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Antifungal activities of origanum oil against *Candida albicans*

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Abstract

The antimicrobial properties of volatile aromatic oils from medicinal as well as other edible plants has been recognized since antiquity. Origanum oil, which is used as a food flavoring agent, possesses a broad spectrum of *in vitro* antimicrobial activities attributed to the high content of phenolic derivatives such as carvacrol and thymol. In the present study, antifungal properties of origanum oil were examined both *in vitro* and *in vivo*. Using *Candida albicans* in broth cultures and a micro dilution method, comparative efficacy of origanum oil, carvacrol, nystatin and amphotericin B were examined *in vitro*. Origanum oil at 0.25 mg/ml was found to completely inhibit the growth of *C. albicans* in culture. Growth inhibitions of 75% and >50% were observed at 0.125 mg/ml and 0.0625 mg/ml level, respectively. In addition, both the germination and the mycelial growth of *C. albicans* were found to be inhibited by origanum oil and carvacrol in a dose-dependent manner. Furthermore, the therapeutic efficacy of origanum oil was examined in an experimental murine systemic candidiasis model. Groups of mice (n = 6) infected with *C. albicans* ($5 \times LD_{50}$) were fed varying amounts of origanum oil in a final vol. of 0.1 ml of olive oil (vehicle). The daily administration of 8.6 mg of origanum oil in 100 µl of olive oil/kg body weight for 30 days resulted in 80% survivability, with no renal burden of *C. albicans* as opposed to the group of mice fed olive oil alone, who died within 10 days. Similar results were obtained with carvacrol. However, mice fed origanum oil exhibited cosmetically better clinical appearance compared to those cured with carvacrol. The results from our study encourage examination of the efficacy of origanum oil in other forms of systemic and superficial fungal infections and exploration of its broad spectrum effect against other pathogenic manifestations including malignancy. (Mol Cell Biochem **228**: 111–117, 2001

Key words: antifungal activity, *in vitro, in vivo*, female BALB/c mice, origanum oil, *Candida albicans*, carvacrol, nystatin, amphotericin B

Introduction

The antimicrobial properties of volatile aromatic oils from edible plants have been recognized since antiquity. Origanum oil, which is used as a food flavoring agent, has been shown to possess a broad spectrum of antimicrobial activity due to its high content of phenolic derivatives such as carvacrol and thymol [1–3]. Earlier studies have demonstrated the ability of origanum oil to retard and inhibit the growth of various food spoiling organisms including the species of *Aspergillus* (mycotoxinogenic filamentous fungi) and *Hansenula* (industrial yeasts) [4–7]. While the oil and many of its constituents have been demonstrated to be antifungal *in vitro* against non-pathogenic yeasts [8, 9], a few studies have suggested a potential therapeutic effect against experimental infections in rats due to *Trichophyton rubrum,* a human dermatophytic filamentous fungus) [10]. Nevertheless, very little information is available on its compara-

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tive antifungal activity on the growth and physiology of human pathogenic yeasts or filamentous fungi either *in vitro* or *in vivo*. Furthermore, its direct therapeutic use either in superficial or systemic infections due to bacteria or fungi has not been clearly established. In the present study, we examined the antifungal properties of origanum oil and its major chemical constituent carvacrol against *Candida albicans,* a dimorphic yeast-like fungus. *C. albicans* which resides as commensal in the mucocutaneous cavities of skin, vagina and intestine of humans [11], can cause infections under altered physiological and pathological conditions such as infancy, pregnancy, diabetes, prolonged broad spectrum antibiotic administration, steroidal chemotherapy as well as AIDS [12–18]. We have demonstrated that origanum oil inhibits the growth of *C. albicans in vitro* as well as *in vivo.* We conclude that the daily oral administration of origanum oil may be highly effective in the prevention and treatment of candidiasis.

Materials and methods

Animals and treatment

Female BALB/c mice (15–18 g) were obtained from Taconic Farms (Germantown, NY, USA). The animals were maintained in a controlled environment at 25°C with a 12 h light and 12 h dark cycle, and were acclimatized for 3–5 days before use. The mice were housed in groups of five, fed commercial rodent pellets and given water *ad libitum* throughout the experiments. The protocol for the entire investigation was approved by the Animal Welfare Board at Georgetown University Medical Center.

Plant oils and chemicals

Origanum (P73 Oreganol™) and olive oil were provided by North American Herbs and Spices, Waukegan, IL, USA. Carvacrol, nystatin and amphotericin B were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud's glucose (S.g) broth and agar media were purchased from Difco Laboratories (Detroit, MI, USA). All other chemicals used in this study were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were of analytical grade or the highest commercial grade available.

Organisms

Standard strain of *Candida albicans* (ATCC No. 48274) obtained from ATCC, Fairfax, VA, USA, was grown and maintained on S.g. agar slants.

Susceptibility testing

A micro-broth dilution technique was employed to determine the susceptibility of the strains of *C. albicans* to oil of origanum and carvacrol [19, 20]. Susceptibility was expressed as minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The stock solutions of origanum oil, olive oil and carvacrol (63% v/v adjusted in olive oil) were dissolved in ethanol-Tween 80 solvent. Nystatin and amphotericin B were dissolved in 50% ethanol, and used as positive controls. Solvent and media controls were also included for reference. The Sabouraud's glucose (S.g) broth containing varying amounts (logarithmic, serially and 2-fold diluted) of origanum oil and carvacrol and the various controls were inoculated with actively dividing *C. albicans* cells. The cultures were incubated for 24 and 48 h at 30°C on a metabolic rotary shaker (200 rev/min), and the growth was monitored both visually and colorimetrically (at 540 nM). The minimum inhibitory concentration (MIC) was defined as the lowest concentration required to arrest the growth of the fungi at the end of 24 h of incubation. Minimum fungicidal concentration (MFC) was determined by sub-culturing a 0.01 ml aliquot of the medium drawn from the culture tubes showing no macroscopic growth at the end of 48 h of culture on S.g. agar plates and incubated further for the appearance of yeast-like growth. The plates were scored for growth of the yeast colonies. The lowest concentration of the antifungal agent from which negative growth or fewer than 3 colonies were recorded was considered as minimum fungicidal concentration (MFC).

Effect on yeast and mycelial forms of C. albicans

The effect of origanum oil on the formation of germ tubes (filament initiation) by *C. albicans* was examined, by incubating the blastospores with various concentrations of origanum oil, carvacrol and other agents in 20% egg albumin at 37°C [21]. The comparative percentage inhibition of germ tube formation was determined by microscopic examination. The effect on the mycelial formation (filament elongation) was carried out by incubating the previously germinated blastospores of *C. albicans* in the presence of varying amounts of antifungal agents at 37°C for an additional 24 h and 48 h. Both the germination and the filament elongation were microscopically monitored. The MIC and MFC were determined as above.

Toxicity tests with mice

Since origanum oil is known to be pungent and corrosive, a titration of the doses of origanum oil was conducted to determine the tolerable dose to be given orally. Both the neat

oil and the dilutions in olive oil (vehicle) were orally administered to the mice according to the body weight using a ball tip gavage needle (Harvard Apparatus, South Natick, MA, USA). Higher concentrations of the oil were given as single bolus dose, while the lower concentrations were given daily for 7 days. The overall health of the animals such as coat color, body weight, other side effects such as scruffiness, and death were recorded for 14 days. At the end of the experiment, mice were sacrificed to visually examine the internal organs for any abnormalities.

Effect on experimental murine systemic candidiasis

A murine systemic candidiasis model developed previously [22], was employed to evaluate the *in vivo* anti fungal activities of origanum oil and carvacrol. Initially, the concentrations of *C. albicans* needed to achieve 50% mortality of the animals (LD_{50}) were determined. Mice were injected *i.v*. with varying doses of actively growing *C. albicans* in a final vol. of 0.1 ml. Mortality of mice was monitored for 30 days. The death of the mice due to *C. albicans* was confirmed by anatomical evidence for organ involvement, i.e. the histological presence of yeasts and/or pseudomycelia. Furthermore, renal burden of *C. albicans* was assessed by culturing an aliquot of the kidney homogenate on S.g. agar plates. The dose titration revealed that 2.5×10^6 *C. albicans* cells were needed to kill 50% of the mice. Subsequently, batches of mice injected with $5 \times LD_{50}$ dose of *C. albicans* were used to assess therapeutic activity.

Treatment protocol

In two separate experiments, groups of mice (6 each) infected with *C. albicans* ($5 \times LD_{50}$) were gavaged, daily with origanum oil or carvacrol in 0.1 ml of olive oil for 8 days and 30 days. The amount of antifungal agents administered was calculated based on the body weight of the mice. Control mice received either olive oil orally alone (negative control), or olive oil orally plus amphotericin B 25 µg *i.p.* (positive control). The experiments were terminated at the end of 30 days. The body weights, the disease status and the overall health of the mice during the experiment were recorded. The pathological status of the mice was determined by visual examination of the internal organs after their death or sacrifice at the completion of the experiment. For histology, kidney smears were examined microscopically after staining with 1% methylene blue for the presence of fungal elements. Renal burden of *C. albicans* was further tested by culturing aliquots of kidney homogenates on S.g. agar plates.

Results

Origanum oil is fungicidal to C. albicans in vitro

In the present study, we have examined the antifungal properties of origanum oil and one of its major constituents, carvacrol and thymol, both *in vitro* and *in vivo*. Using *Candida albicans* in broth cultures and a micro-dilution method, comparative efficacies of origanum oil, carvacrol, nystatin and amphotericin B were examined *in vitro*. Origanum oil at 0.25 mg/ml was found to completely inhibit the growth of *C. albicans* in culture. Growth inhibitions of 75% and >50% were observed at 0.125 mg/ml and 0.0625 mg/ml level, respectively (Table 1). Figure 1 provides a pictorial presentation of the inhibitory effect. A 2-fold higher concentration of carvacrol was required to be fungicidal.

Both the germination and mycelial elongation of C. albicans are inhibited in vitro

Origanum oil and carvacrol both inhibit germ-tube formation by the blastospores of *C. albicans* (Table 2). The production of germ tubes and subsequent mycelial formation when exposed to either serum or hen's egg albumen is an *in vitro* correlate of the *in vivo* tissue invasive capabilities of the pathogenic strains of *C. albicans.* Origanum oil inhibited

Table1. Effect of origanum oil on the growth of *Candida albicans*

Agent	24 h MIC (mg/ml)	48 h MFC (mg/ml)
Origanum oil	0.125	0.25
Carvacrol	0.25	0.5
Amphotericin B	0. 0015	0.0015
Nystatin	0. 005	0.005
Olive oil	No effect	No effect

MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration.

Table 2. Effect of origanum oil on germ tube formation and filament elongation

Agent	Germ tube formation		Filament elongation	
	MIC mg/ml	MFC mg/ml	MIC mg/ml	MFC mg/ml
Origanum oil	0. 062	0.125	0.125	0.125
Carvacrol	0. 125	0.125	0.125	0.25
Olive oil	NE	NE	NE	NE

NE = no effect; MIC and MFC for nystatin and amphotericin B = 2.5 and 5.0 µg/ml respectively.

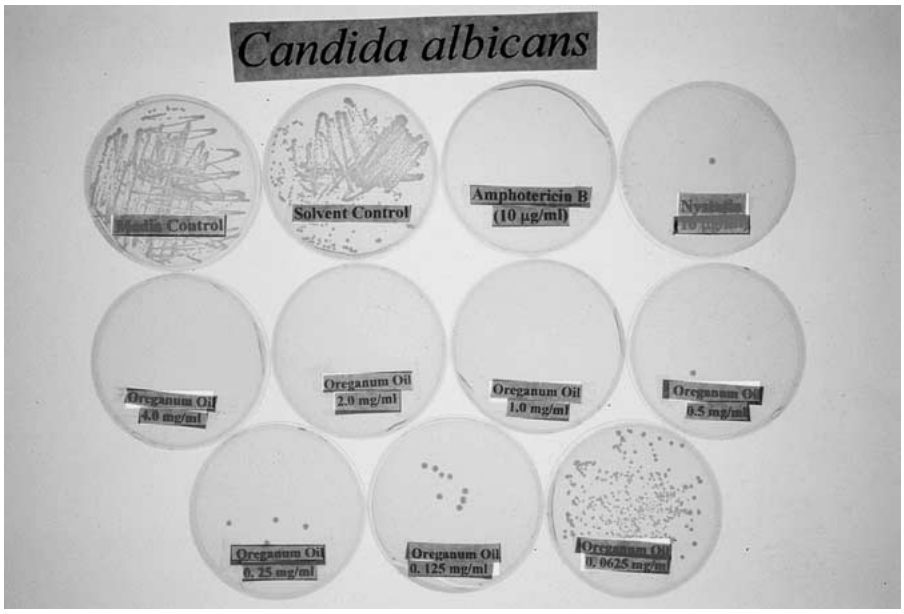

Fig. 1. In vitro antifungal activity of origanum oil against *Candida albicans*. Exponentially growing *C. albicans* cells were cultured with varying concentrations of antifungal agents. Aliquots from the cultures drawn at the end of 48 h of culturing were plated on to Sabouraud's glucose agar. The plates were further incubated for an additional 48 h for the appearance of yeast like colonies.

germ-tube production by blastospores of *C. albicans* in a dose-dependent manner. The MIC required to inhibit the production of germ tube formation was 0.062 mg/ml (24 h) and the fungicidal effect (at 48 h) required double the concentration (MFC = 0.125 mg/ml). Carvacrol exerted effects similar to origanum oil at twice the concentrations. Origanum oil and carvacrol were also found to affect filament elongation. The mycelial forms of *C. albicans* were also inhibited by origanum oil at 0.125 mg/ml (MIC) and 0.125 mg/ml (MFC) levels, and by carvacrol at 0.125 mg/ml (MIC) and 0.25 mg/ml (MFC) levels, respectively.

Origanum oil can protect mice from systemic candidiasis

The therapeutic efficacy of origanum oil was examined in an experimental murine systemic candidiasis model. Toxicity studies to determine the least toxic dose of origanum oil that can be fed safely to mice were conducted (Table 3). A single bolus dose of 650 mg/kg body weight caused uniform mortality. However, daily administration of smaller doses of origanum oil mixed with olive oil was well tolerated, showing no apparent clinical abnormalities (Table 3).

In the first *in vivo* study, the therapeutic ability of origanum oil given only for 8 days to prevent the mortality of mice with systemic infection due to *C. albicans* was tested (Table 4). Groups of mice (n = 6) infected with *C. albicans* ($5 \times LD_{50}$) were gavaged with varying amounts of origanum oil in a final vol. of 0.1 ml. An 8-day treatment with 162.5 mg and 325 mg per kg body weight was found to rescue the mice from mortality with 100% survival rate over the 30 days of observation, similar to mice that received amphotericin B (1 mg/

Table 3. Toxicity of origanum oil to BALB/c mice

	Concentration of oil* (mg/kg)	No. of mice (tested/survived)
Bolus dose	2600	5/0
	1300	5/0
	650	5/0
	325.0	5/5
	162.5	5/5
Daily dose for 1 week	26	6/6
	52	6/6
	78	6/6
	104	6/6
	130	6/6

*Oil was mixed with 100 µl olive oil and gavaged.

Table 4. Therapeutic efficacy of origanum oil: 8-day treatment

Agent	Conc. @ per kg body wt. (mg)	No. of mice tested/survived	No. of mice with renal burden#
Origanum oil	325. 5	6/6	4/6
	162. 5	6/6	6/6
Amphotericin B	1. 0	6/6	2/6
Olive oil (vehicle)		6/0*	–

*Died within 10 days. #Renal burden+ = kidney smear+ culture+.

Table 5. Therapeutic efficacy of origanum oil: 30-day treatment

Agent	Conc. @ per kg body wt. (mg)	No. of mice tested/survived	No. of mice with renal burden[#]
Origanum oil	52. 00	6/6	0/6
	43. 33	6/6	0/6
	31. 00	6/6	0/6
	26. 00	6/6	0/6
	17. 33	6/6	0/6
	8. 66	6/5	0/5
Carvacrol	17. 33	6/6	0/6
	8. 66	6/5	0/5
Amphotericin B	1. 0	6/6	0/6
Olive oil		6/0*	6/6

*Died within 10 days. [#]Renal burden+ = kidney smear+ culture+. One mouse treated with 8.66 mg origanum oil/kg b.w. died on day 15, while one mouse treated with 8.66 mg carvacrol/kg b.w. died on day 13.

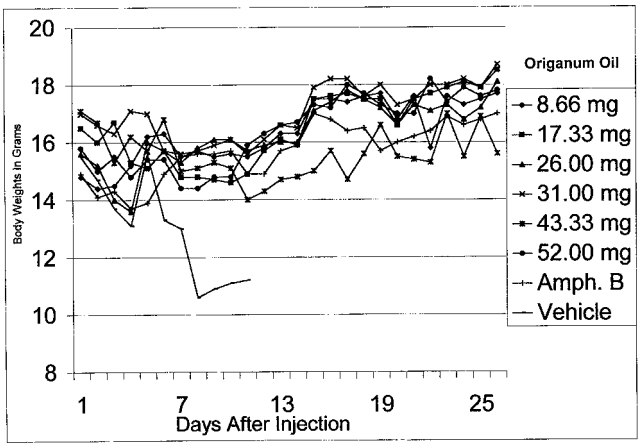

Fig. 2. In vivo effect of origanum oil in experimental murine candidiasis. Infected mice were fed orally with varying amounts of origanum oil in 0.1 ml vol. of olive oil (vehicle). The control animals received either olive oil alone or olive oil plus amphotericin B (*i.p.*). The body weights and the overall health of the mice were recorded daily.

kg body weight). In contrast, the control group of mice who received 0.1 ml of olive oil alone died within 10 days. At the end of 30 days, the surviving animals appeared to be clinically healthy, without scruffiness and with regain of normal coat luster. Internally, no abnormalities of organs were noted. Furthermore, the kidney smears failed to demonstrate the presence of either yeasts or pseudomycelia. However, the culture of their kidney homogenates on S.g. agar revealed the presence of *C. albicans*. (Table 4).

The results from the second experiment to determine the optimal dose over a 30-day treatment course required for clinical cure and to completely eliminate the renal burden of *C. albicans* are shown in Table 5. A daily administration of origanum oil as low as 8.66 mg/kg body weight (equivalent of 1 µl per mouse) was found to cure 80% of the infected mice. Initially all groups of infected mice, exhibited scruffiness, loss of body weight, and lack of coat luster. In the vehicle-control mice, the average body weight was found to decrease 7 days after injection from approximately 16–11 g. In contrast, mice receiving treatment had a gradual increase in body weight after injection from 15–17 to 16–19 g by the end of 30 days (Fig. 2). The daily administration of equivalent amounts (8.66 and 17.33 mg/kg body weight) of carvacrol was found to confer similar therapeutic properties (data not shown). However, the overall clinical appearance of the mice receiving origanum oil was cosmetically better than those receiving carvacrol, as evidenced by the improved coat luster and minimal scruffiness.

Discussion

Aromatic herbal oils used for cooking and flavoring are increasingly claimed to have broad spectrum antimicrobial activities. Origanum oil has been suggested to have potent antimicrobial activity, including anthelmintic properties, due to its phenolic, alcoholic and terpenoid constituents [4]. The objective of the present study was to assess the antifungal properties of origanum oil against C. *albicans* in both *in vitro* and *in vivo*. Furthermore, the comparative efficacy of origanum oil, carvacrol, nystatin and amphotericin B was also examined. The results clearly demonstrate that the origanum oil can act as a potent antifungal agent against C. *albicans,* and can function similar to antifungal antibiotics such as nystatin or amphotericin B.

C . albicans is a harmless commensal yeast-like fungus in healthy humans, which can cause superficial as well as life-threatening systemic infections under immune compromised situations. *C .albicans* can colonize or infect virtually all body sites because of its high adaptability to different host niches by the activation of appropriate sets of genes in response to complex environmental signals [21–25]. Thus, our objective was to assess the possible therapeutic potential of the commonly used origanum oil against this human dimorphic commensal, which can become a facultative pathogen under altered physiological situations.

Previous studies to assess the inhibitory effect of origanum oil against twenty-five different genera of microorganisms including animal and plant pathogens, food poisoning and spoilage bacteria, have demonstrated the growth inhibition properties by origanum oil [26]. Essential oils of oregano (*Origanum vulgare*) were found to inhibit the growth and production of ochratoxin A by *Aspergillus ochraceus* NRRL 3174 up to 21 days in culture [27].

Origanum oil has been shown to delay or inhibit the growth of saprophytic food spoiling fungi such as *Aspergillus flavus* (mycotoxogenic) and industrial yeasts such as *Hansenula*

anoma [8]. Furthermore, origanum oil, and carvacrol have been reported to have some promising effect on rats infected with a human dermatophytic fungus, *Trichophyton rubrum* [10]. However, the essential oils including origanum oil at 100 ppm had no effect on the pseudomycelial formation by *Candida lipolytica* [10,11].

In this paper we have demonstrated, that origanum oil effectively inhibits the *in vitro* growth of *C. albicans*, a human yeast-like fungus which can cause both systemic and superficial infections in debilitated individuals. In addition we have shown that origanum oil directly inhibits germination and filament formation (the two phases required for tissue invasion) by *C. albicans*. Nevertheless, the novel fungicidal property of origanum oil was of superior efficacy as compared to carvacrol against the germination and mycelial elongation of pathogenic strain of *C. albicans in vitro.* Furthermore, selected concentrations of origanum oil demonstrated therapeutic efficacy comparable to the effective examined dose for amphotericin B (1.0 mg) in protecting the mice from systemic candidiasis. The overall clinical appearance of the mice receiving origanum oil was cosmetically better than those receiving carvacrol (data not shown), although this difference in appearance could possibly be attributed to the presence of additional constituent phenols such as thymol, terpenes and flavonoids in origanum oil [4]. It has been demonstrated that some of these components of origanum oil have antispasmodic and antioxidant activities, in addition to their antimicrobial potentials [23, 28, 29]. A comparative study of these components may shed more light not only on the antifungal potentials, but also on the pharmacological effects of each of these components. It is interesting to speculate that secondary infections due to *C. albicans* in debilitated individuals such as those having diabetes and HIV infection may be prophylactically controlled by the daily intake of small amounts of oregano oil, either alone or added to a food.

In summary, the results presented in this paper conclusively demonstrate the antifungal potential of edible origanum oil. Origanum oil is shown to be both fungistatic and fungicidal to *C. albicans*, the human pathogenic yeast. Both the germination and the pseudomycelial phase of the yeast are inhibited *in vitro*. Further, the daily oral administration of as little 1.0 µl of oil for 30 days completely freed 80% of the laboratory mice from experimental systemic candidiasis, which is fatal if not treated. Thus, daily oral administration of origanum oil may be highly effective in the prevention and treatment of candidiasis. The results from our study not only encourage examination of the efficacy of origanum oil in other forms of systemic and superficial fungal infections, but also to explore its broad spectrum effect against other pathogenic manifestations including malignancies.

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