., 1999; Sforcin et al., 2000).

Among constituents with biological activity, flavonoids contribute more than others to the observed effect of propolis (Marcucci, 1995; Burdock, 1998).

The antibacterial and antifungal properties of propolis have been extensively investigated and, although its chemical composition is linked to the phytogeographic origin, the activity of bee glue has always been reported (Krol et al.,1993; Kujumgiev et al., 1999; Drago et al., 2000).

On the basis of some recent studies of the antimicrobial properties of some substances containing flavonoids (Marcucci, 1995; Stepanovic̀ et al., 2003), we have investigated the antimicrobial activity and synergistic effect with some antibiotics of ethanolic extract of propolis (EEP) against 140 *Staphylococcus* spp. and 123 *Streptococcus* spp. strains.

At present, specific studies on propolis capacity to inhibit virulence factors are not reported.

We have studied the effect of sub-inhibitory concentrations of EEP on activity of lipase and coagulase enzymes in some *Staphylococcus* spp. strains and its interaction with the production of biofilm in *Staphylococcus aureus* ATCC 6538P.

Furthermore, we have investigated for a structural damage of bacterial cells, using propidium iodide (PI) uptake, by fluorescence microscopy.

Materials and methods

Propolis and ethanol extract

Crude propolis (kindly supplied by Specchiasol S.p.A. Verona Italy) (Ricchiuto, 1994) was ground, and 6.6 g of fine powder were extracted with a total 100 ml of ethanol 70%, in a rotary shaker for 7 days at room temperature.

The mixture was centrifuged at 3960*g* for 15 min (Beckman GP Centrifuge); the pellet was handled in the same way three times and supernatants collected and used as EEP.

Bacterial strains

A total of 263 bacterial clinical isolates were tested: 140 *Staphylococcus* spp. strains (35 *S. aureus*, 63 *S. epidermidis*, 7 *S. hominis*, 18 *S. haemolyticus*, 10 *S. warnerii*, 4 *S. capitis*, 3 *S. auricularis*) and 123 *Streptococcus* spp. strains (59 *S. faecalis*, 30 *S. viridans*, 15 *S. β-haemolyticus*, 19 *S. pneumioniae*).

Muller Hinton Broth (MHB-Oxoid) overnight cultures were centrifuged at 3960*g* for 10 min, washed in sterile saline and diluted to an optical density equivalent to 0.5 McFarland (1×10^8 CFU/ml).

Antibiotics

Antibacterial drugs: gentamycin (GENT), streptomycin (STREP), ceftriaxon (CFT), erythromycin (ERIT), vancomycin (VANC), chloramphenicol (CLM), ampicillin (AMP) (Sigma-Aldrich S.r.l.Milano), were dissolved in sterile saline at the concentration of 1 mg/ml.

Minimal inhibitory concentration

Minimal Inhibitory Concentrations (MICs) of EEP and of the antibacterial drugs were determined.

Muller Hinton Agar plates (MHA, Oxoid S.p.A. Milano Italy) were supplemented with 2-fold serial dilutions of EEP at concentrations ranging from 2.5 to 0.1 mg/ml and inoculated with 10^5 CFU by multipoint inoculator.

Ethanol at the concentration used did not interfere with bacterial growth.

Two-fold serial dilutions of each antibiotic in a range from 200 to 0.1 μg/ml were added to MHA and MHA+EEP plates (1/2 EEP MIC for each tested strain).

The plates were incubated at 37 °C for 24 h.

The MIC values were defined as the lowest concentration inhibiting completely the bacterial growth.

Lipase test

Eighteen *Sthaphylococcus* spp. strains grown overnight in Brain Heart Infusion (BHI, Oxoid) (condition C_1) and BHI+ EEP at 1/2 MIC for each strain (condition C_2), were centrifuged at 3960*g*, washed two times in PBS. Compared at the same OD_{600} and 1×10^5 CFU were inoculated

into Spirit Blue Agar (SBA, Difco) normal and added with 1/2 MIC value of EEP.

To improve sizing of lipase halos, the lipolytic activity of the strains, was tested under the same experimental conditions, in a medium without Blue Spirit dye.

The lipolytic activity of strains was detected after 18 h of growth at 37 °C.

The inhibition of enzymatic activity was considered total when the halo was absent and partial when the halo diameter was $<50\%$, compared with the positive control without EEP.

Coagulase test

The generally accepted identifying characteristic of *S. aureus* is the ability to produce free and bound coagulase. The coagulase test detects the presence of clumping factor through clumping of fibrinogen-sensitized sheep red blood cells (Flandrois and Carret, 1981).

The specificity of the reaction is ensured by a simultaneous test with a control reagent.

Coagulase activity of 11 *S. aureus*, at sub-inhibitory concentrations of EEP, was estimated by the Staphylase test Kit DR595 (Oxoid).

Propidium iodide uptake

Overnight BHI brothcultures of *S. aureus* ATCC 25923 and of *S. aureus* 408 (clinical isolates), were grown at mid log phase and despensed in equal volume into two vials.

One of these was supplemented with EEP at 1/2 MIC value for each strain for 45 min at 25 °C, successively 0.05 ml of both samples were transferred into Eppendorf vials containing 0.95 ml of phosphate buffer.

Five microliters of staining solution, consisting of 1 mg/ml PI dissolved in 50 mM phosphate buffer pH = 6, were added to Eppendorf vials.

After 15 min at 25 °C the staining solution was removed by centrifuging for 1 min at 3960*g* and the pellet was dissolved in 0.1 ml phosphate buffer.

The cells were then examined under a fluorescence microscope (Leitz Aristoplan × 400, Leitz S.r.l. Milano Italy).

The presence of fluorescent cells was compared to the control.

Prevention of biofilm forming by EEP

Adherence to smooth surfaces was performed using the modified Christensen method (Christensen et al., 1982).

Twenty-four-well tissue cultures plates containing Luria-Bertani broth (LB) and LB+EEP at 1/2, 1/4, 1/8 MIC values were incubated with *S. aureus* ATCC 6538P with overnight broth culture (200 μl).

The adherence was measured after 48 h of incubation at 37 °C.

After aspiration of the supernatants, the wells were washed four times with PBS (pH = 7.2) and stained with safranin for 10 min.

After various washing in PBS, the staining retained by biofilm was extracted with ethanol and measured by spectrophotometer (DU70 Beckman) at 484 nm.

Results

MICs of ethanolic extracts of propolis (EEP)

MIC_{50} and MIC_{90} (Table 1) for 35 strains of *S. aureus* were both of 1.25 mg/ml.

MIC_{50} and MIC_{90} for 63 strains of *S. epidermidis* and 42 strains of *Staphylococcus* spp. were, respectively, of 1.25 and 2.5 mg/ml.

For 123 strains of *Streptococcus* spp. the values of MIC_{50} and MIC_{90} were between 0.31 and 2.5 mg/ml.

Minimal inhibitory concentrations

For most antibiotics, a clear abatement of MIC_{50} and MIC_{90} of 140 strains of *Staphylococcus* spp. was evident in presence of EEP (Table 2).

Especially for STREP, CLM, AMP, GENT, the presence of EEP enhanced the antimicrobial effect of these antibiotics.

Table 1. MIC_{50} and MIC_{90} of ethanolic extract of propolis (EEP) against 140 *Staphylococcus* spp. and 123 *Streptococcus* spp. strains (final concentration of ethanol $\leqslant 2.5\%$)

Strains	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)	Range (mg/ml)
S. aureus (35)	1.25	1.25	0.62–2.5
S. epidermidis (63)	1.25	2.5	0.62–2.5
S. hominis (7), *S. haemolyticus* (18), *S. warnerii* (10), *S. capitis* (4) *S. auricolaris* (3)	1.25	2.5	0.62–2.5
S· β-haemolyticus (15)	0.31	0.62	0.31–0.62
S. viridans (30)	0.31	2.5	0.31–2.5
S. pneumoniae (19)	0.62	1.25	0.31–1.25
S. faecalis (59)	2.5	2.5	2.5

MIC_{90} with STREP+EEP in all 140 strains of *Sthaphylococcus* spp. decreased if compared with STREP used alone.

The best performance was detected with *S. aureus* in which in presence of STREP+EEP, lost their characteristics of resistance decreasing MIC_{90} from 200 to 0.05 μg/ml (Table 2).

The association of AMP+EEP, in the same strains, decreased MIC_{90} 256-fold in *S. aureus*; 250-fold in *S. epidermidis*; 132-fold in other *Staphylococcus* spp.

Table 2. MIC_{50} and MIC_{90} of drugs alone and added with sub-inhibitory concentration of ethanolic extract of propolis (EEP) against 140 *Staphylococcus* spp. strains (final concentration of ethanol ⩽0.8%)

Drug	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)
Staphylococcus aureus (35)			
AMP	12.5	>200	0.05–>200
AMP+EEP	0.1	0.78	0.05–0.78
GENT	12.5	50	0.1–>200
GENT+EEP	0.1	0.19	0.1–0.19
CLM	12.5	25	1.56–100
CLM+EEP	0.1	0.1	0.05–6.25
STREP	50	>200	1.56–>200
STREP+EEP	0.05	0.05	0.05–0.39
VANC	3.12	3.12	1.56–3.12
VANC+EEP	0.19	0.39	0.1–3.12
Staphylococcus epidermidis (63)			
AMP	1.56	25	0.05–50
AMP+EEP	0.05	0.1	0.05–0.78
GENT	0.39	25	0.05–200
GENT+EEP	0.1	0.19	0.05–0.19
CLM	12.5	100	1.56–>200
CLM+EEP	0.1	6.25	0.1–25
STREP	3.12	25	0.78–50
STREP+EEP	0.1	1.56	0.05–3.12
VANC	3.12	6.25	1.56–6.25
VANC+EEP	0.19	3.12	0.05–3.12
Staphylococcus spp. (42): *S. haemolyticus* (18), *S. warnerii* (10), *S. hominis* (7), *S. capitis* (4), *S. auricolaris* (3)			
AMP	6.25	25	0.05–200
AMP+EEP	0.05	0.19	0.05–0.78
GENT	6.25	50	0.05–200
GENT+EEP	0.1	12.5	0.05–50
CLM	12.5	100	0.05–>200
CLM+EEP	0.1	1.56	0.05–3.12
STREP	3.12	12.5	0.78–12.5
STREP+EEP	1.56	1.56	0.05–3.12
VANC	3.12	6.25	0.39–6.25
VANC+EEP	0.19	0.39	0.1–3.12

MIC: minimum inhibitory concentration, EEP: ethanolic extract of propolis, AMP: ampicillin, ERIT: erithromycin, GENT: gentamycin, VANC: vancomycin, CLM: chloramphenicol, STREP: streptomycin.

Also, in this case, in presence of EEP, especially in *S. aureus*, strains lost their characteristics of resistance.

Synergistic effect appeared also in the association of GENT+EEP in which MIC_{90} decreased 263-fold in *S. aureus* and 132-fold in *S. epidermidis*.

A moderate effect has been detected in CLM+EEP association (Table 2).

The antibiotic VANC was the more efficacious drug against *Staphylococcus* and *Streptococcus* spp. than the others antibiotics we used.

In fact, MICs of VANC were between 3.12 and 6.25 μg/ml in *Staphylococcus* spp. and 3.12 μg/ml in *Streptococcus* spp.

The association of VANC+EEP decreased MIC_{90} 8-fold in *S. aureus* and 16-fold in *Staphylococcus* spp.

In the 64 strains of *Streptococcus* spp. MIC_{90} of STREP+EEP and MIC_{90} of GENT+EEP in all strains were reduced 16-fold.

MIC_{90} with AMP+EEP were reduced 16-fold in 59 strains of *Streptococcus faecalis* and 32-fold in remaining *Streptococcus* spp. (Table 3).

Neither synergism has been detected with ERIT and CFT in association with EEP, nor in the *Staphylococcus* spp. (data not shown).

Lipase test

Lipolytic halos, surrounding colonies of 18 strains of *Staphylococcus* spp. have been compared.

In presence of EEP, especially when EEP was continually present in all stage (condition C_2: SBA+EEP), small and faint halos displayed an evident decrease in lipase activity (Fig. 1).

High levels of lipase activity were detected in clinic isolates and in *S. aureus* ATCC 25923.

Lipase activity for each strain was displayed by halos (Fig. 1).

The ratio of diameter of halos without and with EEP pointed out an inhibition of lipase activity between 17% and 50% in tested strains (Table 4).

Coagulase test

In presence of EEP we observed drastic inhibition of coagulase enzyme and using Staphylase test Kit (Oxoid S.p.A) no activity was detected.

Propidium iodide test

An evident suffering state of the cells was evidenced by PI in presence of sub-inhibitory concentrations of EEP.

Table 3. MIC_{50} and MIC_{90} of drugs alone and added with sub-inhibitory concentration of ethanolic extract of propolis (EEP) against 123 *Streptococcus* spp. strains (final concentration of ethanol $\leqslant$0.8%)

Drug	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)
S. viridans (30), *S· β-haemolyticus* (15), *S. pneumoniae* (19)			
AMP	0.78	100	0.1–100
AMP+EEP	0.19	3.12	0.05–3.12
GENT	12.5	25	0.05–25
GENT+EEP	1.56	1.56	0.05–1.56
STREP	50	100	1.56–100
STREP+EEP	3.12	6.25	0.05–6.25
VANC	3.12	3.12	0.78–3.12
VANC+EEP	1.56	3.12	0.05–3.12
Streptococcus faecalis (59)			
AMP	0.78	50	0.39–50
AMP+EEP	0.39	3.12	0.19–3.12
GENT	12.5	25	6.25–25
GENT+EEP	1.56	1.56	1.56
STREP	50	>200	25–>200
STREP+EEP	6.25	50	3.12–50
VANC	1.56	3.12	0.78–3.12
VANC+EEP	0.78	1.56	0.39–1.56

MIC: minimum inhibitory concentration, EEP: ethanolic extract of propolis, AMP: ampicillin, ERIT: erythromycin, GENT: gentamycin, VANC: vancomycin, CLM: chloramphenicol, STREP: streptomycin.

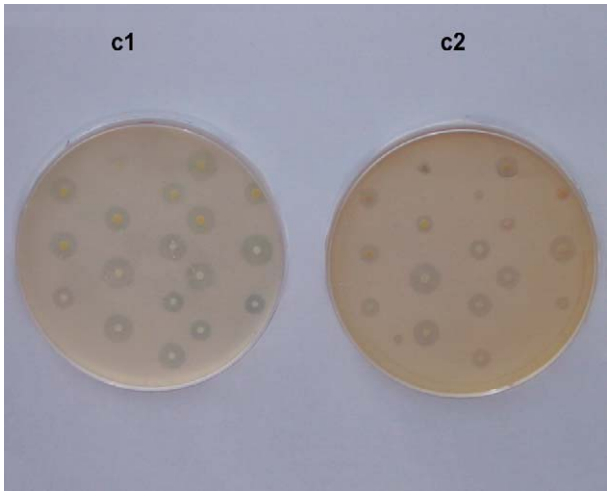

Figure 1. Lipolytic halos in C_1 and C_2 growth conditions (see text for details).

In contrast, in absence of EEP strains of *Staphylococcus* spp. were always negative to the test (Fig. 2).

Table 4. Diameter (cm) of lipolytic halos in growth conditions (C_1, C_2) of 18 *Staphylococcus* spp. strains and % of reduction by EEP on enzymatic activity

Strains	C_1	C_2	% Reduction of lipase activity
S. aureus			
ATCC 25923	0.6	0.4	33
ATCC 6538	—	—	—
001	0.5	0.3	40
89	0.4	0.2	50
1	0.5	0.3	40
408	0.6	0.3	50
32	0.5	0.3	40
S. epidermidis			
30A	0.6	0.5	17
1B	0.5	0.4	20
6A	0.6	0.4	33
27A	0.6	0.5	17
16A	0.6	0.6	0
23B	0.4	0.2	50
33A	0.5	0.4	20
22A	0.5	0.4	20
7A	0.5	n.d.	n.d.
S. hominis			
83A	0.6	0.5	17
S. warnerii			
15B	0.5	0.3	40

C_1: without EEP (100% activity).
C_2: with EEP (1/2 MIC for each strain).

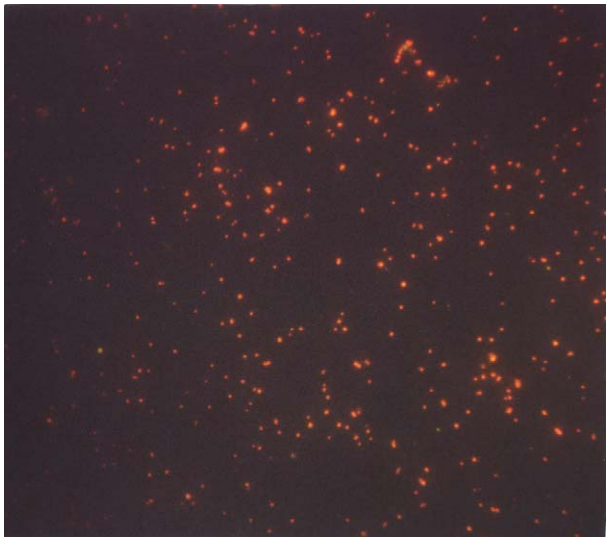

Figure 2. Propidium iodide uptake in presence of EEP (see text for details).

EEP interference with biofilm production

Adding serial dilutions of sub-inhibitory concentrations of EEP in different wells, a proportional

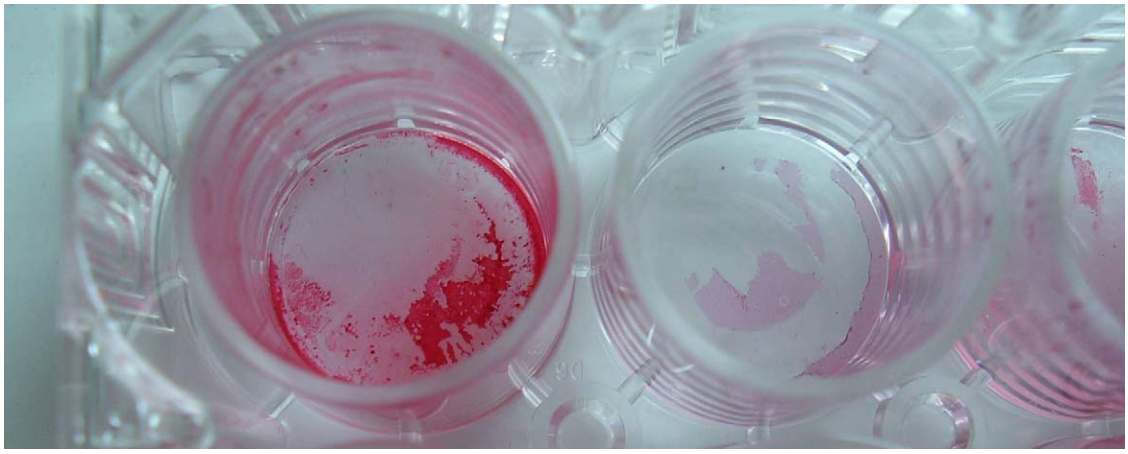

Figure 3. Interference of EEP with biofilm production (see text for details).

decrease of adhesion in *S. aureus* ATCC 6538P, was displayed (Fig. 3), demonstrating a positive correlation between EEP concentration and adhesion.

A maximum decrease of 40% in adhesion, and possibly biofilm formation, in presence of EEP at 1/2 MIC value was observed.

Discussion

The positive effect of natural propolis is well known. (Kujumgiev et al., 1999; Nieva Moreno et al., 1999; Sforcin et al., 2000).

Investigating the antimicrobial activity towards some Gram-positive bacteria, we found propolis to be effective against many virulence factors.

Worthy of notice, these multifactorial aspects have been investigated.

Sub-inhibitory concentrations of EEP displayed a synergistic effect with many antibiotics we used; an evident decrease of MICs in some drugs is of remarkable interest.

Some components present in propolis extract, like flavonoids (quercetin, galangin, pinocembrin) and caffeic acid, benzoic acid, cinnamic acid, probably act on the microbial membrane or cell wall site, causing functional and structural damages (Marcucci, 1995; Cook and Samman, 1996; Mirzoeva et al.,1997; Gatto et al., 2002).

As shown by the PI test, a suffering state of the cells in presence of EEP was evident.

Staphylococcus's virulence factor coagulase was completely suppressed by EEP; lipase, for many Staphylococci was strongly reduced and a dose-dependent prevention of biofilm formation was evident.

As highlighted in many recent works, the reduction of microbial virulence factors is a target of great interest.

Our observations demonstrate a multiple action of propolis against different virulence factors of some Gram-positive bacteria of clinical interest.

Ineffective antibacterial drugs, consequence of emergence of antibiotic resistances, can be reused at the same time with propolis during early stages of infection. The recovery of efficacy of old antibiotics and consequent reduction of economical problems, are important aspects to be considered.

Although it is highlighted, in vitro and in vivo, the property of propolis as a natural treatment in some Gram-positive infections, no in vivo observations of synergism of propolis and antibiotics are reported.

Our data together with the widespread appearance of antibiotic resistance and the increasing interest towards natural therapy, effective and healthy pharmacological compounds, suggest further studies for best comprehension of which propolis compounds are involved in the mechanisms of synergistic interaction with other antibacterial drugs.

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