Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: http://www.tandfonline.com/loi/ienz20

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To cite this article: Simone Carradori, Paola Chimenti, Marina Fazzari, Arianna Granese & Letizia Angiolella (2016): Antimicrobial activity, synergism and inhibition of germ tube formation by Crocus sativus-derived compounds against Candida spp., Journal of Enzyme Inhibition and Medicinal Chemistry, DOI: 10.1080/14756366.2016.1180596

To link to this article: http://dx.doi.org/10.1080/14756366.2016.1180596

Published online: 10 May 2016.

Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–5
 DOI: 10.1080/14756366.2016.1180596

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SHORT COMMUNICATION

Antimicrobial activity, synergism and inhibition of germ tube formation by *Crocus sativus*-derived compounds against *Candida* spp.

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Abstract

The limited arsenal of synthetic antifungal agents and the emergence of resistant *Candida* strains have prompted the researchers towards the investigation of naturally occurring compounds or their semisynthetic derivatives in order to propose new innovative hit compounds or new antifungal combinations endowed with reduced toxicity. We explored the anti-*Candida* effects, for the first time, of two bioactive compounds from *Crocus sativus* stigmas, namely crocin 1 and safranal, and some semisynthetic derivatives of safranal obtaining promising biological results in terms of minimum inhibitory concentration/minimum fungicidal concentration (MIC/MFC) values, synergism and reduction in the germ tube formation. Safranal and its thiosemicarbazone derivative **5** were shown to display good activity against *Candida* spp.

Keywords

Candida spp., checkerboard method, *Crocus sativus* L., germ tube formation, safranal

History

Received 29 March 2016
Revised 15 April 2016
Accepted 17 April 2016
Published online 3 May 2016

Introduction

Candida albicans, a dimorphic opportunistic human pathogen, is the most prominent cause of oropharyngeal or invasive candidiasis in man[1]. In particular, oropharyngeal infections are very common in HIV-infected individuals and patients with AIDS, while deep-seated infections are frequent in neutropenic patients[2]. The incidence of candidiasis has dramatically increased in the last decades and bloodstream infections due to *Candida* spp. are becoming a prime cause of morbidity and mortality in different types of immunocompromised patients[3]. Among the numerous factors associated with virulence in *C. albicans*, hyphal morphogenesis is likely to be one of the most important[4]. Hyphae development from yeast cells is critical for adherence, an essential first step in microbial colonization, which is in turn a key event in the initiation of the pathogenic process[5]. Different classes of antimycotic drugs are available to treat fungal infections. Azoles remain among the most common antifungal drugs, but their intensive clinical use for both therapy and prophylaxis has favoured the emergence of resistant strains[6]. The echinocandins were cytolytic drugs which inhibit cell wall synthesis through the blockage of 1,3-β-glucan synthase and have rapidly become an important therapeutic option in several fungal infections[7]. Although echinocandin resistance is still considered unusual, cases of resistance by using this therapeutic class have become increasingly frequent[8]. Echinocandins display predominantly fungicidal activity against *Candida* spp.[9]. The main resistance mechanism described for echinocandins involves the occurrence of mutations in the FKS1 gene, resulting in conformational changes in the enzyme encoded by this gene (Fks1), decreased affinity between echinocandins and Fks1, and the consequent inefficacy of these compounds[10].

The threat of increasing antifungal resistance (ADR) associated with the relative scarcity of antifungal drugs prompted the development of new compounds. To resolve this emergence, alternatives to conventional antimicrobial therapy and synergism among different classes of antifungal agents have been explored by our research group[11–17]. The concept of antimicrobial synergy is based on the principle that, in combination with other drugs, the formulation may enhance efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose and reduce the development of antimicrobial resistance[18]. Synergism between antimicrobial drugs and compounds of natural origin[19] was generally performed in many studies, but only few reported it among substances of natural origin[20].

Moreover, we also focused our efforts on the biological characterization of bioactive compounds from the dried stigma of *Crocus sativus* L., keeping in mind the importance of screening naturally occurring products for the treatment of fungal infections[21]. This expensive spice (saffron), belonging to the Iridaceae family, has been used as a drug in folk medicine since ancient times for various pharmacological purposes and as colouring agent[22]. The presence of carbohydrates, minerals, mucilages, vitamins and pigments (including anthocyanins, carotenoides, lycopene, zigzantin and flavonoids) has been reported in saffron stigmas, among which, crocins, picrocrocin, crocetin and safranal are regarded as the main active ingredients[23]. In this study, for the first time, we aimed at analysing the antifungal activity of some important bioactive components of saffron, namely crocin 1 (**1**) and safranal (**2**), together with few semisynthetic derivatives (**3–9**) of safranal in order to explore the chemical space around the

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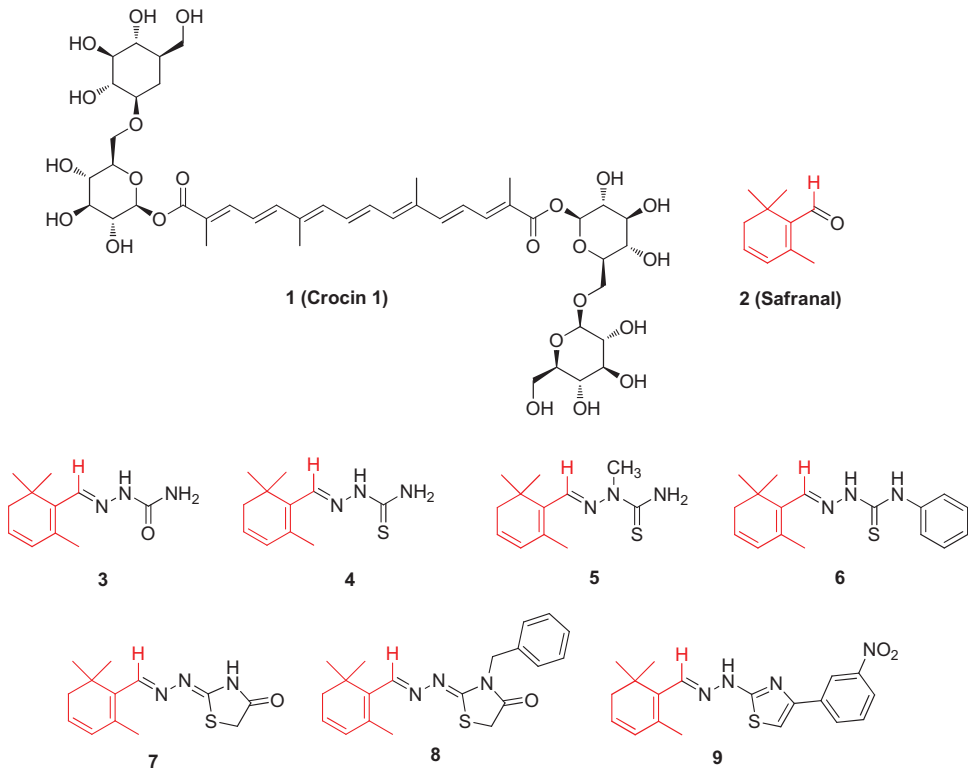

Figure 1. Structures of crocin 1 (**1**), safranal (**2**) and semisynthetic derivatives (**3–9**) of safranal.

carbonylic group of this molecule and to derive structure–activity relationship (SAR) within this small library (Figure 1).

Materials and methods

Chemistry

Commercial samples of crocin 1 (crocetin digentiobiose ester) and safranal (88%) were purchased from Sigma-Aldrich (Milan, Italy). Safranal was further purified by column chromatography on silica gel (230–400 mesh, G60 Merck, ethyl acetate:*n*-hexane 1:3). The other compounds (**3–9**) have been synthesized and characterized to ensure purity as reported in the literature[24,25].

Organisms and growth conditions

The strains used in this study were as follows: 11 strains of *Candida albicans*, seven of *Candida glabrata*, two of *Candida krusei* and two of *Candida tropicalis*. Many of these clinical isolates showed high resistance towards fluconazole. Fungi were grown in Sabouraud dextrose agar (SDA) at an optimal growth temperature of 30–37 °C with aeration for 24 h.

Antimicrobial assay: MIC and MFC

The minimum inhibitory concentration (MIC) was determined by microbroth dilution method (microsterile plate) according to the National Committee for Clinical Laboratory Standards (NCCLS) Approved Standard M27-A3, 2008[26]. The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of compounds at which no microbial growth was observed. Compounds **1–9** were prepared by dissolution in DMSO. 8 mg/mL solutions of each compound were prepared in RPMI 1640. Briefly, to determine the MIC of test compounds, RPMI-1640 supplemented with MOPS at pH 7 was used. Test compounds were diluted in RPMI-1640 supplemented with Tween 80 (final concentration of 0.001% *v*/*v*). The dilutions of compounds, ranging from 0.0078 to 8 mg/mL, were prepared in 96-well plates. The inoculum size was about 2.5×10^3 cells/mL. The plates were incubated at 30 °C for 48 h. To determine the minimum fungicidal concentration (MFC), Sabouraud dextrose agar (SDA) plates were seeded with 10 µL cell suspensions taken from the wells of the MIC assay plates where cell growth was not observed. These plates were incubated at 30 °C for 48 h and colony-forming units (CFU) growth was evaluated.

Checkerboard method used to evaluate the synergic action of compounds

Twelve serial twofold dilutions of **2** and **5** were prepared following the same broth dilution method adopted to assess MICs. A dilution of **2** and **5** was prepared ranging from 0.0078 to 8 mg/mL. All **2** and **5** dilutions were mixed with the appropriate concentration of drug (micafungin, MCFG) or the other compound, thus obtaining a series of different solutions. The analysis of the combination of the substances was carried out calculating the fractional inhibitory concentration (FIC) index. The FIC index (FICI) was calculated dividing the MIC of the combination of compound and the antifungal reference drug by the MIC of compound or antifungal reference drug alone:

FIC of compound = MIC of compound in combination with antifungal drugs/MIC of compound alone;

FIC of antifungal drug = MIC of antifungal in combination with compound/MIC of antifungal drug;

FICI = FIC of compound + FIC of antifungal drug.

The FICI, obtained by adding both FICs, was interpreted as indicating a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was >0.5 and ≤ 2, and as antagonistic when it was >2[27,28].

Germ tube formation

The induction of germ tube formation was conducted by a preculture in 50 mL of Winge broth at 28 °C for 24 h[29]. Subsequently, the cells were recovered and resuspended in 5 mL

of RPMI supplemented with serum (10% v/v) at O.D. 0.35 nm in the absence or in the presence of **2** or **5** at concentrations from 0.0039 mg/mL to 1 mg/mL and incubated with agitation to 37 °C. After 0, 90 min, 240 min and 24 h, an aliquot of cells was taken and counted with a phase contrast microscope using a 40x objective. The inhibition rate of hyphae formation was obtained by the ratio between the number of germ tubes observed in presence of compound **2** or **5** and with those found in the control without **2** or **5**. The number of germ tubes was evaluated using a Thoma camera.

Results and discussion

Antimicrobial activity of *Crocus sativus*-derived compounds against *Candida* spp.

Antimicrobial activity of nine (natural and semisynthetic) compounds: compounds **1**–**9** against a sensitive strain CO23 of *Candida albicans* were tested. Table 1 shows that only safranal (**2**) and **5** (thiosemicarbazone derivative of safranal) were active against *C. albicans* with MIC values of 1 mg/mL, whereas other compounds (crocin 1, **3**, **4**, **6**, **7** and **8**) showed MIC values >8 mg/mL. Similar values to MICs for fungicidal activity (MFC) were reported. To confirm the obtained antifungal activity, safranal (**2**) and **5** were also tested against 22 strains of *Candida* spp. as reported in Table 2. Safranal was more active than its thiosemicarbazone derivative **5** with MIC_{50}, MIC_{90}, and MFC values between 1 and 2 mg/mL for all *Candida* spp. In particular, *C. krusei* was more sensitive to safranal with MIC_s and MFC values of 1 mg/mL. Conversely, compound **5** was less active with values between 1 and 8 mg/mL. In many cases, compound **5** was not fungicidal.

At present, the antifungal activity of saffron on *C. albicans* was only reported by C.-J. Zheng et al.[30], who studied the inhibitory activity of ethanol extracts constituted with many compounds and the values of MICs were lower when compared with ours. Conversely, in this study, for the first time, the antimicrobial activity has been evaluated with two bioactive single compounds, so the values of MIC are higher, confirming a synergistic effect among *Crocus sativus* constituents.

Table 1. Antimicrobial activity of test compounds against *Candida albicans*.

Compound	MIC (mg/mL)	MFC (mg/mL)
Crocin 1 (**1**)	>8	>8
Safranal (**2**)	1	4
3	>8	>8
4	>8	>8
5	1	1
6	>8	>8
7	>8	>8
8	>8	>8
9	>8	>8

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration.

The fractional inhibitory concentration index (FICI)

To explore the possibility of developing a more powerful combination therapy of safranal and its derivative **5** together or with micafungin (MCFG), the checkerboard microtitre test was performed. Table 3 shows the results obtained in terms of the minimal inhibitory concentration (MIC). By treatment with safranal or **5**, inhibition of the growth of *C. albicans* was achieved at 1 and 2 mg/mL, respectively. In comparison, the MIC of MCFG was 4 μg/mL.

The FICIs calculated from the results of the checkerboard microtiter assays (Table 3) revealed the following: treating *C. albicans* in combination with safranal and its derivative **5** caused a significant decrease in the MIC, compared to their individual MIC values. For example, the MIC of safranal alone against *C. albicans* was lowered from 2 to 0.25 (mg/mL) in the presence of **5**. The MIC of **5** alone also decreased from 4 to 1 (mg/mL). Synergistic effects were obtained only using various combinations of safranal and **5**. Nevertheless, the results indicated that only an additive effect was obtained with the combination of safranal or **5** with MCFG. These results are in agreement with other authors that reported the synergistic effect of three essential oils against *Pseudomonas syringae* pv. *actinidiae*[20].

Inhibition of germ tube formation in presence of safranal or compound 5 in *C. albicans*

Hyphal growth is an intriguing morphological feature of *Candida albicans* and represents an important virulence factor that contributes to the forming biofilm. The inhibition of germ tube formation against six strains of *C. albicans* in the presence of different safranal or **5** concentrations was evaluated at several

Table 3. Fractional inhibitory concentrations (FICs) and indices (FICIs) of antifungal drug Micafungin (MCFG) combined with safranal and its thiosemicarbazone derivative **5** against *Candida albicans*.

	MIC_a	MIC_c	FIC	FICI
Safranal-Micafungin				
Safranal (mg/mL)	1	1	1	1.25
Micafungin (μg/mL)	4	1	0.250	
Compound 5-Micafungin				
Compound **5** (mg/mL)	2	2	1	1.50
Micafungin (μg/mL)	4	2	0.5	
Safranal–Compound 5				
Safranal (mg/mL)	2	0.25	0.125	0.375
Compound **5** (mg/mL)	4	1	0.250	

MIC_a: MIC of the sample alone; MIC_c: MIC of the sample of the most effective combination. FIC of compound = MIC of compound in combination with antifungal drugs/MIC of compound alone. FIC of antifungal drug = MIC of antifungal in combination with compound/MIC of antifungal drug. FICI = FIC compound + FIC of antifungal drug.

Table 2. Antifungal activity (mg/mL) of safranal (**2**) and its derivative **5** against twenty-two clinical isolates of *Candida* spp.

	Safranal (**2**)				**5**			
Candida spp. (n° strains)	MIC_{50}	MIC_{90}	MFC_{50}	MFC_{90}	MIC_{50}	MIC_{90}	MFC_{50}	MFC_{90}
C. albicans (11)	2	2	2	2	1	4	1	>4
C. glabrata (7)	1	2	1	2	4	4	>8	>8
C. krusei (2)	1	1	1	1	1	1	8	8
C. tropicalis (2)	1	1	1	2	2	2	>8	>8

MFC_{50} and MFC_{90}: minimum fungicidal concentration of 50 and 90% of the strains.

Table 4. Percentage of inhibition of germ tube formation in presence of safranal and compound **5** in *C. albicans*.

	90 min		4 h		24 h	
mg/mL	Safranal	**5**	Safranal	**5**	Safranal	**5**
0	0	0	0	0	0	0
1.00	100	100	100	100	100	100
0.500	100	100	100	100	100	100
0.250	100	100	100	100	100	100
0.125	100	100	100	100	100	100
0.0625	100	60.50 ± 26.16	100	25.40 ± 25.31	100	10.70 ± 3.88
0.0312	58.50 ± 14.8	44.0 ± 15.55	46.75 ± 3.88	15.05 ± 18.31	41.52 ± 15.60	19.0 ± 9.90
0.0156	48.50 ± 0.70	35.00 ± 9.90	42.30 ± 17.40	11.60 ± 10.46	27.50 ± 15.60	23.0 ± 21.90
0.0078	5.0 ± 7.07	21.50 ± 19.10	5.0 ± 7.07	5.0 ± 7.07	15.68 ± 3.21	17.50 ± 24.74
0.0039	0	5.0 ± 7.07	0	5.5 ± 7.70	0	0

± Standard deviation.

time intervals. As the production of germ tubes usually requires 3 h of incubation, it would not be correct to assume that the inhibition of germ tube formation occurred at 90 min, but the experiment clearly demonstrated that this event is inhibited at an early stage of transition at least 90 min after the onset of incubation. Nevertheless, it has been confirmed the inhibition until 24 h. It is noteworthy that germ tube inhibition occurred at the subinhibitory concentration as reported in Table 4. In particular, after 24 h of induction, safranal (**2**) was found to inhibit hyphae formation at a rate about 41.52 ± 15.60% at a concentration of 0.0312 mg/mL and completely at a concentration of 0.0625 mg/mL equal to 1/32 MIC. At the same concentrations of safranal (**2**), derivative **5** was found to inhibit hyphae formation about 19.0 ± 9.9 and 10.7 ± 3.88, respectively. The total inhibition was obtained at concentration of 0.125 mg/mL also in this case, corresponding to 1/32 MIC. Values of SD reported in Table 4 were sometimes higher than value of means, but this discrepancy strongly depends on hyphae that make heaps and can be counted difficultly.

The literature includes many studies assessing the susceptibility of yeasts of the genus *Candida* to medicinal plant derivatives; however, few studies have investigated the plant derivatives' effects on *C. albicans* morphology and germ tube formation[31]. According to Silva et al.[32], a compound's ability to inhibit germ tube formation could be a means to assess its antifungal activity. The ability to reduce yeast viability is a desired quality in candidate products and/or compounds that could be developed into novel therapeutic agents.

SAR studies on naturally occurring and semisynthetic derivatives as antifungal agents

Collectively, crocin 1 was not active as antifungal agent (MIC >8 mg/mL), whereas the most important component of the volatile fraction of saffron (safranal) displayed a promising inhibitory activity against the selected *Candida albicans* strain. Among its semisynthetic derivatives, obtained by transformation of the carbonylic function into semicarbazone (**3**) and (substituted) thiosemicarbazone derivatives (**4**–**6**), only the thiosemicarbazone **5** was endowed with the same MIC value and a lower MFC value. The successive cyclization of the thioamidic portion of compound **5** led to inactive derivatives (thiazolidinones (**7** and **8**) and thiazole (**9**)), suggesting a pivotal role for this moiety. This is confirmed by several studies in the literature showing that thiosemicarbazone derivatives usually act as potent anti-*Candida* agents[11–17]. In detail, researchers focused their attention on the effect of these compounds as potent iron chelators and as selective inhibitors of dihydrofolate reductase (DHFR) of *Candida albicans*[36,37]. For these reasons, compound **5** was further explored along with its parent compound safranal against twenty-two clinical isolates of *Candida* spp. and for the determination of their FIC index and inhibition of germ tube formation.

Conclusion

The urgency of new compounds with anti-*Candida* activity has increased due to the therapeutic failure, fast development of resistant strains and low bioavailability of current drugs. Moreover, the correct evaluation of the efficacy of a new antifungal compound requires several *in vitro* assays such as MIC/MFC determination, combination therapy and inhibition of Germ Tube formation. We performed, for the first time, this large biological screening for two bioactive compounds of *Crocus sativus* L. (crocin 1 and safranal) and modified the chemical structure of safranal to obtain some semisynthetic derivatives. In conclusion, the obtained results show an interesting antifungal activity of safranal against *Candida* spp. and a synergistic effect when used in combination with derivative **5**. Finally, these two bioactive compounds were able to inhibit completely germ tube formation, an important virulence factor in *C. albicans*, at concentration of 1/32 MIC. Some chemical modifications yielded more potent and promising compounds, which could be used as lead compounds for the search of innovative anti-*Candida* agents.

Declaration of interest

This work was supported by local grants from ''G. D'Annunzio'' University of Chieti to Dr. Simone Carradori. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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