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In vitro Evaluation of the Antifungal Activity of Propolis Extract on *Cryptococcus neoformans* and *Candida albicans*

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The antifungal activities of propolis on *Cryptococcus neoformans* and *Candida albicans* were evaluated. In microbroth culture assay, the MIC (minimum inhibitory concentration) of propolis for *C. neoformans* and *C. albicans* were 2 and 16 mg/ml, respectively. In propolis-included solid medium assay, the MIC of propolis for *C. neoformans* and *C. albicans* were 4 and 16 mg/ml, respectively. Propolis showed fungicidal activity against *C. neoformans*, whereas propolis possesed fungistatic activity against *C. albicans*. The MFC (minimum fungicidal concentration) for *C. neoformans* was 8 mg/ml. Cell morphology of *C. neoformans* was affected by treatment of propolis. In scanning electron microscope, the appearance of cell rupture was observed.

KEYWORDS: Antifungal activity, *Candida albicans*, *Cryptococcus neoformans*, Propolis

Propolis is a plant resinous substances collected by bees for use in the hive. Bees utilize propolis in protecting their hives against invasion by other insects and weather (Thomson, 1990). Historically, propolis has been used by man for various purpose, and especially as a medicine because of its antimicrobial properties. In mid-century of Europe, there was a record, referring that propolis has been used for the treatment of mouth and thorat infection, and dental cares (Krell, 1996). Presently, in addition to antimicrobial activity, propolis were found to have several other medicinal properties such as anticancer, immunostimulating agent and wound healing effect (Matsuno *et al.*, 1997; Manolova and Maksimova, 1987; Krell, 1996). A large number of studies have been carried out about the effect of propolis on a variety of human pathogenic bacteria (Bankova *et al.*, 1995; Christov *et al.*, 1999). However, studies about the effects of propolis on medically important yeasts were limited. Holderna and Kedzia (1987) found that propolis showed synergistic effect on *Candida albicans* when used with other antibiotics in combination. Ota *et al.* (2001) studied the antifungal activity of Brazilian propolis on different species of *Candida* and demonstrated that the degree of inhibitory activity of propolis on Candida vary, depending on species. Hegazi *et al.* (1999) reported high activity of European propolis against *C. albicans*.

Cryptococcus neoformans is a yeast-like fungus which cause life-threatening meningoencrphalitis in immunocompromised individual, particularly AIDS patients. The treatments of existing drugs are known to be toxic and develop the drug-resistant strain of *C. neoformans*. Therefore, identification of a new therapeutic agent is important for prevention of disease.

In this study, we evaluated the antifungal activity of Korean propolis on *C. neoformans*, and compared it with that of *C. albicans*. This study is the first report on the antifungal activity of propolis against *C. neoformans*.

Materials and Methods

Propolis extract. Propolis extract was obtained from Honeybee World Co. in Korea. The preparation of propolis is described according to manufacturer's method. Propolis extract was made by extracting 400 g of natural propolis source in 1600 m*l* of 73% ethanol for the period of 30 days.

Antifungal test

Disc diffusion assay. *C. neoformans* ATCC 2344 and *C. albicans* KCTC 7965 were obtained from American Type Culture Collection (ATCC) and Korean Collection for Type Culture (KCTC), respectively. Strains were maintained on Sabouraud dextrose agar (SDA) at 26°C. Disc diffusion assay was carried out using SDA medium by the method of Bauer *et al.* (1966). Sterilized Whatman filter paper disc of 6 mm diameter were impregnated with 20 μl of various concentration of propolis extracts (2, 4, 8, 16, 32 mg/m*l*) and placed over the center of the surface of SDA plate seeded with yeast cells. The culture was incubated for 72 h at 37°C to obtain maximum growth in the culture media. The diameter of inhibition zone of growth subtracting the diameter of disc was measured to estimate the degree of antifungal activity of propolis.

In order to investigate the fungicidal activity of propolis, the whole surface of SDA was inoculated with yeast cell suspension using cotton swap. Plates were incubated

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at 37°C untill SDA was fully covered with yeast cells. Paper disc impregnated with propolis extract (32 mg/m*l*) was placed on the surface of SDA plate. Plate was incubated for 48 hr at 37°C and observed for the appearance of clear zone around paper disc.

Microbroth culture assay. For inoculum preparation of *C. neoformans* and *C. albicans*, one loop of colony from 3 day-old culture was suspended in malt extract broth. A concentration of inoculumn cell suspension was adjusted to 1×10^6 cells/m*l* by cell counting. Eight hundreds microliter of malt extract broth was dispensed into each well of 24-well plates. Then each well was inoculated with 100 μl of inoculum suspension prepared as above. One hundred microliter of propolis extract diluent was added to each well at a final concentration from 1 to 64 mg/m*l* (two-fold dilution). Plates were incubated at 37°C for 48 h. As a control, ethanol diluent was added to each well instead of propolis. Inhibition of growth was determined by counting cell number. The MIC (minimum inhibitory concentration) reading criteria was the lowest concentration that caused 100% inhibition of growth. For the measurement of minimum fungicidal concentration (MFC), 50 μl of broth taken from each tubes in the above static test were subcultured onto SDA without propolis for 72 h. The MFC was defined as the concentration at which no growth was observed after subculture.

Propolis-included solid medium assay. Propolis extract was incorporated into SDA to obtain a dilution from 1 mg to 64 mg of propolis per ml. Each plate was inoculated with 20 μl of yeast cell suspension and incubated for 72 h at 37°C. The results were described as MIC.

Scanning electron microscope of propolis-treated cells. Cell suspension of *C. neoformans* was treated with propolis and incubated for 48 h at 37°C. After harvesting cells, samples were prefixed in 1.5% glutaraldehyde in potassium phosphate buffer (pH 6.0) for 2 h, and washed with phosphate buffer. Samples were dried in a critical point dryer. After coating samples with fine gold particles, samples were scanned using SEM (S-2500C, Hitachi, Japan).

Result and Discussion

The susceptibility of *C. neoformans* and *C. albicans* to propolis was evaluated in solid and liquid culture. In a disc diffusion assay, both *C. neoformans* and *C. albicans* were sensitive to propolis. As the concentration of the propolis extract loaded on the disc increased, the diameter of the zone of inhibition around the paper disc also increased (Fig. 1). *C. neoformans* was more sensitive to propolis than *C. albicans*.

In a microbroth culture assay, MIC value of propolis

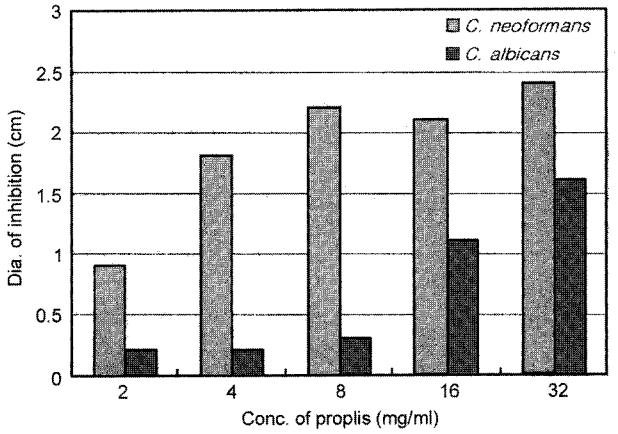

Fig. 1. Disc diffusion assay of propolis against *C. neoformans* and *C. albicans*.

for *C. neoformans* and *C. albicans* were 2 mg and 16 mg/ml, respectively. In propolis-included solid medium assay, MIC value of propolis for *C. neoformans* and *C. albicans* were 4 and 16 mg/m*l*, respectively. From these results, we observed that MIC vaue of *C. neoformans* was significantly higher than that of *C. albicans*.

In order to investigate the fungicidal activity of propolis, paper disc impregnated with propolis extract was placed on the surface of SDA covered with fully grown *C. neoformans* or *C. albicans*. After 24 h incubation, clear zone was observed around paper disc in *C. neoforamns* whereas clear zone was not formed in *C. albicans*.

In a microbroth culture, when compared with the inoculated initial cell number, the cell number of *C. neoformans* was significantly reduced in propolis-treated broth culture after 24 h incubation whereas the cell number of *C. albicans* was not significantly changed at 4 and 16 mg/m*l*, respectively (data not shown). These results demonstrated that propolis possess fungicidal activity rather than fungistatic activity. In assay for determining MFC, the MFC of propolis for *C. neoformans* was 8 mg/m*l*.

We also found that cell morphology of *C. neoformans* was affected by treatment of propolis. Under SEM, the appearance of cell rupture was observed (Fig. 2). Takaisi and Schilcher (1994) suggested that inhibition of cell division was the possible mechanism of the antimicrobial action of propolis. In this study, however, our results showed that *C. neoformans* was killed rather than inhibited by propolis.

Ota *et al.* (2001) tested antifungal activity of Brazilian propolis against several different species of *Candida* and observed that *C. albicans* was the most sensitive strain. Holderna and Kedzia (1987) found that combinations of antibiotics with propolis was capable of increasing their effrect on *C. albicans*. It has been reported that antimicrobial activity of propolis varied according to the propolis origin. Since bees are collecting propolis from a variety of

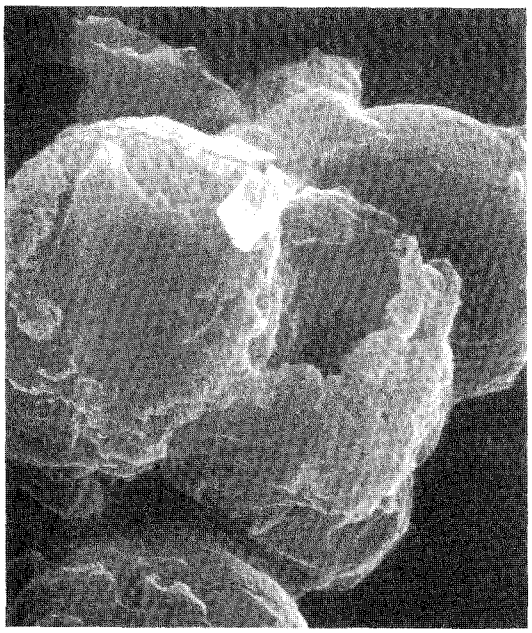

Fig. 2. Scanning electron microscopic structure of cell surface of *C. neoformans* after propolis treatment. Apperance of cell rupture was observed (×5,000).

plant species, it is very likely that propolis prepared from different locations in same country as well as from different countries may be different in their qualitative and quantitative chemical compositions. In this study, Korean propolis possesses both anti-cryptococcus and anti-candida activity. Especially, the propolis extract possesses potent anti-cryptococcal activity. The antifungal activity of propolis against *C. neoformans*, therefore, could provide novel therapeutic tool for the immunocompromised pateints with meningititis since propolis has been widely used for the treatment of several disease as a ethnomedicine and has not been known to contain toxic effect. At present, an investigation into the anti-cryptococcal effect of propolis on experimental animal is in progress.

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