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Antimicrobial activity of propolis special extract GH 2002 against multidrug-resistant clinical isolates

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The need to discover and develop alternative therapies to treat multidrug-resistant bacterial infections is timely. The aim of this study was to examine the antimicrobial potential of propolis, as a purified and concentrated special extract GH 2002, against clinical isolates of *Streptococcus pyogenes*, methicillin-resistent *Stapylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and *Candida*. Minimal inhibitory concentrations (MICs) and minimal microbicidial concentrations (MMCs) of propolis against microbial pathogens were evaluated using the broth microdilution method. Propolis extract GH 2002 revealed low MICs in the range of 0.03 to 2 mg/ml against *S. pyogenes, S. aureus, E. faecium* and *Candida*. A high bactericidal activity of propolis extract in the range of 0.06 to 1.0 mg/ml was determined for *S. pyogenes* and *S. aureus*, however propolis was not bactericidal against *E. faecium*. Propolis concentrations between 0.6 and 2.4 mg/ml displayed fungicidal activity against different *Candida* species. Whereas all tested MRSA strains were highly susceptible against propolis, only minor activity was found against VRE strains. Time-kill curves demonstrated a high antimicrobial activity at low MICs already after few hours of incubation against reference strains, clinical antibiotic-susceptible strains, clinical antifungal susceptible strains as well as all tested clinical MRSA strains, but not against VRE strains. In conclusion, clinical drug-sensitive as well as some clinical multidrug-resistant microbial isolates, i.e. MRSA, were susceptible to propolis with different degrees of susceptibility. These results suggest that the special propolis extract GH2002 might be used in the development of alternative products for therapy of microbial infections.

1. Introduction

Propolis is manufactured by bees while adding their saliva to the resinous plant exudate, subsequently the partially digested material is additionally mixed with beewax (Burdock 1998; Banskota et al. 2001). Bees use propolis (bee glue) as a building material and also to keep pathogenic bacteria and fungi out of the hive. Thus, action against microorganisms is an essential characteristic of propolis. Raw propolis undergoes secondary processing to remove resins and other impurities before being used in a variety of pharmaceutical and natural health care products. Propolis mainly consists of resins (40–55%), beewaxes and fatty acids (20–35%), essential oils (about 10%), pollen (about 5%) and some other components such as minerals, vitamins, and sugar. The chemical components of propolis are qualitatively and quantitatively variable, depending on the geographic origin and regional plant ecology. It has a long history in folk medicine (Ghisalberti 1979; Banskota et al. 2001). Since propolis possesses a broad spectrum of biological activities, there is a renewed interest in its antimicrobial potential.

Pharmacological properties of different propolis preparations have been reported as antihepatotoxic (Gonzales et al. 1995), anti-inflammatory (Borrelli et al. 2002), antimicrobial, (Kujumgiev et al. 1999; Scheller et al. 1999; Marcucci et al. 2001; Abd El Hadi and Hegazi 2002; Kartal et al. 2003; Boyanova et al. 2005; Papova et al. 2005; Reichling et al. 2009). A pharmacological activity against several viral infections has been demonstrated, e.g. influenza (Serkedjieva et al. 1992), HIV (Ito et al. 2001), and herpes simplex virus (Huleihel and Isanu 2002; Schnitzler et al. 2010; Nolkemper et al. 2010). Therapeutic benefits have been reported for propolis extracts against respiratory tract infections in children (Cohen et al. 2004). In addition propolis was shown to immunomodulate Toll-like receptors 2 and 4 expression and pro-inflammatory cytokines production in mice (Orsatti et al. 2010). Antiviral activity of natural products, e.g, essential oils or plant extracts have demonstrated high antiviral activity against herpetic infections (Schnitzler et al. 2007; Astani et al. 2010; 2011; Schnitzler and Reichling 2011; Astani et al. 2012). The number of methicillin-resistant *Staphylococcus aureus* (MRSA) infections and other multidrug-resistant infections is still increasing and treatment with antibiotics is problematic (Shelburne et al. 2004; Traczewski et al. 2009).

In the current study we have investigated the antimicrobial activity of the special ethanolic propolis extract GH 2002 against

Table 1: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of special propolis extract against bacterial reference strains and clinical bacterial isolates

Microorganism	Number	Source	Antibiotic treatment	Propolis	
				MIC (mg/ml)	MBC (mg/ml)
S. pyogenes	ATCC 12344	susceptible	0.03	0.13	
S. pyogenes	AL 106013	abscess	susceptible	0.03	0.06
S. pyogenes	BL 103909	wound	susceptible	0.06	0.13
S. pyogenes	BL 103935	wound	susceptible	0.06	0.13
S. pyogenes	BL 103943	leg	susceptible	0.06	0.25
S. pyogenes	BL 207198	face	susceptible	0.06	0.25
S. pyogenes	BL 207341	catheter	susceptible	0.06	0.13
S. *pyogenes*	BL 207382	ear	susceptible	0.06	0.13
S. pyogenes	BL 207497	urethra	susceptible	0.06	0.13
S. pyogenes	BL 207758	abscess	susceptible	0.03	0.06
S. pyogenes	BL 207760	wound	susceptible	0.03	0.13
S. pyogenes	KL 211239	throat	susceptible	0.06	0.13
S. aureus	ATCC 25923	susceptible	0.25	0.5	
S. aureus	ATCC 10442	MRSA	0.13	1	
S. aureus	MR 105709	throat	MRSA	0.5	1
S. aureus	MR 105996	perianal	MRSA	0.25	0.5
S. aureus	MR 106091	nose	MRSA	0.25	1
S. aureus	MR 106188	hip	MRSA	0.25	1
S. aureus	MR 106642	nose	MRSA	0.25	1
S. aureus	MR 106804	nose	MRSA	0.25	0.5
S. aureus	MR 112793	nose	MRSA	0.25	1
S. aureus	MR 209340	nose	MRSA	0.13	1
S. aureus	MR 209442	nose	MRSA	0.13	1
S. aureus	MR 209481	nose	MRSA	0.25	0.5
E. faecium	ATCC 29212	susceptible	1	> 4	
E. faecium	ATCC 51299	VRE	2	> 4	
E. faecium	KL 211353	wound	VRE	0.5	> 4
E. faecium	VR 102056	rectum	VRE	0.5	> 4
E. faecium	VR 102072	rectum	VRE	0.5	> 4
E. faecium	VR 102105	rectum	VRE	0.13	> 4
E. faecium	VR 102172	rectum	VRE	0.13	> 4
E. faecium	VR 102201	rectum	VRE	0.5	> 4
E. faecium	VR 205835	swab	VRE	1	> 4
E. faecium	VR 206060	swab	VRE	0.5	> 4
E. faecium	VR 206092	rectum	VRE	0.5	> 4

S. pyogenes: *Streptococcus pyogenes*; *S. aureus*: *Staphylococcus aureus*; *E. faecium*: *Enterococcus faecium*; MRSA: methicillin resistant *Stapylococcus aureus*; VRE: vancomycin resistant *Enterococcus faecium*

drug-sensitive and drug-resistant clinical isolates of microbial pathogens. This extract was prepared from propolis, well characterized in respect to its botanical and geographical origin as well as chemical composition. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) were determined and time-kill curves were assessed for propolis against different microorganisms.

2. Investigations and results

To study the potential application of propolis extract against *S. pyogenes,* multidrug-resistant bacteria, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE), MICs and MBCs for propolis were determined. MICs and MBCs of propolis tested against clinical isolates of *S. pyogenes*, *S. aureus* (MRSA), and Vancomycin-resistant *E. faecium* (VRE) are shown in Table 1. *Streptococcus pyogenes* was the most sensitive strain to propolis (MICs 0.03−0.06 mg/ml), followed by antibiotic-sensitive as well as methicillin-resistant *S. aureus* (MRSA) at drug concentrations of 0.13 – 0.5 mg/ml. Higher MICs were observed for all *E. faecium* strains, regardless their susceptibility or resistance to antimicrobial drugs. A similar pattern was detected for the MBC values, with low MBCs for *S. pyogenes*, between 0.5 and 1 mg/ml for all *S. aureus* strains, and no bactericidal activity against *E. faecium*. All tested *Candida* strains, either reference strains or clinical isolates, were susceptible to propolis as well (Table 2). However MICs and MFCs were higher when compared to bacterial isolates.

In a time-kill study, the kinetics of propolis' antimicrobial activity against several *Streptococcus*, *Staphylococcus*, *Enterococcus* and *Candida* strains were investigated in detail. Results are presented as $\log_{10}$ cfu/ml change in the viable colony number. The results obtained are shown in Fig. 1 for *Streptococcus* (reference strain and clinical isolate), in Fig. 2 for *Staphylococcus* (reference strain and three MRSA clinical isolates), and Fig. 3 for *Enterococcus* (reference strain and three VRE clinical isolates). For *Sreptococcus pyogenes*, a rapid initial decrease in the number of viable bacteria was observed within the first 6 h for the clinical isolate KL 211239 at all MIC concentrations tested (Fig. 1). A fourfold MIC concentration of propolis was bactericidal for all tested clinical MRSA strains (Fig. 2). However, for all *E. faecium* strains, either reference strain or VRE clinical isolates, only bacteriostatic but no bactericidal (reduction of 3 x $\log_{10}$ cfu/ml) activity was detectable, even at twofold or fourfold MIC concentrations for 24 h of incuba-

Table 2: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of special propolis extract against fungal reference strains and clinical fungal isolates

Microorganism	Number	Source	Propolis Mic (mg/ml)	MFC (mg/ml)
C. albicans	ATCC 90028	0.6	1.2	
C. glabrata	ATCC 2950	0.6	1.2	
C. albicans	EL 203719	stool	0.6	1.2
C. albicans	KL 105328	sputum	0.3	0.6
C. albicans	KL 105366	tracheal secr.	0.15	1.2
C. albicans	KL 211374	prothesis	0.6	1.2
C. glabrata	AL 210444	ascites	1.2	2.4
C. glabrata	KL 105410	abdomen	1.2	2.4
C. glabrata	KL 105413	perianal	0.3	0.6
C. glabrata	KL 211385	sputum	1.2	2.4
C. krusei	KL 105394	tracheal secr.	0.6	1.2
C. krusei	KL 211374	prothesis	0.6	1.2

C. albicans: *Candida albicans*; *C. glabrata*: *Candida glabrata*; *C. krusei*: *Candida krusei*

tion (Fig. 3). Concerning the yeast *Candida*, time-kill curves of propolis exhibited a strong decrease of cell viability of about 3 x $\log_{10}$ cfu/ml within 6 h for the reference strain and no viable microorganisms for the clinical isolate KL 211374, indicating a fungicidal effect (Fig. 4).

3. Discussion

The antimicrobial activity of the special ethanolic propolis extract GH 2002 was evaluated against clinical isolates of drug-sensitive and drug-resistant microbial pathogens, i.e. *Streptococcus*, *Staphylococcus* (MRSA), *Enterococcus* (VRE) and *Candida*. MICs and MMCs were determined and time-kill curves for propolis were assessed against these microorganisms. A time-dependent and concentration-dependent activity of propolis was demonstrated against these pathogens. Only a marginal activity could be demonstrated for propolis against VRE. However, propolis inhibited MRSA strains and displayed bacteriostatic as well as bactericidal activity against MRSA. The pharmaceutical industry is increasingly targeting natural products with the aim of identifying lead compounds, focusing particularly on suitable alternative antimicrobial agents. Resistance toward antibiotics is increasingly observed in pathogenic microorganisms including Gram positive like MRSA and VRE and presents a tremendous problem on a global scale. These bacteria play an important role in nosocomial infections (Coates et al. 2002; Taubes 2008), a molecular mechanism of drug-reistance in VRE had been described (Schnitzler et al. 2011). To overcome resistance, many antimicrobial agents have been investigated and propolis was included as alternative antimicrobial agent.

According to Kim et al. (2011), Korean propolis revealed similar MICs as GH2002 propolis and was bactericidal against clinical isolates of *Streptococcus mutans*. Low MIC against MRSA might be advantageous in some therapeutic applications such as topical treatment with regard to toxicity and stability of formulations. In a study of Moncla et al. (2012), *Enterococcus faecium* was susceptible to MIC values of 1.6 mg/ml of Brazilian propolis, however VRE strains were not investigated. A clinical study demonstrated that propolis is very active against yeasts from patients with superficial mycoses (Silici and Koc 2006). In a recent report, MIC and MFC against fluconazole-resistant *Candida glabrata* were about five times higher than GH2002 (Shokri et al. 2011). MIC is an endpoint determination which allows only limited insight into the toxicological process during the incubation period in the presence of potentially antimicrobial agents. Besides MICs, reliable information on the microbicidal property of an antimicrobial agent is essential for a successful treatment of infectious diseases. As demonstrated in animal models and with patients, microbicidal results of time-kill assays highly correspond with clinical efficacy (Drake and Sande 1983;

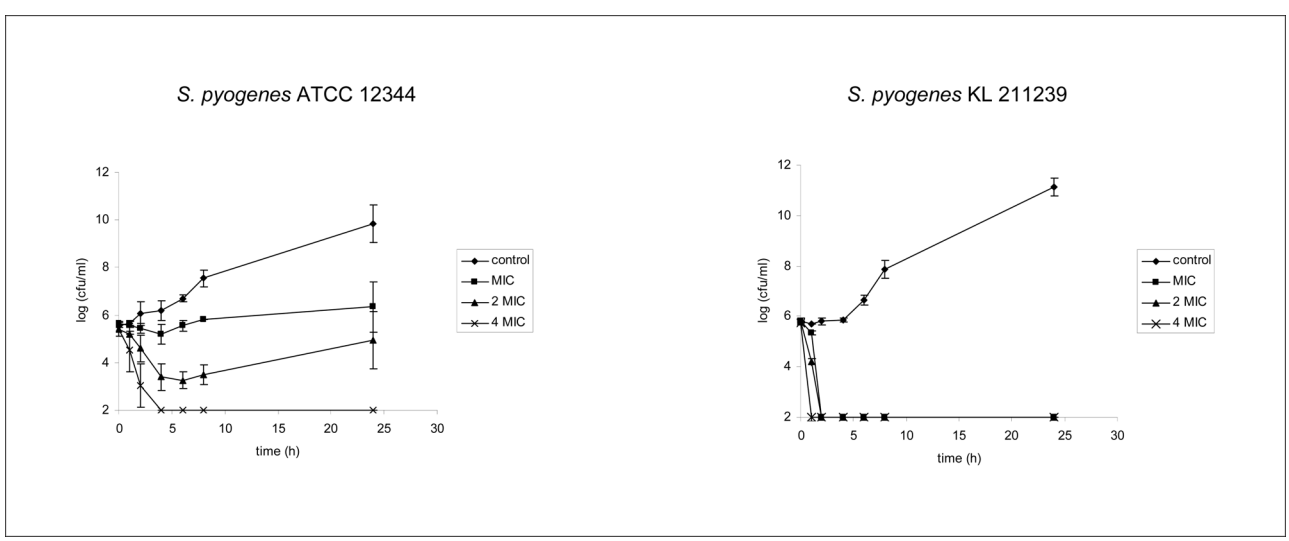

Fig. 1: Time-kill curves of propolis extract GH2002 against *Streptococcus pyogenes* reference strain ATCC 12344 and clinical isolate KL 211239. *S. pyogenes* was incubated with different propolis concentrations, i.e. control (no propolis), MIC, 2 MIC and 4 MIC

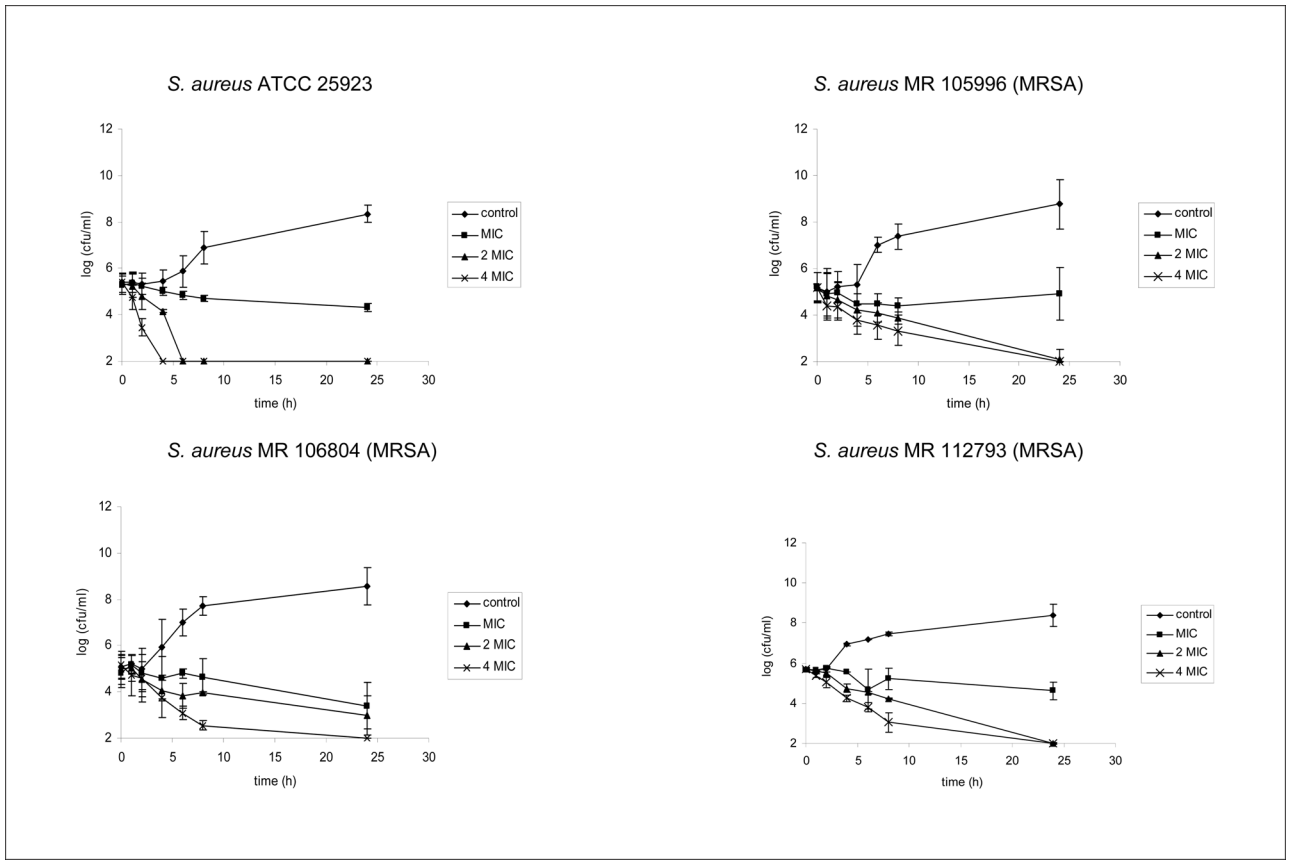

Fig. 2: Time-kill curves of propolis extract GH2002 against *Staphylococcus aureus* reference strain ATCC 25923 (antibiotic-susceptible) and clinical multidrug-resistant isolates MR 105996 (MRSA), MR 106804 (MRSA), and MR 112793 (MRSA). *S .aureus* was incubated with different propolis concentrations, i.e. control (no propolis), MIC, 2 MIC and 4 MIC

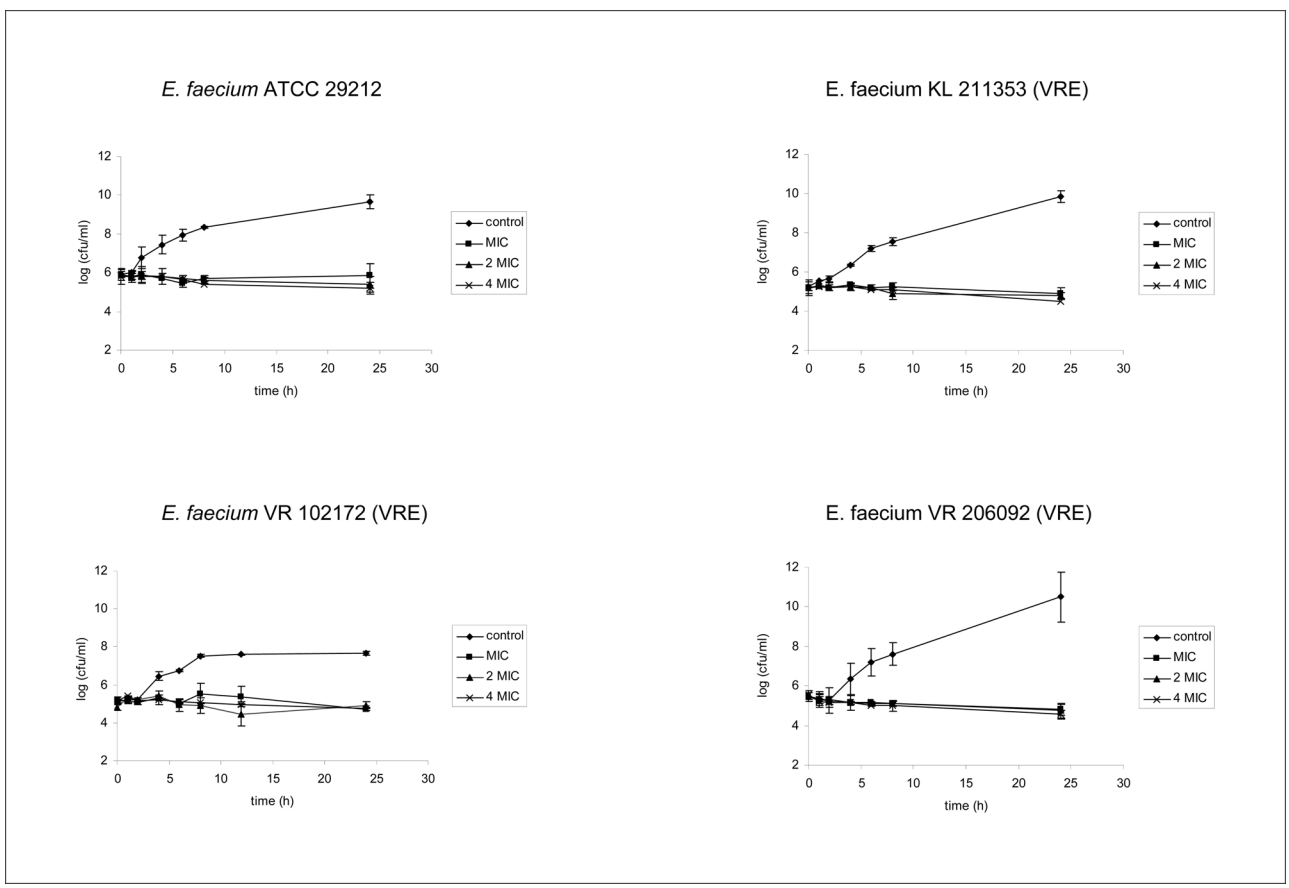

Fig. 3: Time-kill curves of propolis extract GH2002 against *Enterococcus faecium* reference strain ATCC 29212 (antibiotic-susceptible) and clinical multidrug-resistant isolates KL 211353 (VRE), VR 102172 (VRE), and VR 206092 (VRE). *E. faecium* was incubated with different propolis concentrations, i.e. control (no propolis), MIC, 2 MIC and 4 MIC

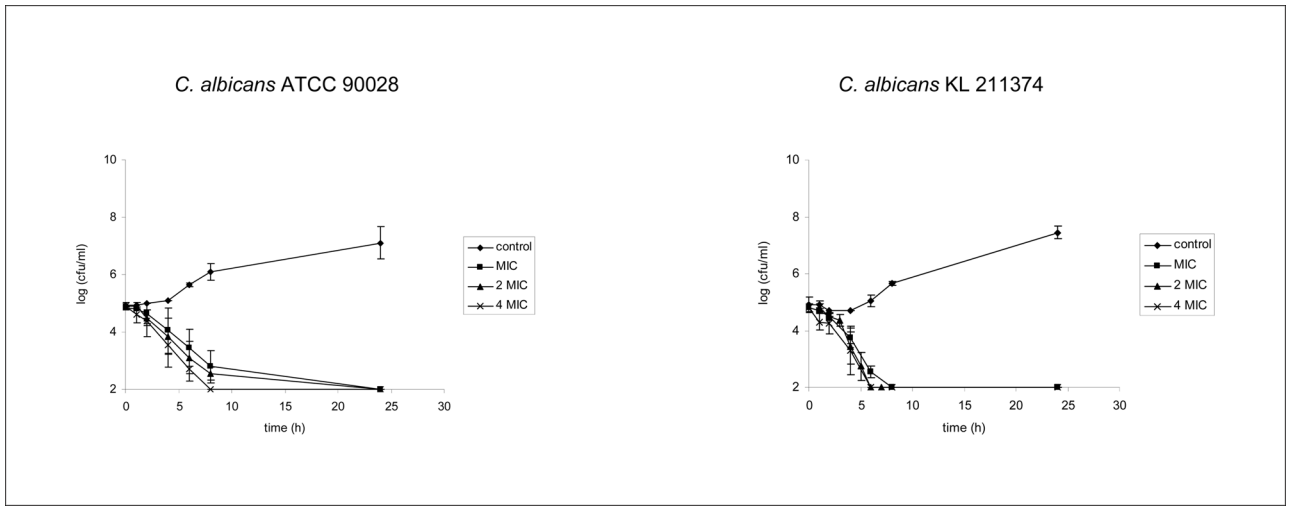

Fig. 4: Time-kill curves of propolis extract GH2002 against *Candida albicans* reference strain ATCC 90028 and clinical isolate KL 211374. *C. albicans* was incubated with different propolis concentrations, i.e. control (no propolis), MIC, 2 MIC and 4 MIC

Chandrasekar et al. 1987). Our results demonstrated a high antimicrobial activity at concentrations of 4 MIC against *Streptococcus*, MRSA and *Candida*. For appropriate treatment of bacterial and fungal infections, the drug concentration should not only reach MIC level, but should exceed MIC fourfold to ensure maximum efficacy (Aiyegoro et al. 2008; Ogunmwonyi et al. 2010).

For propolis from different geographic regions, some of the active antibacterial compounds have been investigated. Kartal et al. (2003) found propolis activity mainly due to caffeic acid and its esters, high biological effect against *S. aureus* was attributed to *p*-coumaric acid (Salomao et al. 2008). Anti-MRSA active compounds in pacific propolis were the prenylflavanones propolin H, G, D and C (Raghukumar et al. 2010), MIC against MRSA of propolin D was 8−16 μg/ml and propolin C with 8−32 μg/ml. Some inhibitory effect of quercetin on the growth of *Escherichia coli in vitro* has been demonstrated recently (Kallio et al. 2012). However, in a study of Drago et al. (2000), propolis showed high antimicrobial activity against *Staphylococcus*, but not against *Enterobacteriaceae*. It seems that propolis has general pharmacological values as a natural mixture, different substance combinations are essential for the biological activity (Kujumgiev et al. 1999). However some authors reported a higher activity of the natural propolis compared to single isolated compounds. According to Santos et al. (2002), none of the assayed fractions was more active than the total propolis extract, suggesting that the antibacterial activity is probably due to the synergistic effect of several compounds. Additive antimicrobial effects of natural compounds as well as synergism of propolis with topical mupirocin against MRSA has been reported (Iten et al. 2009; Onlen et al. 2007). Actichelated propolis, a multicomposite material obtained with mechano-chemical activation, has proven to possess antibacterial and antiviral activity and interfered with bacterial adhesion to human oral cells (Drago et al. 2007). An alternative approach to antibiotics is the development of anti-pathogenic agents to control the bacterial virulome. Such anti-pathogenic agents could target a phenomenon known as quorum sensing. Propolis suppresses quorum sensing and is valuable for further development of therapeutics to disrupt quorum sensing signalling systems which regulate the virulome in many pathogenic bacteria (Bulman et al. 2011).

In conclusion, propolis exerted a marked inhibition against drug-sensitive and multidrug-resistant microorganisms like MRSA. Thus, propolis, alone or in combination with other antibmicrobial agents, might provide a new promising regime in antimicrobial therapy.

4. Experimental

4.1. Microbial strains

Antimicrobial activity of the propolis special extract GH2002 was studied against bacterial and fungal strains. In addition to reference strains, antibiotic-sensitive and antifungal-sensitive clinical isolates as well as multidrug-resistant clinical isolates were studied. *Streptococcus pyogenes* reference strain: ATCC 12344. *Streptococcus pyogenes* clinical isolates: AL 106013, BL 103909, BL 103935, BL 103943, BL 207198, BL 207341, BL 207382, BL 207497, BL 207758, BL 207760, KL 211239. *Staphylococcus aureus* reference strains: ATCC 25923, ATCC 10442 (methicillin-resistant *Stapyhlococcus aureus*, MRSA). *Stapylococcus aureus* clinical isolates: MR 105709 (MRSA), MR 105996 (MRSA), MR 106091 (MRSA), MR 106188 (MRSA), MR 106642 (MRSA), MR 106804 (MRSA), MR 112793 (MRSA), MR 209340 (MRSA), MR 209442 (MRSA), MR 209481 (MRSA). *Enterococcus faecium* reference strains: ATCC 29212, ATCC 51299 (vancomycin resistant *Enterococcus faecium*, VRE). *Enterococcus faecium* clinical isolates: KL 211353 (VRE), VR 192056 (VRE), VR 102072 (VRE), VR 102105 (VRE), VR 102172 (VRE), VR 102201 (VRE), VR 205835 (VRE), VR 206060 (VRE), VR 206092 (VRE). *Candida albicans* reference strain: ATCC 90028, *Candida glabrata* reference strain ATCC 2950. *Candida* clinical isolates: EL 203719 (*C. albicans*), KL 105328 (*C. albicans*), KL 105366 (*C .albicans*), KL 211374 (*C. albicans*), AL 210444 (*C. glabrata*), KL 105410 (*C. glabrata*), KL 105413 (*C. glabrata*), KL 211385 (*C. glabrata*), KL 105394 (*C. krusei*), KL 211374 (*C. krusei*). All microorganisms were stored at −80 °C.

4.2. Cultivation of bacteria and fungi

The culture media for bacteria included Columbia Agar supplemented with 5% sheep blood (Becton Dickinson, Heidelberg, Germany), Mueller-Hinton broth (Sigma-Aldrich, Munich, Germany), and brain heart infusion (Merck, Darmstadt, Germany). *Candida* species were cultivated on CHROMagar *Candida* medium (Becton Dickinson, Heidelberg, Germany) and Sabouraud Dextrose broth (Merck, Darmstadt, Germany). Several bacterial or fungal colonies from an 18 to 24 h over night agar plate culture were suspended in saline to a turbidity matching 0.5 Mc Farland (about 1×10^8 colony forming units/ml for bacteria and about 1×10^6 colony forming units for yeasts). Then 100 μl of the bacterial suspension was diluted 1:100 with broth and fungal suspension was diluted 1:10 with broth.

4.3. Propolis

Propolis, the bee glue of *Apis mellifera*, was collected at Moravia, Czech Republic and has a defined composition, quality and provenance and contains flavonoids and phenylcarboxylic acids. The ethanolic extract GH 2002 was prepared with a special procedure to remove the wax and resin components. Subsequently, propolis was extracted with 90% ethanol resulting in a drug-viscous extract ratio of 2:1; the viscous extract corresponds to about 50% of the primary raw propolis material. For experiments, an aliquot of GH 2002 was dissolved in 90% (v/v) ethanol to obtain a 10% (v/v) stock solution. For culture experiments, ethanolic propolis extract was further diluted resulting in a final ethanol concentration below 1% which is not toxic for microorganisms.

Analytical HPLC of the ethanolic propolis extract GH2002 identified polyphenoles, flavonoids and phenylcarboxylic acids as main components. The ethanolic extract displayed a relatively high flavonoid content including quercetindihydrate, chrysine, pinocembrine and galangine (Schnitzler et al. 2010). In propolis samples from temperated zones, flavonoids (e.g. quercetine, kaempferol, pinocembrine, chrysine, and galangine), phenolic acids and esters of phenolic acids are the main components (Marcucci, 1995; Burdock, 1998; Abd El Hady and Hegazi 2002). Propolis extracts or preparations of high standard must be free of toxic contaminants, display a low content of beewax and insoluble materials, the botanical and geographical origin should be defined and a high content of active compounds (e. g. flavonoids, phenolic acids) should be guaranteed.

4.4. Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)

MICs were determined with the broth microdilution method according to CLSI (Clinical and Laboratories Standards Institute 2006). Each sample was tested three times. Propolis was dissolved in two-fold serial dilution with broth in 96-well plates to obtain a range of different concentrations. The final DMSO concentration in the test system did not exceed 1.0% (v/v). Fifty microliters of microbial suspension were added to give a final concentration of 5 x 10^5 cfu/ml and the plates were incubated at 37 °C for 24 h (bacteria) and at 25 °C for 48 h (yeasts), respectively. MICs were determined as the lowest concentration at which no growth occurred. The MMC was determined by subculturing 3 µl from each well without apparent microbial growth on an appropriate medium and incubation for 24 h (bacteria) or 48 h (yeasts) as described previously (Mulyaningsih et al. 2010; Hamoud et al. 2012). MMC was determined as the lowest concentration that yielded no microbial growth on agar after incubation at 37 °C for 24 h (bacteria) or 25 °C for 48 h (fungi). Each plate included a growth control in 1.0% DMSO and a sterility control.

4.5. Time-kill assay

Propolis extract was prepared in duplicate at several concentrations (MIC, 2 x MIC, 4 x MIC) in the appropriate medium with 1.0% DMSO as solvent. Mixtures were incubated with an overnight culture of the test strain adjusted to give a final concentration of approximately 5 x 10^5 cfu/ml. Medium with 1% DMSO was used as growth control. Aliquots of 50 µl were removed after defined time intervals (0, 1, 2, 4, 6, 8, and 24 h), and diluted serially using sterile saline. Three times 20 µl of each dilution was spread onto agar plates and viable colonies were counted after appropriate incubation in order to calculate the cfu in the test medium at corresponding time points (Iten et al. 2009). An antimicrobial agent is defined as microbicidal if it reduces the number of cfu/ml by $\geq$ 3 log (99.9%).

4.6. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed by SPSS software (SPSS for Windows, 11.0, 2001, SPSS Chicago, Illinois). The means and standard errors were recorded.

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