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 (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 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 < 0.001). 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 < 0.001). With moist propolis discs, inhibition was detected in more strains (92.1 %) than with dried discs (78.2 %, P < 0.05), 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.

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 cefzulodin 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 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.
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 colonies, lack of aerobic growth and testing for the presence of urease, 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* < 0.001, 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* < 0.05). 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* < 0.001).

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* < 0.05). 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**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agar-well diffusion method</th>
<th>Disc diffusion method</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30 µl EEP*</td>
<td>60 µl EEP*</td>
</tr>
<tr>
<td>Corresponding propolis concentration (mg)</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>No. of <em>H. pylori</em> strains tested</td>
<td>94</td>
<td>69</td>
</tr>
<tr>
<td>Mean diameter of growth inhibition (mm)</td>
<td>16.8</td>
<td>19.2</td>
</tr>
<tr>
<td>Growth inhibition diameter (mm)</td>
<td>7–48</td>
<td>7–56.5</td>
</tr>
<tr>
<td>Strains with no growth inhibition (%)</td>
<td>13.8</td>
<td>7.2</td>
</tr>
<tr>
<td>Strains with growth inhibition diameter ≥15 mm (%)</td>
<td>48.9</td>
<td>52.2</td>
</tr>
<tr>
<td>Strains with growth inhibition diameter ≥20 mm (%)</td>
<td>26.6</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*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., 2002).

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.

**References**


