The Inhibitory Effect of Iraqi Propolis Extract Against Three Isolates of 
Candida Albicans

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Abstract
The antifungal effect of Iraqi propolis was evaluated by an in vitro study testing the growth, colonies diameter, yeast cell dimensions and chlamydospores formation of Candida albicans in media containing different concentrations of propolis extract. The results demonstrated that propolis (0.5-10 mg/ml) showed no significant effect on Candida growth and the shape of yeast cell. It was found that propolis extract at a concentration of 15 mg/ml and above exerted inhibitory effects on the cells of Candida. Based on these results, we suggest that propolis could directly activate mechanism of the antifungal action. However, the increasing of the inhibitory effect showed that propolis could have antifungal properties in high concentrations and further work is needed in order to reveal the active compounds in Iraqi propolis.

Introduction
Propolis, a resinous substance collected by Apis mellifera bees from various plant sources and mixed with secreted bees wax, is a multifunctional material used by bees in the construction, maintenance, and protection of their hives. Propolis is a pro-choice natural product with a complex chemical composition (1, 2). Since ancient times propolis has been widely used for diverse purposes. Currently, the activity, effects and possible applications of propolis in biology and medicine are being investigated, with emphasis on their use as a dietary supplement, as well as their possible applications within the pharmaceutical industry. The ethanolic extract of propolis has some activities such as local-anesthetic (3), anti-inflammatory (4, 5), antioxidant (6, 7), hepatoprotective (8), immunostimulating (9), antifungal (10), antibacterial (11, 12, 13) and antitumoral (9, 14). Literature survey revealed that flavonoids, aromatic acids, terpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis extracts. The flavonoids in propolis (mainly pinocembrin) have been considered to be responsible for its inhibitory effect on Candida (15), but only traces of these compounds have been found in propolis of south American (16), European (17) and Egyptian origin (18), indicating that this effect could be due to a different class of compounds. Several authors have reported on the inhibitory effect of the ethanolic extract of propolis on Candida using propolis of temperate and tropical region. Different microbiological tests have been used to evaluate this effect, serial dilution in tubes, agar dilution in plates, agar diffusion in plates and bioautography. Although Candida species are commensal inhabitants of the surfaces of animal and human they also cause local or systemic infection in patients with immunodepressive diseases, in patients who are using certain kinds of drugs such as broad spectrum antibiotics, anti-tumor agents who use oral prosthesis or orthodontic material without adequate care (19, 20). Thus, present study was conducted to evaluate the antifungal activity of Iraqi propolis against isolates of C. albicans using different growth parameters.

Materials and methods
Iraqi propolis (15) samples were collected from an apiary located in Tarmiya (north east of Baghdad, Iraq) in May 2001 and stored at 4°C.

Extraction and sample preparation
One gram of propolis sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol using ultrasonic bath (Denton FS 300, England) 5 periods for each period 50 minutes. This suspension was kept at room temperature and was shaken every day for 30 seconds for a period of 2 days and was subsequently filtered twice with Whatman filter papers (No. 1). The ethanolic extract was evaporated at 50°C until dryness. The dry extract was then redisolved in certain volume of 20% ethanol (50 ml) to obtain stock solution. All tests were performed in 10 measurements using 20% ethanol, and without propolis as control.

Yeast isolates
Two clinical isolates of C. albicans were originally isolated from oral and vaginal infections were used, in addition to a standard isolate (ATCC: 10231).

Assessment the inhibition of yeast growth
Yeast cell suspension was prepared by taking a single colony grown for 26-48 h on Sabouraud dextrose agar (SDA) and added to 10 ml of sterile
saline solution, shaken gently and adjusted with sterile saline to a concentration of 1 × 10⁵ colony forming units (CFU) ml⁻¹, evaluated by a haemocytometer. A 0.1 ml portion of each cells suspension was spread evenly with a sterile glass rod on the surface of a sterile Petri dish containing sterile SDA plates. Plates were left for 20 min to ensure diffusion of the suspension. Four (4) mm diameter holes were made in each Petri dish using cork borer, holes were filled with 50 µl of different concentration of propolis ranged from 0.5-20 mg ml⁻¹. The plates were incubated at 30 °C and observed after 24 and 48 h.

Determination of the minimal inhibitory concentration (MIC) of propolis

The minimum inhibitory concentration of propolis was measured by using the dilution method. Different concentrations of propolis ranging from 0.5-20 mg ml⁻¹ were made in SDA broth (9 ml). Each tube was inoculated with 0.1 ml of cell suspension prepared as described earlier. MIC values were incubated at 30 °C for 24 h. Cells survival was determined by plating on a duplicate of 0.1 ml from each tube on to SDA plates, incubated for 48 h at 30 °C. MIC was defined as the lowest concentration of propolis that killed 99.9% of the given counts of the test organism. Diameters of the survival colonies and diameters of single cells from survival colonies were also determined to observe the effect of propolis on these criteria using ocular micrometer with 20-25 reps.

Effect of propolis on chlamydocoele formation by C. albicans

Chlamydocoele formation by C. albicans is one of the virulence factors of this organism, therefore effect of different concentration of propolis on chlamydocoele formation was carried out on corn meal agar (CMA). Few drops of the medium were spread on a clean sterile slide, overlaid on a shape glass rod in a Petri dish containing wet filter paper in the bottom. The medium was incubated with the test isolate by streaking, plates were incubated at 30 °C for 3-4 days, and examined later by microscopy after adding few drops of lactophenol cotton blue and cover the growth with the cover slide.

Statistical data analysis

Data were statistically analyzed using SPSS statistical software (version 11.5) by analysis of variance (ANOVA) test. The values are given as mean ± standard error.

Results and Discussion

Ethanolic solution of propolis showed inhibitory effects on Candida isolates tested at concentrations 15 and 20 mg ml⁻¹, as shown in table 1. The most susceptible isolate was the standard isolate which showed strongly inhibited zone (20 ± 1.5 mm) followed by the vaginal and nail isolates (18 ± 0.4 and 14 ± 0.5 mm), respectively at concentration of 20 mg ml⁻¹. Insignificant inhibition (p<0.05) was observed at concentrations ranged from 0.5 mg ml⁻¹ to 10 mg ml⁻¹ as compared to the control treatment containing 20% ethanol.

Table (1): Antifungal properties of Iraqi propolis (inhibition zone in millimeters including diameter of 8 mm hole)

<table>
<thead>
<tr>
<th>Concentration of Iraqi propolis mg ml⁻¹</th>
<th>Control</th>
<th>6.3 - 10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td></td>
<td>Diam. of inhibition zone ± standard error, mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>6</td>
<td>8</td>
<td>10 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Nail</td>
<td>8</td>
<td>8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>8</td>
<td>12.4</td>
<td>14 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Viable cell counts of Candida isolates using broth dilution method with different concentrations of propolis ranged from 0.5 mg ml⁻¹ to 20 mg ml⁻¹ showed gradual reduction in growth (represented by CFU ml⁻¹) with the increasing concentration of propolis. MIC of propolis was 18 mg ml⁻¹, no growth was observed at concentration 19 and 20 mg ml⁻¹ [Fig. 1].

Figure (1): Propolis effect against cell viability of Candida (Nail)

Variations in colonies diameters of the viable cells due to treatment with different concentration lower than the MIC were clear obvious compared with the control, as shown in table 2. Results showed gradual increasing in diameter with the increasing concentration of propolis; maximum colony diameter (2.294 ± 0.164 mm) was at 17 mg ml⁻¹.

Table (2): Effect of Iraqi propolis on diameters of Candida colonies (Standard)
Assessment of the effect of propolis concentration on the ability of chlamydospores formation by C. albicans grown on CMZ after 3-4 days incubation at 30 °C showed the ability of these organisms to produce chlamydospores at concentration lower than 15 mg ml⁻¹. Higher concentrations inhibited chlamydospore formation, and the yeast remained as single cells as compared to the control. (Figure 2).

**Table (3):** Effect of Iraqi propolis on dimensions of Candida cell (Vaginal)

<table>
<thead>
<tr>
<th>Concentration of Iraqi propolis mg ml⁻¹</th>
<th>Dimensions</th>
<th>Control</th>
<th>10</th>
<th>15</th>
<th>17</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>22.70 ± 1.59</td>
<td>Not tested</td>
<td>30.37 ± 1.41</td>
<td>34.66 ± 1.15</td>
<td>No growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>Width</td>
<td>28.80 ± 1.55</td>
<td>Not tested</td>
<td>25.50 ± 1.79</td>
<td>31.33 ± 1.05</td>
<td>No growth</td>
<td>No growth</td>
<td></td>
</tr>
</tbody>
</table>

*Dimension of Candida cell ± stand. error, μm*

The results obtained by the agar diffusion method were conclusive, indicating that propolis has exerted an inhibitory effect on Candida while the isolates of Candida grew on the control plates containing 20% ethanol, indicating that the solvent did not exert any inhibitory effect on the yeast under these conditions. High concentrations (15-20 mg ml⁻¹) were used in this study achieved inhibitory effect on the growth of the Candida isolates. These values are similar to those obtained by Pepejnjak et al. (21) they found that, for pure propolis extracts, a concentration of 15 - 30 mg ml⁻¹ was needed to inhibit the growth of C. albicans. Antimicrobial activity of plant extracts, Esculetin, Pentelidium viridimum and P. roselle. But other authors found the MIC values of Brazilian , European and Egyptian propolis ranging between 5 and 15 mg ml⁻¹ (22, 23, 18). These variations in the antifungal activity seem to be due to the differences in the chemical composition of different propolis samples. Flavonoids are well known for their antimicrobial, antifungal and antiviral action and are thought to be responsible for the beneficial properties of propolis (24, 25). Extracts of phenolic acids and especially caffeates and ferulates have been identified as antibacterial, antifungal and antiviral principles of propolis, too (15, 26). We suggest that the antifungal effect of our propolis is the result of their flavonoid contents and volatiles. Volatiles is known to reduce the aerobiosis within the spores (24). Different authors have found that propolis volatile oils possess good to moderate antimicrobial action (27). Perić et al. (28) found that propolis sample from 20 locations in Hungary contained from 0.3 to 1.6% essential oils. The components of the oil fraction were the same for all samples, but the ratio of the components differed in microbiological tests, propolis oil showed good to moderate activity against Gram-positive and Gram-negative bacteria and against 3 of fungi. Holdeman and Kedzie (29) studied the combined action of antimicrobial drugs and propolis on C. albicans and found that this combination increased the inhibitory effect on C. albicans. Kovalik (30) investigated 12 patients (55-62 years old) suffering from chronic candidiasis, caused by C. albicans. In vitro tests the fungi was sensitive to propolis in 8 cases, weak in 2 while 2 were resistant. Ota et al. (31) found inhibitory activity on 75 strains of Candida yeasts, 20 strains of C. albicans, 20 strains of C. tropicalis, 20 strains of C. krusei, and 15 strains of C. parapsilosis. Central European propolis (Germany, France and Austria), with similar qualitative compositions and a predominance of
trans-p-coumaric acid, show activity against C. albicans (32), while Mediterranean varieties (Bulgaria, Turkey, Greece and Algeria), that contain flavonoids, esters of caffeic acid and ferulic acids, possess antifungal activity to a lesser extent (33). Egyptian propolis from Dakahlia, with two caffeic esters and two pteroprenoids, is more active against C. albicans than the variety from Ismailia, which does not contain aromatic acids, esters, or flavonoids (18). Studies on the incidence of Paracoccidioides in Latin America suggest that independency of geographical origin, macrophages stimulated with propolis increase fungalicidal activity (14). Propolis shows, in varying degrees, fungicidal effects against numerous species such as C. albicans, Aspergillus niger, Botrytis cinerea, Ascosphaera apicola and Plasmopara viticola (34). The highest degree of inhibition observed, 50% in all of the species studied, corresponded to a propolis concentration of 4% and the most affected microorganisms are Alternaria alternata and Penicillium digitatum (35). The highest degree of inhibition on pathogenic fungi was observed in Trichophyton mentagrophytes, Trichophyton, Malassezia pachydermatis and on Candida genus (36, 37). Effect of different concentrations of propolis on dimensions of single cells may reflect the effect on colony diameters which increased with the increasing concentration of propolis and that effect could be due to effect of propolis on growth rate and reproduction of the yeast cells. In the other side, the decrease of colonies diameters showed that propolis at high concentration (18-20 mg ml⁻¹) indicates that propolis is an antifungal agent. The possible mechanism of the antibacterial/antifungal action of propolis was studied by Takisaki-Kikuni and Schilcher (38). They observed an inhibition of cell division in the presence of propolis and this fact suggested that propolis might act by inhibiting DNA replication and indirectly, cell division; although a simple analogy to the mode of action of classic antibiotics could not be made. Propolis, as with other live products, varies within a given area, the time of collection and amount of wax contents (25). This could possibly explain why many authors have given variation in their reports. According to our results, all of C. albicans isolates were shown to be sensitive to propolis and further work is needed in order to reveal the active principles in Iraqi propolis.

References


فلاحة

تم توجيه تأثير الطبيعة الطرقية للعصر المعاصر في معرض في الزجاج على دواء وقائي للمستفيدين والعلاجية للعمرية، و̀كتم نورت طفرات النباتات في مساحة واسعة: على بعض الكائنات مثل مستخدمي الكريات، وقد عالج بعض الأمراض بأن هذا التعرف على تأثيرها تكون من 0.5-10 مجم مللي-ري. لا يظهر أن تكون بالرغم من مساحة البحث حول الخلايا الحيوية، بينما تظهر التعرفات من 15-15 مجم مللي-ري في المثال. على هذه الطريقة، فعلى الرغم من أن ذلك، فإن الخلايا الحيوية تظهر بالعثور بان يمتلك أن تكون علاقة مباشرة بين الشكل الجيولوجي والكيميائي، خاصة في هذه المراحل، ومن ثم، يمكن أن يوجد حالة تظهر شكلها الواضح، ونسبة نشاط للمادة الطبيعية التي تتكاثف من النشاطات المختلفة في الجسم العضو.