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

Ghassan M. Sulaiman[1], Summaya N. Howar[2] and Batool Z. Ali[2]

[1] Bio-chemical Technology Division, Applied Sciences Department, University of Technology.
[2] Department of Biology, College of Education- Ibn Al Haitham, University of Baghdad.

Abstract

The antifungal effect of Iraqi propolis was evaluated by an in vitro study testing the growth, colonies diameters, 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-1) 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-1 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 nontoxic 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 an 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-anaesthetic (3), anti inflammatory (4, 5), antioxidant (6, 7), hepatoprotective (8), immunostimulating (9), antiviral (10), antibacterial (11, 12, 13) and antifungal (9, 14). Literature survey revealed that flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis samples. The flavanoids 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 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 commensally inhabitants of the surfaces of animal and human they also cause local or systemic infection in patients with immunodepressive diseases, in patients who 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 (Ip) samples were collected from an apiary located in Tarmyia (north east of Baghdad, Iraq) in May 2004 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, (Decon HS 300, England) 3 periods for each period 30 minutes. This suspension was kept at room temperature and was shaken every day for 30 second for a period of 4 days and was subsequently filtered twice with Whatman filter papers (No. 3). The ethanolic extract was evaporated at 50 °C until dryness. The dry extract was then redissolved 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 a control.

Yeast isolates

Two clinical isolates of *C. albicans* were originally isolated from nails and vaginal infections were used, in addition to a standard isolate (ATCC 102301)

Assessment the inhibition of yeast growth

Yeast cell suspension was prepared by taking a single colony grown for 24 - 48 h on Sabouraud dextrose agar (SDA) and added to 10 ml of sterile

ISSN: 1814-5922

saline solution, shaked gently and adjusted with sterile saline to a concentration of 1×10^5 colony forming units (CFU) ml^{-1}, evaluated by a haemocytometer. A 0.1 ml portion of each cells suspension was spreaded evenly with a sterile glass rode on the surface of a sterile Petri dish containing sterile SDA, plates were left for 30 min to ensure diffusion of the suspension. Four (8) 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 serial broth dilution method, different concentrations of propolis ranged from 0.5-20 mg ml^{-1} were made in SD broth (9 ml). Each tube was inoculated with 0.1 ml of cell suspension prepared as described earlier. MIC cultures were incubated at 30 °C for 24 h. Cells survival was determined by plating on a lignate 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 killed (no growth or survival at the given conc.) of the test organism. Diameters of the survival colonies and dimensions of single cells from survival colonies were also determinated to observe the effect of propolis on these criteria using ocular micrometer with 20-25 reps.

Effect of propolis on chlamydospore formation by *C.albicans*

Chlamydospore formation by *C.albicans* is one of the virulence factor of this organism, therefore effect of different concentration of propolis on chlamydospore formation was carried on using corn meal agar (CMA) .Few drops of the medium were spreaded on a clean sterile slide overlaid on U-shape glass rode in a Petri dish containing wet filter paper in the bottom .The medium was inoculated 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 slip.

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)

	Concentration of Iraqi propolis mg ml^{-1}			
	Control	0.5 - 10	15	20
Isolate	Diameter of the inhibition zone ± standard error , mm			
Standard	8	8	14 ± 0.3	20 ± 1.5
Nail	8	8	12 ± 0.2	14 ± 0.5
Vaginal	8	8	14 ± 0.1	18 ± 0.4

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).

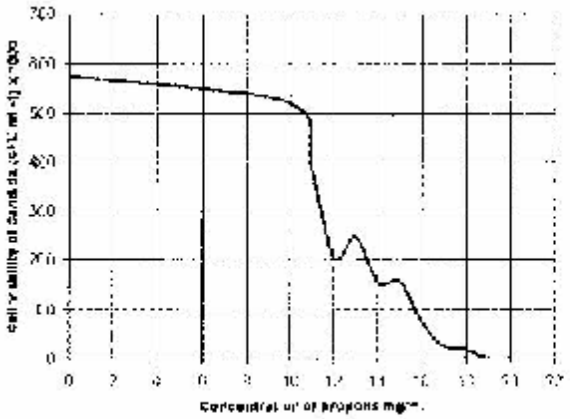

Figure (1): Propolis effect against cell viability of *Candida* (Nail)

Variations in colonies diameters of the viable cells due to treatment with different concentration lower than the MIC was 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 (2.2942 + 0.164 mm) was at 17 mg ml^{-1}.

Table (2). Effect of Iraqi propolis on diameters of *Candida* colonies (Standard)

Propolis Conc. mg ml^{-1}	Antifungal activity (Diameter of *Candida* colony stand. error. mm)*
Control	.7512 ± 0.0290
10	0.8660 ± 0.061
11	1.0657 ± 0.053
12	1.4309 + 0.076
13	1.2965 ± 0.053
14	1.4278 ± 0.102
15	1.5645 + 0.103
16	1.8791 ± 0.113
17	2.2942 + 0.164
18	1.8774 ± 0.082
19	No growth
20	No growth

* Mean of ten measurements

Assessment of the effect of propolis concentration on the ability of chlamydospores formation by *C. albicans* grown on CMA after 3-4 days incubation of 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).

Figure (2): Chlamydospores formation in control sample of *Candida* (Nait) S: Spore

Results also revealed differences in the dimensions (length and width) of the single yeast cell. Usually, the yeast cell is oval to spherical in shape, while those treated with propolis tended to be elongate (Table 3).

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

Dimensions *	Concentration of Iraqi propolis mg ml^{-1} Control	10	15	17	19	20
Length	28.70 . 1.59	Not tested	30.37 ± 1.43	34.66 ± 4.15	No growth	No growth
Width	28.80 − 1.35	Not tested	25.50 + 1.79	31.33 + 1.05	No growth	No growth

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

The results obtained by the agar diffusion method were conclusive, indicating that propolis have 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 an inhibitory effect on the yeast under these condition . High concentrations (15-20 mg ml^{-1}) were used in this study succeeded in inhibition the growth of the *Candida* isolates .These values are similar to those obtained by Pepeljnjak *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, Aspergillus flavus, A ochraceus, Penicillium viridicatum and P. notatum. But* ,other authors were found the MIC values of Brazilian , European and Egyptian propolis ranging between 6 and 12 mg ml^{-1} (22, 23.18) .These variation in the antimicrobial activity seems to be due to the differences in the chemical composition of different propolis samples . Flavonoids are well known for their antibacterial, antifungal and antiviral action and are thought to be responsible for the beneficial properties of propolis (24, 25). Esters 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 apiary (24). Different authors have found that propolis volatile oils possess good to moderate antimicrobial action (27). Petri *et al.* (28) found that propolis sample from 20 locations in Hungary contained from 0.3 to 1.5 % essential oils. The .components of the oil fraction were the same for all samples, but the ratios 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. Holderna and Kedzia (29) studied the combined action of antimycotic drugs and propolis on *C. albicans* and found that this combination increased the inhibitory effect on *C. albicans*. Kovalik (30) investigated 12 patients (35-62 years old) suffering from chronic sinusitis, caused by *C. albicans*. *In vitro* tests the fungus 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. krusi* and 15 strains of *C. guilliermondii*. 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 nod ferulic acids, present antifungal activity to a lesser extent (33). Egyptian propolis from Dakahlia, with two caffeate esters and two triterpenoids, is more active against *C. albicans* than the variety from Ismailia, which does not contain aromatic acids, esters, or flavanoids (18). Studies on the incidence of paracoccidioidomycosis in Latin America suggest that, independently of geographical origin, macrophages stimulated with propolis increase fungicidal activity (14). Propolis shows, in varying degrees, fungicidal effects against numerous species such as *C. albicans*, *Aspergillus niger*, *Botrytis cinerea*, *Ascosphaera apis* 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 mentogrophytes*, *T.rubrum*, *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 Takaisi-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 hive 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

1. Burdock, G. A. (1998). Review of the biological properties and toxicity of propolis. Food Chem. Toxicol., 36:341–363.
2. Ghisalberti, E. L. (1979). Propolis: a review. Bee World, 60:59–84.
3. Paintz M. and Metzner J. (1979), Zur Lokalanästhetiprogressschen Wirkung von Propolis und einigen Inhaltsstoftoth. Fen. Pharmazie, 34: 839-841. (Cited from reference 13)
4. Strehl E., Volpert R. and Elstner E. F. (1993). Biochemical activities of propolis extracts III. Inhibition of dihydrofolate reductase. Z. Naturforsch., 49c: 39-43.
5. Miyataka, H.; Nishiki, M.; Matsumoto, H.; Fujimoto, T.; Matsuka, M. and Satoh, T. (1997). Evaluation of propolis. I. Evaluation of Brazilian and Chinese propolis by enzymatic and physico-chemical methods. Biol. Pharm. Bull. , 20: 496-501.
6. Sun, F.; Hayami ,S.; Haruna, S.; Ogiri ,Y.; Tanaka, K.; Yamada, Y.; Ikeda, K.; Yamada, H.; Segimoto, H.; Kawai, N. and Kojo, S. (2000). In vivo antioxidative activity of propolis evaluated by the interaction with vitamin C and vitamin E and the level of lipid hydroperoxides in rats. J. Agric. Food Chem., 48(5): 1462-1465.
7. Isla, M. I.; Moreno, M. I. N.; Sampietro, A. R. and Vattuone, M. A. (2001). Antioxidant activity of Argentina propolis extracts. J. Ethnopharm., 76: 165-170.
8. Gonzales, R.; Corcho, I.; Remirez, D.; Rodriguez, S.; An- cheta, O.; Merino, N.; Gonzales, A. and Pascual, C. (1995). Hepatoprotective effects of propolis extract on carbon tetrachloride-induced liver injury in rats. Phytoth. Res., 9: 114-117.
9. Dimov, V.; Ivanovska, N.; Manolova, N.; Bankova, V.; Nikolov, N. and Popov, S. (1991). Immunomodulatory action of propolis. Influence on anti-infectious protection and macrophage function. Apidologie, 22: 155-162.
10. Amoros, M.; Lurton, E.; Boustie, J.; Girre, L.; Sauvager, F. and Cormier, M. (1994). Comparison of the anti-Herpes simplex virus activities of propolis and 3-methyl- but-2-enyl caffeate. J. Nat. Prod., 57: 644-647.
11. Rhajaoui, M.; Oumzil, H.; Faid, M.; Lyagoubi, M.; Elyachioui, M.and Benjouad, A. (2001). Antibacterial activity of a Moroccan propolis extracts. Science Letters, 3(3): 1-7.
12. Sulaiman, G.M. and Nima, Z. A. (2005). Comparison of free radicals scavenging activity, phenolics levels and antibacterial activities by Iraqi propolis from different sources. J.Coll. Sci. Wom. In press
13. Silici, S. and Kutluca, S. (2005). Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region.J.Ethnopharmacol. , 99: 69-73.
14. Murad , J. M.; Calvi, S. A.; Soares, A. M. V. C., Bankova, V. and Sforcin, J. M. (2002). Effect of propolis from Brazil and Bulgaria on fungucidal activity of macrophages against *Paracoccidioides brasiliensis*. J. Ethnopharm., 79: 331-334.
15. Metzner, J.; Bekemeier, H.; Paintz, M. and Scheidewind, E. (1979). On the antimicrobial activity of propolis and propolis constituents. Pharmazie, 34(2): 97-102.
16. Salatino, A.; Teixeira E. W.; Negri, G. and Message, D. (2005). Origin and Chemical

Variation of Brazilian Propolis. eCAM, 2 (1) : 33–38.
17. Bankova,V. ; Popova,M. ; Bogdanov,S. and Sabastini,A. (2002).Chemical composition of European propolis: expected and unexpected results , Z. Naturforsch., 57c: 530-533.
18. Abd El Hady, F. K. and Hegazi, A. G. (2002).Egyptian propolis: 2. chemical composition, antiviral and antimicrobial activities of east N.le delta propolis. Z. Naturforsch., 57c: 386-394.
19. Zambon, J. J.; Reynolds, H. S. and Genco, R. J. (1990). Studies of the subgingival microflora in patients with acquired immunodeficiency syndrome. J.Periodontol., 60 :699-704
20. Olsen, I. (1974). Denture stomatitis. Occurrence and distribution of fungi. Acta Odontol. Scand., 32: 329-333.
21. Pepeljnjak, S ; Maysinger, D.and Jalsenjak, I. (1982). Effect of propolis extract on some fungi. Scientia. Pharmaceutica., 50 (2):165-167.
22. Sforcin, J.M.; Fernandes, A. Jr; Lopes, C.A.M.; Bankova, V.; Funari, S.R.C. (2000). Seasonal effect on Brazilian propolis antibacterial activity. J. Ethnopharmacol., 73: 243-249.
23. Metzner, J.; Bekemeier, H.; Paintz, M. and Schneidewind, E. (1979). Zur antimikrobiellen Wirksamkait von Propolis und Propolisinhaltsstoffen. Pharmazie, 34, 97 - 102. (Cited from reference 26)
24. Bankova,V. (2005). Recent trends and important departments in propolis research. eCAM, 2(1): 29-32.
25. Marcucci, M. C. (1995). Propolis chemical composition, biological properties and therapeutic activity. Apidologie 26, 83 - 99.
26. Kujumgiev, A.; Tsvetkova, I.; Serkedjieva, Y.; Bankova, V.; Christov, R. and Popov, S. (1999). Antibacterial, antifungal and antiviral activity of propolis from different geographic origins. J. Ethnopharmacol., 64: 235 - 40.
27. Petri, G., Lemberkovics, E. and Foldvari, M. (1986). Examination of differences between propolis (bee glue) produced from different floral environments. In: Flavours and Fragrances: A World Perspective (Lawrence, B. M., Mookhedjee, B. D. and Willis, B. J., Eds.) Elsevier, Amsterdam, pp. 439 - 446.
28. Petri, G, Lemberkovics, E.; Foldvari, M. (1988). Examination of differences between propolis (bee glave) produced from different floral environments Elsevier Science publishers 439-446.
29. Hodema, E. and Kedzia, B. (1987). Investigation upon the combined action of propolis and antimycotic drugs on *Candida albicans* Herba pol., 33:145-151.
30. Kovalik, P.V. (1979). The use of propolis in the treatment of patients with chronic fungal sinusitis. Vestnik otorhindaritgologii, 6: 60 62.
31. Ota, C.; Unter kircher, C ; Fantinato, V. and Shimizo,M.T. (2001). Antifungal activity of propolis on different species of *Candida*. Mycosis, 44:375-378.
32. Hegazi, A.; Abd El Hady, F. and Abd Allah, F. (2000). Chemical composition and antimicrobial activity of European propolis. Z Naturforsch, 55(1-2):70-75.
33. Velikova, M.; Bankova, V.; Sorkun, K.; Houcine, S.; Tsvetkova, I. and Kujumgiev, A. (2000). Propolis from the Mediterranean region: chemical composition and antimicrobial activity. Z. Naturforsch, 55(9-10):790-793.
34. Krell R. (1996). Value-added products from bee keeping. FAO Agricultural Services Bulletin No. 124. Rome.
35. Ozcan M. (1999) , Antifungical properties of propolis. Grasas y Aceites, 50(5):395-398.
36. Koc, A. N.; Silici, S., Ayangil, D.; Ferahbas, A. and Cankaya, S. (2005). Comparison of in vitro activities of antifungal drugs and ethanolic extract of propolis against Trichophyton rubrum and T. mentagrophytes by using a microdilution assay. Mycosis, 48:205-210.
37. Heinze, W.; Holz, J.; Nattermann, H. and Blankenstein, P. (1998). Effects of ethanol extracts of propolis against common bacteria, fungi and viruses of veterinary importance. Tierarztl Umsch, 53 (6):321-326.
38. Takaisi kikuni,N.B. and Schiteher, H. (1994). Electron microscopic and microcalorimetric investigation of the possible mechanism of the antibacterial of a defined propolis provenance. Planta Med., 60: 222-227.

الخلاصة

تم تقييم تأثير التضاد الفطري للعكبر العراقي بدراسته في الزجاج على نمو و أفطار المستعمرات و أبعاد الخلية الخميرية وتكوين الميورات بفطر المبيضات في وسط زرعي يحتوي على تراكيز مختلفة من مستخلص العكبر. بينت النتائج بأن التراكيز التي تتراوح بين 0.5– 10 ملغم ملليلتر^-1 للعكبر لم تظهر أي تأثير هام على نمو فطر المبيضات وشكل الخلية الخميرية. بينما أظهرت التراكيز من 15 ملغم ملليلتر^-1 فما فوق تأثيرا ملحوظا على خلايا فطر المبيضات البيض. على هذه النتائج، فقد تم بأن العكبر يمكن أن ينشط الآلية المضادة للنمو الفطري عن طريق زيادة الفعالية التثبيطية لخميرة بأن العكبر يمكن أن يستخدم كمضاد مضاد للفطريات عند تراكيز المائية و نحن بحاجة لدراسة طبية لكي نكشف عن المركبات الفعالة في العكبر العراقي.