Morphological and ultrastructural changes induced by essential oil of *Melaleuca alternifolia* on drug-sensitive and drug-resistant strains of *Candida albicans*

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Summary

The essential oil of *Melaleuca alternifolia* (Tea Tree Oil: TTO) exhibits broad-spectrum antimicrobial activity. Here, we report the effects of TTO treatment against the yeast *Candida albicans*.

Previous proliferation study showed that TTO was effective both on fluconazole-susceptible (3153) and on resistant isolate (AIDS68) strains of *C. albicans*. The cells of the two strains were treated with 1% TTO. The analysis was carried out by transmission and scanning electron microscopy to assess the changes induced by treatment both on the wall and the cell membrane of yeasts.

Key words: *Candida albicans*, natural products, multidrug resistance, tea tree oil, electron microscopy.

Introduction

Infectious diseases are caused by bacteria, viruses, parasites and fungi, and they are due to a complex interaction between the pathogen, host and the environment. The discovery of antibiotics had controlled the infections that once ravaged the humankind. But their indiscriminate use has led to the development of multi-drug resistant pathogens. Besides the most common bacterial infections, there is high prevalence of fungal infections, the majority of which are caused by *Candida spp*. In fact, it is an important human pathogen in hosts with various risk factors which compromise their immune competence. Special host settings with high morbidity and mortality rate of fungal infection are cancer patients, transplant recipients (particularly stem cell transplant) and human immunodeficiency virus (HIV)-infected patients. Other severe risk factors for invasive candidiasis are the ever increasing use of broad-spectrum immune-suppressive and antibiotic therapies. Candidiasis is the most common invasive fungal infection in ill non-neutropenic and neutropenic patients (Eggimann *et al.*, 2003). In Italy and the USA, *Candida* is the third or fourth most common isolate in nosocomial bloodstream infections (Méan *et al.*, 2008; Rueping *et al.*, 2009). Fungal infections are also affected by antimycotic resistance threats. Overall, bacterial and fungal infections are still a major issue in medicine. The increasing emergence and spread of antimicrobial and antifungal drug resistant pathogens in Europe and the rest of the world recall a multi-disciplinary approach through the development of effective infection prevention and control strategies as the identification of new antimicrobial compounds (Kauffman, 2006; Gomez-Lopez *et al.*, 2008).

To contrast the antibiotic-resistance phenomenon and the current scarcity of new synthetic antibiotics, renewed attention is being focused on natural products as a source of novel antimicrobial therapeutics. In particular, essential oil and their components obtained from the distillation of some vegetable portions proved to have various properties, such as antimicrobial, antitumoral and anti-

inflammatory activity (Hammer *et al.*, 2000; Cox *et al.*, 2000; Calcabrini *et al.*, 2004). Tea tree oil (TTO), extracted from the leaves of *Melaleuca alternifolia*, has shown a broad spectrum of biological activities. It has been used medicinally in Australia for more than 80 years, with uses relating primarily to its antimicrobial and anti-inflammatory properties. It contains approximately 100 components, which are mostly monoterpenes, and their related alcohols. Moreover, TTO showed antibacterial, antifungal, antiviral and anti-inflammatory properties *in vitro* (Carson and Riley, 1995; Hammer *et al.*, 1996).

In this *in vitro* study the mechanism of action of TTO has been evaluated on yeast cells of *C. albicans* in a drug sensitivity strain (3153) and in its azole-resistant counterpart (AIDS68), isolated from HIV-positive subject with oral candidiasis, by transmission (TEM) and scanning (SEM) electron microscopy analysis.

Materials and Methods

Strains and growth conditions

AIDS68 is a strain of *C. albicans* isolated from HIV-infected patient, and resistant to antifungals, particularly to fluconazole, while 3153 is a fluconazole-sensitive strain, isolated from a patient with recurrent vaginitis (MIC <1 μg/mL). Both strains of *Candida* were cultured on Sabourand dextrose agar (Difco, Detroit).

Transmission electron microscopy

Both sensitive and drug resistant strains (3153 and AIDS68) were treated with 1% TTO at different times (from 2 min to 60 min) and then prefixed for 20 min at room temperature with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After 3 washes in the same buffer, the cells were post-fixed with 1% (w/v) OsO_4 solution overnight at 4°C. The cells were then dehydrated in acetone gradient and embedded in epoxy resin (TAAB Laboratories Equipment Limited, Aldermarton, UK). Ultrathin sections, were obtained with a LKB Ultramicrotome Nova, stained with uranyl acetate and lead citrate (Stringaro *et al.*, 1998) and finally examined with a Philips 208 transmission electron microscope (FEI company, Eindhoven, The Netherland).

Scanning electron microscopy

C. albicans cells (3153 and AIDS68) were grown in glucose supplemented YNB medium, treated with 1% TTO at different times (from 2 min to 60 min) and deposited on glass coverslips of 12 mm diameter. After washing twice in calcium and magnesium-free phosphate-buffered saline (PBS), the cellular pellets resulting from centrifugation were fixed for 20 min at room temperature with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 2% (w/v) sucrose. After 3 washes in the same buffer, the cells were post-fixed with 1% (w/v) OsO_4 for 1 hour, dehydrated on an ethanol gradient, critical point dried in CO_2. The coverslips were attached to aluminium stubs, mounted with silver print and coated with gold in a sputter coater (Arancia *et al.*, 1998). The samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, UK).

Results

In this study we investigated the effects of TTO on two strains of *C. albicans*, the drug-sensitive strain 3153 and the azole-resistant counterpart AIDS68, isolated from a HIV-positive subject. In particular, we evaluated the presence of alterations induced by the treatment both in the cell wall and in the plasma membrane of the dimorphic microorganism.

Control *Candida* cells of both strains 3153 and AIDS68 (Figure 1a and 1b, respectively), observed by TEM after ultrathin sectioning, showed their typical morphology characterized by ovoidal shape, dense and compact cytoplasmic matrix, well preserved plasma membrane and cell wall. In particular, the cell wall appeared to be very uniform in thickness (about 150 nm), with regular structure and delimited in the outer side by a thin and more electron dense capsular layer. No significant difference could be detected between sensitive and resistant cells.

The treatment with 1% TTO induced significant ultrastructural changes in both cell types. The observed alterations appeared to be very similar; however their extent was different between 3153 and AIDS68 strains, depending on the time of exposure. In fact, in 3153 sensitive strain, after a short TTO treatment (10 min) (Figure 1c), most of the cells showed rarefied cytoplasmic matrix with the

presence of numerous small and electron-transparent vacuoles (arrowheads), plasma membrane invaginations (arrows and insert in Figure 1c) and enlarged cell wall (200-300 nm).

Conversely, in resistant AIDS68 cells no significant change was observed after 10 min of TTO treatment, their structure being very similar to that of control cells. However, virtually identical alterations were observed after 60 min of treatment with 1% TTO (Figure 1d). The insert in figure 1d shows at higher magnification the cytoplasmic vacuoles induced by the essential oil.

Control and TTO treated yeast cells were also analyzed by SEM. This microscopical approach did not reveal particular changes induced by the treatment concerning either dimension and shape or the surface morphology of both sensitive (Figure 2a and 2c; control and TTO treated, respectively) and resistant (Figure 2b and 2d; control and TTO treated, respectively) *Candida* cells.

Only after long time of treatment (60 min), some cells of the sensitive 3153 strain exhibited small protrusion on the cell surface (Figure 2c, arrow).

Discussion

Severe yeast infections, especially candidaemia, represent a significant health problem in patients at high risk of infection, leading to increased morbidity and mortality, greater healthcare costs and increased duration of hospitalization (Bouza and Munoz, 2008; Pappas *et al.*, 2009). *Candida albicans* is the most common species associated with candidaemia.

The data here reported indicate that the essential oil TTO induces significant ultrastructural alterations on fluconazole-susceptible and resistant populations of the dimorphic fungus *C. albicans*, even at short treatment times.

Among the observed alterations, the invaginations of the plasma membrane seem to play an important role. In fact, plasma membrane is the first target of terpenens, the main components of TTO (Hammer *et al.*, 2003) because they increase yeast cell permeability and membrane fluidity. Terpenes can insert between the fatty acyl chains that make up the membrane lipid bilayer, disrupt-

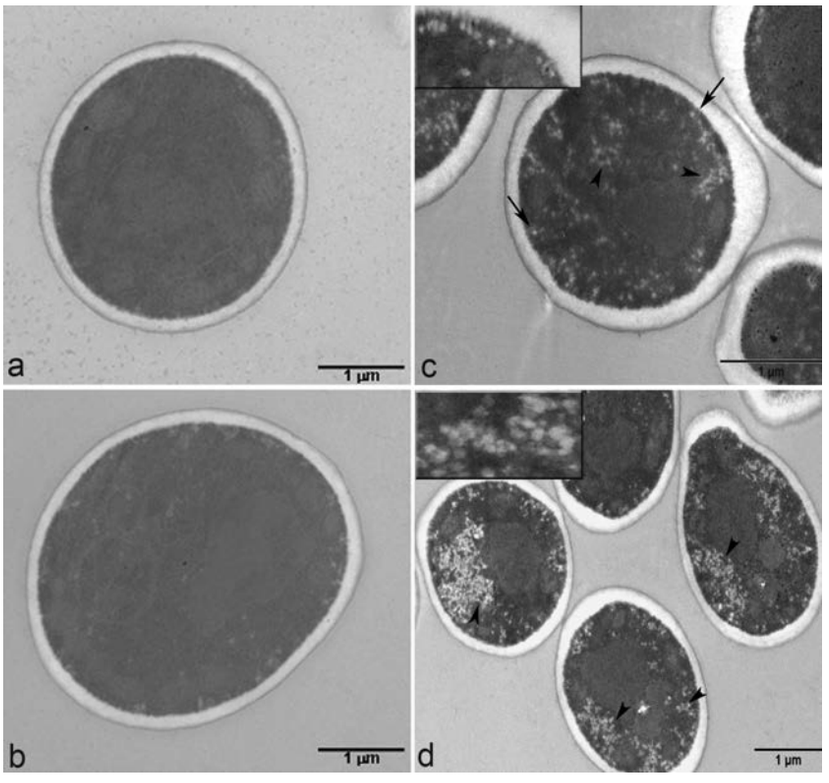

Figure 1. Control and TTO treated yeast cells observed by TEM. a) Control drug-sensitive 3153 cells; b) control drug-resistant AIDS68 cells; c) 3153 cells treated with 1% TTO for 10 min. Numerous plasma membrane invaginations (arrows and insert) and cytoplasmic vacuoles (arrowheads) are well visible. d) AIDS68 cells treated with 1% TTO for 60 min. In the resistant cells the cytoplasmic vacuolization (arrowheads and insert) could be observed only after long time of treatment.

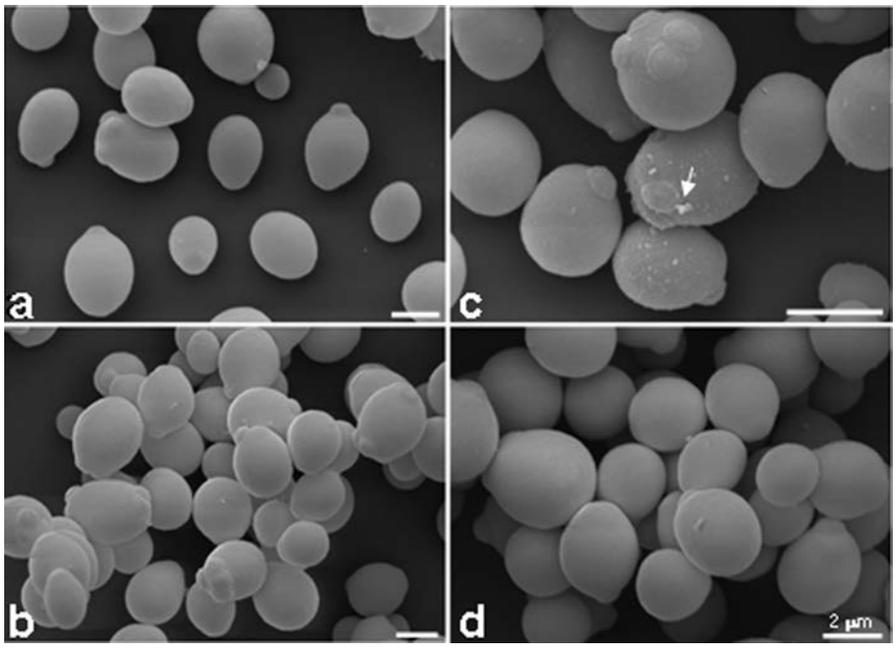

Figure 2. Control and TTO treated yeast cells observed by SEM. a) Control drug-sensitive 3153 cells; b) control drug-resistant AIDS68 cells; c) 3153 cells treated with 1% TTO for 60 min. Some cells exhibit small protrusions on the cells surface (arrow). d) AIDS68 cells treated with 1% TTO for 60 min. No significant morphological changes were observed in the resistant cells after the treatment with the essential oil.

ing lipid packing and cause change to membrane properties and functions (Sikkema *et al.*, 1995; Giordani *et al.*, 2006). The effects on membrane functions induced by other natural products on *C. albicans* have been previously described (Bard *et al.*, 1988).

The antimicrobial activity of TTO has been attributed to its interactions with cellular membranes (Hammer *et al.*, 2003). At relatively low concentrations, these interactions may result in alteration in permeability while at higher concentrations in total loss of homeostasis, gross membrane damage and cell death (Carson *et al.*, 2002).

The different components of tea tree oil vary in their modes of action against *Candida* and thus TTO may have several mechanisms of antifungal action. In fact, we observed numerous cytoplasmic vacuoles especially in the sensitive strain 3153 (Figure 1c and 1d, arrowheads). The hypothesis is that TTO is able to exert its non well-known antimicotic activity through a complex mechanism of action which involves some cytoplasmic proteins able to activate transduction signals and regulate cellular homeostasis.

These molecules could be involved in cell death (such as cell division cycle gene [CDC48], apoptosis-inducing factor [AIF], Ras pathway signaling, yeast metacaspase [MCA1]). In particular, Ras pathway signaling, which plays a key role in the determination of cell fate in mammalian models, accelerates cell death in *C. albicans* (Almeida *et al.*, 2008).

The transduction signals activated by TTO treatment appear to be different between the sensitive strain (3153) and the resistant variant (AIDS68). In fact, 3153 strain reflects in its modifications the quickly effects exerted by the essential oil to the biological structures, while the resistant strain (AIDS68) is able to tolerate the short treatments.

In future, we would like to evaluate the effects of TTO treatment on different resistant strains of *C. albicans* by a time course analysis prolonging the time of the treatments (from 4 to 48 hours). These studies will allow to obtain useful information to understand if TTO could be used in therapy, especially in the presence of recurrent fungal infection caused by resistant strains. More importantly, TTO may be used as chemosensitizer, administered in sub-inhibitory dose, for final testing against fungal infections, both in immunocompetent and in immunocompromized patients.

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