

***In vitro* activity of bergamot natural essence and furocoumarin-free and distilled extracts, and their associations with boric acid, against clinical yeast isolates**

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Objectives: There is very little information, to date, on the antifungal activity of bergamot oil. In this study, we investigated the *in vitro* activity of three bergamot oils (natural essence, furocoumarin-free extract and distilled extract) against clinically relevant *Candida* species. We studied the two derivatives, components of Italian pharmaceutical products, that are supposed to be less toxic than the essential oil.

Methods: *In vitro* susceptibility of 40 clinical isolates of *Candida* spp. (*Candida albicans*, $n = 20$; *Candida glabrata*, $n = 13$; *Candida krusei*, $n = 4$; *Candida tropicalis*, $n = 2$; *Candida parapsilosis*, $n = 1$), associated with symptomatic and asymptomatic vulvovaginal candidiasis, was determined using a modification of the NCCLS M27-A2 broth microdilution method. MICs were evaluated for each of the oils alone and combined with sub-inhibitory concentrations of the well-known antiseptic, boric acid. To boric acid, all isolates had MIC values ranging from 0.094% to 0.187% (w/v).

Results: At 24 h readings, the MIC₉₀s (for all isolates) were (v/v): 5% for natural essence of bergamot, 2.5% for the furocoumarin-free extract, and 1.25% for the distilled extract. At the 48 h reading, these values increased to >10%, 5% and 2.5%, respectively. At both readings, MIC₉₀s for all oil + boric acid combinations were significantly lower than corresponding values for the oils alone ($P < 0.05$).

Conclusions: These data indicate that bergamot oils are active *in vitro* against *Candida* spp., suggesting their potential role for the topical treatment of *Candida* infections.

Keywords: bergamot oils, yeasts, MICs

Introduction

Recently, the potential antimicrobial effects of certain plant oils have attracted serious attention within the scientific community, largely as a result of the growing problem of multidrug resistance among bacteria.¹ Indeed, several studies have documented the antimicrobial effects of *Melaleuca alternifolia* (tea tree) oil and other essential oils, which are generally used as topical agents.^{2,3}

In contrast, there is very little scientific information on the antimicrobial properties of the essential oil of bergamot (*Citrus bergamia*), a fruit grown almost exclusively in the southern Italian region of Calabria. The natural essence, a clear yellow-green liquid, is extracted from the peel of the fruit by a

cold-pressing procedure. It consists of a volatile fraction (93–96%), whose main components are, in approximate percentage, limonene (40%), linalool (8%) and linalyl acetate (28%), and a non-volatile fraction (4–7%) formed essentially by coumarins and psoralens (i.e. bergamottin, citroptene, bergaptene, etc.). To bergaptene is ascribed the main phototoxic activity of furocoumarins present in bergamot oil, so that furocoumarin-free extracts are often used, instead of natural essence, to prepare commercial products.

Popular tradition has always attributed anti-infective properties to bergamot oil, and these properties were investigated in the 19th century by the Calabrian physician, Francesco Calabrò. Since then, however, the medicinal effects of bergamot oil have received only limited attention.^{4,5}

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Anti-*Candida* activity of bergamot oil

In this study, we evaluated the *in vitro* activity of three bergamot oils (the natural essence and furocoumarin-free and distilled extracts of the former), alone and in combination with boric acid, against *Candida* spp. vaginal isolates.

Materials and methods

Yeast isolates

Forty isolates of *Candida* spp., recovered from vaginal swabs of 40 patients with symptomatic ($n=22$) and asymptomatic ($n=18$) vulvo-vaginal candidiasis, were studied. They included *Candida albicans* ($n=20$), *Candida glabrata* ($n=13$), *Candida krusei* ($n=4$), *Candida tropicalis* ($n=2$) and *Candida parapsilosis* ($n=1$). Of the symptomatic patients, 13 were infected with *C. albicans*, seven with *C. glabrata* and two with *C. krusei*. The isolates were identified to the species level by standard procedures.⁶ Among the isolates, fluconazole resistance was documented in one isolate of *C. glabrata* and the four isolates of *C. krusei*, and six other isolates of *C. glabrata* displayed dose-dependent susceptibility to fluconazole. *C. albicans* ATCC 90028 reference strain was also included, and *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strains.

Test compounds and susceptibility testing assays

Natural essence of bergamot (NE) and furocoumarin-free (FF) and distilled (DE) extracts of the former, produced by the Consorzio del Bergamotto of Reggio Calabria, Italy, were supplied by Bergamon S.r.l. (Rome, Italy). As previously described, the NE was shown to contain: 39.72% limonene; 27.95% linalyl acetate; 8.35% γ -terpinene; 7.75% linalool; 7.52% β -pinene; 1.87% bergamottin; 1.35% α -pinene; 1.28% sabinene; 1% myrcene; 0.42% β -bisabolene; 0.38% geranyl acetate; 0.36% α -thujene; 0.36% geranial; 0.35% terpinolene; 0.35% β -caryophyllene; 0.34% neryl acetate; 0.30% trans- α -bergamottene; 0.23% neral; 0.22% citroptene; 0.21% bergaptene; 0.13% 7-methoxy-5 geranoxycoumarin. FF was bergaptene-free, whereas DE was absolutely devoid of non-volatile

residues.⁷ These percentages were found to be similar to those reported in the literature recently.⁸ *In vitro* antifungal susceptibility testing was carried out using the NCCLS M27-A2 broth microdilution method with slight modifications.⁹ Each oil was diluted (v/v) in RPMI 1640 medium (with L-glutamine without sodium bicarbonate) (Sigma, Milan, Italy) containing 2% glucose and buffered to a final pH of 7.0 with 0.165 M MOPS (Sigma). Tween 80 (Sigma) (final concentration 0.001% v/v) was included to enhance oil solubility, with no inhibitory effect on yeast growth. Boric acid (Sigma) was dissolved (w/v) in RPMI 1640 medium. Serial two-fold dilutions of each test substance were placed in 96-well microtitre plates. Final concentrations were:

- (i) Test 1. NE, FF and DE, each tested at concentrations ranging from 10% to 0.02% (v/v);
- (ii) Test 2. Boric acid alone, 3.0–0.0059% (w/v). The choice of 3% as starting concentration was consistent with previous studies;¹⁰
- (iii) Test 3. NE, FF and DE, 10–0.02% (v/v), +boric acid at a fixed concentration of 0.047% (w/v) for each oil;
- (iv) Test 4. NE, FF and DE, 10–0.02% (v/v), +boric acid at a fixed concentration of 0.0235% (w/v) for each oil.

All isolates were tested twice, and testing was repeated a third time if the first two MIC values were discordant. Each isolate was suspended in 5 mL of sterile distilled water and vortexed thoroughly. The isolate suspension, adjusted to a turbidity equivalent to that of a 0.5 McFarland standard, was then diluted twice with RPMI 1640 medium and distributed among the wells of the microtitre plate (100 μ L/well). The final concentration of the inoculum was $0.5\text{--}2.5 \times 10^3$ cfu/mL, as confirmed by determining viable counts on Sabouraud dextrose agar (SDA). Plates were sealed and incubated aerobically at 35°C. The first visual reading was carried out after 24 h of incubation, and the minimum inhibitory concentration (MIC) value was recorded as the lowest concentration that inhibited visible growth; after 48 h of incubation, the plates were agitated on a rotating platform to simplify reading of the end-points, and the lowest concentration that reduced turbidity by $\geq 50\%$, compared with that in the control-growth well, was recorded as the MIC. The minimum concentration of substance that inhibited 90% of the isolates was

Table 1. *In vitro* susceptibility of *Candida* spp. isolates to three bergamot oils and boric acid alone

Organism (n)	NE MIC (% v/v)		FF MIC (% v/v)		DE MIC (% v/v)		Boric acid MIC (% w/v)	
	range	MIC ₉₀	range	MIC ₉₀	range	MIC ₉₀	range	MIC ₉₀
<i>C. albicans</i> (20)								
24 h	2.5–10	5	1.25–5	5	0.625–2.5	1.25	0.094–0.187	0.187
48 h	2.5–10	5	1.25–5	2.5	0.625–2.5	2.5	0.094–0.187	0.187
<i>C. glabrata</i> (13)								
24 h	0.625–10	5	0.625–1.25	1.25	0.312–1.25	0.625	0.094–0.187	0.187
48 h	2.5–>10	>10	1.25–10	5	0.312–1.25	1.25	0.094–0.187	0.187
<i>C. krusei</i> (4)								
24 h	2.5–5	ND	1.25	ND	0.625–1.25	ND	0.094	ND
48 h	2.5–5	ND	0.625–1.25	ND	0.625–1.25	ND	0.187	ND
<i>C. tropicalis</i> (2)								
24 h	5	ND	1.25	ND	0.625–1.25	ND	0.094	ND
48 h	10	ND	5	ND	1.25–2.5	ND	0.187	ND
<i>C. parapsilosis</i> (1)								
24 h	1.25	ND	1.25	ND	0.625	ND	0.187	ND
48 h	1.25	ND	1.25	ND	0.625	ND	0.187	ND

NE, natural essence of bergamot; FF, furocoumarin-free bergamot extract; DE, distilled bergamot extract; ND, not determined (fewer than 10 isolates).

Table 2. *In vitro* susceptibility of *Candida* spp. isolates to three bergamot oils plus boric acid

Organism (n)	NE MIC (% v/v)			FF MIC (% v/v)			DE MIC (% v/v)		
	range	MIC ₉₀	FICI	range	MIC ₉₀	FICI	range	MIC ₉₀	FICI
Plus boric acid 0.0235% (w/v)									
<i>C. albicans</i> (20)									
24 h	0.156–1.25	1.25		0.156–0.625	0.625		0.156–0.625	0.312	
48 h	0.625–1.25	1.25	0.37	0.312–1.25	1.25	0.62	0.312–1.25	0.625	0.37
<i>C. glabrata</i> (13)									
24 h	0.156–0.625	0.625		0.156–0.312	0.312		0.008–0.312	0.156	
48 h	1.25–5	5	NC	0.312–0.625	0.625	0.25	0.156–0.625	0.625	0.62
<i>C. krusei</i> (4)									
24 h	0.156–1.25	ND		0.156–0.625	ND		0.156–0.312	ND	
48 h	0.625–1.25	ND	0.37 (4)	0.312–1.25	ND	0.62 (2); 1.12 (2)	0.312–0.625	ND	0.62 (4)
<i>C. tropicalis</i> (2)									
24 h	1.25	ND		0.312	ND		0.312	ND	
48 h	1.25	ND	0.25 (2)	0.625	ND	0.25 (2)	0.625	ND	0.37 (1); 0.62 (1)
<i>C. parapsilosis</i> (1)									
24 h	0.625	ND		0.312	ND		0.312	ND	
48 h	1.25	ND	1.12	0.625	ND	0.62	0.625	ND	1.12
Plus boric acid 0.047% (w/v)									
<i>C. albicans</i> (20)									
24 h	<0.02–0.625	0.312		<0.02–0.625	0.312		<0.02–0.625	0.156	
48 h	0.08–0.625	0.625	0.37	<0.02–0.312	0.312	0.37	0.08–0.625	0.625	0.5
<i>C. glabrata</i> (13)									
24 h	<0.02–0.156	0.156		<0.02–0.312	0.156		<0.02–0.312	0.156	
48 h	0.625–2.5	1.25	NC	0.312–0.625	0.625	0.37	0.156–0.625	0.625	0.75
<i>C. krusei</i> (4)									
24 h	<0.02–0.312	ND		<0.02–0.156	ND		<0.02–0.156	ND	
48 h	0.156–0.625	ND	0.31 (2); 0.37 (2)	<0.02–0.312	ND	0.28 (1); 0.5 (3)	0.156–0.625	ND	0.5 (3); 0.75 (1)
<i>C. tropicalis</i> (2)									
24 h	0.312	ND		0.156	ND		0.156	ND	
48 h	0.312	ND	0.28 (2)	0.312	ND	0.31 (2)	0.312	ND	0.37 (1); 0.5 (1)
<i>C. parapsilosis</i> (1)									
24 h	0.156	ND		0.156	ND		0.156	ND	
48 h	0.312	ND	0.5	0.312	ND	0.5	0.312	ND	0.75

NE, natural essence of bergamot; FF, furocoumarin-free bergamot extract; DE, distilled bergamot extract; NC, not calculated; ND, not determined (fewer than 10 isolates). FICI values were calculated as specified in the Materials and methods section. For species with less than 10 isolates, the number of isolates with the same FICI value is indicated in parentheses.

Anti-*Candida* activity of bergamot oil

defined as MIC₉₀. Minimum fungicidal concentrations (MFC) were determined for each oil by inoculating 10 µL of broth from the wells without microbial growth onto SDA. After incubation for 48 h at 35°C, the cfu were counted and MFC was defined as the lowest concentration resulting in the death of 99.9% or more of the initial inoculum, as described previously.³

Evaluation of drug interactions

For each bergamot oil + boric acid association tested, the nature of the interaction between the two agents was quantitatively defined by means of the fractional inhibitory concentration index (FICI), which was calculated as follows:

$$\text{FICI} = [(\text{MIC}_{90} \text{ A in combination})/\text{MIC}_{90} \text{ A}] \\ + [(\text{MIC}_{90} \text{ B in combination})/\text{MIC}_{90} \text{ B}]$$

For species other than *C. albicans* and *C. glabrata* (with less than 10 isolates), MIC values were used instead of MIC₉₀ values in the formula.

The FICIs were classified according to the following interpretive scheme: FICI ≤ 0.5, synergic effect; FICI >0.5–4.0, indifferent effect; and FICI > 4.0, antagonistic effect.¹¹

Statistical analysis

The difference in the distributions of MICs was determined by the Kruskal–Wallis test, using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). A *P* value of less than 0.05 was considered statistically significant.

Results and discussion

Tables 1 and 2 show the MIC values of the three bergamot oils and of their associations with boric acid at 0.0235% and 0.047% (w/v) for each *Candida* spp. isolate studied. The two boric acid concentrations corresponded to sub-inhibitory concentrations, as all isolates had an MIC value of this antiseptic ranging from 0.094% to 0.187% (Table 1). At 24 h, when bergamot oils were tested alone against *C. albicans* and *C. glabrata*, DE presented the lowest MIC₉₀s. Among the species with less than 10 isolates, the *C. parapsilosis* isolate had similar MICs of DE, FF and NE; for *C. krusei* and *C. tropicalis*, MICs of DE were similar to those of FF, but both were lower than those of NE. With one exception, all 48 h MIC values were identical to or slightly higher than those recorded at 24 h. Overall, at 24 h readings, the MIC₉₀s (for all isolates) were (v/v): 5% for NE, 2.5% for FF, and 1.25% for DE. At the 48 h readings, these values increased to >10%, 5% and 2.5%, respectively.

At the MIC, all the three oils were also fungicidal for each isolate, as determined by MFC. The MICs of NE, FF and DE for *C. parapsilosis* ATCC 22019 were 1.25%, 1.25% and 0.625%, respectively; for *C. krusei* ATCC 6258 were 2.5%, 2.5% and 0.625%, respectively; and for *C. albicans* ATCC 90028 were 2.5%, 1.25% and 0.625%, respectively. Also for the reference strains, MIC and MFC values coincided.

With regard to the results observed when each bergamot oil was tested with boric acid, 24 h MICs for NE + boric acid 0.0235% decreased, with respect to the corresponding values for NE alone, by one to three dilutions. The most marked reduction was seen with *C. glabrata* isolates. When NE was combined with boric acid 0.047%, there were more substantial decreases

ranging from three (*C. parapsilosis*) to five dilutions (*C. glabrata*). The same basic trend emerged when FF and DE were combined with the two sub-inhibitory concentrations of boric acid. Similar improvements were noted at 48 h readings. Overall, at 24 h readings, MIC₉₀s (for all isolates) for all oil + boric acid combinations were significantly lower (*P* < 0.05) than corresponding values for the oils alone: 1.25% for NE, 0.625% for FF and 0.312% for DE when associated with 0.0235% boric acid, and 0.312% for NE, 0.312% for FF and 0.156% for DE when associated with 0.047% boric acid. At the 48 h readings, these values increased to 5%, 1.25% and 0.625% when the oils were associated with 0.0235% boric acid, and to 1.25%, 0.625% and 0.625% when the oils were associated with 0.047% boric acid. Once again, a significant difference between MIC₉₀s of oils alone and those of oils in combination with boric acid was observed (*P* < 0.05).

Interestingly, the susceptibility of the 11 fluconazole-resistant or -susceptible dose-dependent isolates to the bergamot oils, alone or in association with boric acid, was no different from that observed among the isolates that were fully susceptible to fluconazole. These findings were in agreement with those reported by Mondello *et al.*,³ who tested the activity of tea tree oil against azole-susceptible and -resistant yeasts.

FICI values for each oil tested in association with boric acid are shown in Table 2. At 48 h, for the association with 0.0235% boric acid, NE showed a synergic interaction against *C. albicans*, *C. tropicalis* and *C. krusei*, while an indifferent effect against *C. parapsilosis* was shown; FF was synergic against *C. glabrata* and *C. tropicalis*, while being indifferent against *C. albicans*, *C. parapsilosis* and *C. krusei*; DE showed a synergic effect against *C. albicans* and one *C. tropicalis* isolate, while an indifferent effect was observed against *C. glabrata*, *C. parapsilosis*, *C. krusei* and the other *C. tropicalis* isolate. At 48 h, for the association with 0.047% boric acid, NE and FF were synergic against all the species; DE showed a synergic effect against *C. albicans*, *C. tropicalis* and *C. krusei* (three isolates), while an indifferent effect was observed against *C. glabrata*, *C. parapsilosis* and *C. krusei* (one isolate).

The use of essential oils extracted from various plant species in complementary medicine as antimicrobial agents for topical treatment of infections is often based on popular tradition. However, the efficacy and safety of these products must be validated by means of consistent clinical trials and accurate *in vitro* testing. In our study, a modification of the NCCLS M27-A2 broth microdilution method was used to assess the antifungal activity of the essential bergamot oil and two derivatives, the furocoumarin-free extract and distilled extract, which are components of licensed Italian products for prevention and treatment of vulvo-vaginal mycosis. Our results indicate that the three bergamot oils we tested all exerted antimycotic effects against the yeast strains tested. Their efficacy was evident when used alone and in association with low concentrations of boric acid. The highest activity was observed with DE, followed by FF. The superiority of DE over NE has already been noted by Focà *et al.*⁴ and by Pizzimenti *et al.*⁵ The only other data in the literature on the antimycotic action of bergamot oils are those of Hammer *et al.*,² who evaluated the susceptibility of a single isolate of *C. albicans* to NE. The MIC they reported was 1% (v/v).² In this context, it is important to underline that the best antimycotic action was observed with DE, which is absolutely devoid of non-volatile residues, in particular, the phototoxic bergapten.¹² In addition,

due to the improved efficacy of the associations of the three oils with boric acid, their therapeutic use at lower concentrations could be suggested, thereby reducing costs and toxic risks.

Definition of the mode of action of the bergamot oil was beyond the scope of this study. To our knowledge, although some investigators have suggested that most essential oils target microbial cell membranes, affecting their integrity or permeability or compromising membrane-associated functions (primarily respiration), the mechanisms of antimicrobial activity of bergamot oil, as well as of boric acid, are unknown.¹³ For this reason, further studies are needed to elucidate their activity against fungi and other microorganisms and to better define the therapeutic potential of these oils.

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