Synergism Between Ethanolic Extract of Propolis (EEP) and Anti-Tuberculosis Drugs on Growth of Mycobacteria

Stan Scheller[a,*], Szymon Dworniczak[a], Krystian Waldemar-Klimmek[a], Marek Rajca[a], Anna Tomczyk[a] and Jashovam Shani[b,**]

[a] Department of Microbiology and Immunology, Silesian Academy of Medicine, Zabrze-Rokitnica, Poland. Fax: # ++48-32-272-2554. E-mail: mikroimm@friko3.onet.pl
[b] Department of Pharmacology, The Hebrew University School of Pharmacy, Jerusalem, Israel

* Author for correspondence and reprint requests

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Ethanolic extract of propolis exerts a strong anti-bacterial activity, in addition to antifungal, antiviral and antiprotozoal properties. In previous studies from these laboratories we have demonstrated that the intensity of the bactericidal activity of EEP is correlated with the virulence of the mycobacteria tested, and that EEP has a synergistic effect with antibiotics on growth of staphylococcus aureus. In the present study we investigated whether the same synergism and correlation exists between EEP and some anti-tuberculosis drugs on tuberculosis mycobacteria with different degrees of virulence. Six standard strains and 11 wild strains of mycobacteria were exposed for 30 days to EEP, with or without streptomycin, rifamycin, isoniazid or ethambutol. Out of the 17 strains, 8 were resistant to at least two standard antibiotics, and were considered "multi-resistant strains". The rest were either susceptible or resistant to only one of the antimycobacterial drugs. Antagonism was recorded only in one case, when *Staphylococcus aureus* were treated with a mixture of EEP and ethambutol, suggesting that a chemical bond could have been formed between this anti-tuberculosis antibiotic and one of the active components of the ethanol extract of propolis.

Introduction

Propolis is a natural resinous material, gathered and produced by honeybees, and used by them in order to protect their nests against intruders, such as bacteria and insects. It also helps them isolate and disinfect the nests. The ethanolic extract of propolis (EEP) exerts antibacterial, antifungal, antiviral and antiprotozoal activities, as well as the following properties: antioxidant, free radical scavenger, anticarcinogenic, immunostimulatory, radioprotective and ability to heal damaged tissues (Frankiewicz and Scheller, 1984; Grange and Davey, 1990; Matsuno, 1995; Scheller *et al.,* 1968, 1977a,b,c, 1978, 1988, 1989a,b,c, 1990, 1997, 1998; Starzyk *et al.,* 1977).

EEP is rich in various flavonoid aglycones, phenolic compounds, sesquiterpens, diterpens, steroids, amino acids, carboxyl-acids, aromatic alcohols and about 30 inorganic-, including trace-elements (Bonvehi *et al.,* 1994; Gabrys *et al.,* 1986; Matsuno, 1995; Miller and Leowski, 1997; Scheller *et al.,* 1989b; Krol *et al.,* 1993). Some of these polyphenols inhibit synthesis of reverse transcriptase and protease of retroviruses (Burke *et al.,* 1995; Critchfield *et al.,* 1996; Fesen *et al.,* 1994; Nakane and Ono, 1990; Ono *et al.,* 1990).

The majority of the chemical compounds in the ethanolic extract of propolis are strong hydrophobic entities, with high affinity towards lipids. As lipids are the most abundant and distinct in the outer cell surface of mycobacteria, they easily bind such components. In previous studies we have demonstrated that the intensity of the bactericidal activity of EEP is correlated with the virulence of the mycobacteria, and that when such virulence is augmented, the antimycobacterial activity of EEP is significantly increased (Scheller *et al.,* 1968, 1998).

Also, in a previous study from our laboratories we have shown that EEP has a synergistic effect with antibiotics on the growth of staphylococcus aureus (Krol *et al.,* 1993). We decided, therefore,

** Affiliated with the David R. Bloom Center for Pharmacy at the Hebrew University.

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to investigate whether the same synergism and correlation exists between EEP and some anti-tuberculosis drugs on tuberculosis mycobacteria that possess different degrees of virulence.

Materials and Methods

Mycobacteria and their inoculation

Six standard laboratory strains and eleven "wild" strains of mycobacteria were used. The six standard strains were *$H_{37}Rv$, $H_{37}Ra$, M. kansasii, M. xenopei, M. intracellulare* and *M. BCG*. Eight of the wild strains were isolated from patients pre-treated with anti-tuberculosis drugs: M. 208, M. 223, M. 214, M. 241, M. 252, M. 260, M. 323 and M. 333. Three wild strains were isolated from patients that were not pre-treated with anti-tuberculosis drugs: M. 2563, M. 2583 and M. 2976.

All mycobacteria strains were isolated from pathological specimen, that were obtained fresh from the Tuberculosis Institute in Warsaw (Poland) or from the Clinic for Tuberculosis and Pulmonary Diseases of the Silesian Academy of Medicine in Zabrze-Biskupice (Poland). Six-week old mycobacteria were suspended in distilled water with the aid of a Weigls mortar, and were incubated on a Löwenstein-Jensen solid medium, containing KH_2PO_4, $MgSO_4$, magnesium citrate, L-asparagin, glycerol, potato flour, egg albumin and malachite green. The concentration of the suspension was established as 1 mg/ml, and the inoculation dose was always 5×10^{-5} mg/0.2 ml.

Propolis and EEP

Propolis was collected manually in the beehive of the University's farm (bees using mainly *Populus* buds), and was kept desiccated pending processing. It was extracted in 95% v/v ethanol, in a 1:10 ratio, in hermetically-closed glass vessel, for 10 days at 37 °C, under frequent shaking. The ethanolic extract was then filtered through a Whatman #4 filter paper, and evaporated under vacuum. The dark-brown material (EEP; yield 45–60% of the raw material) was dissolved in the medium prior to its use. The medium containing various concentrations of EEP was coagulated three times, on three consecutive days, at 75 °C.

Antimycobacterial drugs

The following four basic antimycobacterial drugs were used in this study: streptomycin, rifamycin, isoniazid and ethambutol. Each of these drugs was introduced in the Löwenstein-Jensen solid medium in the following concentrations:

*Streptomycin	0	0.125	0.25	0.50	1.0	2.0 μg/ml
*Rifamycin	0	0.6	1.25	2.50	5.0	10.0 μg/ml
*Isoniazide	0	0.025	0.05	0.10	0.2	1.0 μg/ml
*Ethambutol	0	0.03	0.06	0.12	0.25	0.5 μg/ml

Susceptibility of mycobacteria to antibiotics

Mycobacteria were inoculated on the solid medium containing antibiotics at a concentration of 5×10^{-5} bacteria per test-tube and at 37 °C. Growth rate was recorded once weekly, and the final reading was performed on the 30th day after inoculation. Mycobacteria that would not grow in EEP concentrations of 1,800 μg/ml and lower, were designated as "susceptible to EEP" and marked "S". Mycobacteria that managed to grow in EEP concentrations of 1,800 μg/ml and higher, were designated as "resistant to EEP" and marked "R". A strain was considered resistant ("R") if 1% of the mycobacteria would be resistant to 4 μg/ml streptomycin, 0.2 μg/ml isoniazid, 40 μg/ml rifamycin or 2.0 μg/ml ethambutol.

Results

Out of the 17 strains of mycobacteria that were investigated in this study, eight were resistant to at least two standard antibiotics. Six wild (M. 208, M. 260, M. 241, M. 252, M. 323, M. 333) and two standard strains of mycobacteria (*$H_{37}Ra$* and *M. intracellulare*) and were considered as "multi-resistant strains". The rest of the mycobacteria strains appeared either to be totally susceptible or resistant to only one of the investigated antimycobacterial drugs.

EEP partially inhibited the growth rate of 14 out of the 17 mycobacterial strains tested, as could easily be observed by a significant decrease in the size and number of their colonies. Six of the mycobacterial strains reached the ultimate goal of susceptibility to EEP, when less than 10% of them were resistant to an EEP concentration of 1,800 μg/ml or lower. Some wild strains (one of them originating from a patient that had not been treated with anti-mycobacterial drugs) and one

Table I. Sensitivity of mycobacterial strains to anti-tuberculosis drugs and EEP.

Bacterial strain	Anti-tuberculous drug				EEP
	SM	RMP	INH	EMB	
M. 2583	R	S	S	S	R
M. 2976	R	S	S	S	R
M. 223	S	R	S	S	S
M. 241	R	R	R	S	R
M. 252	S	R	R	R	R
M. 260	R	R	R	R	S
M. 323	R	R	R	R	R
M. 333	R	R	R	S	R
M. kanasi	R	S	S	S	R
M. 2563	S	S	S	S	S
M. 208	S	R	R	S	S
M. 214	S	S	R	S	S
M. intracellulare	R	S	R	R	R
M. $H_{37}Rv$	S	S	S	S	S
M. BCG	S	S	R	S	R
M. $H_{37}Ra$	R	R	R	R	R
M. xenopei	S	S	S	S	R

R = resistant; S = susceptible; SM = streptomycin; RMP = rifamycin; INH = isoniazid; EMB = ethambutol.

standard strain (*M. $H_{37}Rv$*) was considered "multi-resistant". Only three mycobacterial strains were totally unaffected (resistant) to EEP: *M. BCG, M. xenopei* and *M. $H_{37}Ra$*, all three belonging to the mycobacteria having "modified (attenuated) virulence" (Table I).

Addition of EEP to medium containing anti-tuberculosis drugs markedly potentiated, in some of the strains, the anti-mycobacterial activity of some of the drugs. Enhanced sensitivity of mycobacteria to their antibiotic treatment was observed in 12 of the mycobacterial strains, even if an initial resistance to some of these antibiotics was recorded during the first weeks of their exposure. In only one strain (*M. $H_{37}Rv$*) an antagonism between the antibacterial activity of EEP and the bacteriostatic efficacy of etambuthol was noticed. The synergism between EEP and some anti-tuberculosis drugs is clearly demonstrated in Table II.

Discussion

Ethanolic extract of propolis is a natural mixture of a variety of chemical components, with multi-directional properties, including antibacterial activity. The present study demonstrates potentiation of the antibacterial property of EEP exerted on 14 mycobacterial strains out of the 17 strains tested, when such activity (as evaluated by a diminution in the number of bacterial colonies) was recorded only in a single bacterial strain.

The synergistic effect of EEP was demonstrated in the present study with different bactericidal

Table II. Sensitivities of mycobacteria to anti-tuberculosis drugs and EEP, added separately or in combination to the bacterial inoculum.

Bacterial strain	Anti-tuberculous drug				EEP	Anti-tuberculous drug + EEP			
	SM	RMP	INH	EMB		SM	RMP	INH	EMB
M. 2583	R	S	S	S	R	S+	+	+	S
M. 2976	R	S	S	S	R	S+	S	S	S
M. 223	S	R	S	S	S	S	S+	S	+
M. 241	R	R	R	S	R	S+	S+	S+	+
M. 252	S	R	R	R	R	S	S+	S+	S+
M. 260	R	R	R	R	S	R	R	S+	R
M. 323	R	R	R	R	R	S+	S+	S+	R
M. 333	R	R	R	S	R	+	R	S+	+
M. kanasi	R	S	S	S	R	S+	S	+	S
M. 2563	S	S	S	S	S	+	S	S	+
M. 208	S	R	R	S	S	S	R	R	+
M. 214	S	S	R	S	S	S	S	R, D	+
M. intracellulare	R	S	R	R	R	R	+	R	R
M. $H_{37}Rv$	S	S	S	S	S	+	+	+	–
M. BCG	S	S	R	S	R	S	S	R	S
M. $H_{37}Ra$	R	R	R	R	R	R	R	R	R
M. xenopei	S	S	S	S	R	S	S	S	S

R = resistant; S = susceptible; + = synergism; – = antagonism; S+ = susceptible as a result of EEP activity; D = decrease in number of colonies; SM = streptomycin; RMP = rifampicin; INH = isoniazide; EMB = ethambutol.

anti-tuberculosis drugs, including streptomycin, rifamycin and isoniazide. No synergism was recorded when the myco-bacteriostatic agent etambuthol was mixed with EEP. We consider this finding as an exception rather than the rule in our synergism studies between EEP and established antibacterial agents, and it may be explained by a chemical bond that occurs between etambuthol and one of the EEP components, thus diminishing the active level of etambuthol in the medium (Danysz and Jeljaszewicz, 1976).

A synergistic effect between EEP and well-established anti-tuberculosis drugs was recorded in the present study also in samples from patients that had not been treated with ant-tuberculosis drugs. Two strains of tuberculosis bacilli that were found resistant to some of the anti-tuberculosis drugs tested, lost part of their resistance when treated with a drug+EEP mixture. The possible antagonism between an EEP component(s) and some anti-tuberculosis drugs is rare and warrants further studies.

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