

# Effect of a novel phyto-compound on mucosal candidiasis: Further evidence from an *ex vivo* study

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**OBJECTIVE:** To isolate, identify and determine the prevalence of yeasts in the oral cavity of individuals and to test the minimum inhibitory dilution (MID) of Kolorex against the yeasts isolated.

**METHODS:** Twenty-nine individuals of both sexes aged on average 61.3 years were evaluated at the dental clinic in order to isolate and identify yeasts from their oral cavity, with and without lesions, and to determine the MID of the commercial phyto-product Kolorex against the strains isolated. The antifungal activity of the product tested was determined by the technique of dilution on a solid medium. Monocyte chemoattractant protein 1 (MCP-1) was measured by biotinylated antibody assay by an enzyme-linked immunosorbent assay (ELISA).

**RESULTS:** Yeasts of the genus *Candida* were detected in the saliva of 45.4% of the 11 individuals with a clinically healthy mouth and in 88.2% of 17 individuals with oral lesions. In the group with oral candidiasis we isolated in tongue and lesion, respectively, for each species: *C. tropicalis* (5.8% and 11.7%), *C. glabrata* (5.8% and 5.8%) and *C. parapsilosis* (0% and 5.8%), in

addition to *C. albicans* as the only species or in association with others, respectively (64.7% and 70.5%). The total clonal formation unit (CFU) (counts/mL) in the saliva showed a higher mean value in the group with oral candidiasis ( $158.3 \times 10^3$ ) than in the control group ( $64.6 \times 10^3$ ). Most of the 70 test strains (95.7%) were sensitive to Kolorex by presenting a MID of 1:20. Sixty percent of strains from the 70 healthy sites showed results similar to those obtained with strains from oral lesions. Different results were mainly observed among different species. Patients with oral lesions showed a significant time-course increase of the level of monocyte chemoattractant protein 1 (MCP 1) as compared to those without lesions or to healthy people in whom *Candida* has not been detected ( $P < 0.05$ ). Co-culture with Kolorex using aliquots from the same patients with oral lesions inhibited such event ( $P < 0.05$ ).

**CONCLUSION:** Although this study was focused on oral cavity candidiasis, the results indicate the possibility of a broader use of the antifungal Kolorex in the prevention and treatment of mucosal candidiasis located elsewhere.

**KEY WORDS:** *ex vivo*, Kolorex, MCP-1, oral candidiasis, saliva.

## INTRODUCTION

*Candida albicans* are opportunistic fungi which generally exist in the oral cavity, skin, vagina and intestinal organs<sup>1,2</sup>. They are dimorphic fungi which undergo a transition from a yeast form to a hyphal form depending

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on the growth conditions.<sup>3,4</sup> In physiological condition, such yeast-like fungi represent a biological link with the host which guarantees their saprophytic condition by establishing an ecologic equilibrium known as amphibiosis. When this equilibrium is altered due to different endogenous and/or exogenous factors, such amphibionts turn into opportunistic microorganisms, eventually causing multiple oral infections which, if unresolved, may become generalized, leading to more severe systemic mycoses.<sup>5</sup> The lesions due to oral candidiasis are more frequent on the tongue, cheeks and palate,<sup>6</sup> sites that may be more frequently and densely colonized in subjects harboring yeasts of the genus *Candida*.<sup>7</sup> Although the most frequent etiologic agent of oral candidiasis is *C. albicans*, other species such as *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis*, among others, may be responsible for this type of mycosis.<sup>7</sup> Antifungal prophylaxis may be indicated for the prevention of colonization or multiplication of *Candida* spp. in patients susceptible to primary infection and also for the prevention of recurrence in patients who have previously submitted to anti-mycotic treatment. The *in vitro* evaluation of the yeast sensitivity to antiseptics has been little studied, even though the application of oral antiseptics deserves to be considered at least as a preventive measure or as an alternative or a complementary procedure in treatment. On the other hand, mucosal mycosis, wherever located, often implies a reduced systemic biological equilibrium, therefore a broader therapeutic strategy has to be considered while avoiding and harmful unrestricted use of chemotherapies. In recent years a polygodial-anethole compound has been employed in clinical practice since it has been found that anethole, a natural compound isolated from *Pimpinella anisum*, enables an over 30-fold increase of the antifungal activity of polygodial against *C. albicans*.<sup>8,9</sup> The aims of the present study were: (i) to isolate, identify and determine the prevalence of yeasts in the oral cavity of individuals with and without lesions, and (ii) to test the minimum inhibitory dilution (MID) of Kolorex against the isolated yeasts.

## MATERIALS AND METHODS

The study was conducted on 29 adults (13 women, 15 men) aged on average 61.3 years (42–67), 11 of whom (37.9%) presented thorough oral health (the control group) and 17 (58.6%) had some type of intra-oral lesion with suspected oral candidiasis. Approximately 2.0 mL of non-stimulated saliva was collected from each patient into 20 × 150-mm sterilized tubes containing glass beads. The tubes were then shaken until a uniform suspension was obtained to be used for serial dilutions

in phosphate buffered saline (PBS). Aliquots of 0.1 mL of pure saliva from each of three dilutions ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ) were added to a 15 × 100-mm petri dish containing agar Sabouraud plus chloramphenicol and then seeded with a sterilized L-shaped glass rod. The plates were then incubated at 37°C for 24–48 h and stored at room temperature for the subsequent tests. Material was collected aseptically from the dorsum of the tongue of each patient. After a clinical examination of the individuals with lesions clinically suspected to be caused by yeasts, material was collected from the lesions in cooled platinum loop and seeded onto agar. After seeding, all tubes containing samples from the tongue or from the lesions were incubated at 37°C for 4 days, re-isolated in agar Sabouraud and distributed into different plates by the depletion technique. The yeasts were identified by classical methods<sup>10</sup> using the following tests: formation of germinative tubes, study of micromorphology, assimilation of carbon and nitrogen sources, fermentation, urea hydrolysis and triphenyltetrazolium reduction.

The inocula for the identification tests were obtained from recent cultures (24–48 h at 37°C) on agar Sabouraud after the culture purity was confirmed. For the detection of the MID of Kolorex, 70 yeast strains of different species of the genus *Candida* isolated from the oral cavity of subjects without lesions were tested. The Kolorex sample tested was supplied by Named srl, Lesmo, Italy in the form of tablets (100 mg composition: *Pseudowintera colorata* 50 mg, *Pimpinella anisum* 41.5 mg, *Lactobacillus acidophilus* 2.9 mg and vitamin C 5.6 mg).

The antifungal activity of the product tested was determined by the technique of dilution in a solid medium.<sup>11</sup> Serial dilutions of the compound were prepared in duplicate in sterilized distilled water, corresponding to 1:20–1:200. An adequate amount of Mueller Hinton agar medium (MHA-Difco, Difco Laboratories, Detroit, MI) to provide a final volume of 20 mL was added to the serial dilution tubes and to the control tubes, followed by homogenization and distribution among sterilized 15 × 100-mm petri dishes. Suspensions in sterilized physiological saline containing approximately  $1 \times 10^6$  cells/mL were applied with Steers replicator onto the series of agar plates. Incubation was carried out for 24 h at 37°C and the results were evaluated by observing the possible occurrence of microorganism growth at the corresponding dilution of the compound.

Moreover, monocyte chemoattractant protein 1 (MCP-1) was measured by biotinylated antibody assay by enzyme-linked immunosorbent assay (ELISA) on separate

coculture supernatants at the entry and at the fourth day observation either alone and incubated with Kolorex at 1:20 dilution.

## RESULTS

Sixteen (55.1%) of the samples obtained from the oral cavities of 29 individuals were positive for yeast. Yeasts of the genus *Candida* were isolated from 45.4% of the saliva samples from the 11 clinically healthy individuals and 88.2% of the samples from the 17 patients suspected to have oral candidiasis. *C. albicans* was the most prevalent among the species isolated and was the only one detected in all types of samples analyzed, occurring at a frequency of 51.7% (15/29) in saliva, in 28.0% (3/10), 28.6% (2/07) and 12.5% (1/08) of the normal, fissured and coated tongues, respectively, and in 67.8% of the 14 samples from oral lesions. Other species were also isolated, mainly from saliva and lesions, such as *C. tropicalis*: 6.9% of the 29 saliva samples and 10.7% of the nine lesions, and *C. glabrata*: 3.4% of the 29 saliva samples and 3.6% of the 28 lesions (Table 1).

The study of the salivary levels of yeasts of the genus *Candida* by the methods of serial dilution showed a mean number of total CFU/mL saliva of  $158.3 \times 10^3$  in the group of patients with lesions (range:  $0.42 \times 10^3$ – $2760 \times 10^3$  CFU/mL). Such levels were higher than in the groups of patients with no lesions (control) with values of  $66.5 \times 10^3$  CFU/mL. Among the species

Table 1. *Candida* spp. isolated from the oral cavity: frequency and species

Species	Saliva	Normal	Lesions
<i>C. albicans</i>	48	28	67
<i>C. tropicalis</i>	8	–	10
<i>C. glabrata</i>	4	–	3.6
<i>C. guillemondii</i>	2	–	–
<i>C. parapsilosis</i>	2	–	3.6
<i>C. krusei</i>	4	–	–

Table 2. Maximum inhibitory dilution of phyto-compound Kolorex: number and frequency of genus *Candida* isolated from oral lesions

MID	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. glabrata</i>	<i>C. guillemondii</i>	<i>C. parapsilosis</i>	<i>C. krusei</i>
1:20	76	75	50	100	100	100
1:40	21	12	10	38	50	50
1:80	3	0	8	0	0	0
1:100	3	0	0	0	0	0
1:200	0	0	0	0	0	0
Resistant	1.8	12	50	0	0	0

isolated from the three study groups, *C. albicans* was the most frequent with a mean level of  $216.3 \times 10^3$  CFU/mL in the groups with lesions and  $70.9 \times 10^3$  CFU/mL in the group without lesions. Yeasts of the species *C. tropicalis* and *C. glabrata* were isolated only from patients with lesions, at respective levels of  $19.5 \times 10^3$  CFU/mL and  $36.1 \times 10^3$  CFU/mL saliva.

In the assessment of the action of Kolorex against the whole strains of yeasts of the genus *Candida* isolated from the oral cavity of all 70 individuals it appeared that most strains (95.7%) were sensitive. A large number of strains were inhibited by the 1:20 dilution of Kolorex (77.1%) while all the strains tested showed a different degree of growth when submitted to Kolorex dilutions higher than 1:40. Of all strains tested in the 70 individuals, (41.4%) were sensitive to a MID of 1:20 of Kolorex. Of these strains, 96.5% (28/29) were of the species *C. albicans*. *C. albicans* was sensitive to a MID of 1:20–18/24 (75.0%) or 1:40–5/24 (20.8%), whereas the remaining species were sensitive to a MID of 1:20 (Table 2). The three species that were resistant to all dilutions of Kolorex tested were *C. albicans*, *C. tropicalis* and *C. glabrata* isolated from oral lesions.

As shown in Figure 1, patients with oral lesions showed a significant time-course increase of the level of

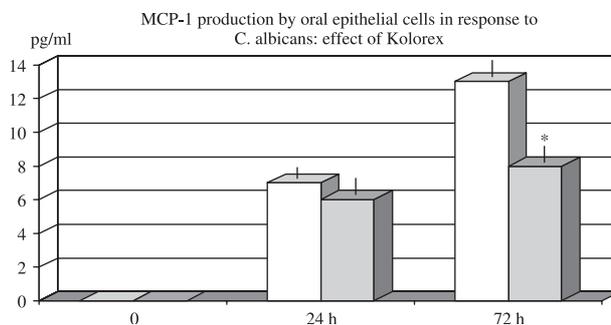


Figure 1. MCP-1 production by oral epithelial cells in response to *C. albicans*: effect of Kolorex. (■): Kolorex-treated samples, (□): control. \* $P < 0.05$ .

MCP-1 as compared to those without lesions or healthy people in whom *Candida* was not detected ( $P < 0.05$ ). Co-culture with Kolorex using aliquots from the same patients with oral lesions inhibited such an event ( $P < 0.05$ ).

## CONCLUSION

Although several chemically synthesized antifungal molecules have proven their efficacy in clinical practice, nonetheless they also pose possible drawbacks, such as toxic effects as well as opportunistic bacterial overgrowth.<sup>12</sup> Moreover, during the past decade, fluconazole has been largely employed in high risk populations such as neutropenic patients and, together with its unquestionable benefits against systemic candidiasis, this has also led to a selection-driven shift from highly susceptible to less susceptible *Candida* species. The density of yeasts in the oral cavity is usually high and more than one species is frequently isolated so that it is difficult to determine the relative role of individual species in the disease process.<sup>1,2,13</sup>

The total quantification of yeasts of the genus *Candida* varied in the two groups studied, particularly in patients with oral candidiasis ( $171.5 \times 10^3$  CFU/mL saliva) compared to the control group ( $72.6 \times 10^3$  CFU/mL). With respect to the species isolated, *C. albicans* showed the highest levels in the group with lesions among individuals with oral candidiasis. *C. albicans* was detected in 59.1% of the saliva samples and in 67.8% of the lesion samples. This is lower than what has been reported by Davenport<sup>13</sup> in saliva (70.0%) and by Rindum *et al.*<sup>14</sup> in oral mucosa lesions (94.3%). In contrast to what has been observed with common antiseptic solutions such as chlorhexidine<sup>15</sup> (data not shown), the antifungal action of Kolorex was more constant against all species tested, with a MID of 1:20 for most of them, whether or not they were isolated from lesions. Most of the strains tested (77.1%) were sensitive to Kolorex at a dilution of 1:20 and, to a lesser extent, also to a higher dilution. Patients with oral lesions showed a significant time-course increase of the level of monocyte chemoattractant protein 1 as compared to those without lesions or healthy people in whom *Candida* was not detected ( $P < 0.05$ ). Finally, from our parallel co-culture of saliva with Kolorex using aliquots from the same patients with oral lesions, it appeared that this phyto-compound inhibited such an

event ( $P < 0.05$ ). Even considering the fact that *ex vivo* data cannot be directly extrapolated to *in vivo* effects, our results indicate that yeasts of different species of the genus *Candida* could be inhibited by the application of Kolorex to the oral cavity, and possibly to any mucosal surface, for therapeutic and/or preventive purposes, as has recently been shown.<sup>16,17</sup>

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