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ANTIMICROBIAL EVALUATION OF SESQUITERPENE α-CURCUMENE AND ITS SYNERGISM WITH IMIPENEM

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ABSTRACT

α-Curcumene was isolated from the fresh aerial parts of *Senecio selloi* Spreng. DC. and its activity against bacteria, yeasts and an alga was inspected by the applying the microdilution method. The strongest effect was manifested against *Saccharomyces cerevisiae* with estimated values of MIC and MFC 0.8 mg/mL. The *α*-curcumene synergism in the concentrations of 1 mM and 5 mM, respectively, with selected antibiotics (ciprofloxacin, imipenem, ceftazidim and a combination of amoxicillin and clavulanic acid) was investigated against *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae*, *Staphylococcus haemolyticus* and *Klebsiella pneumoniae* by the disk diffusion assay. The results have shown the occurrence of synergism of *α*-curcumene with imipenem against the clinical isolate *E. cloacae* with a significance level of p> 0.05. Based on these informations it can be concluded that fungal strains are more sensitive for *α*-curcumene than the bacterial ones and the synergism of *α*-curcumene with imipenem can improve the antibiotic efficiency against the *E. cloacae*.

Keywords: *Senecio selloi*; *α*-curcumene; antimicrobial activity; *Enterobacter cloacae*; imipenem, synergism

INTRODUCTION

In accordance with World Health Organization (WHO), the antimicrobial resistance threatens, the effective prevention and treatment of an ever-increasing range of infections caused for instance by bacteria and fungi. A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria. Futhermore, the resistance to one of the most widely used antibacterial drugs for the oral treatment of urinary tract infections caused by *Escherichia coli*, is very widespread **(WHO, 2014)**. Therefore greater efforts must be made toward the discovery of new antimicrobial compounds. Constituents isolated from plants may be an alternative such as species from the genus *Senecio* that even crude extracts are known to possess an antimicrobial activity, the antibacterial and the antifungal ones **(Yang *et al.*, 2011)**. The genus *Senecio* (Asteraceae) is constituted of about 2000 species worldwide, 85 of which are found in southern Brazil and of these, 25 are native of the state of Rio Grande do Sul **(Matzenbacher, 1998)**. Besides pyrrolizidine alkaloids **(Tundis *et al.*, 2007)**, *Senecio* species are rich in diterpenes **(Ndom *et al.*, 2010)**, sesquiterpenes **(Xie *et al.*, 2010)**, triterpenes **(Rücker *et al.*, 1999)** and flavonoids **(Niu *et al.*, 2013)**. Some of these are components of the essential oils, which are goals of synergic studies with antibiotics, in a view to evaluating and enhancing antimicrobial efficacy **(Rosato *et al.*, 2007)**. The ability of sesquiterpene to increase the bacterial susceptibility to a number of clinically important antibiotics was investigated with nerolidol, bisabolol, and apritone as well enhancing the activities of all six antibiotics tested against *S. aureus*. Nerolidol and farnesol also sensitized *E. coli* to polymyxin B **(Brehm-Stecher and Johnson, 2003)**. In this context, the present report describes the isolation, the identification, an antimicrobial evaluation and the synergism with antibiotics of *α*-curcumene (Figure 1), a sesquiterpene obtained from the aerial parts of *Senecio selloi* Spreng DC.

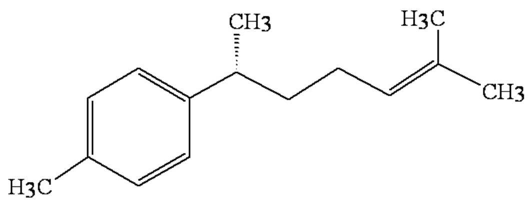

Figure 1 Chemical structure of sesquiterpene *α*-curcumene.

MATERIAL AND METHODS

Plant material

Plant material was identified by Prof. Dr. Nelson Ivo Matzenbacher (Graduate Botany Program, UFRGS). The aerial parts were collected in Guaíba, RS, Brazil. Voucher specimen Nº SMDB 10206 is preserved in the Herbarium of the Department of Botany, UFSM, RS, Brazil.

Extraction, isolation and identification

Fresh aerial parts of *Senecio selloi* (550 g) were divided and extracted with CH_2Cl_2 by the maceration for 8 days (d), twice. The macerate was concentrated *in vacuo*, yielding 15 g of crude extract. The amount 5 g of them was chromatographed on a silica gel column (335 g) using CH_2Cl_2, yielding 41 fractions. The fractions 5-8 (400 mg) were rechromatographed on a silica gel column with 10% $AgNO_3$ using hexane, obtaining 50 fractions. The fractions 11-13 (17 mg of *α*-curcumene) were analyzed by NMR and MS spectra. The extraction, isolation and identification were done based on previous research of **Silva *et al.* 2010**. General experimental: Optical rotation in $CHCl_3$: Perkin-Elmer 241; EI-MS at 70 eV: Kratos MS 50; NMR Spectra in $CDCl_3$: Varian XL 300 MHz.

α-Curcumene: R_f 0.35 (Petrol), [α]D + 59.95° (c= 8.18). EI-MS *m/z* (%): 202 (30), 187 (2.1), 159 (4.5), 145 (29.1), 132 (93), 119 (100), 105 (50.9), 95 (6.6), 91 (29.4), 77 (11.3), 69 (16), 65 (7.2), 55 (26.4), 41 (51.1). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.14 (4 H, m, H-2, H-3, H-5, H-6), 5.15 (1 H, ddsept, J 1.5, 7 and 7 Hz, H-10), 2.72 (1 H, tq, J 7 and 7 Hz, H-7), 2.38 (3-H, s, Me-15), 1.94 (2 H, m, H2-9), 1.74 (3 H,d, J 1.5 Hz, Me-13), 1.66 (2 H, m, H2-8), 1.58 (3 H, d, J 1.5 Hz, Me-12), 1.28 (3 H, d, J 7 Hz, Me-14). ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 144.5 (C4), 135.0 (C1), 131.2 (C11), 128.9 (C2), 128.9 (C6), 126.8 (C3), 126.8 (C5), 124.5 (C10), 39.1 (C7), 38.5 (C8), 26.3 (C9), 25.8 (C13), 22.6 (C14), 21.1 (C15), 17.8 (C12).

The *in vitro* antimicrobial assay

The antimicrobial activity of *α*-curcumene was determined by the microdilution method, based on documents M27-A2 for yeasts (*Candida albicans* ATCC 44773, *Saccharomyces cerevisiae* ATCC 28952 and *Candida glabrata* (clinical isolate fluconazole resistant)) and M100-S12 for bacteria (*Staphylococcus aureus* ATCC 27923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 2792 and *Pseudomonas aeruginosa* ATCC 27853). The alga *Prototheca zopfii* was also assayed by M27-A2 methodology. Previous to test, the bacterial cultures were activated by subculture on Mueller-Hinton agar during 24 h at 37 °C; the yeasts and *Prototheca* isolated were subcutured on Sabouraud´s dextrose agar during 48 h at 35 °C. After being subcultured, the bacterial inoculum was prepared in order to achieve the turbidity of the suspension was similar to the 0.5 Mc Farland standard (1×10^8 CFU/mL). Such suspension was immediately diluted in the ratio of 1:10 (1×10^7 CFU/mL) in sterile saline. The volumes of 10 µL were deposited in the wells containing 200 µL of the medium plus different concentrations of the *α*-curcumene, resulting in a final inoculum concentration varying from 2×10^5 to 5×10^5 CFU/mL. Similarly, the initial inoculum suspension of yeasts was obtained and afterwards, in order to be adjusted according to the turbidity of Mc Farland No. 0.5 standard, the suspensions were successively diluted at 1:50 and 1:20 on the RPMI buffered broth. The assays were incubated at 37 °C for 48 h for yeasts species and *Prototheca zopfii*. The assays with bacteria were incubated at 37 °C for 24 h. In all cases the results were visually compared to a positive growing control in RPMI 1640 broth or Mueller-Hinton broth containing Tween 80 1:20 diluted in the medium at the same proportion that could be found on the highest concentrations 6400 µg/mL; 3200 µg/mL; 1600 µg/mL; 800 µg/mL; 400 µg/mL; 200 µg/mL; 100 µg/mL for yeasts and *Prototheca* and 12800 µg/mL, 6400 µg/mL; 3200 µg/mL; 1600 µg/mL; 800 µg/mL; 400 µg/mL; 200 µg/mL for bacteria. The minimum inhibitory concentrations (MICs) were determined as the lowest concentrations which completely inhibited the growth of the microorganisms. The experiments were repeated at least three times.

Assessment of the synergism of *α*-curcumene with antibiotics

The synergism of *α*-curcumene with antibiotics was evaluated for imipenem, ciprofloxacin, combination of amoxicillin and clavulanic acid and, finally, for ceftazidime against *S. aureus* ATCC 25923, *E. coli* 25922, *E. cloacae* (a clinical isolate), *S. haemolyticus* (SCN4) and *Klebsiella pneumoniae* ATCC 700603 by the disk diffusion assay. The cells were grown overnight (from 18 to 20 h) at 35 °C in 10 mL of Trypticase soy broth (Becton Dickinson and Company, Franklin Lakes, N.J.) and diluted to a concentration of 10^7 CFU/mL in neutral phosphate buffer. 0.5 mL aliquot of this suspension was combined with 4.5 mL of an overlay agar prepared from Iso-Sensitest broth (Oxoid, Ogdensburg, N.Y.) plus 0.7 % agar, tempered to 50 °C. *α*-Curcumene 10 % was added to the cell overlays mixture which was vortexed. After vortexing, treated cell overlays were poured over hardened Iso-Sensitest agar plates (2 % agar). The plates were then swirled thoroughly in alternating clockwise and counterclockwise motions to help ensure an even distribution of *α*-curcumene throughout the overlay. Antibiotic disk was placed on hardened agar overlays, and plates were incubated at 35 °C ± 2 °C for 24 h. Diameters of zones of inhibition were measured with a ruler from the bottom of each plate **(Brehm-Stecher and Johnson, 2003)**. The experiments were repeated at least three times.

Statistical analysis

The data were analyzed statistically by one-way ANOVA, followed by Tukey's multiple range tests. The results were considered statistically significant for $p < 0.05$. The data were calculated by using the Statistical Package for Social Science (SPSS) program.

RESULTS AND DISCUSSION

The identity of isolated *α*-curcumene was verified by spectral analysis. This natural product is present in many essential oils obtained from plants, several of them with significant antimicrobial activity **(Schwob *et al.*, 2002; Govinden-Soulange *et al.*, 2004)**. For given sesquiterpene different biological activities are related, such as anti-tumor with mechanism of action suggested by the apoptotic effect of *α*-curcumene on SiHa cells that may converge caspase-3 activation through the release of mitochondrial cytochrome c **(Shin and Lee, 2013)**. In current study, its antimicrobial activity was inspected against eight species of the microorganisms with variable susceptibility to some antimicrobial agents. The bacterial species chosen were those usually recommended for studies with antibacterials: Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*). The fungi species included have shown different susceptibility profiles to antifungal agents: species usually sensitive such as *S. cerevisiae* **(Kohler *et al*., 2004)** and related species with significant development of the resistance to azoles such as *C. albicans* **(Marichal *et al*., 1999)**. The alga belonging to *Prototheca* species (among them *P. zopfii*) have shown worldwide distribution and are responsible for several diseases affecting both animals and man. In dairy cattle, they may cause a mastitis **(Thiele and Bergmann, 2002)**.
The results in Table 1 have shown that *α*-curcumene is active against all bacterial species, at the same concentration (13 mg/mL), although Gram-negative bacteria are usually more resistant to essential oil constituents than the Gram-positive ones **(Deuschle *et al*., 2006)**. In the current research, *α*-curcumene was regarded to be equally effective against both kinds of investigated microorganisms. These results are significant mainly for *P. aeruginosa*, a Gram-negative anaerobic bacterium which has shown high resistance to the conventionally used antibiotics **(Mesaros *et al.*, 2007)**.
Althouth *α*-curcumene did not display an antimicrobial efficiency against *C. glabrata* at the tested concentrations (until 6.4 mg/mL) as given in Table 1, concerned sesquiterpene exhibited an antimicrobial activity against *C. albicans* and *S. cerevisiae*, permitting postulate that fungal strains are more sensitive than the bacterial ones. So far the mechanism of an antimicrobial action of the sesquiterpene is incompletely understood. The antimicrobial effectiveness of cyclic hydrocarbons depends upon a variety of factors, and some information about the chemical features that promote their activity. An important characteristic of *α*-curcumene is its hydrophobicity, which can enable it to distort the lipids of the microbial cell membrane, disrupting the cell structures and rendering them more permeable **(Sikkema *et al.*, 1992)**. An extensive leakage from microbial cells or the exit of critical molecules and ions can lead to death **(Prabuseenivasan *et al.*, 2006)**. Synergism between essential oil components has been observed in the studies aiming to elucidate their mechanism of the antibiotic action **(Burt, 2004)**.
The results of synergy between antibiotic imipenem and *α*-curcumene against *E. cloacae* are presented in Table 2. Other applied antibiotics did not act by the synergy with *α*-curcumene. Imipenem, a *β*-lactam, was the first carbapenem antibiotic selected for development. Its action mechanism is through inhibition bacterial cell wall synthesis by binding to and inactivating relevant transpeptidases, known as penicillin binding proteins (PBPs) **(Rodloff *et al.*, 2006)**. The *Henry – Michaelis* complex and acyl-enzyme adduct formed between imipenem and *E. cloacae* P99 was reported by the molecular-mechanics study. Part of the results showed that the carboxy group of the imipenem plays a prominent role in the binding of the substrate to the active site, and activates Ser^{64} through interaction with the phenolic OH group of Tyr^{150} **(Ferrer *et al.*, 2005)**.
Current paper can confirm that cyclic hydrocarbons, such as terpenes, interact with biological membranes through the acting of *α*-curcumene by the synergism with imipenem against *E. cloacae* (concentration 5 mM). These interactions lead to the changes in structure and function of the membranes which, in turn, may impair growth and activity of the cells **(Sikkema *et al.*, 1992)**. These findings suggest a general role for the use of *α*-curcumene as the enhancers of non-specific bacterial permeability to imipenem.

Table 1 MIC values of the *α*-curcumene against bacteria, yeasts and an algae.

Microorganisms	MIC (mg/mL)	MBC (mg/mL)
Gram-positive		
S. aureus	13	13
B. subtilis	13	13
Gram-negative		
E. coli	13	13
P. aeruginosa	13	13
Yeast	MIC (mg/mL)	MFC (mg/mL)
C. albicans	6.4	6.4
S. cerevisiae	0.8	0.8
C. glabrata	>6.4	ND
Alga		
P. zopfii	>6.4	ND

Legend: ND=Not Determined.

Table 2 Evaluation of the synergism of *α*-curcumene with antibiotics by the disk diffusion against *E. cloacae* (a clinical isolate).

Antibiotic	**Diameter of zone of inhibition (mm)**		
	Control	1 mM	5 mM
Imipenem	24[a]	24[a]	27[b]
Ciprofloxacin	R	R	R
Combination of amoxicillin and clavulanic acid	R	R	R
Ceftazidime	R	R	R

Legend: R=Resistant. Different letters superscripts are significantly different ($p < 0.05$).

CONCLUSION

In this study, a sesquiterpene, *α*-curcumene, was obtained from aerial parts of *Senecio selloi*. Following the antimicrobial evaluation, the best efficiency was found against yeasts, *S. cerevisiae* and *C. albicans*. The activity of *α*-curcumene against *in vitro* tested bacteria was observed only in the highest concentration. It can be concluded that fungal strains are more sensitive for *α*-curcumene than the bacterial ones. Since relatively high MIC values for bacteria species, the investigation of the *α*-curcumene synergism with antibiotics was inspected. Among the four antibiotics (or their combination) and five bacteria tested; only the synergism of *α*-curcumene with imipenem occurred against the clinical isolate of *E. cloacae*. Current study can contribute to information for the development of potent antimicrobial compounds derived from natural sources with antibiotic synergism.

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