Journal of Medical Microbiology (2005), **54**, 481–483 DOI 10.1099/jmm.0.45880-0

Short Communication

Activity of Bulgarian propolis against 94 *Helicobacter pylori* strains *in vitro* by agar-well diffusion, agar dilution and disc diffusion methods

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Propolis exhibits antimicrobial, anti-inflammatory and other biological effects. The aim of this study was to evaluate the activity of 30 % ethanolic extract of Bulgarian propolis against 94 *Helicobacter pylori* strains by three methods. By the agar-well diffusion method, only 13·8 % of the strains exhibited no inhibition by 30 µl propolis extract (containing 9 mg propolis) and all isolates were inhibited to some extent by 90 µl of the extract (27 mg propolis) per well. The mean diameters of growth inhibition by 30, 60 or 90 µl propolis extract or 30 µl 96 % ethanol per well were 16·8, 19·2, 27·5 and 8·3 mm, respectively. The propolis extract was more active than the ethanol ($P < 0{\cdot}001$). With 90 µl propolis extract per well, 69·4 % of the strains exhibited large diameters of growth inhibition (⩾20 mm) versus 26·6 % with 30 µl per well ($P < 0{\cdot}001$). With moist propolis discs, inhibition was detected in more strains (92·1 %) than with dried discs (78·2 %, $P < 0{\cdot}05$), with mean inhibitory diameters of 18·7 and 13·8 mm, respectively. By the agar dilution method, 100 and 300 µg propolis ml^{-1} inhibited the growth of 57·1 % and 76·2 %, respectively, of the 21 strains tested. In conclusion, Bulgarian propolis had a strong and dose-dependent activity against most of the *H. pylori* strains tested. Although the effect of propolis on *H. pylori in vitro* is promising, further microbiological, pharmacological and clinical trials are required.

Received 26 August 2004
Accepted 24 January 2005

Introduction

Propolis (bee glue) is a resinous hive product collected by honey bees from living plants. In temperate zones, the main sources of propolis are the buds of poplars (Bankova *et al.*, 2000). It is important to know the plant sources because if no suitable plants are available for the honeybees, toxic substances may be included in the propolis (Bankova *et al.*, 2000). Bee glue is composed of resins (flavonoids and related phenolic acids), wax, essential oils, pollen and organic compounds (Burdock, 1998). Propolis exhibits antimicrobial, antioxidant, anti-inflammatory, anaesthetic and other properties (Bankova *et al.*, 2000). Synergism between propolis and antibacterial agents has been observed (Krol *et al.*, 1993, Stepanovic *et al.*, 2003). The antimicrobial properties of propolis are related to the synergistic effect of its compounds (Santos *et al.*, 2002). The bee glue affects the cytoplasmic membrane and inhibits bacterial motility and enzyme activity (Mirzoeva *et al.*, 1997). Propolis exhibits bacteriostatic activity against different bacterial genera and can be bactericidal in high concentrations (Drago *et al.*, 2000, Mirzoeva *et al.*, 1997). Although allergic reactions following propolis use have been reported, the bee glue is relatively non-toxic according to Burdock (1998).

There are only limited data concerning the activity of bee glue on *Helicobacter pylori* (Banskota *et al.*, 2001). The aim of the present study was to evaluate the activity of 30 % ethanolic extract of Bulgarian propolis against a large number of clinical *H. pylori* isolates *in vitro* by agar-well diffusion, agar dilution and disc diffusion methods.

Methods

A total of 94 *H. pylori* strains, isolated from antral biopsy specimens of patients with gastroduodenal diseases, were included in the study. The specimens were transported in Stuart transport medium (Merck) for less than 5 h. A smear was prepared from one part of each specimen for modified Gram staining, and a part of each specimen was used for a rapid urease test. The remaining part of the specimen was homogenized in 0·1 ml sterile saline and inoculated onto Columbia agar (Becton Dickinson) containing 10 µg vancomycin, 5 µg trimethoprim, 5 µg cefsulodin and 5 µg amphotericin B ml^{-1} and/or 10 % defibrinated sheep blood, and 1 % Isovitalex (Becton Dickinson).

Selective and non-selective media were used for primary culture of the specimens. Plates were incubated microaerophilically (Helico-Campy Pack gas-generating envelopes, National Centre of Infectious and Parasitic Diseases, NCIPD or Campy Pak envelopes, Becton Dickinson) at 35 °C for 3–12 days. *H. pylori* was identified by Gram staining of the colonies, lack of aerobic growth and testing for the presence of urease, oxidase and catalase. Stock cultures were maintained in 15 % glycerol broth at −70 °C. They were subcultured onto blood Mueller–Hinton agar (NCIPD) with 1 % Isovitalex and incubated microaerophilically at 35 °C for 48–72 h.

The activity of 30 % ethanolic extract of Bulgarian propolis (w/v, purchased from Hygitest, Sofia, Bulgaria) was tested against 94 *H. pylori* strains by the agar-well diffusion method. Ethanol (96 %) was used as a control. *H. pylori* inocula (McFarland turbidity standard 2) were prepared in Mueller–Hinton broth (NCIPD) and were plated onto Mueller–Hinton agar with 5 % sheep blood and 1 % Isovitalex in three directions by sterile swabs. Wells (7 mm diameter) were punched in the plates using a sterile stainless steel borer. The wells were filled with 30, 60 or 90 μl propolis extract (containing 9, 18 or 27 mg propolis per well, respectively) or 30 μl 96 % ethanol per well. The plates were incubated microaerophilically at 35 °C for 72 h. The diameters of the inhibitory zones were measured in millimetres.

The activity of 300, 100, 30 and 10 μg propolis ml^{-1} was tested against 21 *H. pylori* strains by an agar dilution method using blood Mueller–Hinton agar with 1 % Isovitalex and *H. pylori* inocula corresponding to McFarland turbidity standard 2 (1 μl per spot). The plates were incubated microaerophilically at 35 °C for 72 h. If *H. pylori* growth appeared on the plate, the isolate was considered to be resistant to the corresponding concentration.

The disc diffusion method, using paper discs containing 5 μl of either 30 % ethanolic extract of Bulgarian propolis (1·5 mg of propolis per disc) or 96 % ethanol, was performed for 87 *H. pylori* strains. Moist propolis discs were prepared immediately before testing and dry propolis discs were prepared in the same way and left to dry for 2–3 days. *H. pylori* colonies were suspended in Mueller–Hinton broth and adjusted to a density equal to McFarland turbidity standard 2. Suspensions were spread onto the plates with sterile cotton swabs and then the discs were added. The plates were incubated microaerophilically at 35 °C for 72 h. The diameters of the inhibitory zones were measured in millimetres.

The agar-well diffusion and disc diffusion methods were performed on fresh *H. pylori* isolates, and the agar dilution method was carried out on stock cultures. Isolates were tested in duplicate and mean values of growth inhibition for each strain were taken into account. Chi-square with Yates' correction was used as a statistical method to determine significance.

Results and Discussion

In the agar-well diffusion test with 30 μl volumes per well, the propolis extract inhibited more strains than the ethanol (86·2 % versus 35·6 %, $P < 0{\cdot}001$, Table 1). With 90 μl volumes per well, the propolis extract inhibited all of the *H. pylori* strains tested, versus 86·2 % with 30 μl per well ($P < 0{\cdot}05$). The effect of propolis extract on *H. pylori* growth was dose-dependent. With 90 μl propolis extract per well, 69·4 % of the *H. pylori* strains exhibited large diameters of growth inhibition (⩾20 mm), versus 26·6 % with 30 μl per well ($P < 0{\cdot}001$).

The effect of propolis against Gram-positive bacteria and yeasts is much greater than that against Gram-negative bacteria (Drago *et al.*, 2000; Stepanovic *et al.*, 2003). However, as only 7·2 % of the *H. pylori* strains exhibited no inhibition by the agar-well diffusion method using 60 μl propolis extract per well, and all the isolates were inhibited by 90 μl of the extract per well, Bulgarian propolis seems to possess a marked antibacterial activity against *H. pylori in vitro*.

Similar results were obtained by the disc diffusion method. More than 60 % of the *H. pylori* strains exhibited considerable growth inhibition (diameter ⩾15 mm) with moist propolis discs. Ethanol exhibited a slight inhibitory effect on *H. pylori*, with inhibitory zone diameters ⩾15 mm in only 23·1 % of isolates. Propolis in dried discs retained antibacterial activity, resulting in a considerable growth inhibition (⩾15 mm) in 46 % and strong inhibition (⩾20 mm) in 27·6 % of the *H. pylori* strains. Moist propolis discs inhibited more strains (92·1 %) than dried propolis discs (78·2 %, $P < 0{\cdot}05$). It is known that the flavonoid levels in aged propolis are 20 % lower than those in fresh propolis and that some labile propolis compounds are highly active (Bonvehi & Coll, 2000; Mirzoeva *et al.*, 1997). However, in the present

Table 1. Activity of 30 % ethanolic extract of propolis and 96 % ethanol against *H. pylori* strains by agar-well diffusion and disc diffusion methods

Parameter	Agar-well diffusion method				Disc diffusion method		
	30 μl EEP*	60 μl EEP*	90 μl EEP*	30 μl Ethanol	5 μl EEP* (moist disc)	5 μl EEP* (dried disc)	5 μl Ethanol
Corresponding propolis concentration (mg)	9	18	27	–	1·5	1·5	–
No. of *H. pylori* strains tested	94	69	36	45	63	87	26
Mean diameter of growth inhibition (mm)	16·8	19·2	27·5	8·3	18·7	13·8	9·0
Growth inhibition diameter range (mm)	7–48	7–56·5	9–60	7–18	6–40	6–32	6–18
Strains with no growth inhibition (%)	13·8	7·2	0	64·4	7·9	21·8	42·3
Strains with growth inhibition diameter ⩾15 mm (%)	48·9	52·2	77·8	6·7	60·3	46·0	23·1
Strains with growth inhibition diameter ⩾20 mm (%)	26·6	33·3	69·4	0	44·4	27·6	0

*EEP, 30 % ethanolic extract of propolis.

study, the effect of dried propolis discs on most *H. pylori* strains, with a mean inhibitory zone diameter of 13·8 mm, strongly suggests the presence of relatively stable antibacterial compounds in the agent.

The effect of Bulgarian propolis on *H. pylori* growth, detected by both the agar-well diffusion method and the disc diffusion technique, was confirmed by the agar dilution method. Even 10 μg propolis ml^{-1} inhibited 14·3 % of the 21 *H. pylori* isolates tested, whereas 30, 100 and 300 μg propolis ml^{-1} inhibited 47·6 %, 57·1 % and 76·2 % of the strains, respectively.

Many factors may influence the antibacterial activity of bee glue (the propolis origin, bee species and extract preparation). Flavonoids (pinocembrin and galangin) and esters of phenolic acids have been associated with the antibacterial activity of European propolis (Grange & Davey, 1990). The chemical composition of bee glue exhibits considerable geographic differences. Propolis from Bulgaria, Turkey, Greece and Algeria usually contains mainly flavonoids and esters of caffeic and ferulic acids (Velikova *et al.*, 2000). According to Hegazi *et al.* (2000), Austrian propolis has exhibited a high activity against *Candida albicans* and German propolis has been very active against *Staphylococcus aureus* and *Escherichia coli*. The effect of Brazilian propolis on *H. pylori* has been associated with lambdane-type diterpenes and some prenylated phenolic compounds (Banskota *et al.*, 2001).

It is interesting that the effect of Bulgarian propolis on *H. pylori* was similar to that of Brazilian propolis fractions against oral anaerobic bacteria (MIC, 64–1024 μg ml^{-1}) (Santos *et al.*, 2002), as well as to the effect of the Bulgarian propolis on Gram-negative anaerobic rods. Sixteen clinical strains within the genera of *Prevotella* (15 strains) and *Porphyromonas* (1 strain) were evaluated by the agar-well diffusion method (30 μl propolis extract per well) and growth inhibition was observed in 87·5 % of the strains, with considerable inhibition (⩾15 mm diameters) in 31·2 % (L. Boyanova, unpublished results).

In conclusion, Bulgarian propolis has a strong and dose-dependent activity against most of the *H. pylori* strains tested. The synergism between propolis and antimicrobial agents, as well as the anti-inflammatory, anaesthetic and tissue-regenerative properties of the bee glue (Bankova *et al.*, 2000), can be additional advantages for evaluating propolis as a possible candidate in the treatment of *H. pylori* infection. Although the effect of propolis on *H. pylori in vitro* is promising, further microbiological, pharmacological and clinical trials are required.

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