Abstract: The aim of the present study was to compare the effects of some disinfectants, including ethanol extract of propolis (EEP), on the adhesion of Candida albicans to denture base resins. Seventy-two acrylic resin samples were prepared, half of which was polished and the other half was roughened. C. albicans strain ATCC 10231 was incubated on Sabouraud dextrose agar (SDA) at 37°C for 48 h. The adhesion period was completed by keeping the cells in this suspension for 90 min at 37°C. Specimens were then immersed in the following solutions: 1%, 2%, and 5% sodium hypochlorite; 4% chlorhexidine gluconate; and 10% EEP. Quantification of the antifungal activity of the chemical solutions was performed using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay test. One-way ANOVA and post-hoc Tukey tests were performed to evaluate the effectiveness of chemical agents. Polished and roughened surfaces were compared using independent sample t-test. The mean surface roughness value was 0.35 (±0.04) µm for the polished group and 1.2 (±0.2) µm for the roughened group. The contact angles of both surfaces showed statistically significant difference, and 10% EEP solution exhibited significantly less removal of adherent viable C. albicans cells in both groups. All forms of sodium hypochlorite solutions yielded higher efficiency than 4% chlorhexidine gluconate and EEP solutions ($P < 0.05$). (J Oral Sci 58, 431-437, 2016)

Keywords: Candida albicans; sodium hypochlorite; chlorhexidine; propolis.

Introduction

Various epidemiological studies have reported that denture stomatitis is one of most common fungal infections amongst denture wearers, with incidence rates varying between 11-67% (1). Candida albicans, which has the ability to adhere and proliferate on both soft and hard tissues (2), has been widely associated with the etiology of denture stomatitis (3,4). Since adherence of C. albicans to polymethyl methacrylate (PMMA) resin surfaces is an essential and necessary step for successful colonization and development of pathogenesis, (3) treatment of denture stomatitis should target reduction of initial fungal adherence (5).

It has been stated that microbial adherence of C. albicans to the PMMA surface may be attributed to non-specific interactions (electrostatic or hydrophobic interactions) initially, followed by subsequent specific interactions (receptor ligand binding) (6,7). Although some studies have suggested that the adhesion ability of yeasts to polymeric surfaces is modulated by hydrophobic forces, others found no correlation between hydrophobicity and Candida adhesion (8-10). It has also been reported that an increase in surface roughness enhances C. albicans retention (11-14) as it increases the surface area and/or shear forces (5), thus allowing easier
microbial attachment and difficult detachment even when the prosthesis has been cleaned with antimicrobial agents (10). However, most of these studies focused on the adherence of *C. albicans* to rough denture surfaces (3,11-13), but failed to investigate the efficiency of antimicrobial agents in removing yeasts from these surfaces. Problems related to *C. albicans* adherence can be minimized by using mechanical and/or chemical disinfection procedures on the dentures (15). Disinfection with chemical solutions is especially mandatory for geriatric or handicapped denture wearers with compromised manual dexterity (16,17). In fact, it has been recommended as an essential procedure for prevention of cross-contamination and maintenance of a healthy oral mucosa (18).

Various disinfectants have been suggested for this purpose including sodium hypochlorite solutions and chlorhexidine, which have been reported to efficiently reduce adhesion of *Candida* species and microbial growth on dental prostheses (18). Sodium hypochlorite-based denture cleansers are fungicidal, and are known to dissolve mucin and other organic substances easily (19). At low concentrations, chlorhexidine accumulates on the cell surfaces of yeasts, causing cell membrane disorganization and leakage of cytoplasmic components, while higher concentrations produce coagulation of cytoplasmic constituents in those microorganisms (15).

On the other hand, chemical solutions may also have undesirable effects such as changes in color of dentures, increased roughness, and reduction in hardness of the denture acrylic resin surface (20,21). Furthermore, immersion of dentures in chemical solutions may lead to absorption of these liquids by the acrylic resin, which can then be released into the oral cavity (22) and leave an unpleasant residual taste (23). Therefore, chemical solutions are not recommended for daily prosthesis disinfection, and natural products have become increasingly popular as an alternative for the prevention of oral diseases. Of these, Propolis, which is a resin extracted from plants by bees (7,24), is considered to be the most promising option. Its various biological activities and therapeutic properties have recently become the focus of research (25), with several studies reporting the inhibitory effects of ethanol extract of propolis (EEP) on *Candida* species (7,24-26). Santos et al. stated that 10% EEP was as effective as nystatin in the treatment of patients with denture stomatitis (26). However, the effectiveness of EEP as a disinfectant solution for dentures is still unknown.

Thus, the aim of the present study was to compare the effects of various disinfectants, including EEP, on the adhesion of *C. albicans* to polished or roughened denture base resin surfaces.

**Materials and Methods**

**Specimen preparation**

Stainless steel molds measuring 10 mm in diameter and 2 mm in thickness were used to prepare wax patterns of the test samples. They were flasked conventionally and the wax patterns were eliminated. Thereafter, the plaster surfaces were painted with a separating medium, and the denture base resin (Acron Duo, Associated Dental Products Ltd., Swindon, UK) was packed into the molds after mixing according to the manufacturer’s instructions. The resin was processed at 65°C for 60 min and 100°C for 30 min and then allowed to cool at room temperature for 8 h. To simulate clinical conditions, half of the acrylic resin specimens were polished to resemble the upper surface of dentures by manual abrasion with emery paper (400 grit) in a figure-of-eight motion 10 times and then polished for 60 s using a brush disc and felt cone with pumice slurry. The other half of the specimens were roughened to simulate the inner tissue-fitting surfaces of dentures with the help of a metal burr and manual abrasion with emery paper (400 grit). The speed of the hand piece was maintained at 15,000 rpm, and a total of 72 specimens were prepared, with six specimens of each surface texture in each experimental group (Table 1). The acrylic resin specimens were stored for 24 h in sterile distilled water at 37°C to remove any residual monomer.

**Surface roughness measurement**

The surface roughness (Ra) of each polished/roughened PMMA disc was measured with a profilometer (Perthometer M2, Mahr GmbH, Göttingen, Germany). The stylus of the profilometer passed along the specimen surface in a line, and the arithmetic roughness average (Ra) was calculated in microns. This procedure was repeated twice on different areas of the resin surface, and the mean of the two readings were used for data analysis. The test conditions used were as follows: cut off length = 0.8 mm; stylus speed = 0.25 mm/s; and diamond stylus tip radius = 5 µm. All measurements were carried out by the same investigator.

**Contact angle measurements**

The contact angle of the specimens was measured using the sessile drop method along with the drop shape analysis system (DSA 100, Krüss GmbH, Hamburg, Germany). A 20 µL droplet of distilled water was delivered on to the surface of specimens using a micro-syringe, and a high-speed camera was used to record changes in its
contour. The contact angle was measured using the image of a sessile drop, and at the point of intersection between the drop contour and the projection of the surface. Each specimen was measured three times and the mean value calculated.

Prior to microbiologic evaluation, the acrylic specimens were sterilized in an autoclave for 18 min under 120 kPa at 121°C (Charisma vacuum TD, Mediline, Cavriago, Italy).

Yeast preparation and adhesion

*C. albicans* strain ATCC 10231 was incubated on Sabouraud dextrose agar (SDA) at 37°C for 48 h. Standard amounts of this culture were then inoculated into 2 mL of liquid SDA and incubated at 37°C for 24 h. The culture was then centrifuged (IKA KS 4000i, IKAWerke GmbH, Staufen, Germany), and the resultant cell pellets were washed twice with phosphate-buffered saline (PBS) solution. After dilution with this solution, a final yeast suspension was standardized to a concentration of $1 \times 10^6$ cell/mL.

**Adhesion Assay**

Two mL of the standardized *C. albicans* cell suspension was added to each well containing the specimen. The adhesion period was completed by keeping the cells in this suspension for 90 min at 37°C, after which the non-adherent cells were removed by gently washing the specimens twice with 2 mL PBS.

**Chemical disinfection procedures**

A 2 mL solution of each agent was prepared, and six polished or roughened specimens were immersed in each of the experimental groups, as follows: 1% sodium hypochlorite (Aklar Kimya, Ankara, Turkey); 2% sodium hypochlorite (Aklar Kimya); 5% sodium hypochlorite (Aklar Kimya); 4% chlorhexidine gluconate (Marmara Ecza, Istanbul, Turkey); and 10% EEP (Ordu Institute of Apiculture, Ordu, Turkey). The specimens immersed in Phosphate Buffered Saline (PBS) (Department of Biology, Hacettepe University, Ankara, Turkey) were considered as the control group. The specimens were kept in the solutions at 37°C for the recommended disinfection time of each chemical agent, and then washed with PBS.

**Preparation of ethanol extract of propolis solution**

Crude propolis of Apis mellifera bees was obtained, dried, and then ground into fine powder. Twenty g of fine propolis powder was then mixed with 180 mL ethanol at a concentration of 80% in a test tube for 24 h. An EEP solution [10% (w/v)] was obtained using methods previously suggested by Koo and Park (27), and the mixture was centrifuged in order to obtain the supernatants. This suspension was kept in the dark for 7 days at room temperature.

**MTT assay**

Quantification of the antifungal activity of the chemical solutions was carried out using the colorimetric MTT assay test [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide). This test measures cellular metabolic activity using a yellow soluble tetrazolium salt, which is converted into a soluble purple crystal by metabolically active cells. Twenty μL of MTT reagent was added to each well and incubated at 37°C for 4 h in the dark. Thereafter, 200 μL of solubilization solution was added into each well, and the colorimetric change of the supernatant was determined at 550 nm after 15 min using an ELISA Reader (ER 2005, Equipar Diagnostic, S.r.l., Saronno, Italy).

**Statistical analysis**

All data were analyzed using a statistical package (SPSS 16.0, SPSS Inc., Chicago, IL, USA). Data collected from the adherent yeast cells were logarithmically transformed to the base 10. Kolmogorov Smirnov test was used to assess the normality of data, and Levene’s test was used to test homogeneity of variance. The data for *C. albicans* adherence was found to be normally distributed and exhibit homogeneity of variance ($P > 0.05$). Therefore, one-way ANOVA and post-hoc Tukey tests were performed to evaluate the effectiveness of chemical agents on the adherence of *C. albicans*. The comparison of chemical agent efficiency between polished and roughened surfaces was carried out using independent sample t-test. All statistical analyses were performed at a significance level of 0.05. The difference in contact angle measurements between polished and roughened surfaces was analyzed using one-way ANOVA. Considering the limited number of specimens in each group, a post-hoc

| Table 1 Experimental groups |
|-----------------------------|
| Surface texture | Immersion time (min) |
| Polished | Roughened | 10 |
| 5% Sodium hypochlorite | 5% Sodium hypochlorite | 10 |
| 2% Sodium hypochlorite | 2% Sodium hypochlorite | 5 |
| 1% Sodium hypochlorite | 1% Sodium hypochlorite | 5 |
| 4% Chlorhexidine gluconate | 4% Chlorhexidine gluconate | 10 |
| 10% EEP | 10% EEP | 15 |
| PBS (Control) | PBS | 10 |

$n = 6.$
Surface roughness and contact angle measurements of both surface textures are shown in Table 2. The mean surface roughness value (Ra) was 0.35 (±0.04) µm for polished acrylic resins and 1.2 (±0.2) µm for the roughened surface group. The contact angles of both surfaces were significantly different, with values of 58.15 ± 2.17° for polished acrylic resins and 71.1 ± 1.42° for roughened acrylic resin surfaces, respectively. This was relatively more hydrophobic properties of these surfaces along with higher contact angles (71.1 ± 1.42). This was observed in the efficiency of all disinfection protocols in removing adhered C. albicans cells with propolis solution compared to polished acrylic resin surfaces is an important initial step of microbial colonization and subsequent invasion of host surfaces (29), and irregularities on these surfaces can increase the accumulation of microorganisms even after the prosthesis has been cleaned (13). Therefore, the roughness of PMMA surfaces may promote the rate of microbial colonization (30). In agreement with these studies, the results of the present study indicate that roughened specimens with higher surface roughness values showed higher counts of adherent viable C. albicans cells than the polished specimens in the control group.

The microbial adhesion ability of C. albicans to acrylic resin surfaces is also associated with the hydrophobicity of the microorganisms (8,31). In the presence of more hydrophobic surfaces, attractive hydrophobic interactions result between the resin surface and C. albicans (31). In the present study, increased C. albicans adherence to roughened surfaces may be attributed to relatively more hydrophobic properties of these surfaces along with higher contact angles (71.1 ± 1.42). This was

### Table 2 The mean values of contact angle and surface roughness of polished and roughened acrylic resin specimens

|                        | Polished | Roughened |
|------------------------|----------|-----------|
| Contact angle (°)      | 58.15    | 71.1      |
| Surface roughness (Ra) | 0.35     | 1.22      |

### Table 3 The mean value (standard deviation) of adherent C. albicans after the application of tested disinfectant solutions

| Chemical solutions | PBS | 10% EEP | 4% Chlorhexidine gluconate | 1% Sodium hypochlorite | 2% Sodium hypochlorite | 5% Sodium hypochlorite |
|--------------------|-----|---------|----------------------------|------------------------|------------------------|------------------------|
| Polished           | 0.22 (0.07) | −0.90 (0.06) | −1.66 (0.02) | −1.84 (0.07) | −1.83 (0.06) | −1.85 (0.13) |
| Roughened          | 0.35 (0.05) | −0.85 (0.11) | −1.59 (0.04) | −1.82 (0.05) | −1.79 (0.05) | −1.95 (0.08) |

n = 6. Same superscript letters indicate no statistically significant difference (P > 0.05, Tukey test).

### Results

Surface roughness and contact angle measurements of both surface textures are shown in Table 2. The mean surface roughness value (Ra) was 0.35 (±0.04) µm for polished acrylic resins and 1.2 (±0.2) µm for the roughened surface group. The contact angles of both surfaces were significantly different, with values of 58.15 ± 2.17° and 71.1 ± 1.42° for polished and roughened surfaces, respectively.

The mean adherent viable C. albicans counts are shown in Table 3. C. albicans adhesion on roughened surfaces was statistically higher than polished surfaces in the control group (P < 0.05). Comparison of the polished and roughened surface with regard to the effectiveness of each disinfection agent showed no differences, except for 4% chlorhexidine gluconate which resulted in lower adhesion of C. albicans to polished surfaces than roughened surfaces (P < 0.05). Significant differences were observed in the efficiency of all disinfection protocols in removing adhered C. albicans between the polished and roughened surface groups [P < 0.05, f and df (degree of freedom) values of polished groups: 539.7 and 5, respectively; f and df (degree of freedom) values of roughened groups: 781.8 and 5, respectively]. Post-hoc analysis revealed significantly decreased removal of adherent viable C. albicans cells with propolis solution compared to the other chemical solutions in both groups. All forms of sodium hypochlorite solutions yielded higher efficiency than 4% chlorhexidine gluconate and propolis solutions (P < 0.05), and 5% sodium hypochlorite was found to be the most efficient in both surface textures. However, it was only statistically more effective than 2% sodium hypochlorite in the roughened surface group (P < 0.05), while no significant differences between all forms of sodium hypochlorite solutions were observed in the polished surface group (P > 0.05).

### Discussion

Numerous studies have reported that C. albicans is one of the most important etiological agents in the pathogenesis of denture stomatitis (28). In addition, ill-fitting denture bases may act as reservoirs for infections, and insufficient hygiene may contribute to oral candidiasis (29). Moreover, it has been pointed out that the adhesion ability of C. albicans to acrylic resin surfaces is an important initial step of microbial colonization and subsequent invasion of host surfaces (29), and irregularities on these surfaces can increase the accumulation of microorganisms even after the prosthesis has been cleaned (13). Therefore, the roughness of PMMA surfaces may promote the rate of microbial colonization (30). In agreement with these studies, the results of the present study indicate that roughened specimens with higher surface roughness values showed higher counts of adherent viable C. albicans cells than the polished specimens in the control group.

The microbial adhesion ability of C. albicans to polymeric surfaces is also associated with the hydrophobicity of the microorganisms (8,31). In the presence of more hydrophobic surfaces, attractive hydrophobic interactions result between the resin surface and C. albicans (31). In the present study, increased C. albicans adherence to roughened surfaces may be attributed to relatively more hydrophobic properties of these surfaces along with higher contact angles (71.1 ± 1.42). This was
in accordance with Minagi et al. who observed a nearly linear relationship between the adherent C. albicans cell number and contact angle measurement (9).

In both surface texture groups, all forms of sodium hypochlorite solutions were found to be more efficient than the other disinfectants used. This was in agreement with the results of many previous studies where sodium hypochlorite solutions effectively reduced C. albicans adhesion (32,33). Moreover, higher efficiency of sodium hypochlorite solutions in the removal of Candida species have been also verified by several studies using scanning electron microscope (SEM) analysis (34,35). Although 5% sodium hypochlorite appeared to be more effective than the 2% and 1% sodium hypochlorite solutions in both groups, a statistical difference was only observed in roughened specimens immersed in 2% sodium hypochlorite (P < 0.05). In the polished group, the efficiencies of different concentrations of sodium hypochlorite solutions did not differ significantly (P > 0.05). In both surface texture groups, 2% sodium hypochlorite exhibited the lowest efficiency, and this may have been because the immersion time was only 5 min for 2% sodium hypochlorite and 10 min for 5% sodium hypochlorite. Longer immersion period in 2% sodium hypochlorite may lead to better efficiency.

Sodium hypochlorite solutions exhibited higher efficiency than 4% chlorhexidine, and this may be related to their different effect mechanisms. The antimicrobial activity of sodium hypochlorite is based on its higher pH >11 (36). Schwartz stated that a solution with a pH between 7 and 11 reduces the majority of microorganisms within an immersion time of 10 min (37). The high pH of sodium hypochlorite compromises cytoplasmic membrane integrity with irreversible enzymatic inhibition, biosynthetic alterations in cell metabolism, and phospholipid destruction observed in lipidic peroxidation (34,38). Deviations in the pH of sodium hypochlorite may lead to changes in environmental conditions (39) which, in turn, affect microbial metabolism as well as the surface properties of the microorganisms and solid surfaces. Thus, it is able to increase the electrostatic repulsion between the two units, thus disrupting the adhesion of microorganisms to the surfaces (15).

On the other hand, the antifungal influence of 4% chlorhexidine gluconate is based on the binding of positively charged molecules of chlorhexidine to the negatively charged bacterial cell surfaces, particularly the phosphate groups in lipopolysaccharides and the carboxyl groups in proteins (40). This alters the integrity of the bacterial cell membrane, and chlorhexidine is attracted towards the inner cell membrane. It then binds to phospholipids in the inner membrane, leading to increased permeability and leakage of low-molecular-weight components such as potassium ions. This, in turn, induces structural changes in the cytoplasmic membrane. As the concentration of chlorhexidine increases, leakage of low-molecular-weight cytoplasmic components falls, reflecting coagulation and precipitation of the cytoplasm by the formation of phosphate complexes such as adenosine triphosphate and nucleic acids (41). In contrast to sodium hypochlorite, chlorhexidine is at a neutral pH, which helps maintain the dead cells on the surfaces of acrylic resins. However, the exact mechanism of chlorhexidine is still unclear (42). Jones reported that it can modify the cell surface hydrophobicity of Candida (43). In agreement with the present results, Silva et al. suggested that sodium hypochlorite solutions should be preferred over 4% chlorhexidine gluconate as a denture disinfecting agent. Furthermore, they also used SEM analysis to show that sodium hypochlorite solution not only killed C. albicans biofilms but also removed them from the heat polymerized acrylic resin (15).

In the present study, sodium hypochlorite solutions exhibited the same efficiency in both polished and roughened PMMA surfaces. This result is in good agreement with that of Chao et al. who reported that 5% sodium hypochlorite for 10 min immersion time was effective on the polished surfaces as well as the roughened (inner) surfaces (42). In contrast to the results obtained with sodium hypochlorite solutions, 4% chlorhexidine gluconate solution yielded higher efficiency in the polished group compared to the roughened surface group. This may be related to the action mechanism of chlorhexidine stated before, wherein the irregular pattern of the surface texture prevented the chlorhexidine molecules from reaching the candida cells.

The 10% EEP solution appeared to be effective in controlling C. albicans adhesion in both polished and roughened surface groups. However, unlike sodium hypochlorite and 4% chlorhexidine gluconate solutions, it was unable to completely eliminate them from the acrylic surfaces. Several studies have reported the antifungal activity of propolis (26,27), and it has also been stated that its mechanism may be related to the presence of flavonoids, phenolic acids, and their esters (24). Takaisi-Kikuni and Schilcher claimed that propolis brought about inhibition of DNA replication and, indirectly, cell division (44). Moreover, it disorganized the cytoplasm, cytoplasmic membrane, and cell wall, and also inhibited protein synthesis (41). Silva et al. evaluated the effects of 10% EEP on acrylic resin surfaces, and reported an increase in the hardness of resin accumulating on the
surface. They proposed that this could act as a barrier for adhesion of Candida species (7).

In the present study, the most commonly used concentration of propolis (10% EEP) was selected for testing (7,25,26), and its effectiveness was found to be limited when compared to the other chemical disinfectants. Further studies should evaluate the effectiveness of higher concentrations with longer immersion periods. Nevertheless, it should be noted that propolis is a natural, biological material, and an oral rinse form of it may be used along with other disinfectants for the rehabilitation of oral candidiasis.

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Conflict of interest
The authors declare that this research is not supported by any projects.

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