Protective Activity of Geranium Oil and Its Component, Geraniol, in Combination with Vaginal Washing against Vaginal Candidiasis in Mice

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In order to evaluate an effective administration method of essential oils for vaginal candidiasis, efficacy of vaginal application of essential oils against murine experimental candidiasis was investigated. The effect on vaginal inflammation and Candida growth form was also studied. Vaginal candidiasis was established by intravaginal infection of C. albicans to estradiol-treated mice. These mice intravaginally received essential oils such as geranium and tea tree singly or in combination with vaginal washing. Vaginal administration of clotrimazole significantly decreased the number of viable C. albicans cells in the vaginal cavity by itself. In contrast, these essential oils did not lower the cell number. When application of geranium oil or geraniol was combined with vaginal washing, the cell number was decreased significantly. The myeloperoxidase activity assay exhibited the possibility that essential oils worked not only to reduce the viable cell number of C. albicans, but also to improve vaginal inflammation. The smear of vaginal washing suspension suggested that more yeast-form cells appeared in vaginal smears of these oil-treated mice than in control mice. In vitro study showed that a very low concentration (25 μg/ml) of geranium oil and geraniol inhibited mycelial growth, but not yeast growth. Based on these findings, it is estimated that vaginal application of geranium oil or its main component, geraniol, suppressed Candida cell growth in the vagina and its local inflammation when combined with vaginal washing.

Key words Candida albicans; essential oil; Pelargonium asperum

Vaginal candidiasis is a very common mucosal infectious disease in women and 75% of women are said to have the onset of this disease in their reproductive years. Between 40 and 50% of these women have recurrent episodes.1 Its clinical features are not life-threatening, but are an uncomfortable experience with itching, soreness and vaginal discharge which significantly disrupt a woman’s life. Usually antifungal treatment is very effective under normal conditions, although it does not prevent recurrence2); furthermore, incidence of antifungal-resistant Candida albicans by prolonged antifungal treatment is reported3) so that there is a need to develop new treatment methods other than antifungal agents.

Aromatherapy has been traditionally used for vaginal candidiasis and essential oils have been applied vaginally by suppository and douche. Efficacies of these therapeutic methods are estimated only by anecdotal experiences, and scientific researches on aromatherapy for vaginal candidiasis basically are not sufficient. From in vitro study, essential oils such as lemongrass (Cymbopogon citratus) and geranium (Pelargonium asperum),4) tea tree (Melaleuca alternifolia),5,6) lavender (Lavandula angustifolia)7) and thyme (Thymus vulgaris)8) oils are reported to inhibit Candida mycelial growth. On the other hand, in vivo studies were very limited. Mondello et al. reported that intravaginal injection of tea tree oil9) or its main active compound, terpinen-4-ol,10) just 1 h after intravaginal inoculation of C. albicans reduced significantly, but not remarkably, viable cell number of C. albicans in vaginal cavity in rat models. Chami et al. reported that carvacrol and eugenol11) effectively reduced the cell number, however, these compounds are not suitable for clinical use because of their irritant activity.

As far as we know, there are no report about the efficacious administrative method of essential oils in terms of decrease of Candida cell number and suppression of vaginal inflammation. These situations of basic studies against vaginal candidiasis required us to examine the effect of non-irritable essential oils for vaginal candidiasis including its proper administration methods in vivo.

We recently reported that geranium oil and its component, geraniol, have anti-inflammatory activity.12,13) Geranium oil is one of the most popular essential oils in aromatherapy, is gentle to skin and has traditionally been used for vaginal candidiasis. Baudoux recommended geranium oil as an effective essential oil for therapeutic treatment of vaginal candidiasis.14) Geraniol is one of main components of geranium oil and its content is about 20%.

In this study using a murine model, we investigated effective administration methods of essential oils, especially geranium oil, together with their effect against vaginal inflammation and against Candida growth.

MATERIALS AND METHODS

Essential Oils Geranium Bourbon oil was purchased from Pranarom (Kenso-igakusha, Ltd., Tokyo, Japan). The major components of geranium oil determined by GC analysis were geraniol (22.3%), β-citronellol (19.7%), citronellyl formate (8.6%), and geranyl formate (8.1%). Other essential oils were from Pranarom and Sanoflore (Hyperplants, Ltd., Tokyo).

Agents Polyoxyethylene (20) sorbitan monooleate (Tween 80), carboxymethyl cellulose sodium salt (CMC) and HTAB, human myeloperoxidase (MPO), and tetramethylbenzidine (TMB) were from Sigma-Aldrich Japan (Tokyo).
Animals All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. Six week-old female BALB/c mice (Charles River Japan, Inc., Kanagawa, Japan) were used for all animal experiments. The photoperiods were adjusted to 12 h of light and 12 h darkness daily, and the environmental temperature was constantly maintained at 21 °C. The mice were kept in cages housing 4—6 animals and were given ad libitum access to food and water.

Candida albicans The clinically isolated strains of C. albicans, TIMM1768 and TIMM2640, were maintained in our laboratory and were stored at −80 °C in Sabouraud dextrose broth (Becton Dickinson, MD, U.S.A.) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol until the experiment was performed.

For the in vivo experiment, TIMM2640 was grown on Sabouraud dextrose agar plates (Becton Dickinson) at 37 °C for 18 h. The cells were harvested by centrifugation (1500 rpm, 5 min, 4 °C) and suspended to 3×10⁸ cells/ml in RPMI1640 medium containing 2.5% fetal calf serum (FCS) for vaginal inoculation.

For the in vitro experiment, TIMM1768 was washed with RPMI1640 containing 2.5% FCS by centrifugation (1500 rpm, 3 min, 4 °C) and suspended in 5×10⁵ cells/ml.

In Vivo Activity of Essential Oils by Continuous Vaginal Administration Prior to Candida infection, the mice were injected subcutaneously with 0.125 mg of β-estradiol-17-valerate (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 50 μl olive oil on day −3 and day 1. On day 0, these mice were inoculated intravaginally with 6×10⁶ cells of C. albicans in 20 μl. On day 1 to 3, 10 μl of essential oils suspension or clotrimazole solution was injected vaginally. Essential oils were dissolved in 50 μl of ethanol. Then, 1% CMC solution including 0.01% Tween 80 was added to make up to 1 ml. Clotrimazole (20 mg) was dissolved in 20 μl of ethanol, to which 180 μl of 1% CMC solution including 0.01% Tween 80 was added. For control mice, aqueous solution including 5% ethanol, 0.01% Tween 80 and 1% CMC (control solution) was injected.

On day 4, mice were washed vaginally with 500 μl of saline. The suspension was diluted and cultured on Candida GS plates (Eiken Chemical Co., Ltd., Tokyo) for 18 h at 37 °C. The colony forming units (CFU) were counted, and the total CFU per mouse was calculated (n=5—6).

In Vivo Effect of Vaginal Washing The procedure was almost the same as mentioned above; all mice were injected control solution on day 1 to 3. Mice of washing group were washed vaginally with 500 μl of saline just before the first injection of control solution and 6 h after each injection (n=5—6).

In Vivo Activity of Administration of Geranium Oil or Geraniol Coupled with Vaginal Washing Prior to Candida infection, the mice were injected subcutaneously with 0.125 mg of β-estradiol-17-valerate in 50 μl olive oil on day −1. On day 0, these mice were inoculated intravaginally with 6×10⁶ cells of C. albicans in 20 μl. Two days after inoculation, the vagina was washed with 500 μl of saline. Ten microliters of essential oils were injected vaginally immediately after (0 h) and 3 h after washing. Six, 24 and 96 hours after the first essential oil injection, the vagina was washed with saline.

(a) Measurement of CFU: The suspension was diluted and cultured on Candida GS plates for 18 h at 37 °C, the CFU were counted, and the total CFU per mouse was calculated (n=20—21). The relative viable C. albicans cell numbers of each time were expressed by relative values calculated by the following formula: log[(CFU at each hour)/(CFU at 0 h)].

(b) MPO Activity: The suspension at 96 h was centrifuged at 1500 rpm at 4 °C for 5 min and the supernatant and the precipitate were stored separately at −20 °C until the myeloperoxidase (MPO) assay based on the method of De Young et al.15 and partly modified. Five-hundred microliters of 80 mm sodium phosphate buffer, pH 5.4, containing 0.5% HTAB (0.5% HTAB solution) was added to the precipitate, and it was freeze-thawed 3 times, centrifuged at 1500 rpm at 9 °C for 5 min and the resulting supernatant was collected. Triplicate 30 μl portions of the supernatant or the resulting supernatant were poured into 96 well microtiter plates. For assay, 200 μl of a mixture containing 100 μl phosphate buffered saline, 85 μl of 0.22 M sodium phosphate buffer, pH 5.4, and 15 μl of 0.017% hydrogen peroxide was added to the wells. The reaction was started by the addition of 20 μl of 18.4 mM TMB · 2HCl in 8% aqueous dimethylformamide. Plates were stirred and incubated at 37 °C for 3 min and then placed on ice where the reaction was stopped by addition to each well of 30 μl of 1.46 M sodium acetate, pH 3.0. The MPO value was evaluated by measuring the absorbance of samples at 620 nm (OD value). The total MPO values per mouse were calculated by the addition of each value detected from the supernatant or the resulting supernatant.

(c) Morphological Study: For morphological study, the cell suspensions recovered 6 h after oil treatment were smeared, stained with periodic acid-Schiff (PAS), and mounted in Entellan Neu (Merck KGaA, Darmstadt, Germany).

In Vitro Activity of Geranium Oil and Geraniol against Candida Mycelial Growth The mycelial growth of C. albicans was measured based on the method of Abe et al.16 Geranium oil and geraniol were dissolved in dimethyl sulfoxide (DMSO) in 100 mg/ml solution. Geranium oil was further diluted using RPMI1640 containing 2.5% FCS to 50, 100, 200, 400 μg/ml. Geraniol was diluted to 25, 50, 100, 200 μg/ml. We have checked that DMSO dose not significantly affect Candida growth in these concentrations from our preliminary experiments (data not shown). One hundred microliters of geranium oil or geraniol solution was poured into the wells of 96-well flat-bottomed culture plates, followed by 100 μl of TIMM1768 (500 cells/well). Fifty micrograms per milliliter of the oil solution corresponded to 25 μg/ml of final concentration. Twenty-five micrograms per milliliter of geraniol is equal to 0.16 mm. Then, the mixtures were incubated for 16 h at 37 °C in a 5% CO₂ incubator, after which the supernatants were discarded to remove nonadherent yeast-form cells. The adherent mycelial cells were sterilized with 70% ethanol, washed 3 times with distilled water, and dried. They were then stained for 15 min at room temperature by addition of 100 μl of 0.02% crystal violet, washed 3 times with distilled water, and solubilized by the addition of 150 μl of isopropanol including 0.04 N HCl and 50 μl of 0.25% sodium dodecyl sulfate. The mycelial growth of C. albicans was evaluated by measuring the absorbance of tripli-
cate samples at 620 nm (OD value). The values of mycelial growth were relatively expressed by the ratio to those without oils.

**Statistical Analysis** The results were expressed by the mean ± standard error. The data were statistically compared using the Student's t-test.

**RESULTS**

The Effect of Vaginal Administration of Essential Oils on Vaginal Candidiasis

We first checked the vagina histologically to confirm that mice developed vaginal candidiasis in our protocol. Four days after inoculation, *C. albicans* proliferated in the vaginal cavity of the infected mice and the mycelia invaded epithelium of the vaginal wall as shown in Fig. 1.

Next, we evaluated the effect of essential oils as well as an azole-antifungal, clotrimazole, which is clinically used for vaginal candidiasis, by vaginal administration for 3 continuous days.

Vaginal administration of a topical clotrimazole (1 mg/10 μl/mouse) significantly decreased the number of viable *C. albicans* cells in the vaginal cavity compared with control, while 10 μl of 5 and 1% geranium and tea tree oils did not lower the cell numbers (Fig. 2). The essential oils of lemongrass, oregano and clove oils, which are known to have strong anti- Candida activity in vitro, also did not significantly influence the cell numbers at doses of 0.2 and 1.0% (data not shown). These results indicated that administration of essential oils, but not clotrimazole, are ineffective when used alone against vaginal candidiasis under this experimental condition.

The Effect of Combined Administration of Geranium Oil or Geraniol and Vaginal Washing

We examined the effect of vaginal washing on the infection, since in folk medicine essential oil treatment for vaginal infectious diseases was frequently combined with vaginal washing using a bidet. The vagina was washed once a day for 3 continuous days, and the CFU was measured on day 4. Vaginal washing significantly lowered the number of viable *C. albicans* cells compared with non-washing control (*n* = 6, 3.94 ± 0.93, and *n* = 5, 5.29 ± 0.28 log CFU/mouse, respectively, *p* < 0.05).

As the effectiveness of washing treatment had been shown, we examined the combined effect of essential oils and vaginal washing. Treatments were started from 2 d after *Candida* inoculation to evaluate the therapeutic activity of this combination. The vagina was washed and then oils were applied vaginally immediately after (0 h) and 3 h after the washing. The CFU was measured at the time of the first-washing (0 h) and 6, 24 and 96 h after the first oil application. Deviations in the cell numbers of *C. albicans* of individual infected mice were relatively large, so we compared the relative value of each hour to 0 h.

In a preliminary experiment, the effects of 1 and 5% geranium oils were examined and the two concentrations were found to be similarly effective. Therefore, in subsequent experiments, we evaluated the effects of geranium oil and geraniol administration at 1%. In washing alone of the control mice, the relative cell numbers reached the maximum at 24 h and maintained this level for 96 h (Fig. 3). On the other hand, both geranium oil and geraniol significantly decreased

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**Fig. 1.** Microscopic Observation of a Typical Lesion on Vaginal Tissues from BALB/c Mouse on Day 4

*Candida albicans* was inoculated on day 0. On day 4, tissues around the vaginal cavity were excised and stained with hematoxylin and eosin stain and PAS stain. (a) Non-inoculated mice (b) inoculated mice showing invasion of the filamentous *C. albicans* to epithelium of vaginal wall.

**Fig. 2.** Effects of Continuous Vaginal Application of Essential Oils and Antifungal Agent against Vaginal Candidiasis

Essential oils and clotrimazole were administered vaginally for 3 d. On day 4, mice were washed vaginally and the colony forming units (CFU) were counted. *p* < 0.05.
the relative cell numbers 6 h after the first oil application, and the effect of each oil was enhanced time-dependently. Relative CFU at 96 h decreased to 1/10 compared with the washing control.

To assess the growth form of *C. albicans* in the vaginal cavity, the cell suspensions recovered 6 h after oil treatment were smeared and observed microscopically. Although most of the preparations showed that yeast and mycelial forms were mixed at various ratios, yeast form cells appeared predominantly in many vaginal smears of these oil-treated mice, while the mycelial form seemed more prevalent in the control mice. Typical samples are shown in Fig. 4.

To examine the effects of inflammatory response of essential oils applied vaginally, we measured the total MPO activity of washing fluids recovered from the vaginal cavity. MPO is a marker enzyme of neutrophil granules, and have been used as a parameter of infiltration of neutrophils in various inflammatory experiments.15,17,18) Figure 5 shows the distribution of *Candida* CFU and total MPO activity in vaginal washing fluid obtained from individual mice 96 h after treatment. The whole area, in which the two parameters of each mouse were plotted, was divided into 4 regions (A: low CFU and high MPO; B: high CFU and high MPO; C: low CFU and low MPO; and D: high CFU and low MPO), and the ratio of each group was compared between control and

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**Fig. 3. Effects of Vaginal Administration of Geranium Oil and Geraniol with Vaginal Washing against Vaginal Candidiasis**

White columns, control (n=21); grey columns, 1% geranium oil (n=20); black columns, 1% geraniol (n=20). Vagina was washed with saline 2 d after *Candida* inoculation. Geranium oil and geraniol were administered immediately and 3 h after washing. Six, 24, 96 h after first oil administration, mice were washed vaginally and the CFU were counted. *p<0.05, **p<0.01, *mean±S.E. was calculated from average of $log\{(CFU at each hour)/(CFU at 0 h)\}$.

**Fig. 4. Microscopic Observation of the Smears from Vaginal Washing Fluid Obtained 6 h after Treatment**

(a) Control mouse, (b) geranium-treated mouse. The mycelial form existing predominantly in the control mice was markedly decreased in the treated mice.

**Fig. 5. Distribution of *Candida* CFU and the MPO Activity**

Ninety-six hours after first oil administration, CFU and the MPO activity were measured from vaginal washing fluid obtained from individual mice. The area, in which the two parameters of each mouse were plotted, was divided into 4 regions (A: low CFU and high MPO; B: high CFU and high MPO; C: low CFU and low MPO; and D: high CFU and low MPO). (a) total mice, white circles, washing control mice; black triangles, geranium oil or geraniol treated mice; (b) washing control, (c) geranium oil or geraniol-treated mice.
treated mice. The MPO activity from vaginal washing fluid of non-infected mice was 545.7±414.0 munits/mouse (unpublished data). Mice with lower CFU (less than 10^5 cells/mouse) also had lower MPO activity (1284.8±576.6 munits/mouse) (region C), while mice with higher CFU (more than 10^5 cells/mouse) also had higher MPO activity (3677.5±1753.8 munits/mouse) (region B). In the control mice (Fig. 5b), 65% of the mice belonged to region B with high CFU and high MPO. In the treated mice (Fig. 5c), however, the ratio with low CFU and MPO (region C) was increased. This distribution suggested that essential oils worked not only for reducing the viable cell number of C. albicans, but also for suppressing the infiltration of neutrophiles, that is, improving vaginal inflammation.

These revealed that essential oils might be effective for vaginal candidiasis when combined with vaginal washing. To learn the reason for the efficacy, we checked the effect of geranium oil and geraniol against the Candida growth form in vitro as the next step.

**Inhibitory Effects of Geranium Oil and Geraniol on Mycelial Growth of C. albicans in Vitro** Figure 6a shows that geranium oil and geraniol inhibited the mycelial growth of C. albicans dose-dependently. The activity of geraniol appeared weaker than or only equal to geranium oil. The concentration of 50% inhibition (IC_{50}) was lower than 25 μg/ml for geranium oil and was 26 μg/ml for geraniol. Figure 6b shows C. albicans cultured in the medium with or without geranium oil. This indicated that most C. albicans cultured with 25 μg/ml of this oil was proliferated as the yeast form. Microscopically, geranium oil, in the range of 25 and 50 μg/ml, lowered the number of mycelial forms, but not yeast forms. The oil above 100 μg/ml inhibited the yeast form growth dose-dependently (data not shown). On the other hand, geraniol did not inhibit the yeast form growth in the range of 6.25 and 100 μg/ml (data not shown).

**DISCUSSION**

In this study, we showed that application of geranium oil or its component, geraniol, suppressed Candida cell growth in vaginal cavity only when it was combined with vaginal washing. We also found that, in the case of the mice with decreased Candida CFU, MPO activities as an indicator of neutrophile accumulation were also lowered. As far as we know, this is the first report showing that combination of essential oil treatment and vaginal washing elicits a protective effect on vaginal candidiasis.

In our vaginal candidiasis model, local administration of essential oils without vaginal wash did not decrease CFU of Candida in the vagina. It has already been reported that administrations of tea tree oil (9) and essential oil components, terpinen-4-ol, (10) carvacrol and eugenol, (11) decreased the viable number of C. albicans when the rat vaginal candidiasis model was used. Although these results seem to contradict ours, we think this difference may depend on experimental conditions. In our model, we applied only 10 μl of diluted essential oils vaginally in mouse, while they used a large quantity of essential oil solution (100—500 μl) for one application in rat. It is conceivable that such a large quantity might have a similar effect to that of vaginal washing when applied.

Further clarification is needed on why geranium oil and geraniol were effective only when combined with vaginal washing. At this moment, two possibilities can be pointed out. Firstly, vaginal washing lowers the number of viable Candida cells, and it makes the subthreshold effect of essential oil significant. Secondly, C. albicans treated with essential oils was washed out by vaginal washing. We think the second possibility is the more likely, because even less than 50 μg/ml of geranium oil changes the growth form of C. albicans to yeast, which has less ability to adhere to tissue. (1, 19) The latter possibility can also be supported by the results obtained by our infectious experiments that yeast-form cells were found predominantly in many vaginal smears of the oil-treated mice, while mycelial-form cells were mostly observed in those of control group as shown in Fig. 4, even though it could not be quantitatively estimated.

In our previous experiment using a murine oral candidiasis model, oral application of essential oils improved their symptoms and decreased the viable Candida cell number (unpublished data). We can assume that the action mechanisms of essential oils against candidiasis include “change to” or “maintenance of” the yeast form as a growth form of C. albicans, by application of the oils, and these may facilitate washing out of Candida cells by the flow of salivary fluids, drinking water and so on.
Therefore, it is possible to speculate that treatment with essential oil, which suppresses mycelial growth of *C. albicans* and lowers the adherent ability to tissues, makes easy to wash out yeast form of *C. albicans* in the infected area and thus to give therapeutic effects on mucosal candidiasis when combined with washing. This concept is very important clinically, because in folk medicine essential oil treatment for vaginal infectious diseases has frequently, but not always, been combined with vaginal washing using a bidet.

Another possibility should be discussed. Microscopically, geranium oil inhibited Candida growth including yeast form with high concentration (400 μg/ml) in vitro (data not shown). This direct inhibition of Candida growth may have therapeutic role in this model. However, this speculation cannot explain that main population of Candida cells was yeast-form in vaginal smears of geranium oil treated mice (Fig. 4b).

In this study, we have not tested therapeutic activity of essential oils other than geranium oil in combination with vaginal washing. But, we think that some other essential oils might be effective for vaginal candidiasis.

Although in this study the severity of Candida infection was evaluated by CFU in vaginal cavity, CFU seems insufficient as a parameter of severity degree. Complaints of vaginal candidiasis include vaginal itching and soreness accompanied from vaginal inflammation. Therefore, we checked effects of essential oil on inflammatory response by vaginal MPO activity, a maker enzyme of neutrophils.14 Compared with the MPO activity from vaginal washing fluid of the non-infected mice (545.7±414.0 munits/mouse, unpublished data), that of the infected mouse with CFU more than 10^4 cells/mouse was clearly high (3677.5±1753.8 munits/mouse). In the distribution chart between CFU and MPO (Fig. 5), a large number of “washing control mice” were plotted in region B (high CFU, high MPO). On the other hand, by the combined treatment with essential oil, both CFU and MPO seemed to be decreased. At least, when CFU from vaginal washing fluid was clearly suppressed, its MPO activity was also low (region C). These results also suggest that Candida infection with vaginal inflammation was improved by this combined treatment. In other words, essential oils worked not only to reduce the viable cell number of *C. albicans*, but also to suppress vaginal inflammation. This anti-inflammatory improvement by essential oil might be caused indirectly by decrease of viable Candida cells. But, since geranium oil has an anti-inflammatory effect in the murine acute and chronic inflammatory model as we reported,12,13 this improvement might result from a direct anti-inflammatory effect on a vaginal lesion.

In aromatherapy, several essential oils are used as an aid in therapeutic treatments for vaginal candidiasis in forms of suppository and douche. Our results presented here suggest that vaginal application of geranium oils and its main component, geraniol, might be effective for the treatment of vaginal candidiasis, especially when combined with vaginal washing. In order to confirm this hypothesis, we need to check the growth form of *C. albicans* from vaginal washing of patients who clinically undergo treatment with essential oils and vaginal washing.

REFERENCES