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Microbiological and calorimetric investigations on the antimicrobial actions of different propolis extracts: an in vitro approach☆

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Abstract

The antibacterial action of three different types of propolis extracts: (i) water-extracted propolis (WEP), (ii) propolis volatiles (PV), and (iii) ethanol-extracted propolis (EEP) were investigated by flow microcalorimetry coupled with polarography, and by Petri dish bioassay methods. The water-extracted propolis solution had the weakest antibacterial and antifungal action, compared to the other two extracts, which showed effects nearly similar to each other. Filamentous fungi were generally less sensitive to propolis than bacteria and yeasts, regardless of the type or concentration of propolis.

Propolis displayed both bacteriostatic and bactericidal actions depending on the concentration, type of propolis, and type of bacteria tested. The Gram negative bacterium *E. coli* was insensitive to most treatments, and higher concentrations of propolis were required to achieve bactericidal effects.

Treatments of bacteria with weak propolis concentrations caused a decrease in the calorimetric power-time (p–t) curves to lower levels, at which the curves remained for the rest of the experimental period, decreased to the baseline with the course of time, or revived after some time and attained peaks. The treatment with strong concentrations, however, caused the curves to descend to the baseline immediately.

Keywords: Propolis; Bee glue; Calorimetry; Antimicrobial; Flow calorimetry

1. Introduction

Propolis has been used by man since early times for various purposes, especially as a medicine because of its antimicrobial properties [1,2]. Ancient Greek texts refer to the substance as a “cure for bruises and suppurating sore”, and in Rome propolis was used by physicians to make poultices. Records from twelfth century Europe describe medical preparations using propolis for the treatment of mouth and throat infections, and dental cares [3]. Several antimicrobial activities have been ascribed to propolis including antibacterial [4–14] antifungal [13–17], antiprotozoan [18–21], and antiviral [20–22], among others.

The medicinal use of propolis was nearly forgotten in modern era due to the discovery and effective use of antibiotics. Nowadays, however, since several pathogens are developing resistance to potent antibiotics, and the latter causing side effects in humans, there is an increased need to search and screen for new antimicrobial agents is growing [23,24].

Regardless of the increasing emergence of drug resistant microbes, the pace at which new antimicrobials are discovered and produced is slowing and the so-called new and emerging pathogens are aggravating the problem [25]. The mechanisms of antimicrobial actions of antibiotics and the resistance mechanisms by most microbes to antibiotics are well documented [26,27]. The mechanisms of action of biocides based on natural mixtures, such as propolis, are however,

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poorly understood due mainly to the several target sites they have within a bacterial cell [28,29].

A method that is reliable enough to be used in the study of antimicrobial agents and without severe drawbacks in such applications is calorimetry [30]. Its heat flow signals show in an online manner the bacteriocidic or bacteriostatic effects and the influence of the agent on the growth kinetics of the microbial culture, while many other methods work integratively and render their results only after a rather long time and rather unspecific. One of the aims of the present investigation was, therefore, to apply calorimetry to elucidate the mechanism of action of propolis and to evaluate its credibility compared to the often-used standard microbiological methods in testing the effects of antimicrobials.

Research has been done on the biological activity of propolis against different sorts of ailments, infections and parasites in the past. But most investigations concentrated on only one sample from a special geographic location, one type of extract (usually the ethanol extract, EEP), or derivatives of one type of extract. Almost all used only the Petri dish bioassay method with no, or very little hints about the mechanisms behind the antimicrobial effects. Thus, a second purpose of the present investigations was to compare the efficiency of propolis extracts from different geographic origins, and samples from the same apiary but different hives. Such comparison requires considering the species and subspecies of bees that did the collection. The comparison between samples from different places will be done at the level of extracts, i.e. ethanol-extracted propolis (EEP), water-extracted propolis (WEP), and propolis volatiles (PV).

2. Experimental

2.1. Propolis acquisition and preparation of different extracts

Propolis samples were obtained from different countries by personal contact with beekeepers and scientists in the corresponding countries (C: Colombia, E: Ethiopia, G: Germany, I: Italy, K: Kazakhstan, P: Poland, R: Russia, SA: South Africa). All samples were obtained as solid samples and extracted in 70% ethanol or distilled water according to previous methods [31] to obtain the corresponding propolis extracts. In addition to that samples were extracted by steam distillation using a Lickens-Nickerson apparatus following Kujumgiev et al. [11] to collect the volatile components of propolis. The extracted and dried samples were dissolved and applied in 60% ethanol in case of the ethanol extracted propolis (EEP) or the volatile components (PV), and in water in the case of the water extracted propolis (WEP).

2.2. Biological material

Bioassays of the antimicrobial activities of the different propolis samples were performed using (i) four fungal species: the yeast *Saccharomyces cerevisiae* (DSM 211) and three filamentous fungi (ascomycete), *Aspergillus niger* (DSM 737), *Penicillium chrysogenum* (DSM 844) and *Trichoderma viride* (DSM 63065); (ii) four species of Gram positive bacteria: *Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348), *Bacillus megaterium* (DSM 90), *Bacillus brevis* (DSM 5609); and (iii) two species of Gram negative bacteria: *Escherichia coli* (DSM 31), *Pseudomonas syringae* (DSM 5176). All strains of microorganisms were bought from the German Collection for Microorganisms and Cell Culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig/Germany). The microbes were cultivated on culture media according to the prescription of DSMZ.

2.3. Petri dish bioassay

Minimal inhibitory concentration (MIC) values for the different propolis samples against the various bacterial and fungal species were determined by the agar dilution method, according to the recommendation of the National Committee for Clinical Laboratory Standards guidelines [32].

Corresponding volumes of a 10% propolis solution or of lower concentrations were added to the sterile agar solutions at a temperature of 48 °C, to achieve final concentrations of 0.05, 0.1, 0.5, 1.0, 1.5, and 2% w/v propolis in the fungal growth media and 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1% w/v propolis in the bacterial and yeast growth media. The plates were inoculated with an actively growing standard microbial suspension and incubated at a temperature of 30 °C for 24 h for bacteria and at 25 °C for 48–72 h for fungi. The MIC value was the lowest concentration of propolis that inhibited any visible growth of bacteria, yeast, or fungi.

2.4. Calorimetric bioassay

All calorimetric experiments were conducted with bacteria at a temperature of 30 °C using a flow calorimeter (Type 10700-1, LKB Bromma, Sweden) with a sensitivity of 61.6 μV mW^{-1}. The calibration of the flow-through cell was performed regularly by means of the incorporated Joule heater and only from time to time by the triacetin method proposed by Chen and Wadsö [33]. The calorimetric heat flow signals were divided by volume of the flow-through spiral of 0.587 ml to obtain the specific heat flow rates presented in Figs. 1–3. The calorimeter was connected by a Teflon tube of 1 mm inner diameter and less than 1 m length to an external fermenter, a 50 ml reaction vessel with 20 ml nutrient broth, placed in a water bath at 30 °C. The bacterial culture was circulated from the fermenter to the calorimeter and back using a peristaltic pump (type LKB Pharmacia, Bromma, Sweden) at the outlet of the calorimeter in a sucking mode with a pumping rate of 56 ml h^{-1}. The culture was vigorously stirred with a magnetic stirrer in order to avoid settling of cells and minimize depletion of oxygen in the fermenter and in the flow line. Because of the high pumping rate, oxygen consumption

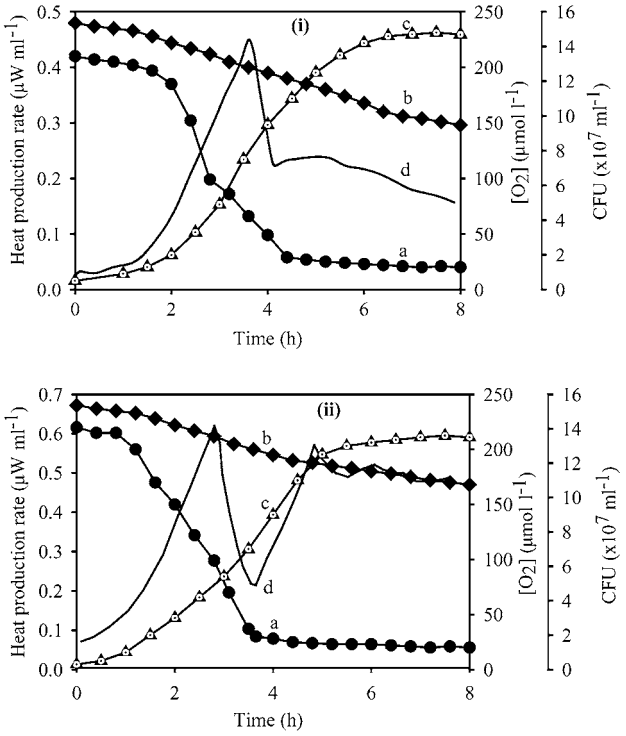

Fig. 1. Simultaneous recording of (a) oxygen tension in the flow line, (b) oxygen tension in the fermenter, (c) number of colony forming units (CFU), and (d) heat production rate of untreated cultures of (i) *B. megaterium* and (ii) *E. coli* in a flow microcalorimetric experiment.

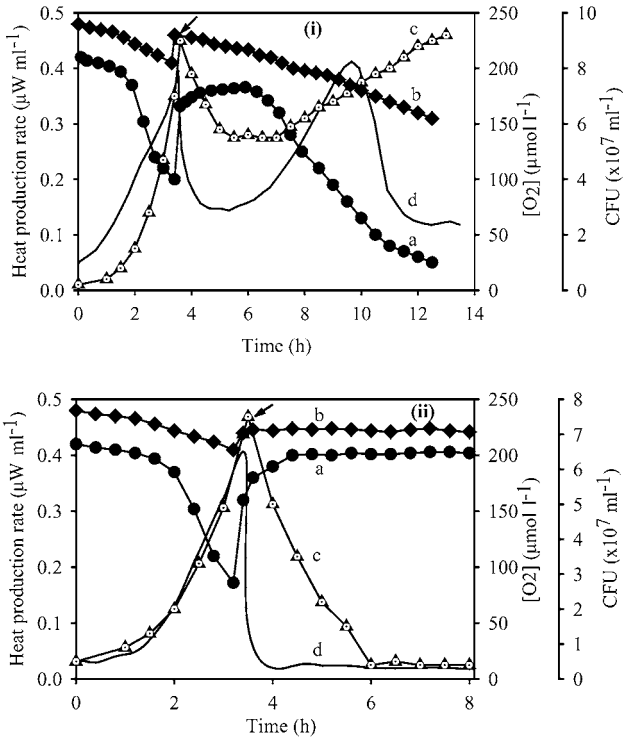

Fig. 2. Simultaneous recording of (a) oxygen tension in the flow line, (b) oxygen tension in the fermenter, (c) number of colony forming units (CFU), and (d) heat production rate of a culture of *B. megaterium* treated with (i) 0.025% and (ii) 0.05% EEP of SA8 in a flow microcalorimetric experiment. Arrows indicate treatment.

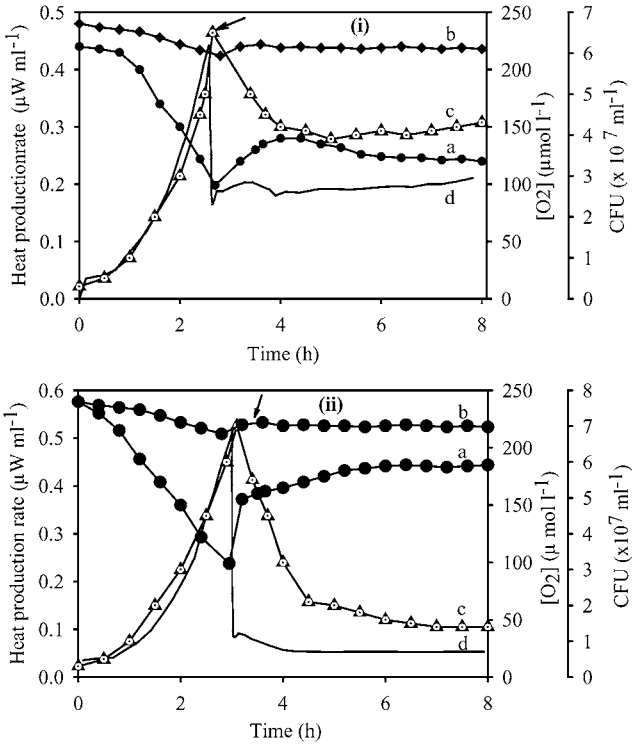

Fig. 3. Simultaneous recording of (a) oxygen tension in the flow line, (b) oxygen tension in the fermenter, (c) number of colony forming units (CFU), and (d) heat production rate of a culture of *E. coli* treated with (i) 0.025% and (ii) 0.05% EEP of P3 in a flow microcalorimetric experiment. Arrows indicate the time of treatment.

in the flow line is mainly due to bacterial respiration and not to leakage through the Teflon walls.

The flow calorimetric line and the calorimetric spiral were sterilized by circulating a sterilizing solution composed of 10% H_2O_2 and 2% H_2SO_4 in 60% ethanol for 30 min before and after each experiment. After the allocated sterilization time the flow calorimetric set up was cleaned with 0.1 M potassium-phosphate buffer of pH 7.0 for 1 h.

2.4.1. Microcalorimetric cultivation of bacteria

The calorimetric investigations were done only with bacteria. Preliminary calorimetric experiments coupled with polarography and the determination of Colony Forming Units (CFU) displayed that all strict aerobes, i.e. *B. megaterium*, *B. subtillis*, *B. brevis*, *M. luteus*, and *P. syringae*, have similar patterns of *p–t* curves, change in oxygen tension in the flow line and of number of CFU, though minor differences exist among the shape of the *p–t* curves. The facultative anaerobe *E. coli*, however, showed a unique *p–t* curve due to the shift of metabolism to the anaerobic phase. For this reason, further calorimetric experiments were done using *E. coli* as a typical facultative anaerobe and *B. megaterium*, randomly chosen as a representative strict aerobe.

The oxygen tension in the flow line and in the fermenter was monitored by incorporating two galvanic oxygen electrodes (WTW Cellox 325, connected to WTW Multi 340i

Table 1
Minimal inhibitory concentrations (MIC) of propolis against bacteria

Propolis type	*B. brevis*	*B. megaterium*	*B. subtilis*	*M. luteus*	*E. coli*	*P. syringae*
WEP	0.500	10.000	10.000	10.000	n.d.	10.000
I1	0.010	0.010	0.060	0.100	0.500	0.060
E1	0.005	0.060	0.040	5.000	n.d.	0.080
C1	0.040	0.040	0.040	0.500	n.d.	0.080
K1	0.005	0.010	0.080	0.060	0.100	0.060
G1	0.010	0.010	0.040	0.040	0.100	0.500
RUS1	0.010	0.010	0.020	0.020	5.000	0.040
P1	0.010	0.010	0.040	0.080	0.100	0.040
P2	0.010	0.010	0.040	0.500	0.100	0.500
P3	0.040	0.010	0.040	0.060	0.100	0.100
P4	0.060	0.010	0.040	0.060	0.100	0.100
SA1	0.005	0.010	0.005	0.020	0.100	0.005
SA3	0.005	0.060	0.005	0.040	5.000	0.060
SA5	0.005	0.005	0.005	0.005	1.000	0.005
SA6	0.005	0.005	0.005	0.005	5.000	n.d.
SA8	0.005	0.005	0.005	0.060	1.000	0.010
SA11	0.005	0.060	0.060	0.080	1.000	0.040

MIC values (% w/v) of EEP from different geographic origins, and of a WEP from Germany, against bacterial species, determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w\v.

Data logger, Wissenschaftlich Technische Werkstätten, Weilheim, Germany) one in the fermenter, and the other in the flow line at the outlet of the calorimeter. The number of CFU was determined by removing 50 μl cultures from the outlet of the calorimeter (inlet of the fermenter) every 30 min. The samples were serially diluted and plated on Standard I nutrient agar, incubated for 24 h at 30 °C, and the number of CFU was counted.

2.4.2. Treatment of bacteria with propolis

Treatment of bacteria with propolis was done in the exponential growth phase by adding corresponding volumes of a 10% EEP stock solution to achieve final propolis concentrations of 0.005, 0.0125, 0.025 or 0.05% (w/v). As the WEP solutions were ineffective at these concentration levels, larger volumes of the stock solution were added to the culture to achieve concentrations of 0.05, 0.125, 0.25 or 0.5% (w/v). The experiments with WEP and PV were done only with G1 due to its sufficient availability. Corresponding volumes of 60% ethanol and distilled water were used as controls.

Table 2
Minimal inhibitory concentrations (MIC) of propolis against fungi

Propolis type	*T. viridae*	*A. niger*	*P. chrysogenum*	*S. cerevisiae*
WEP	n.d.	n.d.	n.d.	5.00
I1	1.00	1.00	1.00	0.10
E1	n.d.	2.50	1.00	0.50
C1	n.d.	0.50	1.50	0.10
K1	0.50	0.50	0.50	0.04
G1	1.50	1.50	1.50	0.10
RUS1	2.00	1.00	1.50	0.50
P1	1.00	1.50	1.00	0.04
P2	1.00	1.00	0.50	0.50
P3	0.50	1.00	0.50	0.04
P4	0.50	1.00	0.50	0.10
SA1	n.d.	1.00	1.00	0.10
SA3	n.d.	1.00	1.00	0.50
SA5	10.00	1.00	1.00	0.06
SA6	n.d.	1.00	1.00	0.08
SA8	2.00	1.00	1.00	0.01
SA11	2.00	1.00	1.50	0.50

MIC values (% w/v) of EEP from different geographic origins, and of a WEP from Germany against various filamentous fungi and a yeast determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w/v.

2.4.3. Determination of calorimetric MIC and MBC values

The minimum concentration of propolis that resulted in a drop of the *p–t* curve, was considered as the MIC value against the corresponding bacteria. The minimal concentration of propolis that killed bacteria and hence caused the heat production rate to decrease to the baseline either immediately or first to a level above the base line and gradually, with incubation time, to the baseline was considered as the minimal bactericidal concentration (MBC).

3. Results

3.1. Differences in the sensitivity of the chosen microorganisms to propolis extracts

Comparison of sensitivity of the different test organisms in view of the MIC values displayed in Tables 1–3 demonstrates that filamentous fungi are generally less sensitive to propolis treatment. The MIC values of the various propolis samples against bacteria lie between 0.005 and 0.5% w/v except for *E. coli* (Table 1), whereas it amounts between 0.5 and 2.5% w/v propolis against filamentous fungi (Table 2).

The yeast showed significantly higher MIC values than most bacteria, but significantly lower ones than the three moulds. Among the bacteria, the Gram negative bacterium *E. coli* was highly resistant to propolis treatment followed by

Table 3
Minimal inhibitory concentrations (MIC) of propolis volatiles (PV)

	E1	C1	G1	P1	P2	SA1	SA3	SA5	I1
B. brevis	0.01	0.10	0.08	0.04	0.04	0.01	0.01	0.01	0.08
B. megaterium	0.10	0.10	0.08	0.04	0.04	0.04	0.10	0.04	0.08
B. subtilis	0.08	0.10	0.08	0.08	0.08	0.01	0.01	0.01	0.08
M. luteus	n.d.	1.00	0.08	0.10	1.00	0.06	0.08	0.02	0.08
E. coli	n.d.	n.d.	0.50	0.50	0.50	0.50	n.d.	5.00	0.50
P. syringae	0.10	0.10	1.00	0.06	1.00	0.01	0.01	0.01	0.50
S. cerevisiae	1.00	0.50	0.50	0.08	1.00	0.50	1.00	0.10	0.50
A. niger	5.00	1.00	5.00	2.50	2.50	5.00	5.00	5.00	2.50
P. chrysogenum	5.00	2.50	5.00	2.50	2.50	5.00	5.00	5.00	2.50
T. viridae	n.d.	n.d	5.00	2.50	2.50	n.d.	n.d	n.d	n.d.

MIC values (% w/v) of propolis from different geographic origins against various bacterial and fungal species determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w/v.

the other Gram negative *P. syringae*. *E. coli* did not show any recognizable response to the 10% treatments with E1, C1, and WEP.

The two moulds *A. niger* and *P. chrysogenum* exhibited similar sensitivities to treatment with all propolis samples. However, *T. viridae* was insensitive to three of the six South African samples (SA1, SA3 and SA5), to the samples from Ethiopia (E1) and Colombia (C1) and the water-extracted propolis (WEP) at a 10% concentration. All filamentous fungi were insensitive to 10% WEP.

The water-extracted propolis (WEP) proved to be significantly less active ($P < 0.05$, *t*-test) than the ethanol-extracted one from the same apiary (G1) as displayed by the higher MIC value against each organism tested. Inferiority of the antimicrobial action of WEP of G1 also holds true when compared to the ethanol-extracted propolis samples obtained from different geographic regions.

The minimal concentrations of propolis needed to inhibit microbial growth were higher in case of the PVs than the EEPs. A two- to ten-fold concentrated PV was needed in order to get a complete inhibition of bacterial growth as would be achieved by the EEP of the same propolis sample (cf. Tables 1 and 2 with Table 3). The filamentous fungi were less sensitive or even insensitive to the volatile components of propolis at lower concentrations, as in the case of the ethanol extracts of propolis. Bacteria that were sensitive to only highly concentrated ethanol extracts of propolis (*M. luteus* to 5% E1 and *E. coli* to 5% SA3) were insensitive even to a 10% PV extract.

3.2. Calorimetric experiments

3.2.1. Calorimetric cultivation of bacteria

The microbial metabolic and growth events taking place in the fermenter during the first few hours of growth were represented by an initial lag phase followed by an exponential rise of the heat production rate and the number of CFU (Fig. 1i). These events continued similarly up to the peak of heat production rate. After the *p–t* peak (0.45 $\mu W\,ml^{-1}$) for *B. megaterium*, the heat production rate dropped steeply whereas the number of CFU increased up to the stationary phase with a cell density of 1.47×10^8 CFU ml^{-1}, about twice as much as that at the *p–t* peak (7×10^7 CFU ml^{-1}) about 2 h before. The heat production rate then remained at a lower level and the number of CFU at a higher level. In case of *E. coli*, however, the nature of the *p–t* curve showed a different pattern. After the *p–t* curve achieved its peak at 0.62 $\mu W\,ml^{-1}$, it descended to a level of about 0.21 $\mu W\,ml^{-1}$, ascended again until it achieved a level at about 0.5 $\mu W\,ml^{-1}$, lower than the first aerobic peak at 0.62 $\mu W\,ml^{-1}$ (Fig. 1ii). It remained at this level for the rest of the experimental period.

The simultaneous monitoring of oxygen tension in the flow line and in the fermenter displayed a big disparity between them at higher cell densities in the middle and late exponential growth phase. At lower cell densities, at the lag phase and early exponential phase of growth, the tension of oxygen in the flow line and in the fermenter were roughly similar, the latter showing a slightly higher value, by about 30 $\mu mol\,l^{-1}$. The beginning of the exponential growth was marked by an increase in the difference of the oxygen tension between that in the fermenter and in the flow line.

3.2.2. Effect of propolis treatment on bacterial culture properties

After treatment with 0.025% SA8, the heat production rate of *B. megaterium* decreased suddenly from 0.39 to 0.15 $\mu W\,ml^{-1}$ (61.5%) and the oxygen tension in the flow line and fermenter rose from 102 to 182 $\mu mol\,l^{-1}$ and from 205 to 230 $\mu mol\,l^{-1}$, respectively. The number of CFU, however, decreased relatively slowly from 9.1×10^7 to 5.5×10^7 CFU ml^{-1} (Fig. 2i). After a period of nearly 2 h both started increasing again. The heat production rate achieved a peak at 0.42 $\mu W\,ml^{-1}$, slightly lower than the peak of a control experiment (0.45 $\mu W\,ml^{-1}$). Correspondingly, the oxygen tensions in the fermenter and in the flow line decreased with different rates, the one in the flow line to a value of 25 $\mu mol\,l^{-1}$. The online oxygen tension, at which the *p–t* curve achieved its peak after treatment with the sublethal propolis dose, was 68 $\mu mol\,l^{-1}$, slightly but not significantly higher than the control experiments, 50–60 $\mu mol\,l^{-1}$. *E. coli* responded similarly to the treatments with sublethal and lethal doses of propolis, as shown in Fig. 3i and ii.

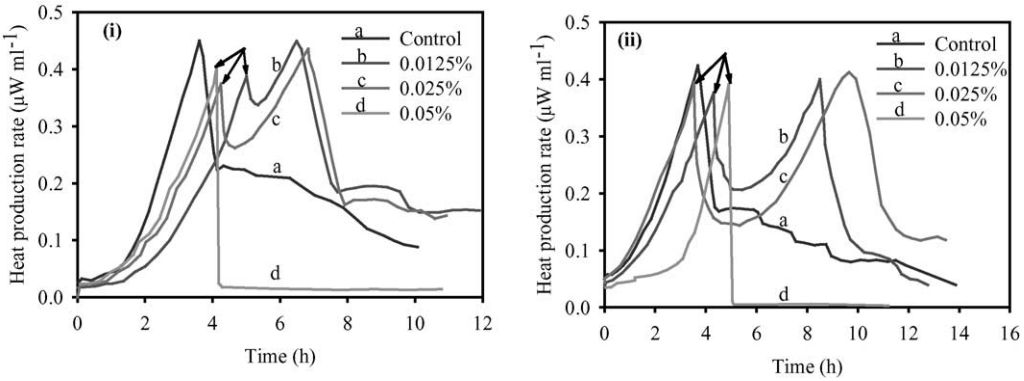

Fig. 4. Effect of different concentrations of EEP from (i) Poland (P3) and (ii) South Africa (SA8) on the structure of the *p–t* curve of *B. megaterium*. Arrows indicate the time of treatment.

3.2.3. Feature of the p–t curve after treatment with different concentrations of propolis

The treatment of an exponentially growing bacterial culture with a concentration of propolis ≥ MIC resulted in a drop of the *p–t* curve to a lower level. Based on the type and concentration of propolis, the curve then either stayed at that level for a certain period of time, and ascended to achieve a second peak or gradually decreased to the baseline, due to bacterial death (Fig. 4i and ii). By measuring the vertical distance between the point on the *p–t* curve, at which treatment was done and the lowest point achieved on the curve due to the treatment of the culture, the dose–response relations were determined, summarized in Fig. 5. No recovery and no second peak occurred in any propolis sample, if the level of the curve was reduced by 80–100%,. The length of time a *p–t* curve needed to revive and come back to the same level as before treatment and to achieve a second peak was positively correlated with the level of the drop of the *p–t* curve (Fig. 6), which is in turn positively correlated with the concentration of propolis for each propolis sample.

3.2.4. Level of the p–t peak achieved after treatment

The peak levels of untreated cultures of *B. megaterium* were very similar to each other with a mean ± SD value of 0.447 ± 0.004 μW ml^{-1}(n = 5). Therefore, comparison of individual values with the mean was considered reliable. The second peaks after treatment lie in the range of 95.5–106.3% of the level of the control peaks with a mean of 99.6%, with no significant difference (Student's t-test, $P > 0.05$). Moreover, no significant differences were observed among the propolis samples or concentrations (2-way ANOVA, α = 0.05) (Table 4).

3.2.4.1. Comparison of the antimicrobial activities of the three propolis extracts. The three different extracts EEP, WEP, PV of the sample G1 were compared using several calorimetric curve parameters to observe if there was any difference in the kinetics of action against *B. megaterium*. The

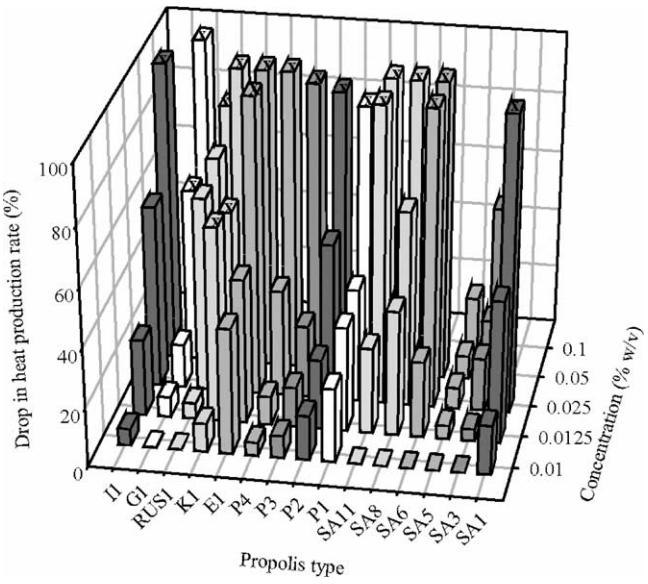

Fig. 5. Effect of EEP treatment on the heat production rate of *B. megaterium* demonstrated by the percentage drop in the level of the *p–t* curve. Control treatments with 60% ethanol and distilled water showed no effect. The "x" at the top of the bars indicates bacterial death and drop of the curves to the baseline suddenly or gradually.

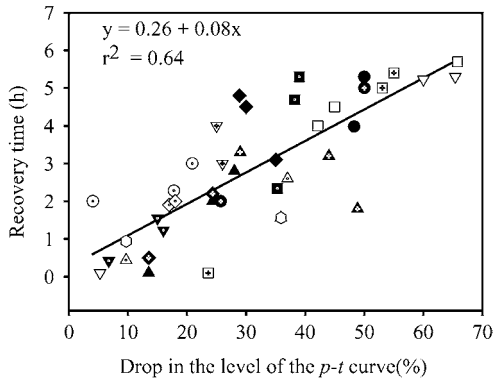

Fig. 6. Relation between pooled percentage drop in the level of the *p–t* curve of *B. megaterium* after treatment with 0.0125, 0.025, and 0.05% w/v EEP from different geographic origins, and the time needed for the curve to recover to the same level as before treatment. Pooled results for the 16 propolis samples investigated.

Table 4
Effect of propolis on the calorimetric power-time curves

Propolis	After treatment with 0.0125% w/v EEP		After treatment with 0.025% w/v EEP	
	l% Slope	l% Peak	% Slope	Peak
E1	33.8	99.5	Declining	None
G1	96.2	100.8	80.4	97.8
K1	138.1(for 0.01%)	96.8(for 0.01%)	None	None
Rus1	100.0	98.2	46.3	98.8
I1	95.5	99.7	97.4	100.3
P1	119.6	101.1	65.6	102.5
P2	187.5	103.2	58.5	102.1
P3	72.3	95.5	66.1	95.8
P4	119.4	95.8	38.9	98.4
SA1	70.4	96.1	74.3	98.2
SA3	97.7	104.0	57.3	106.3
SA5	92.6	106.1	106.3	101.5
SA6	37.7	97.5	Declining	None
SA8	58.5	98.8	58.9	97.7
SA11	47.8	99.6	Declining	None

Effect of the treatment of an exponentially growing culture of *B. megaterium* on the subsequent features of the calorimetric *p–t* curve displayed by (i) the percentage ascend of the slope of the curve after treatment compared to that before treatment, and (ii) the percentage level of the peak after treatment compared to the level of a control peak. Since higher concentrations of K1 caused lethality, the effect of a 0.01% solution on the curve is shown here.

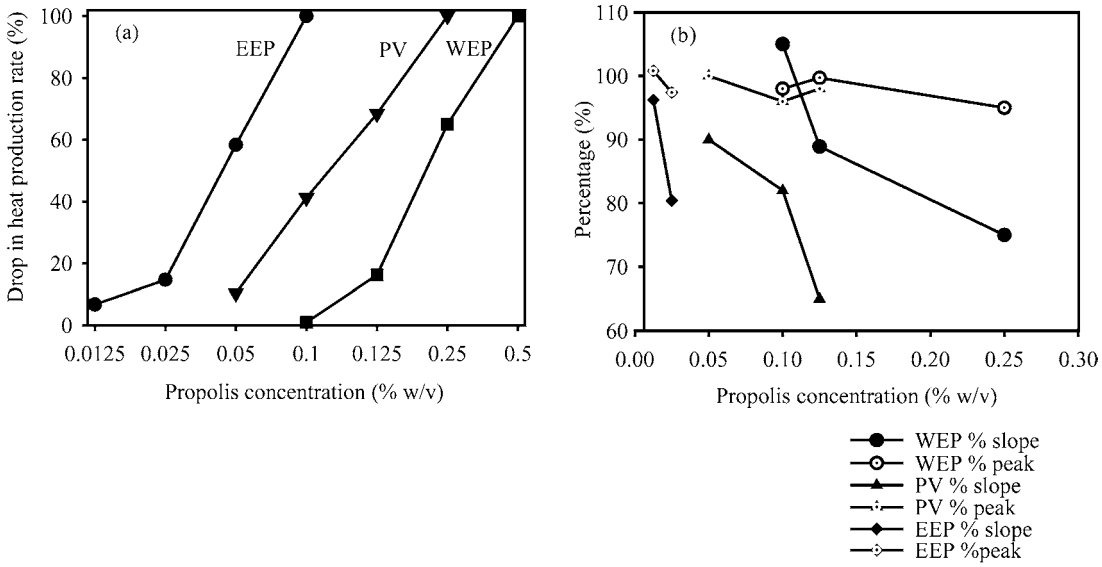

Fig. 7. The effect of treatment of exponentially growing cultures of *B. megaterium* with different concentrations of EEP, of PV, and of WEP on (a) the percentage drop in heat production rate after treatment compared to that before treatment, (b) the percentage ascend of slope of the curve after treatment compared to that before treatment and the percentage level of peak after treatment compared to a control peak.

MIC and MBC values for these extracts were 0.0125 and 0.05 for EEP, 0.05 and 0.25% w/v for PV, and 0.1 and 0.5 for WEP, respectively. EEP required less concentrated solutions to show an inhibitory action and total microbial death than PV, followed by WEP. Apart from these differences, no basic difference in the pattern of kinetics of antimicrobial action could be found. The dose–response curves of concentration versus drop in the level of the *p–t* curves due to treatment with the different extracts showed the same pattern but at different concentrations (Fig. 7a). The resemblance was not only in the level of drop of the curves but also in the slope of ascend of the reviving *p–t* curves, and the level at which the peaks were achieved after treatment (2-way ANOVA, $P > 0.05$, $n = 3$) (Fig. 7b).

4. Discussion

4.1. Petri dish bioassay

The results of antimicrobial tests are unambiguous proofs, that in spite of the great difference in the chemical composition of propolis of different geographical origins and collecting bee races, all of them exhibit significant antibacterial and

antifungal effects. But the strength of antimicrobial activity could differ based on the nature of the specific substances in each sample. Kedzia et al. [34] proposed that the antimicrobial action of propolis is complicated and could be due to the synergism between flavonoids, hydroxyl acids, and sesquiterpenes. It was experimentally demonstrated that not even a single component isolated from propolis showed an activity higher than the total extract [3,6,35]. The synergistic effect between the different components of propolis was already reported by Scheller et al. [18], and latter confirmed in [36]. It is thus obvious that, in different samples, different substance combinations could be essential for the biological activity of propolis, rather than only one, and hence samples of completely different geographic origins may have comparable antimicrobial activities [11].

Regardless of the geographic location, where a plant grows, the purposes for which it secretes resin resemble at least partially. Resin is mainly secreted by plants in order to seal wound, to stop sap loss and protect wounds from infection by microbes, to protect against infection of pollen (it is coated with resin), to stop germination of seeds and sprouting of bud while frost [37]. Though the specific chemicals that are responsible for these actions could differ, the essence of action remains the same, leading to the similar biological activity of different samples.

All of the Gram positive bacteria tested were highly sensitive already to lower concentrations of propolis, but the Gram negative bacterium *E. coli* displayed a reduced sensitivity to most of the samples and was insensitive to 2 of the 16 samples tested. The lower sensitivity of *E. coli* is in agreement with findings by several researchers that this bacterium showed either very low sensitivity or total insensitivity against propolis [8,11,35,38,39]. It cannot, however, be generalized that Gram negative bacteria are insensitive to propolis since the other Gram negative, *P. syringae*, even though it showed relatively higher MIC values, had a sensitivity similar to most Gram positive bacteria at higher concentrations of propolis. The most plausible explanation for the less sensitivity of Gram negative bacteria is their outer membrane that inhibits and/or retards the penetration of propolis at lower concentrations. Another possible reason why the Gram negative bacteria are more resistant to propolis might be the possession of multidrug resistance pumps (MDRs), which extrude amphipathic toxins across the outer membrane [40]. The presence of MDRs in *E. coli* and their role in the insensitivity of the bacterium to antimicrobials was clearly elucidated [41–44].

Fungi are generally less sensitive than bacteria in terms of the MIC values and/or diameter of inhibition zones at higher concentrations, except for the yeast that showed higher diameters like that of the bacteria. Considering the MIC values, the yeast had a sensitivity in between the highly sensitive bacteria and the less sensitive mould.

The presence of propolis at a concentration lower than a critical inhibitory level could enhance the growth of an organism that otherwise would have been inhibited/killed by higher concentrations, a phenomenon known as hormesis [45]. This situation alerts that if propolis is to be used in treatment of infections it has to be used at concentrations far above the MIC values, in order to minimize the risk of encouraging bacterial growth at or in the immediate surrounding of the site of application.

EEP showed the highest antimicrobial activity compared to the other two extracts, even though the differences between EEP and PV were not significant for most samples. The reason why EEP is superior to WEP, and in some case to PV is that extraction of EEP procures all water and ethanol extractable and biologically active components that are also present in the other two extracts. In addition, EEP contains several bioactive components that are not found in WEP and PV.

4.2. Calorimetric experiments

The results of the Petri dish bioassay experiments were affected by the insolubility of propolis in the agar layer, especially while using highly concentrated propolis against the relatively insensitive fungi. The insolubility problem was not serious at lower propolis concentrations tested against bacteria since the highly diluted hydro-insoluble components could diffuse through the agar layer with the excess solvent in which they are dissolved (ethanol). The calorimetric results, however, were not and cannot be affected by this problem since they are done in nutrient broth. Due to vigorous stirring of the culture, the water insoluble components of propolis remain suspended in the medium and show their antibacterial activities.

Because of the unavoidable length of the tubing system between the fermenter and the calorimetric spiral and the metabolic decrease of oxygen tension in the line, calorimetric recording of the heat production rate is a true picture of events taking place in the fermenter only at lower cell densities before the *p–t* peak. If calorimetric data are to be used at higher cell densities, the results have to be compared with other data, such as the oxygen tension in the flow line and the number of CFU, and results have to be interpreted with caution.

The treatment of bacteria with propolis in the calorimetric exponential phase resulted in a decrease of the heat production rate to a lower level, depending on the concentration of propolis. If the concentration is weak, propolis does not kill all bacteria, and hence the survivors do continue to metabolize, maintaining the heat production rate at a certain level above the baseline. This level is kept for some time directly depending on the concentration of propolis, after which the curve revives and ascends again. The most plausible explanation for this behaviour could be that after treatment a certain proportion of the cells are killed, others are inhibited, and some others remain unaffected. The metabolic heat production rate in this case could originate from both, the inhibited cells performing maintenance metabolism, and from unaffected and thus normally metabolizing and growing cells. If the number of the latter is very small and their heat production

rate below the detection limit of the calorimeter, no growth is observed. But with the course of time, the number increases and the change in their metabolic rate could be detected on the *p–t* curve. But it might also be that the inhibition is only temporary and comes to end, allowing the bacteria to start growing. The second hypothesis agrees with the proposal in [46] that bacteriostatic effects achieved by lower concentration of biocides might correspond to a reversible activity on the cytoplasmic membrane and/or impairment of enzyme activity.

The treatment of bacteria with EEP forced the heat production rate to decrease and raised the online and fermenter oxygen tension immediately, while the drop in the number of growing cells (CFU) was gradual. Immediately after treatment, the metabolic rate and thus oxygen consumption presumably drop drastically even though the organisms were not dead, but only weakened. Removal of a sample from the suspension and culturing it on propolis free medium releases the bacteria from the antimicrobial agent and allow them to grow. But with progressive incubation in the fermenter the number of dying bacteria increases and hence the CFU curve declines. The present results are in agreement with those of Sforcin et al. [39].

Since the *p–t* peaks after treatment with weak propolis concentrations occurred at the same levels of heat production rate and of oxygen tension in the flow line, as for the control culture, it can be ascertained that the treatment with propolis did not affect the level of oxygen sensitivity of the survivor bacteria. The lower slope of ascend of the *p–t* curve after the treatment with a higher concentration of propolis indicates a partial inhibition of cell division of the survivors growing and metabolizing at slower rates. An effect of propolis on cell division has already been confirmed [47].

The calorimetric method displayed MIC values lower than the Petri dish bioassay technique. The main reason for this could be that the calorimetric method is an online recording of activity and hence even the slightest and short lasting effects could be detected. But the Petri dish bioassay results are cumulative effects of incubation for 24–48 h. They are influenced by inoculum size, agar layer consistency, incubation temperature, polarity and diffusion potential of the test substance that don't influence the calorimetric output. At a first glance, it seems that the inoculum size affects the calorimetric results too, but since treatment can be done at a level of the exponential phase of the *p–t* curve, which corresponds to an exactly predetermined density of CFU, this plays no role.

The phenomena of change in the structure of the curves after treatment at the calorimetric death phase could be attributed to the shortage of oxygen in the flow line that resulted in a decrease of the *p–t* curve to the minimal point after the peak. The treatments with propolis inhibit/kill some cells in the fermenter and flow line, increasing the availability of oxygen for the survivors, which results in an increased metabolism and ascend of the curve.

The relatively lower sensitivity of Gram negative bacteria compared to the more sensitive Gram positive ones, at least at lower concentrations, could be traced to an activity that involves the Gram positive bacterial cell wall, inhibition of cell wall synthesis and hence distortion of its integrity. Electron microscopic pictures displayed that propolis treated cells possessed defective cell walls and failed to separate after cell division and formed a pseudo-multicellular structure [47]. An experiment with a known antibiotic [48] demonstrated that the formation of a pseudo-multicellular structure after treatment could be due to the blockage of the so-called splitting system of the cross wall.

Antifungal activities of propolis are supposed to be like that of amphothericin B, which forms complexes with sterols (ergestrol) of the fungal membrane [13,49].

It can be concluded that the present results prove that in spite of differences in the chemical composition of propolis from varying geographic locations, all samples exhibited significant antibacterial and antifungal activities. Hitherto investigations of propolis did not point out one individual substance or a particular substance class which could be entirely responsible for this action. Obviously a synergistic action is essential in all samples for the biological activity of bee glue. It seems that the chemical nature of propolis is beneficial not only to bees but have general pharmacological values as an antimicrobial natural product.

References

[1] P. Cheng, G. Wong, Bee World 77 (1996) 8–15.
[2] E. Crane, Bees and beekeeping: Science, Practice and World Resources, Cornstock, Ithaca, NY, USA, 1990.
[3] J. Metzner, E.M. Schneidewind, E. Friedrich, Pharmazie 32 (1977) 730.
[4] J.M. Grange, R.W. Davey, J. Roy. Soc. Med. 83 (1990) 159–160.
[5] K. Ikeno, T. Ikeno, T. Miyazawa, Caries Res. 25 (1991) 347–351.
[6] A. Kujumgiev, V. Bankova, S. Ignatova, Pharmazie 48 (1993) 785–786.
[7] H. Aga, T. Shibuya, T. Sugimoto, M. Kurimoto, S.H. Nakajima, Biosc. Biotech. Biochem. 58 (1994) 945–946.
[8] M. Digrak, O. Yilmaz, S. Ozscelik, Turk. J. Biol. 19 (1995) 249–257.
[9] A. Garedew, I. Lamprecht, Thermochim. Acta 290 (1997) 155–166.
[10] H. Menezes, M. Bacci Jr., S.D. Oliveira, F.C. Pagnocca, Apidologie 28 (1997) 71–76.
[11] A. Kujumgiev, I. Tsvetkova, Yu. Serkedjieva, V. Bankova, R. Christov, S. Popov, J. Ethnopharmcol. 64 (3) (1999) 235–240.
[12] F.A. Santos, E.M.A. Bastos, M. Uzeda, M.A.R. Carvalho, L.M. Farias, E.S.A. Moreira, F.C. Braga, J. Ethnopharm. 80 (2002) 1–7.
[13] S. Pepeljnjak, I. Jalsenjak, D. Maysinger, Pharmazie 37 (1982) 439–440.
[14] J.W. Dobrowolski, S.B. Vohora, K. Sharma, S.A. Shah, S.A.H. Naqvi, P.C. Dandiya, J. Ethnopharm. 35 (1991) 77–82.
[15] C. Ota, C. Unterkircher, V. Fantinato, M.T. Shimizu, Mycoses 44 (2001) 375–378.
[16] A.C.H.F. Sawaya, A.M. Palma, F.M. Caetano, M.C. Marcucci, I.B. da Silva Cunha, C.E.P. Araujo, M.T. Shimizu, Let. Appl. Microbiol. 35 (2002) 203–207.
[17] J. Starzyk, S. Scheller, J. Szaarski, M. Moskwa, A. Stojko, Arzneim-Forsch. Drug Res. 27 (1977) 1198–1199.
[18] S. Scheller, J. Szaflarski, J. Tustanowski, E. Nolewajka, A. Stojko, Arzneim-Forsch. Drug Res. 27 (1977) 889–890.
[19] S.L. De Castro, K.O. Higashi, J. Ethnopharm. 46 (1995) 55–58.
[20] B. König, J.H. Dustmann, Naturwissensch. Rundschau 41 (1988) 43–53.

[21] M. Amoros, F. Sauvager, L. Girre, M. Cormier, Apidologie 23 (1992) 231–240.
[22] R. Krell, FAO Agricultural Services Bulletin No. 124, Food and Agriculture Organization of the United Nations, Rome, 1996.
[23] D. Mc Devitt, D.J. Payne, D.J. Holmes, M. Rosenberg, J. App. Microbiol. 92 (2002) 28S–34S.
[24] C.B. Walker, Periodontology 2000 10 (1996) 79–88.
[25] A.D. Russell, J. Appl. Microbiol. 92 (2002) 1S–3S.
[26] A.D. Russell, I. Chopra, Understanding Antibacterial Action and Resistance, 2nd ed., Ellis Horwood, Chichester, 1996.
[27] H. Nikaido, Curr. Opin. Microbiol. 1 (1998) 516–523.
[28] S.P. Denyer, G.S.A.B. Stewart, Internl. Biodeterior. Biodegrad. 41 (1998) 261–268.
[29] J.L. Rios, M.C. Recio, A. Villar, J. Ethnopharm. 23 (1988) 127–140.
[30] I. Lamprecht, Boll. Soc. Natur. Napoli. 92 (1983) 515–542.
[31] A. Garedew, I. Lamprecht, E. Schmolz, B. Schricker, Apidologie 33 (2002) 41–50.
[32] National Committee for Clinical Laboratory Standards, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A NCCLS, Villanova, PA, 1985.
[33] A.-T. Chen, I. Wadsö, J. Biochem. Biophys. Meth. 6 (1982) 297–306.
[34] B. Kedzia, B. Grppert, J. Iwaszkiewicz, Phytothérapie 6 (1990) 7–10.
[35] S.J. Bonvehi, V.F. Coll, E.R. Jorda, J. Am. Oil Chem. Soc. 71 (1994) 529–532.
[36] W. Krol, S. Scheller, J. Shenai, G. Pietsz, Z. Czuba, Arzneim-Forsch. Drug Res. 43 (1993) 607–609.
[37] W. Ogren, Am. Bee J. 130 (1990) 239–240.
[38] M.C. Marcucci, Apidologie 26 (1995) 83–99.
[39] J.M. Sforcin, A.C. Fernandes Jr., A.M. Lopes, V. Bankova, S.R.C. Funari, J. Ethnopharm. 73 (2000) 243–249.
[40] G. Tegos, F.R. Stermitz, O. Lomovskaya, K. Lewis, Antimicrob. Agents Chemother. 46 (2002) 3133–3141.
[41] O. Lomovskaya, K. Lewis, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 8938–8942.
[42] H. Nikaido, J. Bacteriol. 181 (1999) 4–8.
[43] K. Lewis, O. Lomovskaya, in: K. Lewis, A. Salyers, H. Taber, R. Wax (Eds.), Bacterial resistance to antimicrobials: mechanisms, genetics, medical practice and public health, Marcel Dekker, New York, 2001, pp. 61–90.
[44] H.I. Zgurskaya, H. Nikaido, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 7190–7195.
[45] J.E. Calabrese, L.A. Baldwin, Environ. Health Perspect. 106 (Suppl. 1) (1998).
[46] J.Y. Maillar, J. Appl. Microbiol. 92 (2002) 16S–27S.
[47] N.B. Takaisi-Kikuni, H. Schilcher, Planta Med. 60 (1994) 222–227.
[48] T. Nishino, J. Wecke, D. Kruger, P. Giesbrecht, J. Antimicrob. Chemother. 19 (1987) 147–159.
[49] J. Cizmarik, J. Trupl, Pharmazie 30 (1975) 406–407.