SYNERGISTIC ACTIVITIES OF TWO PROPOLIS WITH AMPHOTERICIN B AGAINST SOME AZOLE-RESISTANT CANDIDA STRAINS. PART II

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Candida strains are opportunistic pathogenic fungi in humans which can cause either septicaemic or mucosal infections. In this study, we aimed to investigate the existence of synergistic activities of two types of propolis (Hatay and Bursa) with Amphotericin B against some azole-resistant Candida strains. To evaluate cytotoxicity of propolis for human cells, the HEp-2 cell line (human larynx epidermoid carcinoma cell line) was used. The cytotoxicity of propolis samples was determined on a conventional haemocytometer using the trypan blue exclusion method. The synergystic activity of Hatay and Bursa propolis samples was assayed against four different azole-resistant Candida isolates (C. albicans, C. tropicalis, C. parapsilosis and C. glabrata). MIC values of Candida spp. were determined as described in the CLSI reference methods. The antifungal activities of a combination of this antifungal agent (Amphotericin B) were assessed by the checkerboard test and time-kill curve study. Bursa and Hatay propolis samples have remarkable antifungal activity against C. albicans, C. glabrata, C. tropicalis and C. parapsilosis. Also, the strong synergistic activity between Amphotericin B and propolis (especially Bursa propolis) was found against the azole resistant candida strains. Propolis may be an important prospect in the treatment of azole-resistant yeast. The effects of these two kinds of propolis against the Candida spp. in vitro are promising.

Keywords: Propolis, candida strains, Amphotericin B.

INTRODUCTION

In recent years, the incidence of both mucosal and invasive opportunistic fungal infections have increased in immunocompromised patients such as HIV-infected individuals, organ transplant patients, in patients receiving immunosuppressive treatment and treated with broad-spectrum antibiotics (Vargas and Joly, 2002).

Although C. albicans is the predominant cause of all types of candidiasis, other Candida species except C. albicans have also been recognised as significant pathogens. Other Candida species such as C. tropicalis, C. Parapsilosis and C. glabrata have emerged as an important pathogen in immunosuppressive patients. Over the past four decades especially rates of systemic infections caused by Candida species have steadily increased.

Candida species are the important pathogenic fungi a common cause of mucosal and invasive fungal infections. Azole antifungal agents have been as a first choice in the treatment of Candida infections for years. The azole class of antifungal agents, like as fluconazole and itraconazole and Amphotericin B are the most useful drugs, are the most frequently prescribed for the treatment of systemic and mucocutaneous candidiasis. Unfortunately, emergence of azole-resistant Candida strains in patients receiving azole treatment has become a serious problem. Besides this, because of their
potential toxicity and acquired drug resistance, the use of these kinds of drugs are limited (Sternberg, 1994; Johnson et al., 1995; Revankar et al., 1996; Rex, J.H., Rinaldi, M.G. and Pfaller, M.A., 1995).

In this study, we aimed to investigate the existence of synergistic activities of Hatay and Bursa propolis with Amphotericin B against some azole-resistant Candida strains.

MATERIALS AND METHODS

Effect of DMSO on Candida species

In order to test the effects of dimethyl sulfoxide [(DMSO); Sigma, USA] against fungal strains, 1x106 yeast cells were inoculated into each well of micro plates containing RPMI medium [(Roswell Park Memorial Institute) (Gibco-BRL)], and yeast cells were allowed to incubate for 48 hours in the presence of decreasing amounts of DMSO (25, 12.50, 6.25, 3.12, 1.56, 0.78, 0.39%). The non-toxic concentration was determined up to 3.12%. DMSO concentrations lower than 3.12% did not inhibit the growth of the yeasts. In experiments, propolis samples were dissolved in 1% DMSO.

Cytotoxicity Test

Preparation of the cell culture

To evaluate cytotoxicity of propolis for human cells, the HEp-2 cell line (human larynx epidermoid carcinoma cell line) was used. The cells were cultured in RPMI medium with 10% (w/v) FCS. The cells were incubated for 48 h at 37°C in an atmosphere with 5% CO2. Propolis samples were solved in DMSO. Stock solutions of propolis samples were prepared in DMSO at the concentration of 1%.

To determine the effects of propolis samples on HEp-2 cells, they were infected with propolis. Then the infected and non-infected Hep-2 (uninfected with propolis) cells were observed under inverted microscopy. To evaluate the effects of propolis on HEp-2 cells, 1x105 cells were seeded into each well micro plates (flat bottomed), and cells were allowed to grow for 6 h at 28°C, and the cells were allowed to grow for an additional 48 hrs. And then, propolis samples were diluted and decreasing amounts (3200, 1600, 800, 400, 200, 100, 75, 50 and 25 µg/mL) were placed per well, and the cells were allowed to grow for an additional 48 hrs. All experiments were performed in triplicate, and the results expressed as log number cells per milliliter and as the percentage of growth inhibition.

The cytotoxicity of propolis samples was determined on a conventional haemocytometer using the trypan blue exclusion method. The highest noncytocidal (on HEp-2 cells) concentration of the tested samples was determined to be 1600 µg/mL (=1.6 mg/mL). Therefore, propolis concentrations were selected lower than 1.6 mg/mL.

Microorganisms

The synergistic activity of Hatay and Bursa propolis samples was assayed against four different azole-resistant Candida isolates (C. albicans, C. tropicalis, C. parapsilosis and C. glabrata). Standard strains were also used: C. albicans ATCC 10231, C. tropicalis ATCC 750 and C. parapsilosis ATCC 22019, C. glabrata ATCC 90030.

Candida isolates were identified by a germ-tube test followed by VITEK Yeast Biochemical Card (bioMerieux, France). Yeast-like fungi were cultured on Sabouraud agar plates for 48 h at 37 °C. The MICs of fluconazole and itraconazol for the Candida spp. isolates were determined by the microdilution broth method according to the guidelines of the Clinical and Laboratory Standards Institute (NCCLS, 1997). In the study, fluconazole- and itraconazole-resistant Candida strains were selected.
Antimycotic assay

The Candida spp. were maintained in Sabouraud dextrose broth (Difco, USA) after incubation for 48 h at 37 °C. Minimal inhibition concentration test (MIC) was carried out in Sabouraud dextrose broth at pH 7.4, and the 2-fold serial dilution technique was applied. The final inoculum size was selected as 105 CFU/mL for the antifungal assay. A set of tubes containing only inoculated broth was used as controls. After incubation for 48 h at 37 °C for the antifungal assay, the last tube with no growth of yeast was recorded to represent the MIC expressed in µg/mL. Each experiment for antimycotic activity was replicated three times in order to define the MIC values.

Determination of Minimal Inhibitory Concentration (MIC)

MIC values of Candida spp. were determined as described in the CLSI reference methods (NCCLS, 1997). The MIC was defined as the lowest concentration of antimicrobial in which no visible growth occurred after incubation at 37°C for 24 h. Fungal killing was determined by cultivating 10 µL of a suitable dilution, made from micro dilution wells, on Sabouraud dextrose agar plates and by counting colony forming units after overnight incubation at 37°C.

Antifungal agents

Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) was obtained as powders and dissolved in dimethyl sulfoxide to make a stock solution that can be held for 6 months at -70°C. Serial twofold dilutions of Amphotericin B was prepared following NCCLS guidelines (NCCLS, 1997). Final dilutions were made in Saboraud dextrose broth (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0.

Checkerboard assay

Checkerboard titration is one of the most preferred method for investigation the drug interactions. Checkerboard testing was performed using 96 well plates. The antifungal activities of a combination of this antifungal agent (Amphotericin B) were assessed by the checkerboard test and time-kill curve study. The checkerboard test was performed according to the method described by Chung (Chung et al., 1999). MIC values were determined for each drug by broth microdilution according to standards of the CLSI (Clinical Laboratory Standardization Institute) (NCCLS, 1997). Each combination assay for antimycotic activity was replicated at three times.

The antifungal combinations assayed included Hatay propolis plus Amphotericin B and Bursa propolis plus Amphotericin B. Serial dilutions of the propolis samples and Amphotericin B were made in Saboraud dextrose broth. Inocula were prepared from colonies grown on Saboraud dextrose agar after overnight culture. The fungal concentration after inoculation was 1x105 CFU/mL. After 24 hours of incubation at 37°C, the MIC values were determined. The fractional inhibitory concentration (FIC) was calculated for each combination that inhibited the growth using the following formula:

1. The FIC value for drug (agent) in a given well is derived by dividing the drug (agent) concentration in the given well by the control MIC of the test organism to that drug.
   \[ \text{FIC}_A = \frac{\text{MIC}_A\text{ combination}}{\text{MIC}_A\text{ alone}} \]  
   \[ \text{FIC}_B = \frac{\text{MIC}_B\text{ combination}}{\text{MIC}_B\text{ alone}} \]  

2. The FIC index value for a well is the sum of the FICs for each of the drugs present in the well:
   \[ \text{FIC}_{\text{index}} = \text{FIC}_A + \text{FIC}_B \]
Synergistic effect was defined as an FIC index of \( \leq 0.5 \). If FIC index is > 0.5 and \( \leq 1 \), it indicates an additive effect. If FIC index is of > 1 and \( \leq 2 \), it considered an indifference effect, and antagonism effect was defined as an FIC index of > 2.

**Statistical analysis**

The mean and standard deviation of at least three experiments were determined. Between-group comparisons were made using the Kruskal-Wallis and Mann-Whitney U-tests. P values of 0.05 or less were considered significant. All statistical analysis in the present study were performed using SPSS for Windows, version 11.5.

**RESULTS AND DISCUSSION**

In the DMSO control group, the yeast cell cultures were incubated with DMSO alone (without any supplement). Effects of DMSO on the viability of cells were compared to control cells. The DMSO control showed no toxic effect at 1% (v/v) for yeast cells. At this concentration of DMSO (Saboraud dextroz broth containing 1% DMSO) did not inhibit the growing of yeast cells. At the end of 48 hours, there was no statistically significant difference in cell number between the control and DMSO containing groups (p>0.05) (Figure 1).

In the experiments, both Bursa and Hatay propolis samples were found to exhibit the antifungal effect in vitro. The cell morphologies and viabilities of the candida spp. were evaluated in the presence of different concentrations of the propolis samples. In this study, the MIC values of Hatay and Bursa propolis samples were found to vary from 64 to 512 µg/mL. The mean quantitative fungal data are presented in Fig. 1 and 2.
Amphotericin B was used as a positive control. In the drug-control group, Candida strains cultured with Amphotericin B, the MIC values of Amphotericin B were determined as follows: for Candida albicans: 0.25 µg/mL; for Candida glabrata: 2 µg/mL; for Candida tropicalis: 1 µg/mL and for Candida parapsilosis: 4 µg/mL (Figure 2 and 3).

When comparing the negative control (without propolis) with the positive control (with Amphotericin B), it was found an important statistically decreasing in the drug group at the end of incubation period (p<0.001).

In checkerboard assay, two different propolis samples showed synergistic activity with the Amphotericin B against C. albicans, C.glabrata, C. tropicalis and C.parapsilosis. 128 µg/ml of Hatay propolis combined effectively with 64 µg/mL of Amphotericin B and showed strong antifungal activity against the isolate of C.parapsilosis (Fractional Inhibitory Concentration, FIC index = 0.1875), (Table 2).

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC values (µg/mL)</th>
<th>MIC for the combination of propolis and Amp.B (µg/mL)</th>
<th>FIC</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td></td>
<td>Hatay propolis</td>
<td>Amp. B</td>
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<td>Amp. B</td>
</tr>
<tr>
<td>C.albicans</td>
<td>256</td>
<td>2</td>
<td>64</td>
<td>0.25</td>
</tr>
<tr>
<td>C.glabrata</td>
<td>512</td>
<td>32</td>
<td>128</td>
<td>2</td>
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<tr>
<td>C.tropicalis</td>
<td>128</td>
<td>8</td>
<td>16</td>
<td>1</td>
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<tr>
<td>C.parapsilosis</td>
<td>128</td>
<td>64</td>
<td>16</td>
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Also, 128 µg/mL of Bursa propolis combined effectively with 64 µg/mL of Amphotericin B. A combination of these ratios showed stronger synergistic activity than Hatay propolis against C.parapsilosis (Table 3), (Fractional Inhibitory Concentration, FIC index = 0.125). Similarly, strong synergistic effects were obtained with the standard candida strains (data not shown).

Ethanolic extract of propolis samples showed good activity against C. albicans, C. parapsilosis, C.glabrata and C. tropicalis. Antifungal activity of Bursa propolis was similar to that observed for Hatay propolis against C.parapsilosis but better against C.albicans, C. tropicalis and C. tropicalis. FIC index for the combination of Bursa propolis and Amphotericin B was stronger than Hatay propolis against C.parapsilosis.
Table 3. Synergistic activity of Bursa propolis with Amphotericin B against Candida strains

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Bursa propolis samples were found to be more effective than Hatay propolis samples against yeast-like fungi. Similarly, in our previous study, Bursa propolis samples had more effective than Hatay propolis samples against leishmania promastigotes (Duran et al., in press). These kinds of components may be responsible for its antifungal activity.

CONCLUSIONS

In conclusion, Bursa and Hatay propolis samples have remarkable antifungal activity against C.albicans, C.glabrata, C.tropicalis and C.parapsilosis. Also, the strong synergistic activity between Amphotericin B and propolis (especially Bursa propolis) was found against the azole resistant candida strains. Propolis may be an important prospect in the treatment of azole-resistant yeast. Although the effects of these two kinds of propolis against the Candida spp. in vitro are promising, further investigation such as microbiological, pharmacological and clinical trials are required.

Acknowledgement

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REFERENCES

Duran, N., Muz, M., Culha, G. et al. (in press), “GC-MS analysis and antileishmanial activity evaluation of two Turkish propolis”, Parasitology res.