Antifungal Effects of Hydrolysable Tannins and Related Compounds on Dermatophytes, Mould Fungi and Yeasts

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Hydrolysable Tannins, Flavonoids, Antifungal Activity

A series of hydrolysable tannins and related compounds was evaluated for antifungal activities against filamentous fungi (Epidermophyton floccosum; Microsporum canis; Microsporum gypseum; Trichophyton mentagrophytes; Trichophyton rubrum; Trichophyton tonsurans; Trichophyton terrestre; Penicillium italicum; Aspergillus fumigatus; Mucor racemosus; Rhizopus nigricans) and opportunistic yeasts (Candida albicans; Candida glabrata; Candida krusei; Cryptococcus neoformans), using the agar dilution method. While all samples had no activity against the filamentous fungi in concentrations of 1.1–5.9 μM (1000 μg/ml), the phenolic compounds displayed significant potencies against all the opportunistic yeasts tested but C. albicans, with minimum inhibitory concentrations ranging from 0.02 to 0.1 μM (16–125 μg/ml). Although the presence of galloyl groups in flavonoids did not necessarily produce activity, this structural element, an HHDP moiety or its oxidatively modified entity proved to be an important structural feature of hydrolysable tannins. Comparison of dilution methods provided strong evidence of dependence of MIC values on the test method. Employing the microdilution broth method, the ellagitannin corilagin (MIC 0.8 μM) was found to be similarly potent as amphotericin B (MIC 0.5 μM) and sertaconazole (MIC 0.9 μM) against Candida glabrata strains. The order of effectiveness observed being 64- and 4–8-fold increased for corilagin and the reference compounds respectively, when compared with that of the agar dilution test.

Introduction

Despite the wide availability of clinically useful antifungal drugs such as imidazole compounds and distinct antibiotics, there is still a current need for effective novel antifungal agents without adverse effects. The dramatic increase in fungal infections is reportedly related to the growing number of immunocompromised patients associated with a great morbidity (Walsh, 1992; Coleman et al., 1999). In the search of the underlying active principle(s) of medicinally used Pelargonium species of therapeutic potential for the treatment of infections of the respiratory tract, we have recently evaluated the antibacterial activity of Pelargonium constituents (Kayser and Kolodziej, 1997), followed by a systematic study on the antimicrobial activities of tannins (Kolodziej et al., 1999). Although tannins are generally recognized as remarkable antimicrobials, our results clearly demonstrated that the potential of chemically defined polyphenols to inhibit the growth of bacteria is less prominent than commonly anticipated, but they also revealed fairly high anticytotoxic properties of hydrolysable tannins. This finding prompted the present investigation to ascertain whether members of this group of phenolic metabolites tend to be broadly potent or very specific fungitoxic agents. It should be noted that antifungal properties of tannins are well documented, but also that some fungi are known to utilize tannins as a unique source of carbon for growth (Scalbert, 1991). However, limited data exist on the susceptibility of the type of fungus to the toxic effects of chemically defined hydrolysable tannins, structure-activity relationships and dependence of antifungal effects on test methods (Paxton, 1991).

In this study a panel of human pathogenic filamentous fungi and yeasts was tested for susceptibility to a series of hydrolysable tannins and related compounds of high purity.
Materials and Methods

Test compounds

Compounds examined in this report were isolated from *Pelargonium reniforme* ssp. *reniforme* (Latté, 1999). The purity and identity of the samples were proven by chromatographic and spectroscopic techniques. Amphotericin B was from Fluka, Germany, and sertaconazole (Zalain®) was a generous gift of Trommsdorff GmbH & Co, Aachen, Germany.

Microorganisms

The following microorganisms used for the antifungal evaluation are either clinical isolates and were kindly provided by Dr. H. P. Seidl (Institut für Mikrobiologie, Ludwig-Maximilians-Universität, München) and Dr. M. Seibold (Robert Koch-Institut, Berlin), or were purchased from DSZM, Braunschweig: dermatophytes (*Epidermophyton floccosum* 1092/96; *Microsporum canis* 160/98; *Microsporum gypseum* 1054/97; *Trichophyton mentagrophytes* 1020/94; *Trichophyton rubrum* 820/98; *Trichophyton tonsurans* 1099/96; *Trichophyton terrestre* 1200/96), moulds (*Penicillium italicum* 2645/97; *Aspergillus fumigatus*; *Mucor racemosus* 548/96; *Rhizopus nigricans* 3122/97), yeasts (*Candida albicans* ATCC 90028; *C. albicans* M924/91 (Seibold and Werner, 1995); *C. albicans* ATCC 10231; *C. glabrata* ATCC 90030; *C. krusei* ATCC 6258; *Cryptococcus neoformans* ATCC 34544; *Cryptococcus neoformans* DSM 70219). Strains were grown on Sabouraud agar for 7 days at 26 °C (filamentous fungi) or 24 h at 37 °C (yeasts) prior incubation.

Antifungal assays

The fungitoxic activity of the samples was evaluated with the agar dilution method by using RPMI 1640 medium supplemented with 2% glucose containing 0.75% agar for both filamentous fungi and yeasts. The assay was carried out in 12-well microtiter plates. Stock solutions of test samples (2%) in the RPMI 1640 medium were diluted to give serial two-fold dilutions. Using a micropipet, an inoculum of 0.5 μl of the yeast cell suspensions (10³ colony forming units/μl) was added to each well. For the filamentous fungi, mycelial in-octa were transferred to the agar wells. The antifungal agents amphotericin B and sertaconazole were included in the assay as positive controls. The final concentration of DMSO, used as solvent for the reference compounds, did not exceed 1% (Seibold and Werner, 1995) and did not affect the growth of any of the microorganisms. The plates were incubated 24 h at 37 °C (yeasts) up to 8 days at 26 °C for dermatophyte strains. Minimum inhibitory concentration (MIC) of the samples was determined as the lowest compound concentration showing no fungal growth after incubation time as determined by direct visual and/or turbidimetric comparison of the test culture with a control (Van Cutsem et al., 1994).

In an independent experiment, samples were subjected to the broth microdilution method for antifungal activity using the liquid RPMI 1640 medium. The protocol of this assay was identical to the semisolid agar dilution method (vide supra).

Fungicidal kinetic assay

The fungicidal kinetic assay for *C. albicans* ATCC 90028 was performed in RPMI 1640 medium with 2% glucose containing gallic acid (1) in concentrations of 1.5 to 11.8 μg (250–2000 μg/ml). The averaged initial inoculum was 54 colony-forming units. Samples were taken after 1 and 5 h of incubations, followed by plating on Sabouraud agar. After 48 h of incubation at 35°C, growth of the fungus was determined by microscopic analysis.

Results and Discussion

Although tannins are known to possess antifungal activity (Scalbert, 1991), the effect of tannin structure on the potency has not been investigated systematically. Following our recent finding that tannin concentration and composition apparently define appreciable toxicity for bacteria in most cases (Kolodziej et al., 1999), a similar more systematic investigation of the antifungal activities of hydrolysable tannins will provide information on the susceptibility of human pathogenic and opportunistic fungi to representatives of this class of tannins and will establish whether the antifungal activity can be ascribed to qualitative or quantitative parameters.
Following various modifications of the RMPI 1640 broth in preliminary tests, addition of 2% glucose (Espinel-Ingroff et al., 1998) to the medium showed excellent growth of the microorganisms under study, while 0.75% agar established satisfactory semisolid medium conditions. Commonly used Casitone medium proved less suitable due to the presence of ferrum ions in significant amount giving rise to polyphenol complexation.

The antifungal spectrum and the minimum inhibitory concentrations (MICs) of the phenolic samples are displayed in Table I. The agar dilution method showed that none of the compounds tested possessed any activity against the filamentous fungi Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton tonsurans, Trichophyton terrestre, Penicillium italicum, Aspergillus fumigatus, Mucor racemosus or Rhizopus nigricans, as reflected by MICs of 1.1 to 5.9 μM (>1000 μg/ml). With reference to reported antifungal activities of tannins (Scalbert, 1991), it should be noted that most of the previous antifungal studies have been carried out with crude tannin fractions. Thus, this finding is highly reminiscent of that of the antibacterial activity of tannins, where polyphenol concentration and composition proved to be crucial parameters. In contrast, different results were obtained for hydrolysable tannins of the series against yeasts, with some compounds displaying strong activities.

As can be seen (Table I), all phenolic samples show prominent antifungal activities against Candida glabrata, Candida krusei and Cryptococcus neoformans with MICs ranging from 0.02 to 0.1 μM (16–125 μg/ml). However, compounds 1–6 were seemingly less potent (MICs of 0.5–10.9 μM, respectively 500 to >2000 μg/ml) against Candida albicans strains, consistent with recent reports on the antifungal activities of hydrolysable tannins (Burapadaja et al., 1995). The benefits of antibiotic therapy in the treatment of Candida albicans as causative agent of candidosis is sporadically counteracted by the concomitant presence of C. glabrata and C. krusei, pathogenic yeasts that are poorly eradicated by current antibiotic therapies (Coleman et al., 1998). Accordingly, this finding of greater susceptibility of the aforementioned opportunistic yeasts to the tested hydrolysable tannins appears of some therapeutic relevance and deserves further attention.

Table I. In vitro antifungal activities of hydrolysable tannins and related compounds using the agar dilution method. (MIC values of duplicates for test compounds and reference samples in μM and μM, respectively; μg/ml in parentheses).

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<td>Gallic acid (1)</td>
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<td>Corilagin (4)</td>
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<td>1.6</td>
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<td>Naringin B (5)</td>
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References

- Sertaconazole
  - (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0) (1.0)
- Amphotericin B
  - (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5) (0.5)

b) 26 °C, 8 days; b) 26 °C, 2 days; c) 37 °C, 8 days; d) 37 °C, 2 days.
As regards structure-activity relationships, it would appear that the presence of pyrogallol elements seems to be crucial to produce the biological response against *C. glabrata* and *C. krusei*. Both the number of hydroxyl groups and the molecular size are apparently no major contributing factors towards antifungal activity. With the exception of gallic acid (1) in a single case, compounds 1–6 display close MIC values of 0.03–0.2 μM (31.2 μg/ml) and 0.1–0.7 μM (125 μg/ml) against *C. glabrata* and *C. krusei*, respectively (Table I).

*Cryptococcus neoformans* preferentially causes infections in individuals suffering from defective T-cell function such as AIDS patients (Levitz, 1991). Compounds 1–6 inhibited this opportunistic pathogen with MICs ranging from 0.02 to 0.2 μM (16–31 μg/ml). Similarly, the prominent anticytotoxic potency of the phenolic samples appears strongly associated with the presence of gallloyl groups in these molecules, while introduction of additional gallloyl groups does not necessarily enhance this particular biological activity, as concluded from preliminary experiments (Kolodziej et al., 1999). Also, the analysis of active ellagitannin structures reveals that the presence of a hexahydroxydiphenoyl moiety or its oxidatively modified entities may be an important structural feature leading to highly potent anticytotoxic candidates such as corilagin (4), pelargoniin B (5) and phyllanthusin C (6) (Table I).

With MICs ranging from 0.02 to 0.7 μM (16–125 μg/ml), the potentially active compounds examined were generally only moderately fungitoxic, when compared with the MICs 0.0003–0.008 μM (0.3–4 μg/ml) of the clinically used agents amphotericin B and sertaconazole. Despite the considerable difference in antifungal effectiveness, lack of toxicity in these concentration ranges and the less side-effect potential of the hydrolysable tannins tested should be meaningful advantages in their possible employment as fungitoxic agents in man, provided bioavailability.

In an additional set of experiments, the antifungal activity of gallic acid (1) and corilagin (4) was tested against *C. albicans* and *C. glabrata* strains (Table II) using the broth microdilution method to evaluate the antifungal response in dependence on the test method. Selection of *C. albicans* was sim...
Table II. In vitro antifungal activities of gallic acid (1) and corilagin (4) against C. albicans and C. glabrata strains using the microdilution broth method (MIC values of duplicates in nM; μg/ml in parentheses).

<table>
<thead>
<tr>
<th>Compound</th>
<th>C. albicans ATCC 90028</th>
<th>C. glabrata ATCC 90876</th>
<th>C. glabrata ATCC 90030</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid (1)</td>
<td>1500 (250)</td>
<td>200 (31.2)</td>
<td>0.5 (7.8)</td>
</tr>
<tr>
<td>Corilagin (4)</td>
<td>800 (500)</td>
<td>0.8 (0.5)</td>
<td>0.8 (0.5)</td>
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</table>

References

1. Amphotericin B 0.3 (0.24) 0.5 (0.5) 0.5 (0.5)
2. Sertaconazole 0.1 (0.06) 0.9 (0.5) 0.9 (0.2)

ploy for convenience, while (1) represents an essential unit of hydrolysable tannins. This comparative study was prompted by differences in MIC values observed during our screenings. As can be seen (Tables I and II), the in vitro antifungal activity of compounds 1 and 4 strongly depend on the selected bioassay, with more powerful MICs observed for the broth microdilution method. Similar observations have recently been made (Shawar et al., 1992), giving credence to this tendency. Here, corilagin (4) did show prominent antifungal properties against the tested C. glabrata strains with MICs of 0.0008 μM similar to the antifungal agents amphotericin B and sertaconazole with MICs of 0.0005 and 0.0009 μM, respectively. This difference in antifungal response is explicable in terms of divergent oxygen supply for a fungus growing on top of agar plates and in liquid RPMI 1640 medium. Also, the microorganism would have come into immediate contact with the full ambient concentration of the sample in the liquid medium. Accordingly, the agar dilution method is well suited for testing compounds against those dermatophytes infecting the skin surface, while employing the broth microdilution method, conditions similar to those in the intestine would be designed. According to our present results, hydrolysable tannins may be regarded as promising antifungal agents for the treatment of various forms of candidosis and cryptocosmosis.

To test the actual role of galloyl structures in the antifungal activities of natural analogs, vitexin (7), isovitexin (11), orientin (9), isoorientin (13) and their 2′-O-galloylated derivatives 8, 10, 12, and 14 were evaluated with the microdilution method. Results indicated that these flavonoids were completely devoid of activity in concentrations ranging from 1.7 to 2.3 μM (MICs >1000 μg/ml) against Candida albicans and Cryptococcus neoformans (data not shown). Although gallic acid (1) itself displayed appreciable antifungal activity (Tables I and II), this finding clearly demonstrated that the presence of pyrogallol elements per se is not enough to produce antifungal activity for any structural analog. With reference to the active ellagitannins, the failure of the detection of gallic acid excluded their hydrolysis.

The fungicidal kinetic assay, using Candida albicans and gallic acid (1), demonstrated the antifungal activity of the test compound to be fungicide, the minimum fungicidal concentration being 5.9 μM (1000 μg/ml) after a 5 h incubation.
These data indicate that hydrolysable tannins might be considered a potential source of promising antifungal agents for treatment of intestinal infections caused by opportunistic yeasts. Also, one might speculate that dietary intake of such polyphenols reduces the risk of some forms of candidosis and related intestinal infections.

Acknowledgements

The authors should like to thank Dr. M. Seibold, Robert Koch-Institut, Department of Mycology, Berlin, Germany, for his valued comments during this work.


