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Short Communication

Inhibition of *Helicobacter pylori* growth *in vitro* by Bulgarian propolis: preliminary report

Lyudmila Boyanova,[1] Sirigan Derejian,[2] Radka Koumanova,[3] Nikolai Katsarov,[4] Galina Gergova,[1] Ivan Mitov,[1] Rossen Nikolov[2] and Zacharii Krastev[2]

Correspondence
Lyudmila Boyanova
l.boyanova@lycos.com
lboyanova@hotmail.com

[1]Department of Microbiology, Medical University of Sofia, Sofia, Bulgaria

[2]Department of Gastroenterology, St Ivan Rilski Hospital, Sofia, Bulgaria

[3]Department of Gastroenterology, Pediatric Hospital, Sofia, Bulgaria

[4]Alexander Hospital, Second Surgery Hospital, Sofia, Bulgaria

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Bee glue (propolis) possesses antimicrobial, anti-inflammatory, anaesthetic and immunostimulating activities. The aim of the study was to evaluate the inhibitory effect of Bulgarian propolis on *Helicobacter pylori* growth *in vitro*. Activity of 30 % ethanolic extract of propolis (EEP) against 38 clinical isolates of *H. pylori* was evaluated by using the agar-well diffusion method. Ethanol was used as a control. In addition, the effect of propolis on the growth of 26 *H. pylori* and 18 *Campylobacter* strains was tested by the disc diffusion method. Mean diameters of *H. pylori* growth inhibition by the agar-well diffusion method, using 30, 60 or 90 μl EEP or 30 μl ethanol per well, were 17·8, 21·2, 28·2 and 8·5 mm, respectively. EEP was significantly more active than ethanol against *H. pylori* ($P < 0{\cdot}001$). The results obtained by the disc diffusion method were similar. The use of moist propolis discs resulted in mean diameters of growth inhibition of 21·4 mm for *H. pylori* and 13·6 mm for *Campylobacter* spp. Dried propolis discs exhibited antibacterial effect against 73·1 % of *H. pylori* isolates, with a considerable zone of growth inhibition (⩾ 15 mm) in 36·4 % of isolates. Using dried propolis discs resulted in mean diameters of growth inhibition of 12·4 mm for *H. pylori* and 11·6 mm for *Campylobacter* spp. In conclusion, Bulgarian propolis possesses considerable antibacterial activity against *H. pylori*, and can also inhibit the growth of *Campylobacter jejuni* and *Campylobacter coli*. The potential of propolis in the prevention or treatment of *H. pylori* infection is worth further extensive evaluation.

Introduction

Propolis is a resinous product of bees with anti-inflammatory, antimicrobial, anaesthetic and immunostimulating activities (Koo *et al.*, 2000). There are few data concerning the antibacterial activity of bee glue, mainly Brazilian propolis, on *Helicobacter pylori* (Banskota *et al.*, 2001). However, several studies have reported that propolis has only a limited inhibitory effect against Gram-negative bacilli (Drago *et al.*, 2000), and the chemical composition of bee glue exhibits geographical differences. There are no data about the effect of bee glue on *Campylobacter* spp. The aim of the study was to assess the inhibitory effect of Bulgarian propolis on the growth of 38 *H. pylori* and 18 *Campylobacter* clinical isolates *in vitro* by two methods.

Abbreviations: AWDM, agar-well diffusion method; DDM, disc diffusion method; EEP, ethanolic extract of propolis.

Methods

Thirty-eight *H. pylori* and 18 *Campylobacter* strains (*Campylobacter jejuni*, $n = 10$; *Campylobacter coli*, $n = 8$) were included in the study. Stock cultures were maintained in 15 % glycerol broth at −70 °C. They were subcultured onto Mueller–Hinton agar with 5 % sheep blood and 1 % IsoVitaleX (BBL), and incubated microaerobically at 35 °C for 48–72 h. Inocula, corresponding to a value of 2 on the McFarland optical density scale, were prepared in Mueller–Hinton broth and were plated onto Mueller–Hinton agar plates in three directions by sterile swabs. The plates were left to dry for 15 min.

Activity of ethanolic extract of Bulgarian propolis (30 % EEP, w/v, Hygitest) was tested against 38 *H. pylori* strains by an agar-well diffusion method (AWDM). Ethanol (96 %) was used as a control. Wells, 7 mm in diameter, were punched in each agar plate using a sterile stainless steel borer. Each well was filled with 30, 60 or 90 μl 30 % EEP or 30 μl 96 % ethanol. In addition, a disc diffusion method (DDM), using paper discs (6 mm in diameter) containing either 5 μl 30 % EEP or 5 μl 96 % ethanol, was performed for 26 *H. pylori* and 18 *Campylobacter* strains. Two kinds of disc were used: 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.

The plates, tested by both methods, were incubated microaerobically (Helico-Campy Pack gas-generating envelopes, NCIPD, Bulgaria) at 35 °C for 72 h. The diameters of inhibitory zones were measured in mm. All isolates were tested in duplicate and mean values of growth inhibition for each strain were taken into account. The χ^2 test with Yates' correction was used as a statistical method to determine significance.

Results

Mean diameters of *H. pylori* growth inhibition by Bulgarian propolis are presented in Table 1. At a volume of 30 µl per well, 30 % EEP exhibited greater activity against *H. pylori* than did ethanol (mean diameters of growth inhibition: 17·8 vs 8·5 mm, $P < 0{\cdot}01$). At volumes of 90 µl EEP per well, around 90 % of *H. pylori* strains exhibited large diameters of growth inhibition (⩾ 15 mm, as shown in Fig. 1), vs 52·6 % by 30 µl EEP per well ($P < 0{\cdot}01$). The results obtained by DDM were similar. The activity of moist propolis discs against *H. pylori* was slightly greater than that against *Campylobacter* isolates (mean inhibitory diameters: 21·4 vs 13·6 mm). Seventeen of 26 *H. pylori* isolates (65·4%) showed considerable growth inhibition (⩾ 15 mm), vs 33 % (6/18) of *Campylobacter* strains ($P>0{\cdot}05$). Ethanol exhibited a slight inhibitory effect on *H. pylori*, with inhibitory zone diameters of at least 15 mm in only 23·1 % of isolates. Although less active than the moist EEP discs, propolis in dried discs retained a residual antibacterial activity, inducing considerable growth inhibition (⩾ 15 mm) in 34·6 % (9/26) of *H. pylori* strains and in 11·1 % (2/18) of *Campylobacter* isolates.

Discussion

In different propolis samples, various substance combinations are responsible for the antibacterial activity of the bee glue. In Bulgaria and several Mediterranean countries, propolis contains mainly flavonoids and esters of caffeic and ferulic acids (Velikova *et al.*, 2000). In propolis samples from the temperate zone, flavonoids and esters of phenolic acids are known to be associated with antibacterial activity (Kujumgiev *et al.*, 1999). Although the inhibitory effect of propolis on Gram-positive bacteria has been demonstrated, the activity of bee glue against Gram-negative bacteria is a matter of controversy (Drago *et al.*, 2000); for example, propolis has shown good activity against *Haemophilus influenzae* and *Moraxella catarrhalis*, but not against *Enterobacteriaceae*. The anti-*H. pylori* activity of Brazilian propolis has recently been reported, labdane-type diterpenes and some prenylated phenolic compounds being the main antibacterial substances (Banskota *et al.*, 2001).

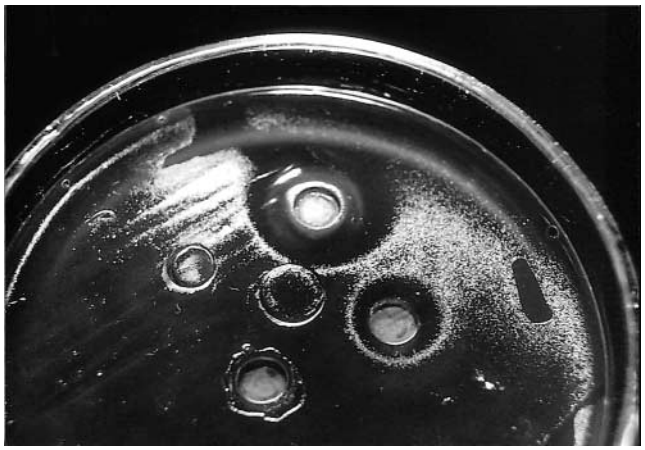

Fig. 1. Inhibitory effect of ethanolic extract of Bulgarian propolis (30 %) on *H. pylori* growth by AWDM. Note the large inhibitory zone around the well containing 90 µl propolis extract.

In the present study, the agar-well diffusion and the disc diffusion methods were used because they have the advantage of showing both inhibition and control growth (outside the inhibitory zone) of fastidious organisms on the same plate. Bulgarian propolis has considerable antibacterial activity against *H. pylori in vitro*: only 21 % (8/38) of the strains exhibited no inhibitory zone by AWDM using 30 µl EEP per well, and all isolates were inhibited to some extent by 90 µl EEP per well. Similar results were obtained by DDM. Only 3·8 % of *H. pylori* strains were not inhibited by moist EEP discs. Mirzoeva *et al.* (1997) have reported the species-dependent antibacterial effect of propolis, with some active, but labile, ingredients showing the highest activity. In the present study, the slight activity of dried propolis discs on *H. pylori* and *Campylobacter* strains, with mean inhibitory zone diameters of 12·4 and 11·6 mm, respectively, also suggests the presence of relatively stable antibacterial compounds.

In conclusion, the eradication of *H. pylori* infection is sometimes difficult because of increasing resistance to clarithromycin and metronidazole, the two major antimicrobial agents used in current triple regimens (Megraud,

Table 1. Antibacterial activity of 30 % EEP and 96 % ethanol against *H. pylori* strains, as measured by AWDM and DDM

Parameter	AWDM (38 *H. pylori* strains)				DDM (26 *H. pylori* strains)		
	30 µl EEP per well	60 µl EEP per well	90 µl EEP per well	30 µl ethanol per well	5 µl EEP (moist disc)	5 µl EEP (dried disc)	5 µl ethanol
Mean diameter of growth inhibition (mm)	17·8	21·2	28·2	8·5	21·4	12·4	9·0
Range (mm)	7–48	7–56	9–60	7–18	6–40	6–30	6–18
Strains with growth inhibition ⩾ 15 mm (%)	52·6	57·9	89·5	7·9	65·4	34·6	23·1

2000). This motivates the search for alternative or additional therapeutic agents. The inhibitory activity of propolis against *H. pylori in vitro* is worth further bacteriological, pharmacological and clinical evaluation. The use of propolis mouthwashes could reduce or eliminate *H. pylori* in the mouth cavity, as a route of transmission of *H. pylori* infection (Megraud & Broutet, 2000). The synergistic effect of propolis and several antimicrobial agents (e.g. cloxacillin and doxycycline) has been demonstrated against *Staphylococcus aureus* (Krol *et al.*, 1993). The presence or lack of synergistic effect of propolis with metronidazole, clarithromycin or amoxicillin against *H. pylori* is worthy of investigation. In addition, the anti-inflammatory and tissue-regenerative properties of propolis (Koo *et al.*, 2000) could be an additional advantage in the prevention or treatment of *H. pylori* infection.

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