Evaluation of carvacrol and eugenol as prophylaxis and treatment of vaginal candidiasis in an immunosuppressed rat model

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Objectives: Anticandidal activity of carvacrol and eugenol, the major phenolic components of oregano and clove essential oils, respectively, were tested in vivo.

Methods: Efficacy evaluation of carvacrol and eugenol in the prophylaxis and treatment of experimental vaginal candidiasis was performed in immunosuppressed rats. The anticandidal activity was analysed by microbiological and histological techniques and was compared with that of nystatin.

Results: Microbiologically, prophylactic treatment with carvacrol eradicated the vaginal fungal burden of infected rats, whereas eugenol reduced the number of colony counts of Candida albicans in vaginas of infected rats by 98.9% 10 days after inoculation. Therapeutic treatment for 7 consecutive days with carvacrol was able to eradicate the vaginal candidal burden in 7/9 of the infected rats and reduced the number of colony counts of C. albicans in vaginas of the two remaining rats by 98%. Treatment with eugenol completely cured 2/9 of the infected animals, but the 7/9 still infected showed an 84% reduction of colony counts of C. albicans in their vaginas. Histologically, in all treated rats, no Candida organisms were found in the lumina of the vagina; this was in contrast to control groups in which many yeasts, strongly stained with periodic acid-Schiff, were observed. The results obtained with nystatin used at 10-fold minimal inhibitory concentration confirm the validity of this model.

Conclusions: Carvacrol and eugenol could be considered as promising products in the treatment of vaginal candidiasis. This work is a preliminary contribution to the development of a new generation of efficient and natural antifungal agents for curative treatment and prophylaxis.

Keywords: anticandidal activity, experimental vaginitis, animal models, antifungals, essential oils

Introduction

Vulvovaginal candidiasis is a widespread common disease affecting about one-third of all women at least once in their lifetime. About 5% of these women are subject to recurrent attacks of the disease.¹ Vaginal candidiasis is often associated with conditions such as diabetes mellitus, antibiotic therapy, corticotherapy and pregnancy, although in many cases, there are no clear predisposing factors.²

In recent years, polyenes and azole agents have been used for treating vaginal candidiasis.¹⁻⁶ Our study aims to find new and effective natural products to treat this fungal infection.

The antifungal effect of essential oils (EO) has been noted in several studies.²⁻⁴ Specific anticandidal activity is also well established.⁹⁻¹¹ The phenolic major components of EO have potent antifungal activity in vitro.¹²,¹³ Carvacrol and eugenol are the phenolic major components of oregano and clove oils, respectively. To evaluate their in vivo efficacy, we have adopted an experimental model of vaginal candidiasis in immunosuppressed rats.¹⁴ Carvacrol has been used previously by oral route as therapy for systemic candidiasis in a model in mice, as described by other authors.¹³

The anticandidal activity of carvacrol and eugenol was evaluated using prophylactic and therapeutic treatment in rats inoculated intravaginally with Candida albicans and compared with nystatin (used as a positive control) by microbiological and histological techniques. To our knowledge, this is the first time the anticandidal activity of carvacrol and eugenol has been investigated in the vaginal candidiasis model.
Materials and methods

Antifungal agents

Carvacrol and eugenol were purchased from Sigma and nystatin was purchased from Bristol-Myers Squibb.

Animals

Oophorectomized, female Wistar rats, body weight 150–200 g, were used throughout this study. They were divided into groups of five and housed in cages (480 mm × 270 mm × 200 mm). 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 ad libitum access to industrial food and water. The research complied with European legislation and with company codes of practice.

To exclude the effect of endogenous ovarian hormones, ovaries were removed before administering a sufficient oestrogen dose necessary to create and maintain the infection. The rats were then maintained under pseudoestrus by giving them estradiol benzoate and were immunosuppressed by giving them dexamethasone, as previously reported by Martinez et al. Each experiment was performed with nine animals in each group and all the experiments were repeated three times. Therefore, the results presented are the mean of the three repetitions.

Microorganism and growth conditions

This study was performed on the C. albicans strain (1E 111PV515) isolated from the vaginal secretions of a woman with acute vaginitis. For the experimental infection, a subculture of the strain from Sabouraud dextrose (Difco) was grown in yeast extract peptone glucose medium for 18 h at 30°C in a shaking water bath at 75 rpm. The obtained cells were harvested by centrifugation at 2500 g. The cells were washed three times in phosphate-buffered saline and approximately adjusted to a final concentration of 6.6 × 10^7 cells/mL. In addition, the viability of the inoculum was confirmed by quantitative cultures of serial 10-fold dilutions on Sabouraud dextrose agar plates at the time of infection, and counts were in accordance with the number of colony forming units (cfu) using the drop count method.

Experimental candidal vaginitis

The rats were inoculated intravaginally with 10^7 cells (in 150 μL of sterile saline solution) of washed blastoconidia of C. albicans, according to the method described by other workers. Three days before inoculation and before the beginning of any treatment, the animals were sampled to confirm the absence of C. albicans organisms in the vaginal cavity.

In vitro determination of minimal inhibitory concentrations (MIC)

The lowest concentration of carvacrol, eugenol or nystatin that caused complete inhibition of growth of C. albicans was determined. This was conducted in triplicate, in a liquid medium that contained the antifungal agent in contact with yeast cells for 24 h at 30°C, according to the method described by Remmal et al.

Vaginal suspension of the antifungal treatment

Preliminary tests using different concentrations of antifungal agents showed that carvacrol and eugenol reduced the number of Candida in the vagina at 2 × MIC and nystatin at 10 × MIC (results not shown). Therefore, these concentrations were used throughout this study. 500 μL of carvacrol or eugenol at 2 × MIC were given twice a day at the final concentration of 2 × 10^3 mg/L (~10 mg/kg per day) and 4 × 10^3 mg/L (~20 mg/kg per day), respectively. 500 μL of nystatin at 10 × MIC, i.e. 54.45 mg/L (~272 μg/kg per day) were also given twice a day. In order to keep the drugs in the vagina without a risk of discharge, all the antifungal drugs used were dispersed in 0.8% agar solution.

Prophylactic treatment

Prior to the infection of the animals, they were separated randomly into six ‘prophylactic groups’ (groups P):

- Group 1 (n=9) positive control group: immunosuppressed, inoculated intravaginally with C. albicans, received excipient (500 μL of agar suspension at 0.8%) twice a day.
- Groups 2, 3 and 4 (n=9 in each group) treated groups: immunosuppressed, infected animals received carvacrol, eugenol or nystatin, respectively. This treatment began 2 days before the inoculation of C. albicans in the vagina and continued 3 days thereafter at a dose of 500 μL twice a day by intravaginal route.
- Group 5: (n=9) infected, untreated but not immunosuppressed animals: this group served to study the impact of immunosuppression on the development of the infection.
- Group 6: (n=9), negative control group: neither infected nor treated animals.

These last two groups served as controls for both prophylactic and therapeutic treatments.

Therapeutic treatment

Before the inoculation, animals (n=36) were separated randomly into four ‘therapeutic groups’ (Groups T):

- Group 1 (n=9) positive control group: immunosuppressed, inoculated intravaginally with C. albicans, received excipient (500 μL of agar suspension at 0.8%) twice a day.
- Groups 2, 3 and 4 (n=9 in each group) received therapeutic treatment with carvacrol, eugenol or nystatin, respectively, at the same concentrations as the prophylaxis. This treatment began 72 h after the inoculation and continued for 7 days thereafter at a dose of 500 μL twice a day by intravaginal route.

Quantification of the infection level and determination of prophylactic and therapeutic efficacy

The quantification of the infection was assessed by microbiological and histological techniques.

Microbiology

For the prophylactic treatment, the evaluation of vaginal burden was performed on samples collected by rolling a sterile cotton swab over the vaginal cavity, the swab was then suspended in 1 mL of sterile saline buffer. This operation was repeated on day 3 and day 10 post-infection to observe the course of infection. Determination of the number of Candida organisms was conducted in duplicate after serial 10-fold dilution of washing fluid and plating on Sabouraud glucose agar containing 0.05% of chloramphenicol. All plates were
For the group of animals treated with carvacrol, only 4/9 of the strain were 10^3 mg/L, 2 C. albicans of each rat were performed and no prior to the initiation of the experiments, vaginal cavity cultures. Microbiological results of prophylactic treatment.

In vitro MIC of carvacrol, eugenol and nystatin against the C. albicans strain were 10^3 mg/L, 2 x 10^3 mg/L and 5.44 mg/L, respectively. The concentrations used for the treatment of rats were 2 x MIC for eugenol and carvacrol. Nystatin at a concentration 10 x MIC was used as reference treatment. The efficacy of carvacrol and eugenol was microbiologically and histologically compared with nystatin’s effect.

Results

In vitro MIC for eugenol and carvacrol. Nystatin at a concentration was used as reference treatment. The efficacy of carvacrol and eugenol was microbiologically and histologically compared with nystatin’s effect.

Microbiological results of prophylactic treatment

Prior to the initiation of the experiments, vaginal cavity cultures of each rat were performed and no Candida organisms were found. After 5 days of treatment, each group of animals was sampled. For the untreated group, the vaginal swabs were all positive with regard to the presence of C. albicans (Table 1). For the group of animals treated with carvacrol, only 4/9 of the rats were colonized with a mean of colony counts significantly lower than the untreated group. In the group treated with eugenol, in comparison with the control, no difference was seen; all animals remained colonized to the same degree as the control, showing no significant difference. The treatment with nystatin allowed accelerated clearance of vaginal infection.

After the prophylactic treatment, animals remained under observation for 1 week in order to monitor the course of the infection. Up to day 10, C. albicans organisms were still detected in the control group (100%). All the animals treated with carvacrol showed negative culture results, whereas only 2/9 of the animals treated with eugenol showed negative culture results and the seven remaining rats showed a low level of infection with significantly less abundant colony counts compared with the control.

In the group of non-immunosuppressed, infected untreated rats, only 1/9 remained infected on day 10.

Table 1. Microbiological study of prophylactic efficacy of carvacrol and eugenol versus nystatin against vaginal candidiasis in rats. The rats were intravaginally infected with 10^7 cells of C. albicans (1E111PV 515). Drugs were topically administered twice a day for 5 consecutive days, starting 2 days before challenge and continuing for 3 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 3</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>infected (%)</td>
<td>log cfu ± s.d.</td>
</tr>
<tr>
<td>P1, control</td>
<td>9/9 (100)</td>
<td>4.66 ± 0.21</td>
</tr>
<tr>
<td>P2, carvacrol</td>
<td>4/9 (44.4)</td>
<td>3.45 ± 0.30*</td>
</tr>
<tr>
<td>P3, eugenol</td>
<td>9/9 (100)</td>
<td>4.48 ± 0.58</td>
</tr>
<tr>
<td>P4, nystatin</td>
<td>1/9 (11.1)</td>
<td>2.74*</td>
</tr>
<tr>
<td>Group 5, non-immunosuppressed rats*</td>
<td>5/9 (55.55)</td>
<td>4.30 ± 0.98</td>
</tr>
</tbody>
</table>

*Group 5, non-immunosuppressed, infected and untreated rats.

*P < 0.05 compared with control treatment (P1).

NA, not applicable.
Table 2. Microbiological study of therapeutic efficacy of carvacrol and eugenol versus nystatin against vaginal candidiasis in rats. The rats were intravaginally infected with 10⁷ cells of C. albicans (1E11PV 515). Drugs were topically administered twice a day for 7 consecutive days, starting 72 h after challenge and continuing until the 10th day. The percentage of cfu reduction indicated is calculated versus log cfu obtained with T1 control group at day 10.

<table>
<thead>
<tr>
<th></th>
<th>Infected animals (%)</th>
<th>log cfu ± S.D.</th>
<th>Infected animals (%)</th>
<th>log cfu ± S.D.</th>
<th>cfu reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1, control</td>
<td>9/9 (100)</td>
<td>4.66±0.21</td>
<td>9/9 (100)</td>
<td>4.40±0.39</td>
<td>NA</td>
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<tr>
<td>T2, carvacrol</td>
<td>9/9 (100)</td>
<td>4.62±0.43</td>
<td>2/9 (22.22)</td>
<td>2.78±0.25*</td>
<td>98.13</td>
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<tr>
<td>T3, eugenol</td>
<td>9/9 (100)</td>
<td>4.44±0.50</td>
<td>7/9 (77.77)</td>
<td>3.73±0.80*</td>
<td>84.83</td>
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<tr>
<td>T4, nystatin</td>
<td>9/9 (100)</td>
<td>3.85±0.45</td>
<td>1/9 (11.11)</td>
<td>2.50*</td>
<td>98.87</td>
</tr>
<tr>
<td>Group 5, non-immunosuppressed rats*</td>
<td>5/9 (55.55)</td>
<td>4.30±0.98</td>
<td>1/9 (11.11)</td>
<td>4.52</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Group 5, non-immunosuppressed, infected untreated rats.
*P<0.05 compared with control treatment (T1).
NA, not applicable

**Histological data**

Prophylactic treatment. In all animals, the presence of yeast was researched on sections of vaginas stained with PAS. In infected untreated rats, both budding yeast and the pseudohyphae form of C. albicans—dark with PAS stain—were found in the luminal vagina. At the surface of the epithelium, we also noticed keratin debris, whereas in the non-infected control group, no Candida was observed in the lumen of the vagina. The histological sections of the infected groups treated with carvacrol, eugenol or nystatin showed the same aspect as the non-infected control group.

In the non-immunosuppressed infected and untreated group, the only remaining infected animal had a few pseudohyphae in the vaginal lumen on day 10.

Therapeutic treatment. Vaginal sections of all the animals were also studied by light microscopy. Infected and untreated rats demonstrated the presence of C. albicans at the surface of the epithelium with desquamation of superficial layers, whereas in the non-infected untreated control group no Candida was found in the vaginal lumina. Therapeutic treatment with carvacrol completely eradicated the vaginal Candida. Eugenol significantly reduced the fungal burden to such an extent that Candida organisms in histological sections were not detectable. Vaginal sections in these last two groups presented the same aspect as the non-infected, untreated control group. Regarding the nystatin group, no Candida was found in the vaginal lumina.

Histologically, the epithelium of the vagina of non-infected rats receiving carvacrol or eugenol was no different from that of non-infected untreated rats.

**Discussion**

The major phenolic components of some EO have been said to have potent antimicrobial and antifungal activity in vitro. The anticandidal effect of these major components has been shown in vitro.

The low values of MIC we obtained in vitro with carvacrol and eugenol encouraged us to test their activity in vivo. These results were similar to those obtained with the same products under the same conditions against bacteria in some of our previous work.

The experimental model of vaginal candidiasis in rats, whose ovaries have been removed, has been shown to be a simple and highly reproducible method of studying the efficiency of the antifungal agents in vivo. In this model, the role of the hormonal milieu is evident because the presence of oestrogen is a very important factor for the persistence of experimental candidal vaginitis.

In this model, the antifungal activity of two major phenolic components of EO, which was compared with that of nystatin, has been investigated and was determined by microbiological tests and histological study. To the best of our knowledge, this work constitutes the first attempt to assess the antifungal role of carvacrol and eugenol in vivo. Our results show that the control group of immunosuppressed, infected and untreated animals remained infected throughout the experiment. By contrast, 10 days after inoculation, the group of non-immunosuppressed, infected and untreated rats showed spontaneous clearing of the yeast. These data confirm that, on the one hand, we managed to induce experimental vaginal candidiasis in the rat and on the other, that immunosuppression is necessary to the success of this model.

To allow optimal adhesion of carvacrol, eugenol and nystatin on the mucosal vagina, a gelatinous suspension of 0.8% agar was used as excipient to treat rats by the intravaginal route. Carvacrol and eugenol were given at double concentration of MIC, that could represent a rational choice for the management of this localized infection. On the basis of preliminary tests, 10-fold MIC of nystatin was chosen as a reference treatment.

Following some authors, who demonstrated the efficacy of azoles in vaginal candidiasis prophylaxis, we investigated the efficacy of eugenol and carvacrol by prophylactic treatment. As regards the in vivo results, the infected untreated control group showed positive Candida cultures throughout the experiment. The mean of colony counts was similar to that reported by other investigators for the same inoculum size.

Under prophylactic treatment, the animals treated with carvacrol remained infected, with Candida counts being, on average, 1 log cfu/swab significantly lower than those found...
Carvacrol and eugenol in a vaginal candidiasis model

in the infected untreated control group. However, 7 days after the interruption of treatment, the results of sample collection showed negative Candida cultures, whereas 100% of the yeast was recovered from vagina samples from all of the infected untreated control animals. The histological examination agreed with the microbiological results concerning the absence of C. albicans among the animals treated with carvacrol.

Prophylactic treatment with eugenol seems to be less effective than that with carvacrol since there was no response to this product immediately after the treatment. However, few animals showed negative culture 7 days after the interruption of the treatment. Furthermore, vaginal burden in the remaining infected animals was significantly less than in the control group, and no C. albicans were detected on vaginal sections. From these results, carvacrol was shown to be more effective than eugenol in preventing Candida vaginal infection, although both seem to have a delayed effect.

Therapeutic treatment with carvacrol for 7 days led to clearance of yeast from the majority of animals exhibiting negative Candida culture, which was confirmed by the histological examination, and the remaining infected animals showed a high percentage of reduction of cfu versus control, with no detectable Candida cells histologically.

Concerning the animals treated with eugenol, in spite of the high number of infected animals, the percentage of reduction of cfu was high compared with that of the control group. Our histological findings confirm microbiological results, given that vaginal sections did not exhibit any hyphae in the vaginal lumina. Therefore, it appears that the therapeutic treatment with carvacrol is more efficient than with eugenol in curing Candida vaginal infection.

The results obtained with the nystatin used as a reference treatment were expected since this drug is known to cure this vaginal infection.

Suresh et al.11 have demonstrated the antifungal activity of whole Santolina oil against vaginal candidiasis in mice. Other workers36,37 have also developed the treatment of vaginal candidiasis in humans with products utilizing whole tea tree oil as an active ingredient. However, the use of whole EOs does not allow the determination of the active principle, because of the complexity of their components. An aim of this work is to demonstrate the antifungal effect of a purified major phenolic component in vivo.

The daily doses of carvacrol and eugenol used in our experiments were so low that no toxicity was observed on vaginal mucosa. Furthermore, these natural components have the advantage of being volatile molecules that can penetrate inaccessible areas. This, however, is not the case with all antifungal drugs used in this kind of mucosal infection.

This work suggests that carvacrol and eugenol are naturally occurring antifungal agents that could be promising drugs for the treatment and prevention of vaginal candidiasis, especially in prophylaxis in patients suffering from AIDS.

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References


