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Study of anticandidal activity of carvacrol and eugenol *in vitro* and *in vivo*

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Background/aims: The mechanism of the anticandidal action of the major phenolic components of oregano and clove essential oils – carvacrol and eugenol – was studied. This activity was also evaluated for the therapeutic efficacy in the treatment of the experimental oral candidiasis induced by *Candida albican*s in immunosuppressed rats.
Methods: *In vitro*, the addition of carvacrol at 0.1% or eugenol at 0.2% during the exponential growth of *C. albicans* was evaluated. The release of substances absorbing at 280 nm by cells treated with these two components was also measured spectrophotometrically. *In vivo,* oral candidiasis in immunosuppressed rats was established by inoculating 3×10^8 cells of *C. albicans* with a cotton swab on three alternate days. The number of colony counts was evaluated from the oral cavity of rats treated for eight consecutive days with carvacrol, eugenol or nystatin and compared to untreated controls.
Results: Carvacrol and eugenol were fungicidal in exponentially growing *C. albicans*. Interestingly, this fungicidal effect was accompanied by the release of substances absorbing at 280 nm. In an immunosuppressed rat model of oral candidiasis, carvacrol or eugenol treatment significantly ($P < 0.05$) reduced the number of colony counts sampled from the oral cavity of rats treated for eight consecutive days compared to untreated control rats. Similar results were obtained with nystatin used as a reference treatment.
Conclusion: The *in vitro* results indicated that both carvacrol and eugenol exerted an anticandidal effect by a mechanism implicating an important envelope damage. Their *in vivo* efficacy on experimental oral candidiasis leads us to consider them as possible antifungal agents.

Key words: *Candida albicans*; carvacrol; eugenol; mechanism of action; experimental oral candidiasis; immunosuppressed rats

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Recurring fungal infections with *Candida albicans* are most frequently described in immunocompromised patients, and the mucosal infections have received a great deal of attention due to the advent of the human immunodeficiency virus (HIV) infection (19). To avoid the many side-effects of the drugs commonly used to treat fungal infections (9), new and effective natural products are being sought. The *in vitro* antimicrobial and antifungal effects of many essential oils have been reported (7, 16, 18). The anticandidal activity of some essential oils has also been established *in vitro* and *in vivo* (13, 21). However, use of the whole essential oil does not allow the determination of the active principle because of the complexity of its components. There is also not enough information on the mechanism of the anticandidal action of these active principles. It is, furthermore, possible that the minor components of essential oils could exhibit some toxic or adverse effects *in vivo*.

In the present work, we tried to determine the mechanism of action of carvacrol and eugenol, the phenolic major components of oregano and clove essential oils, respectively, in comparison with nystatin, a polyene known to be a potent anticandidal agent (2). Two approaches were used in this purpose:

- addition of the antifungal agent during the logarithmic phase of *Candida* growth;
- measurement of leakage of 280 nm absorbing material from the treated washed cells.

The *in vivo* efficacy of these two components was tested on an experimental model of oral candidiasis in immunosuppressed

rats (14, 15). The number of colony forming units (CFU) sampled from the oral cavity of infected and treated rats was compared with that of infected untreated rats.

Material and methods

Yeast cells

Six strains of *C. albicans* were used in the preliminary tests; three of them isolated from the vaginal secretions of a woman with acute vaginitis, and the three from feces. Isolated strains were identified by using the standard yeast determination procedure (11).

Culture media

YEPD (Difco Laboratories, Detroit, MI) containing 0.05% of chloramphenicol was used to determine the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) of yeasts treated with carvacrol, eugenol or nystatin in a liquid medium. YEPD agar (Difco) was used for yeast culture and maintenance.

Preparation of Candida washed cells

A single colony of *C. albicans* from Sabouraud dextrose agar was grown in 50 ml of YEPD for 18 h in a shaker at 30°C. The cells were then recovered by centrifugation at 2500 × *g* for 10 min and the pellet resuspended in phosphate-buffered saline (PBS). This operation was repeated twice to obtain washed cells, which were adjusted to a final number of 10^7cells/ml by using a hematocytometer chamber and the drop count method (4).

Antifungal agents

Carvacrol and eugenol were used in this study as natural antifungal components. They were purchased from Fluka (Steinhein, Germany). These two phenolic major components were dispersed in 0.2% agar suspension. This dispersion method has been improved in our laboratory (17). Nystatin (Bristol-Myers Squibb, Paris, France) was dissolved in 0.2% agar solution to obtain the desired concentration.

Determination of the MIC and the MFC

The MIC was determined in triplicate in a liquid medium by direct exposure of yeast cells to increasing concentrations of the antifungal agent and incubation at 30°C. Twenty-four hours later, the yeast growth was visually observed in the control tube. The MIC of each tube containing the antifungal agents was determined as the lowest concentration leading to total growth inhibition. Fractions of 20 µl from the tubes showing no growth were aseptically transferred into new tubes containing 980 µl sterile YEPD. After an incubation period of 24 h at 30°C, the tubes were examined. The tubes from which the yeast cell fraction had been taken were considered to contain a fungicidal concentration if no growth was observed. The MFC was taken as the lowest concentration of the antifungal agent leading to such an effect (16, 17). This was confirmed by plating, using the drop count method (3).

To determine the appropriate number of cells in the inoculum to be used throughout the study, five amounts of cells in the inoculum were tested, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/ml.

Treatment of exponentially growing *C. albicans* with carvacrol, eugenol or nystatin

A total of 0.5 ml of the overnight preculture of *Candida* (*C. albicans* 1E111PV515) was added to 50 ml YEPD medium and incubated at 30°C with shaking until the OD_{620} reached approximately 0.2, which corresponded to the beginning of the logarithmic phase. Cells were then either sham-treated or treated with different concentrations of the antifungal agents. The suspension turbidity of the cells was measured by spectrophotometry at 620 nm at different growth phases together with the cell viability, evaluated by using the drop count method.

Measurement of 280 nm absorbing material released from treated cells

The leakage of 280 nm absorbing material was measured in this study (5, 18). Washed cell suspensions were adjusted to approximately 10^7cells/ml in PBS as a control. The release of 280 nm absorbing material was measured from the supernatants obtained after centrifugation of the cells suspension at 2000 × *g*. The suspension was treated with various concentrations of the antifungal agent for 1 h. The subpopulation of one cell was not treated and served as a control.

Experimental oral candidiasis

Animals

Male Wistar rats ($n = 41$, 8 weeks; approximately 200 g) were used in this study. They were randomized into groups of three or four animals, housed in large cages. The photoperiods were adjusted daily to 12 h of light and 12 h of darkness. The environmental temperature was constantly maintained at 21°C. The rats were given food and water *ad libitum*.

During the experiment, food composition was complete and equilibrated free from antifungal agent. The research complied with European legislation and company policy on the care and use of animals and related codes of practice.

Organisms and inoculum preparation

The same strain of *C. albicans* (1E111PV515) was used for this *in vivo* study. Washed cells were prepared and adjusted to 3×10^8 CFU/ml as described below. The viability of the inoculum was verified by quantitative cultures of serial 10-fold dilutions on Sabouraud dextrose agar plates.

Oral candidiasis in rat

The experimental oral candidiasis we used here was reported by Martinez et al. (14, 15). In this model, rats are immunosuppressed by giving them dexamethasone with tetracycline. All the experiments were repeated three times and the results were reproducible. The rats were orally infected three times at 48-h intervals (days -7, -5 and -3) with 0.1 ml of saline suspension containing 3×10^8 viable cells of *C. albicans.* Oral infection was performed by means of a cotton swab rolled twice over all parts of the mouth. Just before inoculation, samples were taken from the animals to confirm the absence of *C. albicans* in the oral cavity. Samples were taken again 72 h after the last inoculation. All groups were sampled in the same way to confirm the presence of the fungi and to determine the number of CFU in the oral cavity before the beginning of the treatment.

Oral suspension for topical treatment

Carvacrol and eugenol at final concentrations of 0.2% (2 × MIC) and 0.4% (2 × MIC), respectively, were dispersed in viscous 0.8% agar solution as an excipient for oral treatment. An aqueous solution of 10-fold MIC (50 IU/ml) nystatin was also homogenized in 0.8% agar.

Antifungal treatment

At 72 h post-infection, the animals ($n = 41$) were randomly assigned to one

of six groups. Three groups were treated with a topical application in the oral cavity twice a day for eight consecutive days (day 0 to day 7) with 0.5 ml of 0.2% carvacrol (approximately 10 µg/kg/day), 0.5 ml of 0.4% eugenol (20 µg/kg/day) or 0.5 ml nystatin suspension (approximately 312.5 µg/kg/day).

The control, infected and untreated animals received an oral 0.5 ml sterile saline solution (containing 0.8% agar) twice a day.

An additional group of nonimmunosuppressed animals infected and untreated was added to the experiment to study the impact of dexamethasone/tetracycline treatment on the development of the infection. Animals immunosuppressed but noninfected ($n = 6$) were used as a negative control group.

Quantification of infection level and microbiological determination of therapeutic efficacy

Samples were collected at days 0, 4 and 8 by rolling a sterile cotton swab over the oral cavity and then suspending the swab in 1 ml of sterile saline solution. Samples of 25 ml from this suspension were dropped in duplicate, after serial 10-fold dilution on Sabouraud agar plates containing 0.05% chloramphenicol. All plates were incubated at 30°C for 24 h, and the colonies counted. The number of viable cells was determined using the drop count method to calculate the log of the CFU/ml.

Statistical test

An analysis of variance on ranks was used to statistically compare the numbers of CFU of *C. albicans* isolated from the mouths of the experimental groups. Multiple comparisons of treated groups vs. the control group at days 4 and 8 were performed by Scheffe's method. All statistical evaluations were performed using the analysis of variance program of the S-plus statistical package (S-Plus; version 4.5, 1998 Mathsoft, Inc., Cambridge, UK). *P*-values of 0.05 were considered statistically significant. All mean values given in the text and tables include the standard deviations of the means.

Results

MIC and MFC of carvacrol, eugenol and nystatin

In the preliminary tests, the minimal inhibitory concentration and the minimal fungicidal concentration values against the six strains of *C. albicans* for each antifungal agent were determined according to the number of cells in the inoculum. The MIC and the MFC values were the same for each antifungal agent, and were dependent on the number of cells in the inoculum. When this number was 10^3 to 10^4 CFU/ml, the MIC and the MFC values of carvacrol, eugenol and nystatin were 0.033%, 0.05% and 1 IU/ml, respectively. When the number of cells in the inoculum ranged from 10^5 to 10^7 CFU/ml, the MIC and the MFC values of carvacrol, eugenol and nystatin were 0.1%, 0.2% and 5 IU/ml, respectively. Taking into consideration the above findings, and with the aim of having homogeneous and comparable results throughout this study, 10^6 CFU/ml was chosen as an appropriate number of cells in the inoculum for the *in vitro* tests. The strain *C. albicans* (1E 111 PV 515) has been selected for the rest of this study because of its better growth rate in comparison with the five other strains used in the preliminary tests.

Carvacrol, eugenol and nystatin are fungicidal for exponentially growing Candida cells

Carvacrol at 0.033% stopped the growth of *C. albicans* cells (Fig. 1a). This was translated by a stabilization of the optical density at 620 nm. In terms of cellular viability, treatment with carvacrol killed yeast cells in a dose-dependent manner (Fig. 1b). For all concentrations of eugenol used, the absorbency remained low, whereas the untreated control cells grew exponentially (Fig. 2a). The two highest concentrations of eugenol (0.1%, 0.2%) were able to kill 100% of *Candida* cells (Fig. 2b). Concerning the treatment with nystatin used at its MFC, the optical density at 620 nm was significantly reduced when compared to the untreated control. Under these conditions,

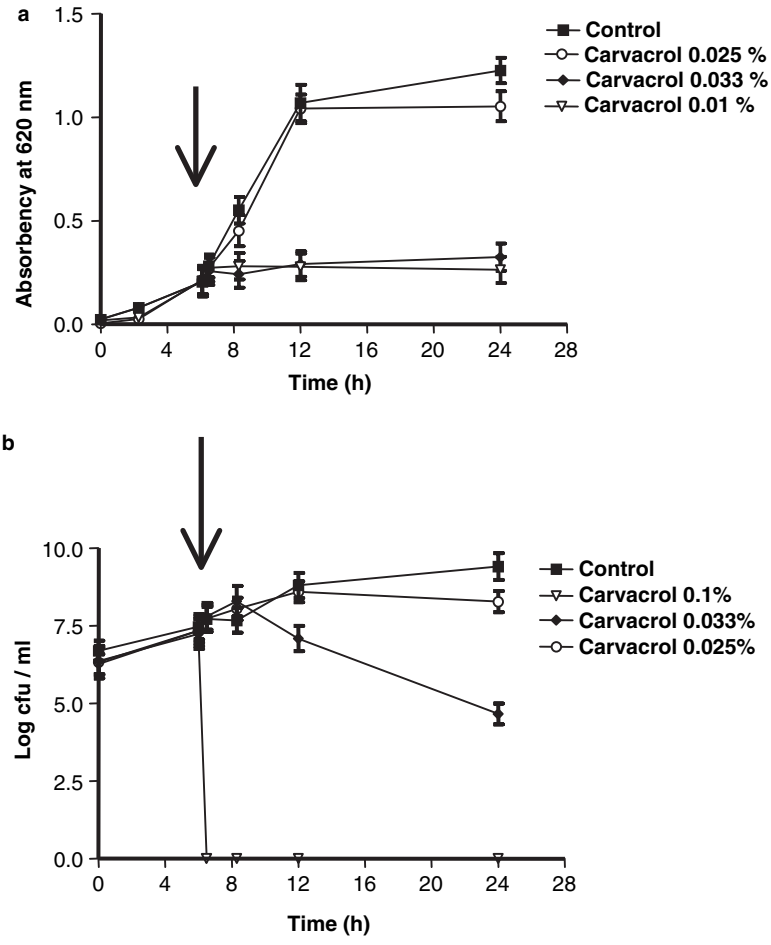

Fig. 1. Effect of carvacrol on exponentially growing *C. albicans.* Cells were treated with different concentrations of carvacrol. The arrows indicate the moment of the addition of carvacrol. (a): The absorbency at 620 nm was measured. (b): The log cfu was determined.

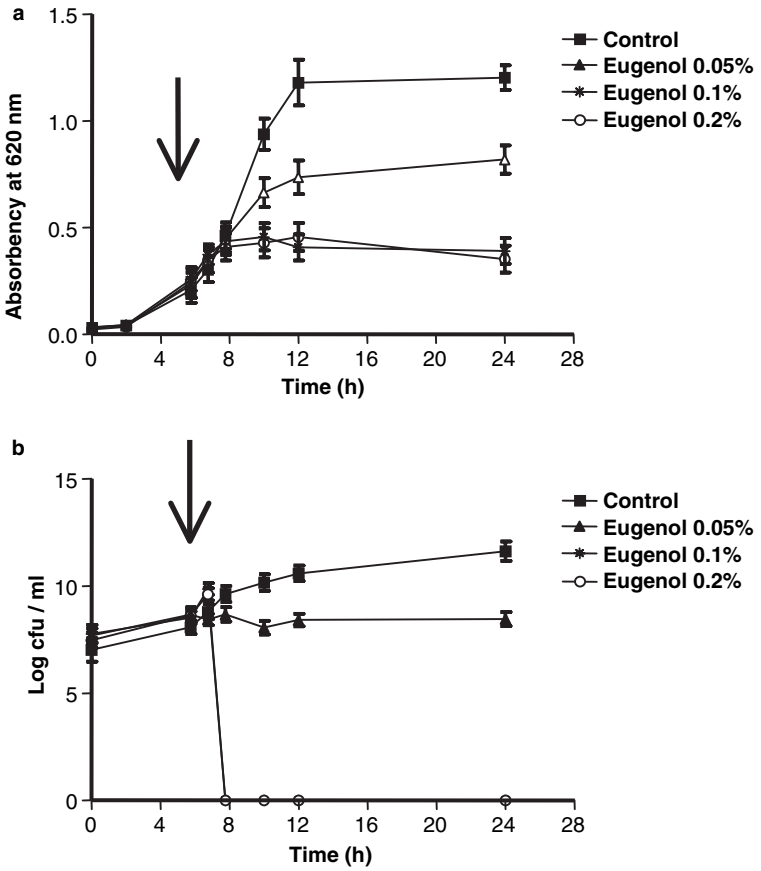

Fig. 2. Effect of eugenol on exponentially growing *C. albicans.* Cells were treated with different concentrations of eugenol. The arrows indicate the moment of the addition of eugenol. (a): The absorbency at 620 nm was measured. (b): The log cfu was determined.

cellular killing was total (results not shown).

Carvacrol and eugenol induced the release of cytosolic constituents

The number of viable cells decreased following treatment with carvacrol at a concentration below 0.1% (Table 1). As also shown in Table 1, in parallel with the increase of cellular killing, the release of the cellular content increased along with the antifungal concentration. Furthermore, for eugenol, the number of viable cells decreased after the treatment with increasing concentrations from 0.03 to 0.20% and became nil at MFC value. However, for nystatin, even there was 100% cell mortality at 5 IU/ml, no leakage of substances absorbing at 280 nm was recorded.

Experimental oral candidiasis

Microbiological data

Prior to initiating the study, oral cavity cultures of each rat were performed, and no *C. albicans* organisms were found. At day 0, i.e. just before the start of the treatment, samples from all groups of infected animals were taken. All the oral swabs were positive for the presence of *C. albicans*, with a mean log CFU/swab of 3.69* ± 0.49 ($n = 28$ rats). The group of nonimmunosuppressed rats was partially infected (Table 2).

Four days after the treatment, oral samples were collected and cultured to quantify the CFU in the oral cavities of the animals of each group. Animals treated with eugenol and carvacrol remained infected but the mean log CFU/swab decreased significantly ($P < 0.05$). In the nystatin group, only 4/7 of rats showed positive *C. albicans* cultures with a significant decrease in mean log CFU/swab. Consequently, the degrees of reduction of CFU vs. control were 95.9% for eugenol, 91.18% for carvacrol and 96.5% for nystatin (Table 2). In the nonimmunosuppressed rats, only 2/7 of animals were still infected.

After 8 days of treatment (day 8), *C. albicans* organisms were detected in the oral cavities of only 2/7 and 3/7 in eugenol- and carvacrol-treated groups, respectively. Moreover, there was a significant percentage reduction of CFU for the eugenol-treated group compared with the control (76.93%). An even larger reduction in the carvacrol-treated group (94.46%) was noted when compared with the control.

Treatment with nystatin, used as reference treatment, was apparently effective in microbiologically eradicating this infection, since only 1/7 of animals remained infected, with a significant reduction of the log CFU/swab.

Table 1. Effect of carvacrol and eugenol concentrations on cell mortality and the release of 280 nm absorbing material

Concentration (%)	Carvacrol		Eugenol	
	10^6 CFU/ml	A_{280}	10^6 CFU/ml	A_{280}
0	6.0 (100%)	0	6.0 (100%)	0
0.033	5.4 (90%)	18.75%	5.6 (93.33%)	32%
0.050	4.0 (66.6%)	21.87%	4.1 (68.33%)	42%
0.066	1.7 (28.33%)	31.25%	3.3 (55%)	53%
0.100	0	62.5%	2.1 (35%)	63%
0.200	0	100%	0	100%

Release values are presented in percentage vs. total release. CFU values are presented in percentage vs. initial number of CFU.

Discussion

Oregano and clove essential oils have been described as having a potent antifungal activity (10, 13). To exclude the potential influence of the minor essential oil components and to avoid their side-effects, pure carvacrol and eugenol, the major components of oregano and clove essential oils, respectively, were used in this study.

Carvacrol and eugenol were dispersed in viscous 0.2% agar solution as previously described by Remmal et al. (16, 17). These authors demonstrated that solvents and detergents, habitually used to disperse essential oils, inhibited their antimicrobial activity.

For the six strains used in the preliminary tests, the MIC and the MFC values we obtained with eugenol and carvacrol were dependent on the number of cells in the inoculum. The same was noted with

Table 2. Microbiological study of therapeutic efficacy of eugenol and carvacrol vs. nystatin against oral candidiasis in rats

	Day 0		Day 4			Day 8		
	infected animals (%)	Log CFU ± SD	positive culture (%)	Log CFU ± SD	CFU reduction (%)	positive culture (%)	Log CFU ± SD	CFU reduction (%)
Positive control	7/7 (100)	3.71 ± 0.48	7/7 (100%)	3.8 ± 0.74		7/7 (100%)	3.71 ± 0.48	
Eugenol	7/7 (100)	3.62 ± 0.29	7/7 (100%)	2.97 ± 0.38	95.91	2/7 (28%)	3.17 ± 0.29*	76.93
Carvacrol	7/7 (100)	3.31 ± 0.46	7/7 (100%)	3.08 ± 0.46	91.18	3/7 (42%)	2.64 ± 0.02*	94.46
Nystatin	7/7 (100)	3.64 ± 0.08	4/7 (57%)	3.07 ± 0.58*	96.62	1/7 (14.2%)	2.71*	91.54
Nonimmunosuppressed rats	5/7 (71.7)	3.09 ± 0.25	2/7 (28%)	3.25 ± 0.35*	92.62	1/7 (14.2%)	2.60*	64.96

The rats were orally infected three times at 48-h intervals with 3×10^8 cells of *C. albicans* (1E111PV515). Drugs were orally administrated twice a day for eight consecutive days starting 3 days after the last infection.
*$P < 0.05$ compared with control treatment.
A supplementary group of animals immunosuppressed but neither infected nor treated ($n = 6$) showed a negative culture throughout the experiment.

nystatin. The relationship between the number of cells in the inoculum and the MIC and MFC is well known for bacteria (12, 16). The anticandidal effect of carvacrol has been reported by Manohar et al. (13), but the MIC and the MFC values obtained by these authors were higher than ours. This is probably due to the inhibition exerted by the ethanol-Tween 80 mixture they used as a dispersing agent, as reported by Remmal et al. (16, 17).

The aim of the present work was to study the mechanism of action of these pure major components of essential oils and to test their efficacy *in vivo by* using an oral candidiasis model in immunosuppressed rats.

In terms of suspension turbidity and cellular mortality, the results obtained after the addition of carvacrol or eugenol at the logarithmic phase of growth of *C. albicans* are comparable to those obtained with nystatin, which was used as an antifungal drug reference. Yeast cell lysis, assessed by the release of substances absorbing at 280 nm, increased in response to the increasing concentrations of carvacrol or eugenol, which may explain the cellular mortality at their MFC values.

Previous results from our laboratory using scanning electron microscope analysis have shown that clove and oregano oils and their major phenolic components, thymol (isomer of carvacrol) and eugenol, induced important damage at the envelope level of gram-negative and gram-positive bacteria (18) and *Saccharomyces cerevisiae* (1, 4).

However, for nystatin, even if 100% cell mortality was shown at 5 IU/ml (MFC), no leakage of substances absorbing at 280 nm was recorded. We conclude from the present results that carvacrol and eugenol may exert their anticandidal effect by inducing more important envelope damage at their MFC than does nystatin. In agreement with that, we suggest an anticandidal mechanism of these major phenolic components, which consistently affect the envelope of *Candida* cells.

These *in vitro* results led us to investigate this antifungal effect on an animal experimental model of oral candidiasis in immunosuppressed rats, which has been shown to be a simple and highly reproducible *in vivo* method of studying the efficacy of antifungal agents (8).

For topical treatment, we used eugenol and carvacrol at 2 × MIC *in vitro*, which could represent a rational choice for the management of this local infection. To validate our experimental conditions, we intentionally used a reference treatment group with 10 × MIC of nystatin to eradicate the infection. For the best adhesion of our products on the oral cavity, a gelatinous suspension of 0.8% agar was used as excipient.

The strain of *C. albicans* used in this study was isolated from patients suffering from acute vaginitis and was passed several times through rat. It demonstrated high pathogenic properties since untreated animals showed persistent *C. albicans* infection. However, in the group of nonimmunosuppressed rats, only 1/7 animals were still infected on day 8, demonstrating the requirement of immunosuppression for a successful model. This result is in agreement with Martinez et al. (15).

The infected untreated group showed positive *Candida* cultures throughout the experiment. The mean log CFU/swab was similar to that obtained by other authors with the same number of cells in the inoculum (6, 15).

Importantly, under the conditions described above, eugenol and carvacrol show no acute toxicity for rats. The doses used (approximately 10 and 20 μg/kg) are far less toxic than the doses used by Manohar et al. (13).

In conclusion, these results suggest that eugenol and carvacrol may be considered potential drugs with better efficacy for the treatment of oral candidiasis, on the condition that toxicologic studies determine whether these two natural phenolic components are safe for the mouth at the concentrations we used. Their potent efficacy in the rat model suggests that it may be of value to investigate the possibility of treating humans suffering from this kind of infection, especially when the infection is refractory to known antifungal drugs.

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