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Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin☆

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Abstract

The phytoalexin resveratrol is commonly found in food and drinks, including red wine, grapes, and peanuts. Many studies have shown that this compound has anti-inflammatory properties, and it has been ascribed as having health benefits that help to prevent cancer and coronary heart disease. A treatment that combines antimicrobial and anti-inflammatory actions may be desirable for alleviating many skin conditions that range in severity. Therefore, we evaluated the antimicrobial activity of resveratrol against bacteria and dermatophytes that are major etiologic agents of human skin infections. Using the broth microdilution protocol of the National Committee for Clinical Laboratory Standards (NCCLS) M7-A5, growth of the bacterial species *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* was inhibited at 171–342 μg/mL of resveratrol in the solvent dimethyl sulfoxide. Using the NCCLS protocol M38-P, activity against the fungal species *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Epidermophyton floccosum*, and *Microsporum gypseum* was also tested. The growth of dermatophytes was inhibited at 25–50 μg/mL of resveratrol. Thus, this study indicates a novel application for resveratrol, a molecule of plant defense, to combat human fungal pathogens. Resveratrol and its analogs may have wide application to skin conditions that afflict a significant portion of our population, and may also have promising clinical potentials in diabetic wounds.

Keywords: Resveratrol; Antimicrobial; Dermatophytes; *Trichophyton* sp; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

1. Introduction

Phytoalexins are low molecular weight secondary metabolites made by plants as a defense response to microbial infections [1,2]. Resveratrol, *trans*-3,4′,5-trihydroxystilbene, a phytoalexin that belongs to the group of compounds known as stilbenes, can be found in dietary items including red wine, grapes, and peanuts. It has been shown to confer many beneficial effects in human and animal models, and it is being studied as a chemopreventive agent for cancer and cardiovascular disease, probably due to its antioxidative and anti-proliferative activities [3–8]. For example, it is well known as an inhibitor of cyclo-oxygenases, and we have shown that it inhibits activation of the inducible nitric oxide pathway in mammalian macrophages [9].

The combination of microbes and the inflammatory response are the cause of many skin conditions. For example, *Staphylococcus aureus* infection of hair follicles, folliculitis, leads to tiny white-headed blemishes surrounded by small red areas (pimples). Redness and itching are the signs of *tinea pedis* (‘athlete’s foot’), *tinea cruris* (‘jock itch’) and *tinea corporsis* (ringworm) [10–13]. A treatment that combines antimicrobial and anti-inflammatory actions would be desirable for alleviating such skin conditions. Hence, extending from our interest in its anti-inflammatory actions, we investigated whether resveratrol has antimicrobial activities against the bacterial and fungal species that are major etiological agents in human skin infections.

The bacteria tested were *S. aureus* and *Streptococcus* group D (*Enterococcus faecalis*), Gram-positive organisms that cause a variety of skin conditions, including folliculitis, impetigo, furuncles (boils), and cellulites, and *Pseudomonas aeruginosa*, a Gram-negative organism that infects burn wounds. The rationale for selecting these organisms is that

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they often infect diabetic foot ulcers and may occur in a nosocomial environment. The fungi tested were *Trichophyton* species, *Epidermophyton floccosum,* and *Microsporum gypseum*, etiological agents in *tinea pedis, tinea corporsis*, and *tinea cruris*. Resveratrol was found to be active against these species of bacteria and dermatophytes that commonly afflict a significant portion of our population.

2. Materials and methods

2.1. Test organisms

All bacterial species were obtained from the American Type Culture Collection (ATCC): *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *P. aeruginosa* ATCC 27853. For dermatophytes, both ATCC strains and clinical isolates were used. The dermatophytes tested included one isolate each of *T. mentagrophytes, T. tonsurans, T. rubrum, E. floccosum*, and *M. gypseum* from the Temple University School of Podiatric Medicine collection, plus the ATCC strains: *T. mentagrophytes* ATCC 18748, *T. tonsurans* ATCC 28942, *T. rubrum* ATCC 18762, *E. floccosum* ATCC 52066, and *M. gypseum* ATCC 14683.

2.2. Antimicrobial susceptibility assays

The antimicrobial activity of resveratrol was determined by following the procedure for the broth microdilution assay of the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility testing. Protocol M7-A5 and M38-P were used for bacteria and dermatophytes, respectively, with some adjustment for concentration of drugs and solvent [14,15]. In brief, cells were added to culture medium containing a series of drug dilutions in wells of microtiter plates, and their growth was assessed after a period of incubation.

For bacteria, from a stock solution of resveratrol in DMSO (both from Sigma), a 10-fold dilution followed by serial 2-fold dilutions was made in cation-adjusted Mueller–Hinton broth (all culture media were obtained from Becton Dickinson). DMSO has been reported as a solvent for resveratrol, but other solvents such as ethanol are also possible [8,9]. The range of resveratrol concentrations tested was 2–512 μg/mL, before the addition of the cells. For a control, a parallel series of dilutions containing the vehicle DMSO alone was made to measure the effect of the solvent. In addition, wells containing medium alone (without DMSO) were used to determine whether the solvent would be inhibitory to the organisms. Further details of the dilution scheme have been described in the NCCLS protocol [14].

S. aureus and *P. aeruginosa* were cultured on plates of Trypticase soy agar. *E. faecalis* was grown on plates of Trypticase soy agar supplemented with 5% defibrinated sheep blood, overnight at 37°. The colonies were harvested, suspended in sterile saline, and adjusted to a concentration that yielded an absorbance similar to that of a 0.5 McFarland standard in a spectrophotometer at 530 nm, the equivalence of 1–2 $\times$ 10^8 cfu/mL. Then, the bacterial samples were further diluted 1:100 in Mueller–Hinton broth, and inoculated at 0.5–1 $\times$ 10^6 cfu/mL at a 1:2 ratio (50–100 μL, v/v) into the drug solutions in 96-well U-shaped tissue culture plates (Corning Inc.). In addition, aliquots of the working suspensions were cultured to confirm the size of the inoculum.

To test the activity of resveratrol on dermatophytes, solutions of the compound were prepared at various concentrations by diluting from a stock solution prepared in DMSO with RPMI 1640 containing 0.165 M 3-(*N*-morpholino) propanesulfonic acid (MOPS) and adjusted to pH 7.0. The range of concentrations tested was 6.25–200 μg/mL before the addition of cells, and the final DMSO solvent concentration was kept constant at 1% of the RPMI-MOPS medium. Additional experiments with various proportions of DMSO in culture medium were also performed.

The size of the inoculum was kept consistent according to the number of cfu [16]. It is known that there are absorbance differences among species of filamentous fungi when McFarland turbidity is used for determining the size of the inoculum [17]. The fungi were cultured at 35° for 7 days on slants of Sabouraud glucose agar, except for *E. floccosum* which was also cultured in Mycosel agar to induce conidium formation. The sample was harvested by pipetting 5 mL of sterile saline into the culture slant, and using a sterile cotton-tipped applicator to dislodge the fungal cells. The suspension was then filtered through a sterile gauze pad to remove large aggregates, and the number of cfu was determined by counting microscopically with a hemacytometer. According to previous reports using the broth microdilution assay for dermatophytes, counted fungal samples were diluted with RPMI-MOPS medium, and 100 μL per well was loaded for testing (with fungal suspensions at 1 $\times$ 10^3 to 1 $\times$ 10^4 cfu/mL) [18,19]. As done with bacteria, the size of the inoculum was confirmed by culturing aliquots on Sabouraud glucose agar plates.

Both bacteria and dermatophyte cultures were incubated at 35° in a humidified incubator as recommended by the NCCLS antimicrobial susceptibility testing procedures. To determine the effect of resveratrol, bacteria were incubated overnight (16–20 hr), whereas dermatophytes were observed daily after the third day [18]. Conventional visual scoring of the degree of growth was done with the aid of a reading mirror [14,15]. In addition, the plates were read spectrophotometrically at 550 nm for quantitative analyses.

3. Results and discussion

The standardized method of the NCCLS for determining susceptibility was used to study the effect of resveratrol on

the growth of *S. aureus, E. faecalis*, and *P. aeruginosa*. These bacteria, among the most commonly found pathogens on the human skin, are etiologic agents for many skin infections and in nosocomial infections and diabetic foot ulcers. Accordingly, the tested compound was dissolved in DMSO, diluted in medium, and added to the culture medium with bacteria. However, this resulted in a concomitant change in the concentration of resveratrol as well as the solvent, as indicated on the x-axis of Fig. 1. Thus, a corresponding series of solvents was set up for comparison to establish that resveratrol conferred the antibacterial effect. DMSO did not reduce the growth of the tested bacteria, except for a slight decrease in *E. faecalis* at 3.3%. When present at 171 μg/mL of resveratrol in 1.7% DMSO,

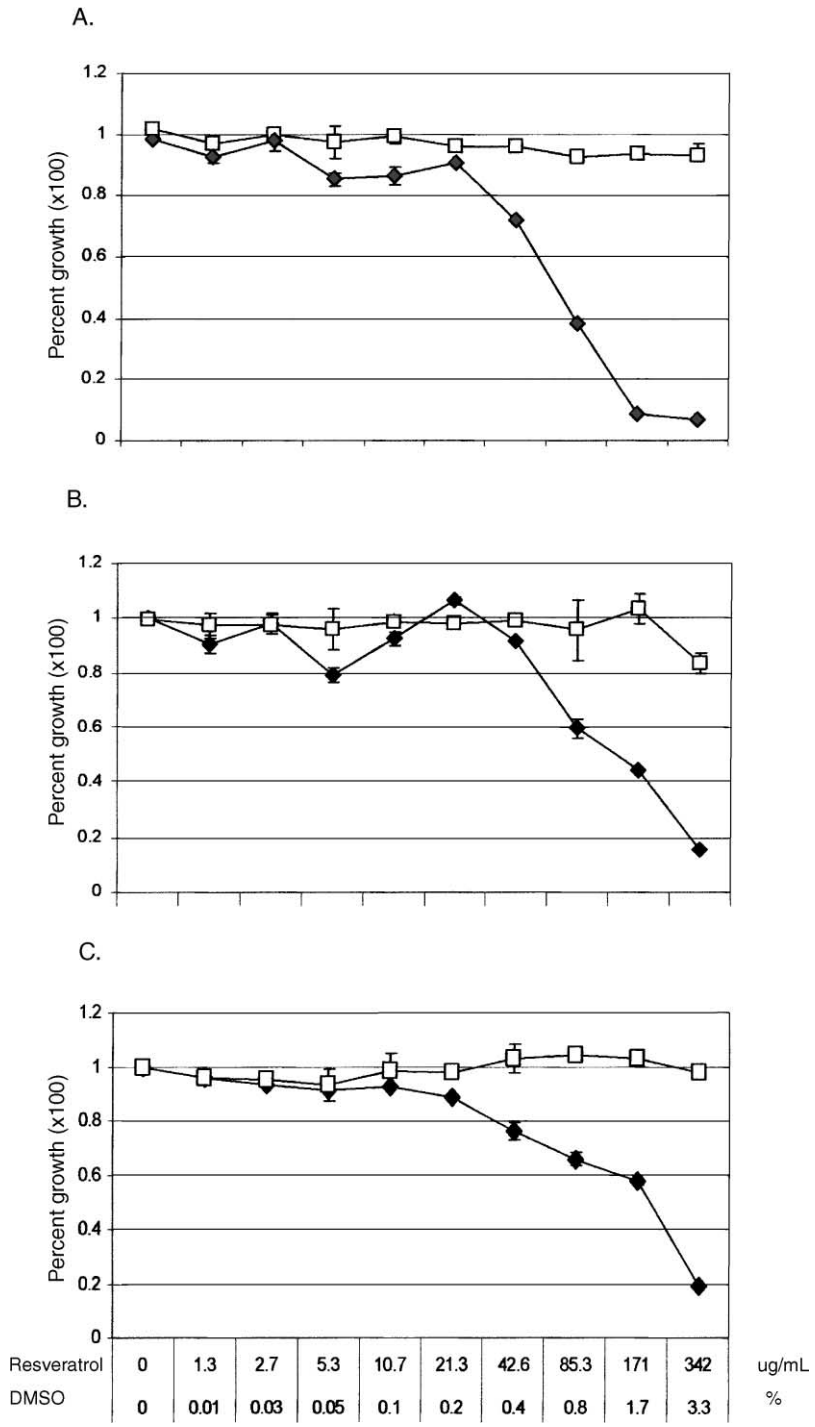

Fig. 1. Effect of resveratrol on bacteria. (A) *S. aureus* ATCC 29213; (B) *E. faecalis* ATCC 29212; and (C) *P. aeruginosa* ATCC 27853. The concentrations of resveratrol (μg/mL) and the DMSO solvent (%) are listed on the x-axis. Open squares are DMSO alone, and black diamonds are DMSO with resveratrol. The percent of growth, based on 550 nm plate reader data, is shown. $N = 2$, and the ranges are indicated by the bars.

the growth of *S. aureus* was inhibited by 80–90%. A similar degree of inhibition was observed for *E. faecalis* and *P. aeruginosa* at 342 μg/mL of resveratrol in 3.3% DMSO.

In addition, the results indicate that solubility may be important for efficacy, as resveratrol has been reported to be inactive against *S. aureus* and *P. aeruginosa* on agar cultures [20,21]. Although our data differ from those reported previously, nonetheless, it is not in conflict. The concentrations of resveratrol used in our study were higher, and the amount of resveratrol attained via diffusion through agar would be substantially less than that applied in liquid medium [22]. Furthermore, corroborative results have shown that resveratrol inhibits the growth of clinically important bacterial species, including *Neisseria gonorrhoeae, Neisseria meningitidis*, and *Helicobacter pylori* [21,23].

Interestingly, unlike bacteria, the dermatophytes were sensitive to DMSO. At 1–5% DMSO in the culture medium, 20–50% inhibition was observed, depending upon the individual fungal species. An example is shown in Fig. 2 for *T. tonsurans*, a species that has become the most frequently isolated dermatophyte from human patients in the United States [24]. Therefore, the drug dilution protocol of NCCLS for fungal susceptibility was modified to establish the effect of resveratrol on species of dermatophytes, so that the cultures were exposed to medium uniformly containing a concentration of 1% DMSO.

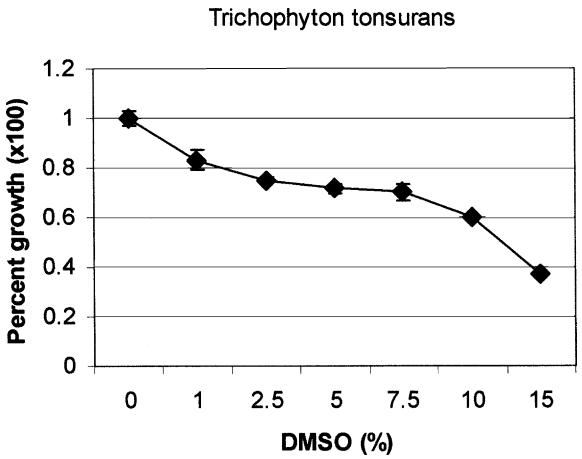

Fig. 2. Effect of DMSO on *T. tonsurans*. The concentration of DMSO is shown on the x-axis. The percent of growth is shown. Values are means ± SD, $N = 4$.

All tested clinical isolates from the Temple University School of Podiatric Medicine were inhibited by resveratrol (Fig. 3). Inhibition of over 75% was observed at 25–50 μg/mL for (A) *T. mentagrophytes*, (B) *T. tonsurans*, (C) *T. rubrum*, (D) *E. floccosum*, and (E) *M. gypseum*. Similar findings were seen with the ATCC strains, for example *M. gypseum* which is shown in Fig. 4. The effective concentration range was similar to that reported for *Helminthosporium carbonum*, which is pathogenic to plants

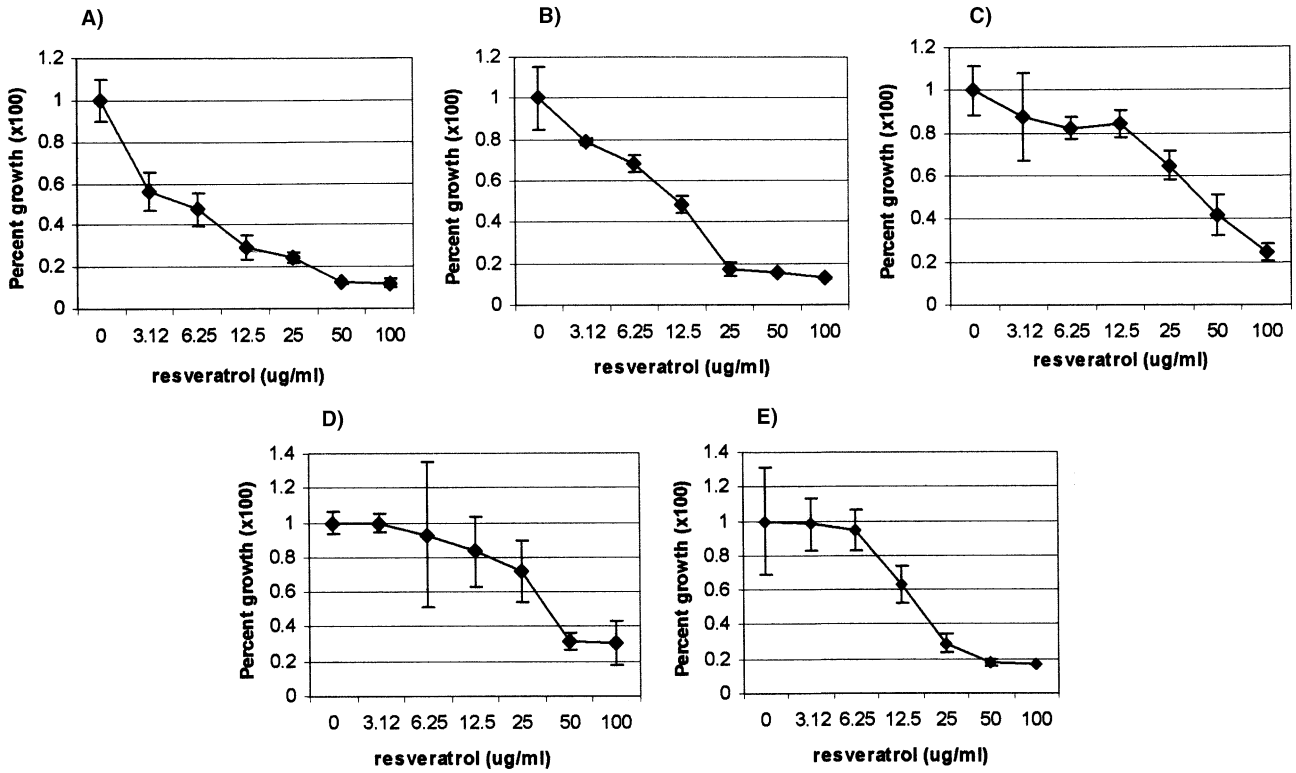

Fig. 3. Effect of resveratrol on dermatophytes: (A) *T. mentagrophytes*, (B) *T. tonsurans*, (C) *T. rubrum*, (D) *E. floccosum*, and (E) *M. gypseum*. The concentration of resveratrol (μg/mL) is shown on the x-axis, and the RPMI-MOPS medium contained 1% DMSO as solvent. The percent of growth, based on 550 nm plate reader data, is shown. Values are means ± SD, $N = 4$.

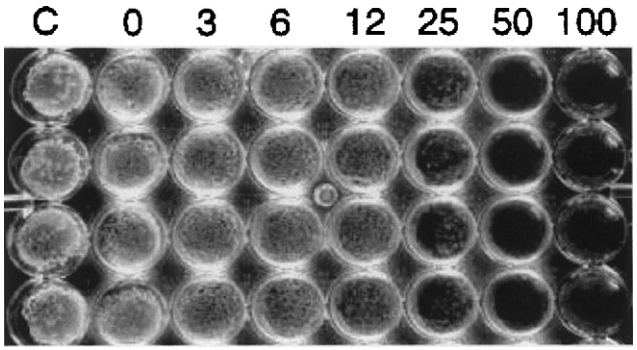

Fig. 4. Effect of resveratrol on *M. gypseum* ATCC 14683. The inhibition by resveratrol on the growth of this dermatophyte in microtiter wells is shown. The concentration range of resveratrol, from 3 to 100 μg/mL (in RPMI-MOPS containing 1% DMSO), is shown. Key: (0) medium with DMSO alone, and (C) (control) medium alone. Four replicate wells are shown.

but not to humans (50 μg/mL) and *Plasmopara viticola*, which causes downy mildew of grapevines (60 μg/mL) [25,26]. It is also in agreement with a study in which the effect of phytoalexins was tested against *T. rubrum* and in which the effective concentrations of 12.5 μg/mL for phaseollin isoflavan, 25 μg/mL for phaseollin, 25 μg/mL for sativan, 25 μg/mL for vestitol, 50 μg/mL for pisatin, 100 μg/mL for maackiain, and over 100 μg/mL for medicarpin were reported [27].

If resveratrol is to be used to treat skin infections, its effect on human keratinocytes and fibroblasts needs to be addressed. Our study showed that the effective dose for dermatophytes was 25–50 μg/mL (about 110–220 μM). At this concentration, resveratrol is not expected to affect fibroblasts, which reside in the dermal layer, as a study has shown that the midpoint cytotoxicity values for a 24-hr exposure are 432–462 μM for normal fibroblasts isolated from the oral cavity [28]. In the epidermis, mitotically active keratinocytes differentiate into cornified cells as they migrate upwards and synthesize increasing amounts of keratin, the protein that is utilized by the dermatophytes. A report has shown that resveratrol is not cytotoxic to normal human epidermal keratinocytes isolated from female breast sections, although it inhibits their proliferation. Up to 95% of keratinocytes are viable after exposure to 75 μM resveratrol for 3 days [29]. In contrast, a different study has shown that, for normal human keratinocytes from foreskin, resveratrol inhibits proliferation and is cytotoxic at 40–100 μM after 3 days of treatment [30]. The apparent discrepancy is explained by a difference in cell density, as 50-fold fewer cells were used in the second study. Nonetheless, in terms of dermatophytic infection, a temporary reduction of keratinocytes and an accumulation of keratin are not necessarily unfavorable for therapy.

Resveratrol represents a novel class of antifungal agents. Antifungal activities of a group of nine stilbenes, including resveratrol, have been compared. Their biological effect is related to the electronic character, the lipophilicity, and the molecular volume of each [31]. Among the antifungal agents currently available, the polyene amphotericin B, the imidazoles (miconazole and ketoconazole), and the triazoles (fluconazole and itraconazole) all target the ergosterol pathway of the fungal cell membrane, leading to membrane instability, and flucytosine is a fungistatic agent that inhibits pyrimidine synthesis [17]. How molecular mechanisms of resveratrol act on human dermatophytes remains to be determined. However, based on a study of interaction of resveratrol with *Botrytis cinerea*, a gray mold that infects grapevines, the mode of action has been proposed as an interference with the functionality of membrane proteins, especially those of the mitochondria. The interaction leads to an immediate decrease in oxygen uptake by the fungal cells. At the ultrastructural level, mitochondrial and nuclear membranes are affected first, followed by a complete disorganization of organelles and disruption of the cell membrane. Cytological changes manifest as production of secondary and tertiary germ tubes, formation of curved germ tubes, cytoplasmic granulation of conidia, and protoplasmic retraction in hyphal tip cells [31,32]. In human cells, resveratrol-induced mitochondrial membrane depolarization leading to apoptosis has been reported for acute lymphoblastic leukemia (ALL) cells [33]. Whether there are similarities among human dermatophytes, plant pathogenic fungi and mammalian cells in their responses to resveratrol needs to be investigated further.

In conclusion, the primary significance of this study is the observation that resveratrol, at effective doses that are achievable by topical application, could inhibit the growth of dermatophytes, fungal infections that have caused patients to seek medical attention in the United States [24]. To our knowledge, this is the first study to apply resveratrol, a molecule of plant defense, successfully to human fungal pathogens. Secondly, the finding that the vehicle DMSO, a popular enhancer for dermal drug delivery, is inhibitory to their growth, and that the two may work synergistically, is also of importance. Moreover, many natural and synthetic analogs of resveratrol have been isolated or synthesized. Thus, we anticipate developing and identifying stilbenes and other related compounds that are more active against dermatophytes than resveratrol, for example, herpes simplex virus replication is inhibited by resveratrol but not by another stilbene [8,26,34].

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