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Original Article

Protection of Oral or Intestinal Candidiasis in Mice by Oral or Intragastric Administration of Herbal Food, Clove (*Syzygium aromaticum*)

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Abstract

We examined the effect of a clove (*Syzygium aromaticum*) administered by two different routes on *Candida albicans* growth, using a murine oral candidiasis model. When the clove preparation was administered into the oral cavity of *Candida*-infected mice, their oral symptoms were improved and the number of viable *Candida* cells in the cavity was reduced. In contrast, when the clove preparation was administered intragastrically, oral symptoms were not improved, but viable cell numbers of *Candida* in the stomach and feces were decreased. These findings demonstrate that oral intake of an herbal food, clove, may suppress the overgrowth of *C. albicans* in the alimentary tract including the oral cavity.

Key words: murine oral candidiasis, clove, intestinal tract, mice

Introduction

Candida albicans is known to be a member of oral or intestinal microbial flora in healthy human individuals[1, 2)]. But overgrowth of *C. albicans* has caused pathogenic symptoms such as oral candidiasis and intestinal candidiasis[3, 4)], which have been postulated to have some pathological relevance to allergic diseases such as atopic dermatitis (AD) and food allergy[5)]. These medical situations around *C. albicans* prompted us to investigate the physiological effects of the overgrowth of *C. albicans* in experimental animal models and to develop some conventional methods for regulation of the intestinal growth of *Candida*[6, 7)]. In a preceding paper, we reported a murine experimental oral candidiasis model which made it possible to estimate the therapeutic efficacy of natural products using two parameters, colony forming units (CFU) of *C. albicans* in the mouth, and scores of the clinical manifestation of *Candida* infected tongues[8)].

Recently we and our colleagues examined anti-*Candida* activity of spices and herbs and reported that a clove preparation inhibited mycelial growth of *C. albicans in vitro*, then suggested its therapeutic availability against *Candida* colonization in mucosal tissues[9, 10)]. Clove is a well known spice, and is used as an aroma additive in food, pharmaceuticals, and cosmetics[11)]. Here, we examined the therapeutic efficacy of a clove preparation in a murine oral candidiasis model[12)].

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Materials and Methods

C. albicans strain

C. albicans strain TIMM 2640[8)], a clinically isolated strain was maintained at the Research Institute of Medical Mycology, Teikyo University. This strain was stored at －80°C in Sabouraud

dextrose broth (Becton Dickinson, MD, USA) containing 0.5% yeast extract (Becton Dickinson), and 10% glycerol (v/v, final concentration) in our laboratory until use. *C. albicans* was grown on a *Candida* GS agar plate (Eiken Chemical Co., Ltd. Tokyo, Japan) for 24 h at 37°C and then the cells were harvested, and suspended in RPMI 1640 medium containing 2.5% fetal calf serum (RP medium) for oral inoculation.

Clove preparations and eugenol

Clove preparations were made at S&B Foods Inc. (Tokyo). Clove was harvested in Indonesia and, after drying, imported to Japan. The dried cloves were milled into fine powder and suspended in distilled water for administration. The content of dry matter in the clove preparation was measured after the weight decreased by drying at 37°C. Eugenol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in dimethylsulfoxide (DMSO) at 10% w/w concentration prior to dilution with RP medium for *in vitro* experiments[13].

Measurement of *in vitro* activities of clove and eugenol against *Candida* growth

Measurement of inhibition of yeast form growth of *Candida* was performed as described previously[14]. *C. albicans* was grown on YPG medium (1% Bacto pepton, 0.5% yeast extract, 2% glucose, pH 6.5) for 16 h at 37°C with shaking at 38 rpm, and the cells were collected by centrifugation (3,000 rpm, 5 min). After the supernatant was removed, the cells were washed twice with RP medium and the cell suspension was prepared in the same medium at 5×10^4 cells/m*l*. Clove preparation, eugenol in DMSO and DMSO (control) were diluted with RP medium, respectively. Each well of a 96-well flat bottom microplate received a mixture of 100 μl of *Candida* cell suspension and 100 μl of dilutions of clove preparation, eugenol in DMSO or DMSO, and the microplate was incubated for 16 h at 37°C in a 5% CO_2 atmosphere.

To determine the extent of mycelial growth of *C. albicans*, the crystal violet (CV) staining assay was performed as described previously[15]. Briefly, the medium in the wells was discarded, and then the adhesive *Candida* mycelia were sterilized by treatment with 70% ethanol. The mycelia were stained with 0.01% CV and washed with water. After the microplate was dried, 150 μl of isopropanol containing 0.04N HCl and 50 μl of 0.25% sodium dodecyl sulfate were added to the wells and mixed. The absorbance at 620 nm of duplicate samples was measured spectrophotometrically. The extent of total growth (including growth forms of both mycelium and yeast) of *C. albicans* was determined using the broth microdilution method. The *Candida* cells obtained by centrifuge (1,500 rpm, 5 min, 4°C) of a culture broth in each well were washed twice and suspended in sterile saline. The cell suspension was inoculated on *Candida* GS agar plate after serial 10-fold dilution, and the plate was incubated for 20 h at 37°C. The CFU were counted and the totals per well were calculated.

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 ICR mice (Charles River Japan, Inc., Yokohama, Kanagawa) were used for all animal experiments. (The average body weight of mice was 25.5 g at the beginning of the 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 5-6 animals and were given food and water *ad libitum*. The experimental procedure of the oral candidiasis model in mice was described previously[8]. Briefly, immunosuppressed mice were induced by subcutaneous treatment with a dose of 100 mg/kg of prednisolone (Mitaka Pharmaceutical Co., Japan) 1 day prior to oral infection. Tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Japan) in drinking water at a dose of 0.08% was given to the mice, beginning 1 day before infection. The animals were anesthetized by intramuscular injection with 100 μl of 0.2% chlorpromazine chloride (Wako Pure Chemical Industries, Ltd.) in the foot. They were orally infected with about 5×10^7 cells/m*l* viable cells of *C. albicans* TIMM 2640 in RP medium. Oral infection was performed by means of a cotton swab (baby cotton buds; Johnson & Johnson Co., Tokyo) rolled in all parts of the mouth. The cell number of *Candida* inoculated in the oral cavity was calculated to be about 1×10^6 cells/mouse by the difference in viable cell number associated with cotton swabs before and after oral inoculation.

Clove treatment

Clove powder suspended in distilled water was applied to *Candida* infected mice using a

top-rounded needle for administration 3 hour, 21 hour, 27 hour, 45 hour, 51 hour and 69 hour after *C. albicans* inoculation by two alternative methods: 1. spreading 50 μl per mouse of the clove suspension on and around the tongue and in the oral cavity, 2. injecting 200 μl per mouse intragastrically.

GC analysis

The eugenol contents of clove preparations were analyzed by gas chromatography (GC) using a Hewlett-Packard model HP 6890, equipped with a DB-WAX column (J&W) (30 m × 0.25 mm, 0.25 μm film thickness) and a flame ionization detector (FID)[16, 17].

Scoring of tongue's fur and squamous layer and quantification of oral infection

Scoring of tongue's fur was performed as described previously[8]. On the 3rd day after inoculation, groups of mice were sacrificed, and the fur of each tongue and squamous disorder was scored as follows: 0, normal; 1, fur in less than 20%; 2, fur in more than 21% but less than 90%; 3, fur in more than 91% and the squamous layer; 4, thick fur in more than 91% and the squamous layer.

Measurement of the number of viable *Candida* cells in each part of a mouse

The oral cavity (i.e. cheek, tongue, and soft palate) was swabbed by a cotton swab. After swabbing, the cotton end was cut off and placed in a tube containing 5 ml sterile saline. The yeast cells were resuspended by mixing on a vortex mixer before culture in 100-fold dilution on a *Candida* GS plate for 20 h at 37°C, the CFU were counted, and the totals per swab were calculated. The stomach was cut out of a sacrificed mouse, washed with sterile PBS to remove its contents and homogenized in a tube containing 20 ml sterile PBS. The homogenates were inoculated on *Candida* GS plates after serial 10-fold dilution with sterile saline. After incubation for 20 h at 37°C, the CFU were counted and the totals per mouse were calculated. Fresh feces from a mouse were placed in a tube containing 1 ml sterile saline and suspended on a vortex mixer. Serial 10-fold dilutions of the suspension were inoculated on a *Candida* GS plate. Using the procedure described above, the total per g of feces was calculated.

Statistical analysis

The data of scores were compared using the non-parametric Mann-Whitney U test. The data of the $\log_{10}$CFU of *C. albicans* isolated from each mouse part were compared using a Student's t test. P values of <0.05 were considered significant. All calculations were performed using a statistical software program (Stat View: Abacus Concepts, Berkeley, Calif.). All mean values given in the text include the standard deviation of the mean.

Histological analysis

The procedure of histological analysis was performed by the method previously described[8]. Tongues were taken from sacrificed animals, fixed in 20% formalin solution and embedded in paraffin. Five-μ sections were cut from the paraffin block and stained with periodic acid-schiff (PAS) stain for histological observation and fungal detection.

Results

Growth inhibitory activities of clove and eugenol against *C. albicans in vitro*

Table 1 shows the activities of clove preparation and eugenol on the mycelial growth of *C. albicans*. The activities were measured by CV staining assay. The IC_{50} of the clove preparation (0.415 - 2.04 mg/ml) was about 10 times larger than that of eugenol (0.0408 - 0.204 mg/ml). Eugenol is known to be the main component of clove's essential oil. The eugenol content in this clove preparation (0.415 mg/ml) was measured by GC analysis to be 0.0615 mg/ml, per gram of dry matter. Table 1 also shows the

Table 1. Activities of clove and eugenol to mycelial and total growth of *C. albicans*

Agent	IC_{50} (mycelial growth) (dry matter or concentration: mg/ml)	IC_{50} (total growth) (dry matter or concentration: mg/ml)
Clove	0.415 - 2.04	0.415 - 2.04
	(0.0615 - 0.3023)	(0.0615 - 0.3023)
Eugenol	0.0408 - 0.204	0.204 - 1.02

Activities were measured by the CV staining method as described in Materials and Methods. The concentration of 50% inhibition (IC_{50}) against *Candida* growth is indicated as the range between two concentrations. The total growth includes both growth forms of mycelium and yeast. The content of eugenol in clove preparations is shown in parenthesis.

activity on the total growth of *C. albicans* measured by counting *Candida* CFU. The IC_{50} of clove preparation on the total growth (0.415-2.04 mg/m*l*) was in the same range as that on the mycelial growth. However, the IC_{50} of eugenol on the total growth (0.204-1.02 mg/m*l*) was equal to or larger than that on the mycelial growth (Table 1). This means that relatively low concentration of eugenol inhibits the mycelial growth of *C. albicans*. In fact, only yeast forms of *C. albicans* were observed microscopically in the cultures at eugenol concentrations above 0.204 mg/m*l* (data not shown).

Protective activity of oral administration of clove preparation to experimental oral *C. albicans* infection

Effect of oral administration of the clove preparation on murine oral candidiasis was examined. Oral candidiasis murine model with the strain, TIMM 2640, was used to evaluate the protective activity of the preparation. Two days after infection, the lingual mucosa of mice infected with *C. albicans* showed pathological symptoms of oral candidiasis. They consisted of patchy areas of smooth mucosa and well-delineated atrophic areas on the dorsal of tongues as depicted in Fig. 1B. The tongues orally treated with the clove preparation (10.38 mg/mouse) appeared normal and healthy (Fig. 1C).

By histological studies, PAS-positive fungi could be observed in the lesions near the oral epithelium of dorsal tongues of the non-treated mice as shown in Fig. 2A.

There was no PAS-positive fungal hypha in

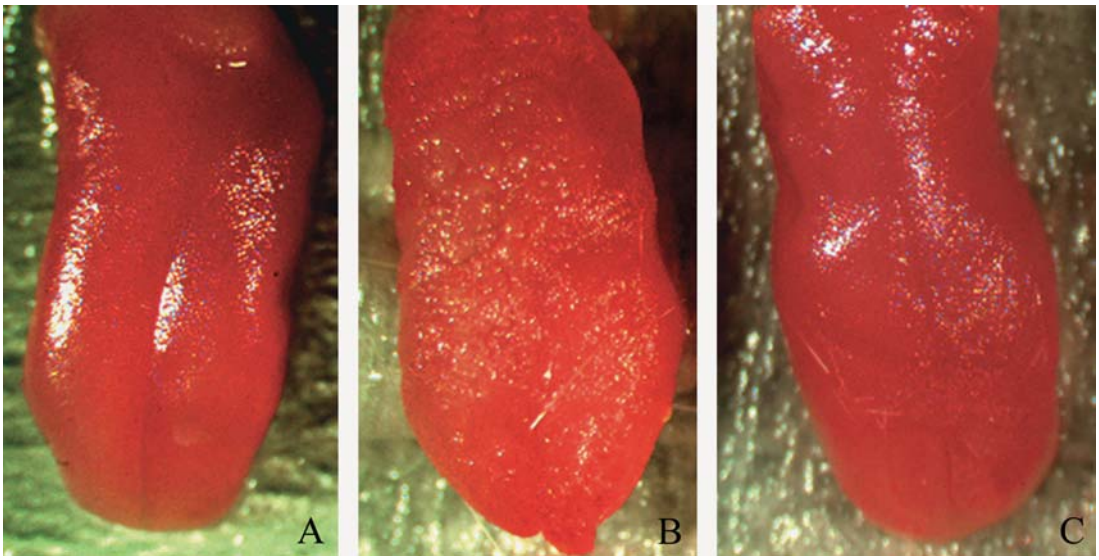

Fig. 1 Macroscopic observation of improvement on tongue of oral candidasis mice by administering clove preparation. A: healthy (control), B: oral candidiasis (white patches showed on the tongue.), C: treated with clove preparation

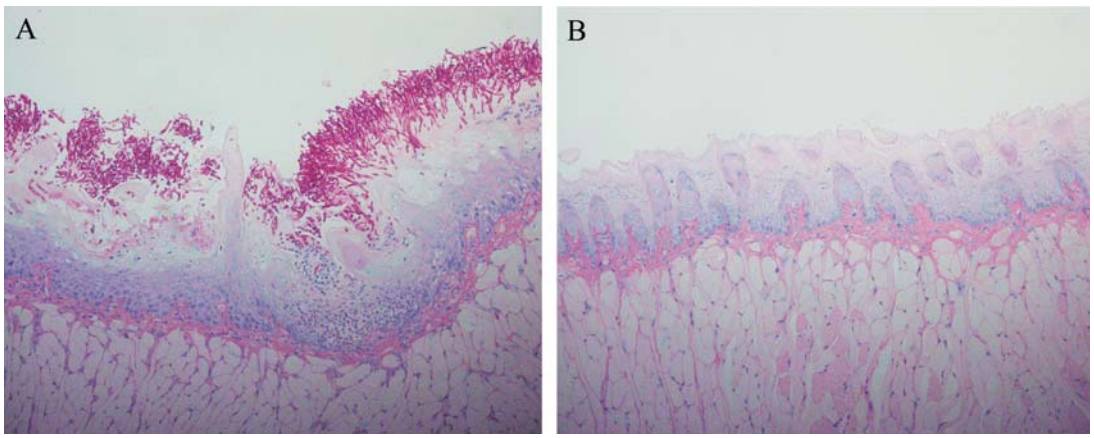

Fig. 2 The improved effect on lingual mucosa of *Candida*-infected mouse by administering clove-preparation. The procedure of this observation was described in Materials & Methods. Samples were stained with PAS stain.

Table 2. The effects of clove administration into oral cavities in oral candidiasis mice

Treatment (dose/day)	Number of mice	Score	Viable *Candida* cells ($\log_{10}$CFU)		
			Oral cavity (per 1 swab)	Stomach (per g weight)	Feces (per g weight)
Control	5	2.00±0.71	4.97±0.30	5.92±0.38	7.14±0.16
Clove 10.38 mg	5	0.20±0.45**	4.05±0.53**	5.56±0.70	7.09±0.38
Clove 2.08 mg	5	1.20±0.45	5.13±0.15	5.94±0.19	7.44±0.23
Clove 0.42 mg	5	1.60±0.55	5.20±0.26	5.87±0.57	6.90±0.37

The clove preparations were administered in oral cavities of oral candidiasis mice at 3 doses.
Scores of the severity of tongue disorder were evaluated by the scoring method described in Materials and Methods.
Viable *Candida* cells in each mouse sample were measured by counting CFU on *Candida*
GS agar plate. *P* values of <0.05 were considered significant (** : *p* values of <0.01, * : *p* values of <0.05).

Table 3. Effects of clove administration into stomachs in oral candidiasis mice

Treatment (dose/day)	Number of mice	Score	Viable *Candida* cells ($\log_{10}$CFU)		
			Oral cavity (per 1 swab)	Stomach (per g weight)	Feces (per g weight)
Control	11	2.00±0.77	4.91±0.44	5.44±0.60	6.85±0.31 (1)
Clove 41.5 mg	10	1.70±0.48	5.09±0.34	4.84±0.48**	6.52±0.33* (1)

The clove preparations were injected intragastrically at the dose of 41.5 mg. Scores of the severity of tongue's disorder were evaluated by scoring method as described in Materials and Methods.
Viable *Candida* cells in each mouse sample were measured by CFU counting on *Candida*
GS agar plate. *P* values of <0.05 were considered significant (** : *p* values of <0.01, * : *p* values of <0.05).
(1) data in feces weight of less than 7 mg were omitted, so that in control group n=8, in clove treatment group n=8.

the tongue of mice treated with the clove preparation (Fig. 2B). Table 2 shows that oral treatment with 10.38 mg of clove preparation protected the mice from oral infection of *Candida*, estimated by the reduced clinical score of tongues and the lower number of CFU recovered from the oral cavity, while a dosage below 2.08 mg/m*l* of clove preparation was less effective. Table 2 also shows that oral administration of clove did not decrease the viable *Candida* cells in stomachs or feces.

Effect of intragastric administration of clove preparation on intestinal growth of *Candida albicans*

To examine its effect on the intestinal tract, clove preparation was administered directly into the stomachs of orally *Candida* infected mice. Table 3 shows that when the preparation was administered intragastrically at a dose of 41.5 mg/mouse to orally *Candida*-infected mice, the number of viable *Candida* cells in stomachs and feces was decreased. However, the clinical scores of tongues and the number of *Candida* of oral cavities were not improved as shown.

Discussion

Here we have shown that oral administration of a clove sample inhibited *Candida albicans* growth and displayed therapeutic effects in a murine oral candidiasis model[14, 18]. Inhibition of *Candida* growth by clove and its therapeutic effects in this model were elucidated by two administration routes. When a clove preparation was administered in the oral cavity of a *Candida*-infected mouse, viable *Candida* cell number in the cavity was decreased. This treatment with clove preparations into the oral cavity not only inhibited macroscopic lesions on the lingual surface but also suppressed invasion of *Candida* mycelia into the lingual tissue microscopically. On the other hand, when the clove preparation was administered intragastrically, the number of viable *Candida* cells in stomach and feces was decreased without reduction in oral *Candida*[10]. This is the first report, as far as we know, to indicate that a conventional food showed an inhibitory effect against intestinal growth of *C. albicans* and improved symptoms of oral candidiasis[9].

The effectiveness of clove preparation was limited locally; when the clove preparation was administered at a dose of 10.38 mg/murine in the oral cavity, there was little effect on *Candida* growth in the stomach. (By this administration route, the dose range was limited below about 10 mg because of the limited volume of the oral cavity.) On the other hand, when administered by the intragastric route, it had no protective effect on *Candida* infection in this cavity. These results suggested that the growth inhibition of *Candida* by clove

preparation was elicited only in tissues with which the clove sample came in direct contact. This suggests that the therapeutic activity of clove samples may be caused by interaction between *Candida* cells and clove preparation, and that active substances in the clove sample may be very easily inactivated or absorbed into the intestinal tract[10].

We investigated the active principle which played a leading role in *Candida*-inhibition *in vitro*. The results of GC analysis of clove preparation showed that eugenol was included at 14.8% of dry weight in the sample. Furthermore, IC_{50} of eugenol (0.0408-0.204 mg/m*l*) for *C. albicans* growth was almost equal to eugenol content of the clove preparation corresponding to IC_{50}[13, 19]. Thus, we theorize that eugenol in the clove sample played a leading role for the inhibitory effect of *Candida* growth, though other components of the preparation might also contribute to this therapeutic efficacy[20, 21].

Apparently further work is needed to establish a therapeutic evaluation of clove preparation for oral candidiasis, such as studies on the optimal conditions in dosage and administration period of clove samples, and on a biological analysis of interaction between clove preparation and lingual mucosa. We wish to emphasize that our studies described here suggest the possible application of clove preparation as a spice in our dietary life to control the overgrowth of *C. albicans* in alimentary tracts including the oral cavity. Now we hope that our study will facilitate the development of a functional food for improvement of intestinal candidiasis in immunocompromised patients or elderly people using spices and herbs.

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