In vitro activity of terpenes against *Candida albicans* and ultrastructural alterations

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Objective. The purpose of this study was to investigate the *in vitro* activity of terpene blends combined with tissue conditioner against *Candida albicans* and the effect on its morphology and sub-micro structure.
Study Design. The minimal inhibitory concentration (MIC) of terpenes, obtained from a by-product of kraft pulping, was determined using broth microdilution against *C. albicans* strains, and the activity of terpenes combined with Coe-Comfort tissue conditioner was assessed. Cell morphologic alterations were evaluated using scanning electronic microscopy and transmission electronic microscopy. Data was analyzed using Student's t test $P < .05$.
Results. The MIC of terpene blends fluctuated between 0.097% and 0.39% (v/v). Coe-Comfort tissue conditioner mixed with terpenes exhibited a total inhibition of *C. albicans* ($P < .05$). Terpenes induced ultrastructural alterations, even at the MIC value, including an increase in size, shape modification, cell wall damage with perforations, pronounced disconnection between cell wall and cytoplasm, and cytoplasmic vacuoles.
Conclusions. Terpenes had pronounced effects against *C. albicans* alone and in combination with Coe-Comfort tissue conditioner, which mainly resulted in cell wall damage. (Oral Surg Oral Med Oral Pathol Oral Radiol 2014;118:553-559)

Candida albicans is identified as the main pathogen in denture stomatitis (DS), an inflammatory reaction of the palatal and alveolar mucosa underlying removable dental prosthesis.[1,2] Different methods of treatment for DS have been proposed, including antifungal agents, relining with tissue conditioners, and tissue conditioners mixed with antifungal agents.[3-6] Moreover, *C. albicans* is the most common agent associated with biofilm-related infections, and this yeast proliferates in resistant biofilms.[2,7-9] The antifungal resistance of biofilms is primarily attributed to an increase in cell number and changes in the genetic, physiologic, and molecular characteristics of *C. albicans* in the biofilm and, secondarily, to restricted penetration of the drug inside the exopolymer matrix or binding of the drug to the biofilm matrix.[8,9] Persistent usage of azole antifungals, which target enzymes involved in ergosterol synthesis, consistently results in the development of multi-drug resistance in *C. albicans*.[8,10,11] Thus, the search for new therapeutic agents is needed because of the increased resistance to conventional antifungal therapy.

Plants provide an excellent source of innovative therapeutic agents that exhibit various effects, including antifungal and antibacterial effects.[6,12-22] The plant byproduct terpenes consist of the combination of several 5-carbon-base units called isoprene and have demonstrated a variety of biological activities, including antifungal, antibacterial, antiviral, and anti-inflammatory activities.[6,12,13-17,19,21] Previous studies using scanning electron microscopy and transmission electronic microscopy have demonstrated that essential oils containing terpenes affect the morphology of *C. albicans* and induce total destruction of fungal cells.[16-18,20,21,23] Changes in permeability and membrane fluidity cause degradation of the cell wall, a decrease in adherence to host surfaces and variable effects, such as the disruption of the cytoplasmic membrane, leakage of cell contents, and coagulation of cytoplasm and cell lysis.[15,18,20,21,23] In addition, terpenes inhibit the respiration of *Candida*, thus resulting in mitochondrial damage.[12]

Pulp mill is one of the most important industries in Chile. One by-product of the kraft pulping process from pinewood is crude sulfate turpentine (CST), which mainly consists of the monoterpenes α-pinene and β-pinene, with lesser amounts of 3-carene, camphene, and dipentene. CST is a cheap and abundant raw material, but its applications are limited because of its contamination with odorous sulfur compounds. In this *in*

Statement of Clinical Relevance

This *in vitro* study shows that the terpenes mixed with Coe-Comfort could be an interesting alternative for treatment of denture stomatitis.

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vitro study, a chemical process resolved this problem, with the main component of the terpenes blend employed in this research being α-terpineol, an ingredient used in decorative fine fragrances, shampoo, toilet soaps.[24]

The toxicity of α-terpineol in animals has been extensively studied.[24-26] The lethal dose (LD_{50}) has been determined by different administration ways like oral exposure (LD_{50} = 4.3 g/kg in rat), dermal contact (LD_{50} = 3.0 g/kg in rabbit), subcutaneous studies (LD_{50} = 1.36 g/kg in mice) and intraperitoneal administration (LD_{50} = 0.260 g/kg in mice). Another important toxicologic test is the irritation assay, performed on rabbit eye (Draize test) and human skin. In the Draize test with 12.5% terpineol, an intense conjunctiva irritation without corneal opacity or iris congestion was observed. The conjunctiva irritation was reversible, disappearing on day 7.[24] Dermal irritation of α-terpineol was determined in human volunteers at a concentration of 20% terpineol, in which no irritation was observed.[27] Another study with 12.5% terpineol in 87% alcohol applied in patches on the skin resulted in little or no irritation.[26] Also, the testing of terpineol in rats showed a negative response for sensitization, in the popliteal lymph node assay.[25]

In as much as the safety of terpenes at low doses has been previously demonstrated, the aim of this study was to investigate the *in vitro* activity of terpene blends combined with tissue conditioner against *C. albicans* and its effect on the morphology and sub-micro structure.

METHODS

Candida isolates

The isolates of *C. albicans* (strains B and E) used in this study were obtained from the palatal mucosa of 2 patients with DS type II examined at the School of Dentistry of the Universidad de Concepción (Chile). Isolation was performed on Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, United Kingdom) at 37 °C, and the strains were stored at −80 °C in medium containing 40% glycerol. Isolates were identified by culture on CHROMagar™ Candida (Difco, Probac, France), a germ tube test, and chlamydospores formation on corn meal agar and growth at 42 °C. *C. albicans* ATCC 10231 was used as the control strain.

Informed consent was obtained from all patients. The Ethics Committee of the University of Concepción, Chile, approved this study.

In vitro antifungal activity testing

Terpenes. Crude sulfate turpentine was obtained from pulp mills in the VIII Region, Chile. The samples were treated by fractional distillation to separate the components and to remove the top of the column body volatile sulfur components. Alpha and β-pinene were separately subjected to hydration reactions in an acid medium, which produced terpene alcohols and dipentene. These components were also purified by fractional distillation, and blends containing terpene alcohol were prepared in a range between 40% and 98%.

The antifungal activity of 6 terpene blends was determined using broth microdilution, according to the Clinical Laboratory Standards Institute M27-A3 document (2008).[28] Briefly, serial two-fold dilutions of terpene blends were prepared in RPMI 1640-MOPS medium (Sigma, St. Louis, MO, USA) buffered to pH 7.0. One-hundred-microliter aliquots were added to 96-well microtiter plates to obtain final concentrations ranging from 0.025% to 1.56% (v/v). To enhance the compound solubility, Tween 80 was included in all assays at a final concentration of 0.05% (v/v). An inoculum of 2.5×10^3 CFU/mL (colony-forming units/mL) was prepared, and 100 μL of the suspension was added to each well. Plates were incubated at 37 °C for 48 hours. The lowest concentration that prevented visible growth was considered as the minimal inhibitory concentration (MIC).

In vitro activity of terpenes in combination with Coe-Comfort tissue conditioner on C. albicans. Coe-Comfort tissue conditioner (GC American Inc. Alsip, IL) was mixed according to the manufacturer's instructions (6 g/5 mL). Adequate volumes of Coe-Comfort liquid were replaced by a terpene blend to prepare tissue conditioners containing concentrations of terpene corresponding to 1 MIC, 2 MIC, 3 MIC, 4 MIC, and 5 MIC. After homogenization in a sterile glass beaker for 30 seconds, the conditioning powder was added and mixed for 40 seconds and poured onto a Petri dish. Once the mixture was solidified, the surface was covered with a thin layer (about 4 mm) of Sabouraud dextrose agar, and 50 μL of a suspension containing approximately 10^3 CFU/mL of *C. albicans* was subsequently spread over the agar. The plates were incubated at 37 °C for 48 hours, and the CFU were quantified. The experiment was performed in triplicate. Sabouraud agar plates were used as the control group.

In vitro activity of nystatin in combination with tissue conditioner Coe-Comfort on C. albicans. Volumes of 1 mL and 2 mL of nystatin (100,000 UI, Micostatin, Bristol-Myers Squibb, Mexico DF, Mexico) were mixed with Coe-Comfort liquid (4 mL and 3 mL). Next, Coe-Comfort powder (6 g) was added. The solution was thoroughly mixed, and the tissue conditioner was spread evenly onto sterile plastic Petri dishes to proceed as previously described.

Analysis of Cell Morphology using scanning electronic microscopy

After exposure to the experimental conditions, a sterile scalpel blade was used to remove a 15×15 mm piece of