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In vitro activity of *Citrus bergamia* (bergamot) oil against clinical isolates of dermatophytes

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***Objectives*: Recently, bergamot oil was shown to be a potent antifungal agent *in vitro* against clinically important *Candida* species. In this study, the activities of bergamot natural essence and its furocoumarin-free and distilled extracts on dermatophytes such as *Trichophyton*, *Microsporum* and *Epidermophyton* species were investigated.**

***Methods*: *In vitro* susceptibility testing assays on 92 clinical isolates of dermatophytes (*Trichophyton mentagrophytes n* = 20, *Trichophyton rubrum n* = 18, *Trichophyton interdigitale n* = 15, *Trichophyton tonsurans n* = 2, *Microsporum canis n* = 24, *Microsporum gypseum n* = 1 and *Epidermophyton floccosum n* = 12) were performed using the CLSI M38-A broth microdilution method, except for employing an inoculum of 1–3 $\times$ 10^3 cfu/mL. MICs were determined at a visual endpoint reading of 80% inhibition compared with the growth control.**

***Results*: MICs (v/v) of all fungi ranged from 0.156% to 2.5% for the natural essence, from 0.02% to 2.5% for the distilled extract, and from 0.08% to 1.25% for the furocoumarin-free extract. The three isolates of *T. tonsurans* and *M. gypseum* exhibited the highest MIC values.**

***Conclusions*: Data from this study indicate that bergamot oil is active *in vitro* against several common species of dermatophytes, suggesting its potential use for topical treatment of dermatophytoses.**

Keywords: MIC, broth microdilution, antifungal susceptibility

Introduction

Unlike other superficial fungal infections, the incidence of dermatophytoses, commonly known as ringworm or tinea, has increased considerably,[1,2] and this trend has paralleled the increased number of individuals with impaired immunity following treatment with cytotoxic drugs, broad-spectrum antimicrobials, or immunosuppressive agents.[3] Some of these infections are still difficult to resolve completely, and remissions and relapses are often observed.[1] Clinical and mycological cure of dermatophytoses may be prevented by the inability of the antifungal drug to penetrate the site of infection or by the intrinsic resistance of the fungus. Cases of infections due to griseofulvin-resistant isolates have been described,[4] as well as a high-level primary resistance to terbinafine displayed by *Trichophyton rubrum* isolates obtained sequentially from a single onychomycosis patient who failed oral terbinafine therapy.[5,6]

The poor availability of antifungals and increasing number of treatment failures have motivated current searches for therapeutic alternatives to include the testing of essential oils (e.g. from *Thymus vulgaris* and *Melaleuca alternifolia*) as potential antimicrobial agents.[7–9] Most of them contain large amounts of phenolic monoterpenes, which are responsible for activity against viruses, bacteria and fungi.[9–11]

The essential oil of *Citrus bergamia*, also called bergamot oil, is primarily produced in Calabria, in southern Italy, and from this country came the first information on the antimicrobial properties of this compound.[12] This oil, termed by us as 'natural essence', is a yellow-green liquid directly obtained from the cold-pressed peels of the fruit, and consists of *c.* 80 volatile (e.g. limonene,

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linalool and linalyl acetate) and non-volatile (e.g. bergamottin, citroptene and bergaptene) components.[13] As a consequence of the phototoxic action of furocoumarins (i.e. bergaptene) present in bergamot oil, furocoumarin-free and distilled extracts are often used, instead of the natural essence, in pharmaceutical products (Bergamon S.r.l., Rome, Italy). Of note, some of these products are empirically used for prevention and treatment of mycoses.

In our previous work, we investigated the antifungal properties of the bergamot natural essence and its furocoumarin-free and distilled extracts against vaginal isolates of several *Candida* species *in vitro*.[14] We established that these preparations were effective agents, mainly when tested in association with boric acid, which suggested that they are potentially active against filamentous fungi as well. Accordingly, the aim of this study was to assess the effects of the three compounds on dermatophytes by the use of *in vitro* susceptibility assays.

Materials and methods

Fungal isolates

Ninety-two isolates belonging to seven species of dermatophytes were tested. They were chosen from the culture collection of clinical isolates maintained at the Mycology Section of the Catholic University Medical Centre, and included 20 *Trichophyton mentagrophytes* isolates, 18 *T. rubrum*, 15 *Trichophyton interdigitale*, 2 *Trichophyton tonsurans*, 24 *Microsporum canis*, 1 *Microsporum gypseum* and 12 *Epidermophyton floccosum*. Isolates had originally been identified to the species level by standard procedures[15] and stored as water suspensions at room temperature. Prior to testing, each isolate was subcultured on a potato dextrose agar (PDA) slant and incubated at 30°C for 4–5 days or until good conidiation was produced. *T. rubrum* isolates were subcultured on rice agar plates to induce conidium sporulation.[2] *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains.[16]

Fungal inoculum preparation

For each dermatophyte isolate, a suspension of conidia was prepared in 0.85% saline by swabbing the colony surface with a sterile swab, as reported recently.[17] After the settling of the larger particles, conidia were counted with a haemocytometer and diluted in RPMI 1640 medium (Sigma, Milan, Italy) to correspond to a final inoculum concentration of 1×10^3–3×10^3 cfu/mL, as described previously.[17] Yeast control strains were subcultured on PDA and incubated at 35°C for 24 h, and the corresponding inocula were prepared to final concentrations of 0.5×10^3–2.5×10^3 cfu/mL.[18]

Test compounds and susceptibility testing assays

Natural essence of bergamot (NE) and its distilled (DE) and furocoumarin-free (FF) extracts, produced by the *Consorzio del Bergamotto* of Reggio Calabria, Italy, were supplied by Bergamon S.r.l. (Rome, Italy). The chemical composition of the three preparations, as determined by gas and gas chromatography/mass spectroscopy analyses, has been reported previously.[14] In particular, the furocoumarin-free extract is bergaptene-free, whereas the distilled extract is absolutely devoid of non-volatile residues.[14] Standard powders of antifungal drugs, such as itraconazole (Janssen, Beerse, Belgium) and griseofulvin (Sigma), were used to prepare stock solutions.[17,18] *In vitro* susceptibility testing of dermatophytes to all compounds was based on a modification of the CLSI M38-A broth microdilution method.[16] Each oil preparation was diluted (v/v) in RPMI 1640 (Sigma), and Tween 80 (Sigma, final concentration 0.001% v/v) was included to enhance oil solubility. At this concentration, the detergent did not show any inhibitory effect on fungal growth (data not shown). Serial 2-fold dilutions of each test compound, prepared in RPMI 1640, were placed in 96-well microtitre plates.[18] The individual ranges of each substance used were as follows: bergamot oil preparations, 0.02–10% (v/v); itraconazole, 0.03–16 mg/L; and griseofulvin, 0.125–64 mg/L. Growth and sterility control wells were included in each plate. After the addition of inocula (prepared as described earlier), plates were incubated for 96 h at 35°C (yeast controls were incubated for 24 h).[17] MIC was determined visually and recorded as the lowest concentration of substance that reduced growth to 80% of that of the control.[17] The minimum concentration of substance that inhibited 90% of the isolates was defined as MIC_{90}. Isolates were tested twice. For the two isolates tested with itraconazole as quality controls, MICs were within expected ranges (for *C. parapsilosis*, 0.25 mg/L; for *C. krusei*, 0.5 mg/L).[18]

Results and discussion

Table 1 shows the MIC values for all the dermatophyte isolates tested against three bergamot oil preparations (NE, DE and FF). MICs ranged from 0.156% to 2.5% for NE, from 0.02% to 2.5% for DE and from 0.08% to 1.25% for FF. Generally, MIC_{90}s were lower for DE and FF compared with NE. Among the species with fewer than 10 isolates, *T. tonsurans* (two isolates) and *M. gypseum* (one isolate) exhibited the highest MICs to NE, DE and FF, but again the MICs of DE or FF were lower than those of NE for both species. Consistent with our previous results,[14] the MICs of NE, FF and DE for *C. parapsilosis* ATCC 22019 were 1.25%, 1.25% and 0.64%, respectively; for *C. krusei* ATCC 6258 were 2.5%, 2.5% and 0.64%, respectively. Griseofulvin and itraconazole gave MICs in the ranges of 0.125–$\geq$64 and 0.03–0.25 mg/L, respectively.

In recent years, proliferation of new classes of drugs, such as the allylamines (e.g. terbinafine) and orally active triazoles (e.g. itraconazole), has represented the most noteworthy trend in dermatophytosis therapy.[1] Many azoles, in particular itraconazole, have been used effectively, often resulting in complete clearance of the lesions.[19] However, treatment with both itraconazole and terbinafine for prolonged times requires periodic laboratory monitoring of liver function.[20] Moreover, these antifungal agents may have drug interactions with other medications.[21] Griseofulvin, which had been for many years the only antifungal available for the treatment of dermatophytoses, is still the long-standing drug of choice for tinea capitis, but there are concerns with resistance and toxicities with this agent.[21]

Even though systemic antifungal therapy is often indicated—especially for tinea unguium, the most resistant of dermatophytoses—topical agents are still frequently used to cure or speed the resolution of uncomplicated lesions.[1] This is in line with the current opinion that systemic therapy should be given consideration when lesions involving a large infected area fail to clear with repeated topical treatment using different drugs.[22] The topical agents, applied to the surface of the skin in the form of creams, lotions or sprays, are known to readily penetrate into the stratum corneum to kill the fungi or render them unable to grow or divide. Fungicidal drugs such as

Table 1. *In vitro* activity of bergamot oils, itraconazole and griseofulvin against 92 isolates of dermatophytes, determined by the microdilution broth method

	NE[a] (% v/v)		DE[a] (% v/v)		FF[a] (% v/v)		Itraconazole (mg/L)		Griseofulvin (mg/L)	
Species (no. of isolates)	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
T. mentagrophytes (20)	0.156–1.25	0.625	0.156–0.625	0.312	0.08–0.156	0.156	0.03–0.125	0.125	0.125–≥64	0.5
T. rubrum (18)	0.156–0.625	0.312	0.08–0.312	0.156	0.156–0.625	0.156	0.03–0.25	0.125	0.125–≥64	0.5
T. interdigitale (15)	0.312–1.25	0.625	0.156–0.625	0.156	0.08–0.312	0.312	0.03–0.125	0.125	0.125–≥64	0.5
T. tonsurans (2)	2.5	ND[b]	1.25	ND	1.25	ND	0.125	ND	0.5	ND
M. canis (24)	0.156–0.625	0.625	0.156–0.625	0.312	0.08–0.625	0.312	0.03–0.25	0.06	0.125–64	0.5
M. gypseum (1)	2.5	ND	2.5	ND	1.25	ND	0.125	ND	0.5	ND
E. floccosum (12)	0.156–0.312	0.312	0.02–0.156	0.156	0.08–0.156	0.156	0.03–0125	0.125	0.125–64	0.5

[a]NE, natural essence of bergamot; DE, distilled bergamot extract; FF, furocoumarin-free bergamot extract.
[b]Not determined (fewer than 10 isolates).

terbinafine are often preferred over fungistatic azoles (miconazole, clotrimazole and ketoconazole) for treatment of dermatophytic fungal infections, since short-term treatments (i.e. one application daily for 1 week) are associated with high cure rates.[23]

Taken together, the improved cure rates, reduced adverse effects, decreased drug interactions and lower cost of topical agents make therapy with these drugs a favourable choice in the management of superficial fungal infections including dermatophytoses.[21] In this context, new antifungal plant derivatives could be useful alternatives for the treatment of dermatophytoses where a topical therapy is required. The advantage of using these natural compounds may be a reduced risk of side-effects and lower cost. It is thus not surprising that, in recent years, there has been growing interest in the use of medicinal plants to cure skin diseases.

In this study, we demonstrated the high *in vitro* activity of bergamot oil against a wide number of clinical isolates of various pathogenic dermatophytes. In general, the three preparations tested had low MICs. However, the two extracts, DE and FF, were more active than NE against all of the species tested. This is of great importance in the light of the fact that the two derivatives are devoid (in part or completely) of non-volatile residues, in particular of the phototoxic bergaptene. Although we found the activities of these compounds against dermatophytes to be superior to the anticandidal effect we observed previously,[14] our data all indicate that bergamot oil can be used as an efficacious antifungal agent against dermatophytes and yeast pathogens.

These results give substantial support to popular or anecdotal beliefs in the effectiveness of treating skin and mucosal infections with bergamot oils. The only other data in the literature on the antimycotic action of bergamot oil are those of Hammer *et al.*,[24] who investigated the susceptibility of a single isolate of *Candida albicans* to NE. Otherwise, *in vivo* and *in vitro* studies conducted on *M. alternifolia* (tea tree) oil[25] have established that some of the anecdotal claims made about natural oils have a scientific basis. In an interesting review, Martin and Ernst[26] critically assessed the evidence, from controlled clinical trials, of the efficacy of antifungal plant oils and extracts. As reported in that systematic review, in some of these studies, plant preparations were compared with conventional antifungal treatments, and in all cases encouraging results were reported. Four trials described the promising use of tea tree oil preparations for treatment of tinea pedis and onychomycosis.[26] Thus, this emphasizes the need for extensive studies to understand the ways in which bergamot oils inhibit fungi, and for clinical trials to prove their effectiveness in the cure of dermatophytoses.

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Transparency declarations

None to declare.

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