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In vitro activity of CAY-1, a saponin from *Capsicum frutescens*, against *Microsporum* and *Trichophyton* species

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Dermatomycoses are among the world's most common diseases and their incidence has increased over recent years, particularly in immunosuppressed patients. In previous studies, the saponin CAY-1 from cayenne pepper (*Capsicum frutescens*), has shown antifungal activities against *Candida albicans* and *Aspergillus* spp. We therefore studied the *in vitro* antifungal activity of CAY-1 against non-germinating conidia and hyphae of clinical isolates of the dermatophytes *Trichophyton mentagrophytes, T. rubrum, T. tonsurans* and *Microsporum canis.* We used a microdilution method to assess the growth inhibitory activities of CAY-1 against conidia (CLSI document M38-A) and a colorimetric procedure (XTT method) to investigate the metabolic inhibitory activity of CAY-1 against hyphae. The minimal inhibitory concentrations (complete visual growth inhibition) of CAY-1 against non-germinating conidia ranged from 10–20 μg/ml for all dermatophyte isolates included in this investigation. In addition, we found >90% inhibition of hyphal metabolic activity of these same isolates with 10–20 μg/ml of CAY-1. Results indicate that CAY-1 merits further investigation as a potential agent for the treatment of dermatomycoses.

Keywords dermatophytes, Capsicum frutenses, saponin

Introduction

Dermatomycoses are among the most widespread and common fungal infections in humans [1]. The increased number of individuals with impaired immunity following treatment with cytotoxic drugs and immunosuppressive agents has contributed to the increased prevalence of these diseases [2]. Although effective antifungal agents have been introduced into clinical practice over the last few years, some of these infections are still difficult to resolve completely and remissions and relapses are often observed, particularly in immunocompromised patients [3,4]. This situation gives impetus to the search for new, safe and effective antifungal compounds.

In recent years, plant peptides have been shown to possess potent antifungal activity [5,6]. A previous report showed that CAY-1 (Fig. 1), a saponin from cayenne pepper (*Capsicum frutescens*), had fungicidal activities against *Candida albicans* and *Aspergillus* species [7]. CAY-1 also enhanced the activity of amphotericin B and itraconazole against the same organisms [7,8]. The aim of this study was to evaluate the *in vitro* activity of CAY-1 against both conidia and hyphae of dermatophytes, since it is not known whether

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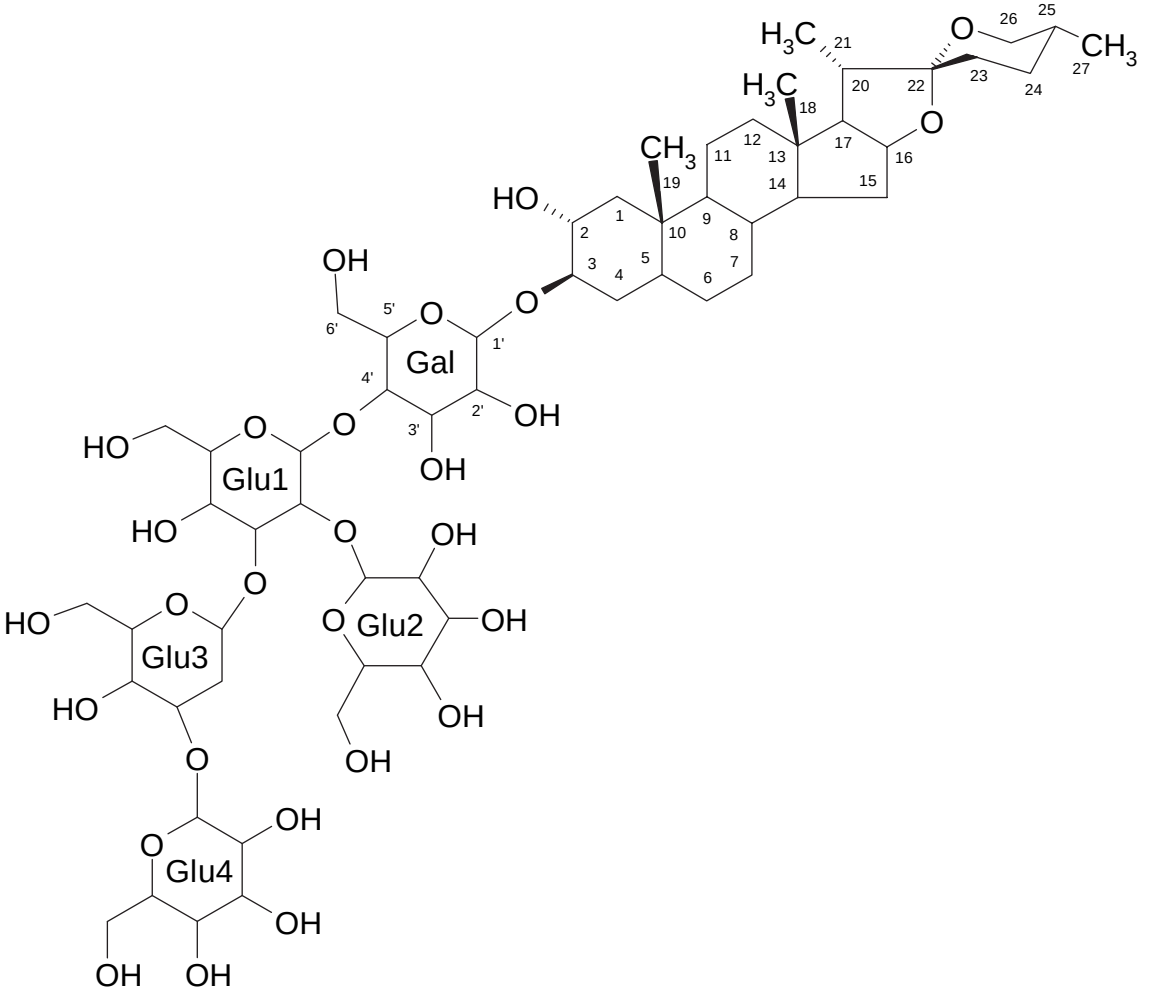

Fig. 1 Chemical structure of CAY-1 molecule.

the antifungal susceptibilities of these two morphological forms are similar.

Materials and methods

Isolates

Five clinical isolates of *Trichophyton mentagrophytes*, three of *T. rubrum*, five of *T. tonsurans* and five of *Microsporum canis* were used in this study. The isolates were stored on potato dextrose agar (PDA) slants at −70°C. Portions of growth from these stocks were transferred to PDA plate and conidia were collected with a wet cotton swab from 7 to 10-day-old cultures that had been incubated for the first day at 37°C and then transferred to room temperature. The tip of the swab was moistened in 5 ml of normal saline and conidial suspensions were prepared. Heavy particles of the suspension (such as small hyphal fragments) were allowed to settle for 5–7 min and the middle homogenous suspension was used for further testing. The conidial suspensions were counted using a hemacytometer and each isolate was tested three times.

Medium. RPMI 1640 (with L-glutamine and without bicarbonate; GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used throughout the present investigations.

XTT and menadione. XTT (2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide; Sigma-Aldrich, St Louis, MO, USA) was dissolved in normal saline. Menadione (Sigma-Aldrich, St Louis, MO), an electron transfer agent, was dissolved in absolute ethanol to a concentration of 10 mM. A working solution of 500 μg/ml of XTT and

125 μM menadione was prepared in order to obtain five times the final XTT and menadione concentrations, i.e., 100 μg/ml and 25 μM, respectively, inside the wells.

CAY-1. CAY-1 (Fig. 1) was isolated and purified to homogeneity from commercial dry, ground fruit of cayenne as previously described [9]. Stock solutions of CAY-1 (40 μg/ml) were prepared in RPMI 1640 medium and then two-fold serially diluted in the same medium in order to obtain two times the final concentrations in the range of 0.02–20 μg/ml. This choice of concentrations was based on previous studies of CAY-1 against *Candida* and *Aspergillus* species [9]. A total of 100 μl of each concentration were added into wells of 96-well flat bottom microtiter plates (Corning Inc., Corning, NY) and stored at -70°C until the day of testing.

Susceptibility testing of dermatophyte conidia. The investigations of the activity of CAY-1 against the dermatophyte conidia was based on CLSI guideline M38-A [10]. RPMI 1640 as noted previously was used throughout the studies. Conidia suspensions were diluted in order to obtain two times the final inoculum, i.e., 3×10^4 conidia/ml of medium. Inoculum quantification was performed by plating 100 μl of a 1:100 dilution of the adjusted inoculum on PDA plates, which were then incubated at 37°C and evaluated on the fifth day. The microtiter plates noted above, were inoculated with 100 μl of the conidial suspension and incubated at 37°C for 144 h. Growth was assessed visually at 96, 120 and 144 h using a reverse reading mirror and spectrophotometrically at a wavelength of 405 nm. The percent of fungal biomass assessed spectrophotometrically was calculated for each concentration as A_{405} of a well – background A_{405}/A_{405} of the drug-free well – background A_{405} of the drug-free well $\times 100$, where the background A_{405} was measured from a plate inoculated with a conidia-free suspension and treated identically as the plates containing conidia. The minimal inhibitory concentration (MIC) of CAY-1 was determined after 96, 120 and 144 h as the lowest concentration showing no visible growth and a 90% decrease of biomass. Each isolate was tested three times by this method.

Susceptibility testing of dermatophyte hyphae. The *in vitro* activity of CAY-1 against hyphae was tested using the colorimetric XTT assay for hyphal metabolic activity. The XTT and menadione solution was prepared as described above. Conidial suspensions of 6×10^4 conidia/ml were prepared from which aliquots (50 μl) were inoculated into the wells of 96-well flat bottom microtiter plates containing 50 μl of medium. The plates were incubated at 37°C for 24 h to allow hyphal formation, the presence of which was confirmed microscopically. Aliquots of 100 μl of CAY-1 dilutions were then added into their respective wells in order to obtain final concentrations of 0.02–20 μg/ml. After incubation at 37°C for 24 h, 50 μl aliquots of XTT-menadione solution were added in each well. XTT conversion by viable hyphae was measured at 450 nm. The percent of metabolic activity was calculated for each concentration as A_{450} of a well – background A_{450}/A_{450} of the drug-free well – background A_{450} of the drug-free well $\times 100$, where the background A_{405} was measured from a plate inoculated with a conidia-free suspension and treated identically as the plates containing conidia. The MIC was defined as the lowest concentration showing $>90\%$ inhibition of metabolic activity compared to that of the growth control.

Regression analysis. Because an MIC endpoint corresponds to a single drug concentration and does not provide information about the antifungal activity of CAY-1 at high and low concentrations, the concentration-effect data against conidia and hyphae obtained from the spectrophotometric readings of fungal biomass after 144 h of incubation and of metabolic activity after 48 h of incubation, respectively, were analyzed by a non-weighted regression analysis using the Emax model (sigmoidal curve with variable slope) (Prism 4.0 Software, GraphPad Inc., San Diego, CA, USA). Goodness of fit was assessed based on R^2 values, standard error of estimates, and visual inspection of the curves. In order to obtain an estimate of EC_{50} and slope for all isolates of each species, the Emax model was fitted globally to all isolates of each species by sharing all four parameters of the Emax model. The EC_{50} is an inverse measure of antifungal potency of CAY-1 (for example, the greater the EC_{50}, the lower is the potency); whereas, the slope describes the steepness of the concentration-effect curve (the larger the absolute value, the steeper the curve) and indicates the gradual change of antifungal activity with increasing CAY-1 concentrations. A steep curve indicates small degree of growth inhibition at concentrations lower than the EC_{50} whereas a shallow curve indicate that significant growth inhibition can be observed at low concentrations.

Results

Sufficient growth was noted with all isolates in microtiter plates within 96 h to allow endpoints to be

measured (ODs for the growth control drug-free wells ranged from 0.1–0.2). The MICs of CAY-1 ranged from 10–20 μg/ml for all isolates (Table 1) and antifungal activities against dermatophyte hyphae was also noted with these isolates. Greater than 90% inhibition was found in the metabolic activity of the hyphae of most isolates with CAY-1 at concentrations of 10–20 μg/ml (Table 2). However, the hyphal metabolism of two isolates each of *M. canis* and *T. tonsurans* was inhibited by only 20–50% at concentrations of CAY-1 as high as 20 μg/ml.

Regression analysis showed that the antifungal activity of CAY-1 against conidia and hyphae followed a sigmoid pattern as the E_{max} model clearly demonstrated the concentration-effect data for each dermatophyte species ($R^2 > 0.81$ for 90% of the fits). The antifungal activity was observed at concentrations 10–20 μg/ml (Fig. 2). The EC_{50} and Hillslope values of the concentration-effect curves of CAY-1 against conidia of *T. mentagrophytes*, *M. canis*, *T. tonsurans*, *T. rubrum* were 9.7 μg/ml, 8.6 μg/ml, 9.2 μg/ml, 6.1 μg/ml and −25.9, −4.0, −18.3, −8.2, respectively. The EC_{50} and Hillslope concentration-effect curves of CAY-1 against hyphae of *T. mentagrophytes*, *M. canis*, *T. tonsurans*, *T. rubrum* were 9.36 μg/ml, 17.30 μg/ml, 10.52 μg/ml, 5.70 μg/ml and −3.52, −5.18, −2.99, −6.50, respectively. There was no significant difference between the EC_{50} values of conidia and hyphae for all species.

Discussion

In this study we demonstrated that CAY-1 has *in vitro* activity against the non-germinating conidia and hyphae of different dermatophyte species. CAY-1 inhibited the growth of all test isolates at concentrations 10–20 μg/ml. There was no significant inter- and intra-species variation in the antifungal activity of CAY-1. CAY-1 also inhibited hyphal metabolism in most isolates at levels of 10–20 μg/ml. Though no growth inhibition was observed below 5 μg/ml, regression analysis revealed a sigmoid concentration effect displaying steep curves at CAY-1 concentrations of 5–20 μg/ml.

Table 2 Medians of concentrations of CAY-1 that caused ≥ 90% inhibition of hyphal metabolic activity of dermatophytes species.

Organisms	No. of isolates	Median (range) of concentrations (μg/ml)
T. mentagrophytes	5	20 (10–20)
T. rubrum	3	10 (10–10)
T. tonsurans	5	20 (10–>20)*
M. canis	5	20 (20–>20)*

*Two isolates each of *Trichophyton tonsurans* and *Microsporum canis* had metabolic activity of 20–50% instead of 10% at 20 μg/ml.

In the recent years, the development of new classes of agents such as the allylamines (e.g., terbinafine) and orally active azoles (e.g., itraconazole) have provided useful alternatives for dermatophyte therapy [1]. However, more effective antifungal agents with fewer adverse effect and short-term therapy are required to treat dermatophytosis. In this context, several studies have been conducted to search for plant compounds as sources of antifungal agents [6,11,12].

CAY-1 is a steroid saponin with potent fungicidal properties against several fungal pathogens [9]. Saponins are glycosides consisting of one or more sugars linked to a steroid or triterpene core [13]. Some saponins exhibit antiviral effects by inhibiting viral DNA and capsid protein syntheses or have immunostimulating properties [14]. There is also evidence of health benefits of saponins as cholesterol lowering and anticancer agents [15,16]. The presence of saponins has been reported in more than 100 plant families [17]. It has been proposed that the detergent properties of CAY-1, which cause lysis of the fungal cell membrane, may be the source of its antifungal nature [9,17]. The complexing of a saponin with membrane sterols results in loss of cell membrane integrity, leading to cell death [18]. This mechanism of action is similar to that of polyene drugs such as amphotericin B.

CAY-1 has broad *in vitro* activity against germinating conidia of *Aspergillus* species, *Pneumocystis jiroveci* and *C. albicans* perhaps due to detergent properties of

Table 1 MICs (range) of CAY-1 against the different species of the dermatophytes.

Organisms	No of isolates	Median MIC (range) (μg/ml)		
		96 h	120 h	144 h
T. mentagrophytes	5	20 (10–20)	20 (10–20)	20 (10–20)
T. rubrum	3	10 (10–20)	10 (10–20)	10 (10–20)
T. tonsurans	5	20 (10–20)	20 (10–20)	20 (10–20)
M. canis	5	20 (10–20)	20 (10–20)	20 (10–20)

*MIC results were the same when determined by visual and spectrophotometric methods.

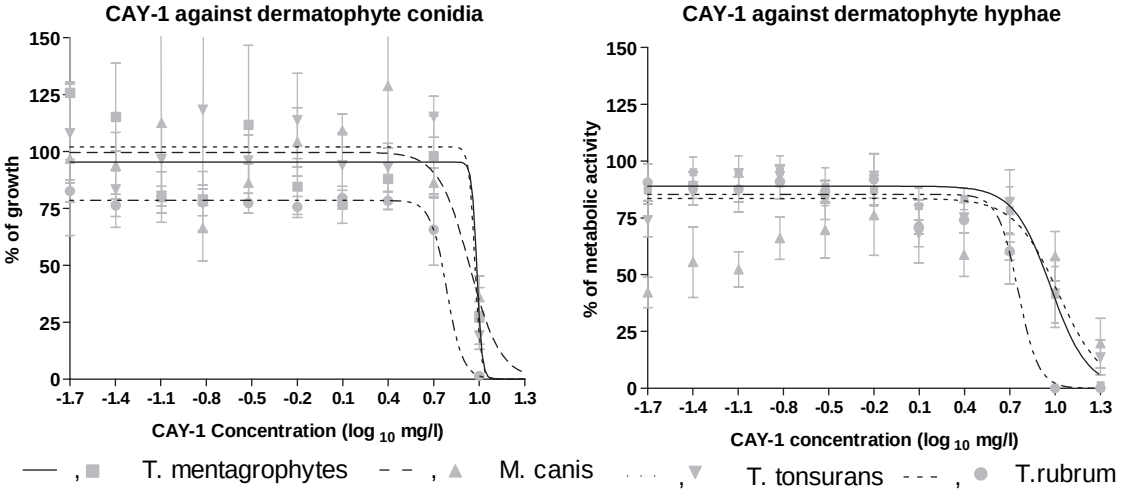

Fig. 2 Concentration-effect curves of CAY-1 against conidia and hyphae of *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans* and *Microsporum canis* clinical isolates.

CAY-1. In a previous report [9], the MICs against the germination of *C. albicans* blastospores and of *Aspergillus* species conidia were within the same range as in the present study against dermatophytes. This activity occurs at concentrations of <20 μg/ml, which is well below the threshold of mammalian cytotoxicity. CAY-1 displays no cytotoxicity at concentrations up to 25 μg/ml against HeLa cells and 10 μg/ml against A549 lung carcinoma cells over three days of exposure. In addition, no significant cytotoxicity was found against 55 mammalian cell lines at CAY-1 concentrations up to 100 μg/ml [8]. As CAY-1 is water soluble, it may be administered parenterally and formulated for topical application. *In vitro* and laboratory animal studies have shown no cytotoxicity at effective concentrations. At higher concentrations, cytotoxic signals were observed *in vitro* in cell lines [8]. Further *in vivo* studies will be conducted in order to investigate the effectiveness of CAY-1, optimal delivery systems, and possible adverse effects. There is currently no information regarding the delivery to or adverse effects in humans.

There is a growing interest in the use of plant-derived compounds to treat dermatomycoses. Controlled clinical trials have been performed to determine the effectiveness of plant extracts against dermatomycoses compared with that of conventional antifungal treatments [19]. In one study, treatment of *tinea pedis* with herbal preparations (tea tree oil, *Solanum chrysotrichum* and oil of bitter orange) was found to be as effective as conventional antifungal therapy, with cur rates of up to 93% [19].

CAY-1 is a saponin with potent *in vitro* antifungal activity against important dermatophytes. However, further studies are needed to prove potential *in vivo* activity against animal models of dermatomycosis.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1 Borgers M, Degreef H, Cauwenbergh G. Fungal infections of the skin: infection process and antimycotic therapy. *Curr Drug Targets* 2005; **6**: 849–862.

2 Mays SR, Bogle MA, Bodey GP. Cutaneous fungal infections in the oncology patient: recognition and management. *Am J Clin Dermatol* 2006; **7**: 31–43.

3 Hainer BL. Dermatophyte infections. *Am Fam Physician* 2003; **67**: 101–108.

4 Weitzman I, Summerbell RC. The dermatophytes. *Clin Microbiol Rev* 1995; **8**: 240–259.

5 Ali-Shtayeh MS, Abu Ghdeib SI. Antifungal activity of plant extracts against dermatophytes. *Mycoses* 1999; **42**: 665–672.

6 Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* 1999; **86**: 985–990.

7 De Lucca AJ, Bland JM, Boue S, *et al*. Synergism of CAY-1 with amphotericin B and itraconazole. *Chemotherapy* 2006; **52**: 285–287.

8 Renault S, De Lucca AJ, Boue S, *et al*. CAY-1, a novel antifungal compound from cayenne pepper. *Med Mycol* 2003; **41**: 75–81.

9 De Lucca AJ, Bland JM, Vigo CB, *et al*. CAY-I, a fungicidal saponin from *Capsicum* sp. fruit. *Med Mycol* 2002; **40**: 131–137.

10 NCCLS. Reference Method for Both Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. 2002; **M38A,** Vol. 22, No. 16.

11 Sanguinetti M, Posteraro B, Romano L, *et al*. *In vitro* activity of *Citrus bergamia* (bergamot) oil against clinical isolates of dermatophytes. *J Antimicrob Chemother* 2007; **59**: 305–308.

12 De Lucca AJ, Bland JM, Vigo CB, *et al*. D-cecropin B: proteolytic resistance, lethality for pathogenic fungi and binding properties. *Med Mycol* 2000; **38**: 301–308.
13 Vincken JP, Heng L, de Groot A, Gruppen H. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry* 2007; **68**: 275–297.
14 Zhang XF, Cui Y, Huang JJ, *et al*. Immuno-stimulating properties of diosgenyl saponins isolated from *Paris polyphylla*. *Bioorg Med Chem Lett* 2007; **17**: 2408–2413.
15 Gurfinkel DM, Rao AV. Soyasaponins: the relationship between chemical structure and colon anticarcinogenic activity. *Nutr Cancer* 2003; **47**: 24–33.
16 Kim SW, Park SK, Kang SI, *et al*. Hypocholesterolemic property of *Yucca schidigera* and *Quillaja saponaria* extracts in human body. *Arch Pharm Res* 2003; **26**: 1042–1046.
17 Guclu-Ustundag O, Mazza G. Saponins: properties, applications and processing. *Crit Rev Food Sci Nutr* 2007; **47**: 231–258.
18 De Lucca AJ, Boue S, Palmgren MS, Maskos K, Cleveland TE. Fungicidal properties of two saponins from *Capsicum frutescens* and the relationship of structure and fungicidal activity. *Can J Microbiol* 2006; **52**: 336–342.
19 Martin KW, Ernst E. Herbal medicines for treatment of fungal infections: a systematic review of controlled clinical trials. *Mycoses* 2004; **47**: 87–92.

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