

Antimicrobial, Anti-Inflammatory and Antioxidant Activities of Polyoxygenated Chalcones

Yesseny A. Vásquez-Martínez,^a Mauricio E. Osorio,^{ib}*^{*,b} Diego A. San Martín,^b Marcela A. Carvajal,^c Alejandra P. Vergara,^c Elizabeth Sanchez,^c Marcela Raimondi,^d Susana A. Zacchino,^d Carolina Mascayano,^e Claudia Torrent,^e Francisco Cabezas,^e Sophia Mejias,^f Margarita Montoya^f and Marcelo Cortez-San Martín^f

^aPrograma Centro de Investigaciones Biomédicas Aplicadas, Facultad de Ciencias Médicas, Universidad de Santiago de Chile, 9170022 Santiago, Chile

^bLaboratorio de Productos Naturales, Departamento de Química, Universidad Técnica Federico Santa María, 2390123 Valparaíso, Chile

^cCentro de Biotecnología CB-DAL, Universidad Técnica Federico Santa María, 2390136 Valparaíso, Chile

^dFarmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina

^eDepartamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, 9170022 Santiago, Chile

^fDepartamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, 9170022 Santiago, Chile

It was synthesized nine polyoxygenated chalcones with a potential and safe use as antioxidant, antimicrobial and anti-inflammatory therapies. Chalcones obtained by Claisen-Schmidt condensation were studied as antioxidant, inhibitors of human 5-lipoxygenase, antifungal, antibacterial and antibiotic resistance modifiers. Two chalcones with catecholic moieties were able to strongly decrease the minimum inhibitory concentration (MIC) of methicillin against methicillin-resistant *Staphylococcus aureus*, increase the antiradical activity and significantly inhibit the human 5-lipoxygenase. Only one of these chalcones was active synergistically with methicillin. Chalcones with methoxyl substituents at different positions displayed the best activities against *Cryptococcus neoformans*. Only one chalcone showed good activity against the plant pathogenic bacteria *Pseudomonas syringae* whose half maximal inhibitory concentration (IC₅₀) value (2.5 µg mL⁻¹) was similar to that observed with the antibiotic streptomycin (2.9 µg mL⁻¹). These simple chalcones have safe potential uses in antioxidant, antimicrobial and anti-inflammatory therapies.

Keywords: antioxidant activity, polyoxygenated chalcones, human 5-lipoxygenase inhibitors, antibacterial, antifungal

Introduction

Chalcones (1,3-diphenyl-prop-2-en-1-ones) essentially differ from other flavonoids by its open C-ring, thus possessing a linear chain of three carbon atoms binding A with B ring (Figure 1).¹ Chalcones have been used as intermediates for the preparation of compounds with therapeutic value. A number of synthetic routes have been reported for the synthesis of chalcones, the Claisen-Schmidt

condensation under homogeneous conditions in the presence of acid or base being the most used. Traditionally, strong alkaline media²⁻⁸ and the use of several Lewis acids⁹⁻¹⁵ have been employed for their synthesis. Some improved strategies include solvent-free conditions,¹⁶ microwave¹⁷ and ultrasound irradiation¹⁸ and the grinding technique.¹⁹ The synthesis of chalcones in acid condition using SOCl₂ in ethanol to generate HCl *in situ* have been used to prepare chalcones with one free hydroxyl group,²⁰ however, the synthesis of chalcones with additional oxygenated groups have not been proven using this method. Chalcones display

*e-mail: mauricio.osorio@usm.cl, osomauricio@gmail.com

a large number of different biological activities, such as antibacterial, antifungal, anti-cancer, anti-inflammatory, analgesic, antiviral, anti-malarial, antipyretic and cytotoxic activities.^{20,21}

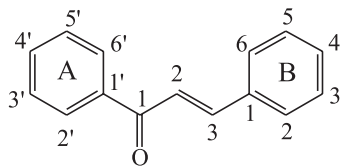


Figure 1. Representative structure of the chalcone backbone.

Multi-drug resistance is one of the major causes of the alarming level of infectious diseases worldwide. The discovery of new drugs with potent antimicrobial activity, particularly against the resistant strains, is, therefore, highly needed. In addition, since many of the currently available antifungal drugs have undesirable side effects, are ineffective against new or re-emerging fungi, or develop a rapid resistance in the pathogen,²² there is an urgent need of a next generation of new antifungal agents that can overcome the above disadvantages. Several reports highlighting the significance of chalcones as antimicrobial and antiparasitic agents have been documented in the literature.²¹ Chalcone derivatives with $-OH$ and $-OCH_3$ in the rings A and B have demonstrated potential anti-leishmanial and anti-trypanosomal activities by acting on a number of molecular targets.²³

Fungal plant pathogens such as *Botrytis cinerea* (*B. cinerea*),²⁴ *Gibberella fujikuroi* (*G. fujikuroi*)²⁵ and *Phytophthora cinnamomi* (*P. cinnamomi*)^{26,27} attack a wide range of agriculturally and ornamentally important plants²⁸ and significantly affect the economy of developing countries causing large economic losses in agriculture.²⁹ Numerous fungicides such as carboxin, captan, thiram, tebuconazole and metalaxyl have been used to control fungal phytopathogens, but all of them possess secondary effects in other non-target microorganisms and living beings.³⁰ Natural and synthetic chalcones with $-OH$ and $-OCH_3$ groups linked to different positions of the A and B rings have shown bactericidal, antifungal, anthelmintic, insecticidal, insect antifeedant, antiviral and phytotoxic activities.³¹

Plant pathogenic bacteria cause serious problems for industrial agriculture because they affect crops of nutritional and commercial interest. *Agrobacterium tumefaciens* (*A. tumefaciens*), *Pseudomonas syringae* (*P. syringae*) and *Erwinia carotovora* (*E. carotovora*) are Gram-negative bacteria that are responsible for a number of economically important diseases. These bacteria usually infect a wide variety of fruits, vegetables, and ornamental plants,³²⁻³⁴

and different types of compounds have been used for their control. However, these control efforts have not always been successful and less toxic new products to replace the existing ones are highly needed. To achieve this goal, the worldwide trend is to explore new compounds against these phytopathogens in order to minimize the risks associated with the development of pathogen populations insensitive to these chemical compounds.³⁵ Also, many countries have limited the use of some commercial antimicrobials based on recommendations from the Codex Alimentarius³⁶ according to the maximum residue limits (MRLs) for residues of pesticides or veterinary drugs in foods.

Endogenous free-radical species like reactive oxygen (ROS) and nitrogen species (RNS) and other reactive small molecules have emerged as important regulators of many physiological and pathological processes. ROS and RNS are essential to maintain homeostasis and health of human beings, but uncontrolled and excess ROS/RNS have been implicated in the pathogenesis of various diseases including cancer, cardiovascular and neurodegenerative diseases as well as ageing. The human organism has developed defense systems to neutralize excessive levels of ROS and RNS and compensate for the oxidative stress.³⁷ When endogenous antioxidant protection is not able to maintain the proper balance of free radicals due to their unlimited or uncontrolled production, several health problems appear. In such cases, additional external antioxidants are required to restore the proper balance between free radicals and antioxidants in the body.³⁸ Chalcone derivatives with an $-OH$ substituent on ring A, and $-SCH_3$ and $-OCH_3$ groups at the *para* position of ring B showed good anti-oxidant activity.²¹

Different natural or synthetic chalcones has been studied for their anticancer activities because they exert cytotoxicity through multiple mechanisms, which include cell cycle disruption as well as inhibition of angiogenesis, tubulin polymerization, apoptosis and cell signaling.³⁹ On the other hand, high expression of 5-lipoxygenase (5-LOX) has been described in cancer cells and its inhibition has been widely characterized to control tumor cell growth.^{40,41} Moreover, ROS also have to be closely linked with tumor initiation, development and progression, by promoting cell survival, cell proliferation and migration.⁴² Thus, inhibition of 5-LOX or the decrease in ROS levels can also have impact on cancer cell survival and/or proliferation. 5-LOX belongs to a group of closely related non-heme iron containing dioxygenases. These enzymes catalyze the addition of molecular oxygen into poly-unsaturated fatty acids (PUFAs) containing *cis*, *cis*-1,4-pentadiene structures to give their hydroperoxy derivatives. LOXs are further classified into 5-, 8-, 9-, 11-, 12-, and 15-LOXs

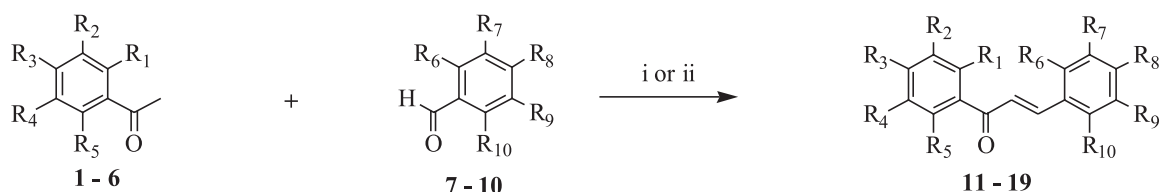
according to the positional specificity of arachidonate oxygenation. In addition, the 5-LOX is the source of potent pro-inflammatory mediators present in a variety of inflammatory and allergic diseases.⁴³ As 5-LOX have been shown to have a major role in the pathogenesis of various inflammatory disorders, including cancer, inhibitors of 5-LOX will have a wide range of therapeutic applications.⁴⁴ Di-*O*-prenylated chalcone derivatives have shown to possess good inhibitory potency of 5-LOX (half maximal inhibitory concentration (IC₅₀) = 4 μM), potent anti-proliferative effects (50% growth inhibition (GI₅₀) = 9 μM) on the MCF-7 breast cancer cell line, and no appreciable effects up to 100 μM on the immortalized nontumorigenic human epidermal (HaCaT) cell line.⁴³

In this paper, we report a facile synthesis of nine chalcones to evaluate their effects against phytopathogens and human pathogenic bacteria and fungi as possible alternatives to antimicrobials currently in commercial use. Additionally, as endogenous free radicals and human 5-lipoxygenase (5-hLOX) have been shown to have major roles in the pathogenesis of various health problems including cancer, cardiovascular and neurodegenerative diseases and inflammatory disorders, the chalcones were tested as 5-hLOX inhibitors and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavengers.

Results and Discussion

Synthesis

Chalcones **11-19** were obtained in poor to good yields (11-90%) via Claisen-Schmidt condensation of acetophenones **1-6** with benzaldehydes **7-10** in acidic or alkaline media (Scheme 1). Acidic medium was mostly



1 R₁=H, R₂=R₃=R₄=OCH₃, R₅=H

2 R₁=R₃=R₄=H, R₂=R₅=OCH₃

3 R₂=R₄=R₅=H, R₁=R₃=OCH₃

4 R₁=R₂=R₅=H, R₃=R₄=OCH₃

5 R₁=R₂=R₃=R₄=R₅=H

6 R₂=R₄=R₅=H, R₁=R₃=OH

7 R₆=R₇=R₁₀=H, R₈=R₉=OH

8 R₆=R₇=R₁₀=H, R₈=OH, R₉=OCH₃

9 R₆=R₇=R₈=R₉=R₁₀=H

10 R₆=R₁₀=H, R₇=R₈=R₉=OCH₃

11 R₁=R₅=R₆=R₇=R₁₀=H, R₂=R₃=R₄=OCH₃, R₈=R₉=OH (i; 44%)

12 R₁=R₃=R₄=R₆=R₇=R₁₀=H, R₂=R₅=R₉=OCH₃, R₈=OH (ii; 75%)

13 R₁=R₅=R₆=R₇=R₁₀=H, R₂=R₃=R₄=R₉=OCH₃, R₈=OH (i; 77%)

14 R₂=R₄=R₅=R₆=R₇=R₁₀=H, R₁=R₃=R₉=OCH₃, R₈=OH (i; 70%)

15 R₁=R₃=R₄=R₆=R₇=R₈=R₉=R₁₀=H, R₂=R₅=OCH₃ (ii; 45%)

16 R₁=R₂=R₅=R₆=R₇=R₁₀=H, R₃=R₄=R₉=OCH₃, R₈=OH (i; 90%)

17 R₁=R₂=R₃=R₄=R₅=R₆=R₇=R₁₀=H, R₈=OH, R₉=OCH₃ (i; 48%)

18 R₁=R₂=R₃=R₄=R₅=R₆=R₇=R₁₀=H, R₈=R₉=OH (i; 57%)

19 R₂=R₄=R₅=R₆=R₁₀=H, R₁=R₃=OH, R₇=R₈=R₉=OCH₃ (ii; 11%)

Scheme 1. Preparation of chalcones **11-19** via Claisen-Schmidt condensation in acidic (i: SOCl₂/EtOH) and alkaline (ii: KOH/MeOH/H₂O) media. Methodologies and yields of reaction are shown in brackets.

preferred when one or more free hydroxyl groups are bound to the aromatic system of the chalcones. When Claisen-Schmidt condensations using other 2'-hydroxy or 2'-methoxy acetophenones were conducted in acidic conditions, chalcones were not produced (**12** and **19**). This was probably due to either the hydrogen-bond formed by the OH or OMe groups on C-2' with the carbonyl group (that could hinder the formation of the enol intermediate) or by problems of steric hindrance with the 2'-methoxy group.

The yields of all the synthesized chalcones were similar or better than those reported in the literature and all were obtained in only one step. Chalcone **12** is the only new molecule in the series. The solid-state reactions (without solvent) were not attempted.

The synthesis of chalcones with a hydroxyl group in 4-position was incompatible with alkaline medium, except for **12**. Chalcones with 2'-OH group can be synthesized in alkaline medium, but with low yield (11% for **19**). Protecting groups resistant to alkaline medium (i.e., methoxymethyl (MOM), tetrahydropyranyl (THP)) should be considered to obtain chalcones in good yields with two or more free hydroxyl groups. Chalcones with 3,4-diOH substituents (catechol group) on the B ring can be synthesized in acid condition with moderate yields (47% for **11** and 57% for **18**).

In vitro antibacterial effects of chalcones **11-19** on bacteria pathogenic to humans

Determination of the minimum inhibitory concentration (MIC) of the chalcones against bacteria

The result of the bacterial growth inhibition test (Table 1) showed that chalcones **11** and **18** were active

Table 1. Results of the *in vitro* antibacterial assay

Compound	Structure	Minimum inhibitory concentration (MIC) / ($\mu\text{g mL}^{-1}$)			
		<i>E. coli</i> ATCC25922	<i>E. coli</i> 33.1	<i>S. aureus</i> NTCC8325-4	<i>S. aureus</i> MRSA 97-7
11		> 100	> 100	70	100
12		> 100	> 100	> 100	> 100
13		> 100	> 100	> 100	> 100
14		> 100	> 100	> 100	> 100
15		> 100	> 100	> 100	> 100
16		> 100	> 100	> 100	> 100
17		> 100	> 100	> 100	> 100
18		> 100	> 100	50	50
19		> 100	> 100	> 100	> 100
Methicillin		–	–	5	50
Ciprofloxacin		1	50	10	80
Ampicillin		60	> 100	–	–

Antibacterial activity of chalcones in reference strain *E. coli* ATCC25922, multi-resistant *E. coli* 33.1, reference strain *S. aureus* NTCC8325-4 and methicillin-resistant *S. aureus* MRSA 97-7 isolates.

against sensitive *S. aureus* strains with MICs of 50 and 70 $\mu\text{g mL}^{-1}$, respectively. These compounds also inhibited the growth of MRSA 97-7 strain (MICs of 50 and 90 $\mu\text{g mL}^{-1}$, respectively).

When comparing the structural characteristics of **11** and **18**, which are weakly active against *S. aureus* with the rest

of the chalcones, it can be observed that both compounds possess a catechol moiety in their structures, which could play a key role in the activity. Then, our results indicated that the presence of catechol group at positions 3,4 on the B ring is necessary to exhibit the anti-staphylococcal activity, decreasing this activity when the hydroxyl at position 3

is methylated (chalcones **11**, **13**, **14**, **16** and **17**). These results are similar to those obtained by Batovska *et al.*,⁴⁵ who reported that the presence of hydroxyl group on ring B alone is not enough for the chalcones to exhibit anti-staphylococcal activity and the introduction of a methoxy group next to *o*- and *p*-hydroxyl groups led to more inactive chalcones, while the activity was kept when the methoxy group was inserted next to the *m*-hydroxyl group. They also showed that the lipophilicity of ring A is important for the antibacterial activity against *S. aureus*.

Chalcones with hydroxyl and methylthio groups on B ring at position 4 are being considered for a next work.

Evaluation of the ability of chalcones **11** and **18** to potentiate the antibiotic activity of methicillin

The detection of what are known as resistance-modifying agents (RMAs) represents an attractive strategy to mitigate the spread of bacterial drug resistance, since it could facilitate the recycling of well-established antibiotics.⁴⁶ Drug repositioning can accelerate the drug development process as the already established compounds are characterized by known safety profiles, pharmacology and administration routes.^{47,48}

Chalcones **11** and **18** showed an important capacity to reduce the MIC of methicillin against MRSA 97-7, re-sensitizing these resistant bacteria 2,500 times methicillin and 2.5 times the synthetic chalcone **18**. Compound **18** was found to act synergistically in conjunction with the commercial antibiotic (Table 2), while compound **11** showed only partial synergy, although the dose was reduced by the same amount as **18**.

These findings add new data to the previously reported⁴⁹ enhancing capacity of chalcones to antibiotics activity showing that five chalcones possessing either 2-OH or 4-OH on the B ring enhanced the antibacterial activity of the non β -lactam antibiotics doxycycline, ciprofloxacin and/or gentamicin. In the present paper, the 3,4-diOH chalcones either unsubstituted (**18**) or 3',4',5'-trimethoxylated (**11**) on the A ring, showed potentiated activity of the β -lactam antibiotic methicillin.

In vitro antifungal effects on fungi pathogenic to humans

In the last decades, fungi have emerged as major causes of human morbidity and mortality, mainly among the immunocompromised and seriously-ill hospitalized patients.⁵⁰ Most of these mycoses-related deaths were associated with *Candida albicans* (*C. albicans*) and *Cryptococcus neoformans* (*C. neoformans*) and, although there are several antifungal drugs in clinical use to treat both opportunistic fungal species, fungal infections remain very difficult to eradicate.

Since chalcones with different substituents than the ones synthesized here have shown antifungal activities in several previous papers,⁵¹⁻⁵⁸ the antifungal properties of these compounds could add new interesting data on the antifungal behavior of chalcones. Thus, the nine chalcones **11-19** were tested for antifungal properties against the clinically important yeasts *C. albicans* (ATCC 10231) and *C. neoformans* (ATCC 32264). To assess antifungal activities, the broth microdilution method M27-A3 for yeasts of the Clinical and Laboratory Standards Institute (CLSI)⁵⁹ was used.

The percentage of inhibition of each fungus was determined for each compound at two-fold dilutions in the range 250-3.9 $\mu\text{g mL}^{-1}$ (Table 3). In the right column, IC_{50} represents the minimum concentration that inhibits fungal growth by 50%. This value is accepted in the literature as representative of the *in vitro* activity of target compounds.⁶⁰

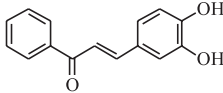
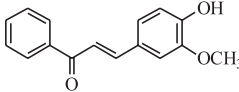
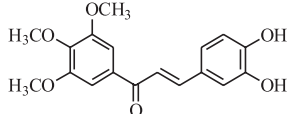
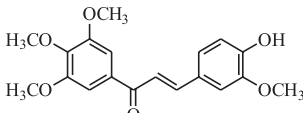
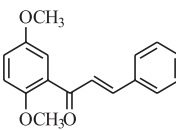
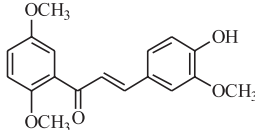
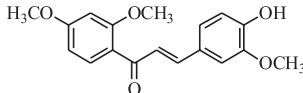
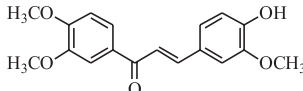
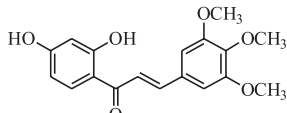
Although Table 3 shows that all compounds inhibit both *C. albicans* and *C. neoformans* at different tested concentrations, it is interesting to note that *C. albicans* is more sensitive than *C. neoformans*. In Table 3, it can be observed that IC_{50} of all compounds against *C. albicans* ranged from 125 to > 250 $\mu\text{g mL}^{-1}$, with five compounds (**11**, **12**, **14**, **16** and **19**) possessing an $\text{IC}_{50} \geq 250 \mu\text{g mL}^{-1}$, one (**13**) with $\text{IC}_{50} = 250 \mu\text{g mL}^{-1}$, two (**15** and **18**) displaying $\text{IC}_{50} = 125 \mu\text{g mL}^{-1}$ and one (**17**) with $\text{IC}_{50} = 50 \mu\text{g mL}^{-1}$. In the case of *C. neoformans* the IC_{50} ranged from 7.8 to > 250 $\mu\text{g mL}^{-1}$ with two compounds (**15** and **17**) displaying an IC_{50} between 7.8-15.6 $\mu\text{g mL}^{-1}$,

Table 2. Synergistic effects of chalcones **11** and **18** with methicillin in MRSA 97-7

Strain	Drug	MIC alone / ($\mu\text{g mL}^{-1}$)	MIC combination / ($\mu\text{g mL}^{-1}$)	FICs of each compound	DRI	FICI	Outcome
MRSA 97-7	18	50	20	0.40	2.5	0.40	synergy
	methicillin	50	0.02	0.0004	2,500		
	11	100	50	0.5	2.0	0.50	partial synergy
	methicillin	50	0.02	0.0004	2,500		

MIC: minimum inhibitory concentration; FIC: fractional inhibitory concentration; DRI: dose reduction index = MIC of each compound alone/MIC in combination; FICI: FIC index.

Table 3. Percentages of inhibition of chalcones **11-19** against *C. albicans* ATCC 10231 (*Ca*) and *C. neoformans* ATCC 32264 (*Cn*) at different concentrations. The compounds were ordered by structural similarity in order to facilitate the structure-activity analysis and not in numerical order

Compound	Structure	Fungi	Concentration / ($\mu\text{g mL}^{-1}$)							IC ₅₀ ^a
			250	125	62.5	31.2	15.6	7.8	3.9	
18		<i>Ca</i>	66.3 ± 1.9	40.6 ± 0.0	24.4 ± 0.1	17.8 ± 0.2	11.1 ± 1.9	8.1 ± 0.7	3.6 ± 0.2	125
		<i>Cn</i>	24.4 ± 2.1	10.9 ± 2.0	0	0	0	0	0	0
17		<i>Ca</i>	100	100	100	45.9 ± 1.5	18.2 ± 0.6	8.8 ± 0.0	5.2 ± 0.5	50
		<i>Cn</i>	100	100	100	100	93.5 ± 0.1	15.4 ± 0.1	8.6 ± 0.4	15.6
11		<i>Ca</i>	25.0 ± 2.8	18.4 ± 1.6	12.6 ± 0.8	8.4 ± 0.4	5.1 ± 1.0	3.4 ± 0.2	2.3 ± 0.9	250
		<i>Cn</i>	45.9 ± 0.2	34.7 ± 2.7	9.1 ± 0.2	7.6 ± 0.5	3.3 ± 0.5	2.8 ± 0.1	1.7 ± 0.5	250
13		<i>Ca</i>	74.6 ± 3.3	27.51 ± 1.35	10.4 ± 1.0	6.7 ± 0.3	3.8 ± 0.7	2.4 ± 1.2	1.8 ± 0.1	250
		<i>Cn</i>	60.6 ± 1.6	46.6 ± 0.4	0	0	0	0	0	0
15		<i>Ca</i>	84.0 ± 2.7	54.5 ± 0.0	31.0 ± 2.3	17.0 ± 2.0	8.0 ± 0.2	3.7 ± 0.0	0	125
		<i>Cn</i>	100	95.6 ± 0.8	95.5 ± 2.9	86.3 ± 0.5	75.3 ± 1.5	61.5 ± 1.0	0	7.8
12		<i>Ca</i>	27.4 ± 8.3	15.1 ± 2.1	13.5 ± 0.3	9.4 ± 1.1	4.9 ± 0.9	3.4 ± 0.3	2.3 ± 0.3	> 250
		<i>Cn</i>	86.0 ± 0.0	80.9 ± 1.9	5.5 ± 0.6	0.00	0.00	0.00	0.00	0.00
14		<i>Ca</i>	42.3 ± 2.1	30.7 ± 1.9	19.6 ± 1.5	13.5 ± 1.2	8.5 ± 1.2	6.0 ± 0.9	3.6 ± 0.0	> 250
		<i>Cn</i>	85.6 ± 2.1	66.5 ± 1.3	27.4 ± 0.0	3.2 ± 0.1	0	0	0	0
16		<i>Ca</i>	27.1 ± 1.3	15.3 ± 0.9	12.1 ± 0.6	8.2 ± 0.7	6.2 ± 0.1	2.5 ± 0.8	0.2 ± 0.5	> 250
		<i>Cn</i>	100	85.8 ± 0.0	77.1 ± 1.3	45.5 ± 0.1	32.0 ± 0.5	19.9 ± 0.0	14.8 ± 0.2	31.25
19		<i>Ca</i>	18.4 ± 1.6	11.0 ± 0.8	5.9 ± 0.2	5.1 ± 0.2	2.6 ± 0.0	1.8 ± 0.3	1.6 ± 0.1	> 250
		<i>Cn</i>	23.7 ± 0.5	7.1 ± 3.7	0	0	0	0	0	0
Amphotericin B		<i>Ca</i>	100	100	100	100	100	100	100	1.5
		<i>Cn</i>	100	100	100	100	100	100	100	1.0

^aIC₅₀ value represents the concentration of each compound that inhibits fungal growth by 50%.

one compound (**16**) with an IC₅₀ = 31.25 $\mu\text{g mL}^{-1}$, three compounds with IC₅₀ \geq 125 $\mu\text{g mL}^{-1}$ (**12**, **13** and **14**) and only two compounds (**11** and **18**) with IC₅₀ > 250 $\mu\text{g mL}^{-1}$.

The comparative behavior of all compounds against *C. albicans* and *C. neoformans* can be clearly corroborated by comparing the dose-responses curves in Figure 2.

Against *C. albicans* (Figure 2a), four (**13**, **15**, **17** and **18**) out of the nine compounds produced an inhibition \geq 50% at 250 $\mu\text{g mL}^{-1}$, one (**15**) produces \geq 50% inhibition at

125 $\mu\text{g mL}^{-1}$ and one (**17**) at 50 $\mu\text{g mL}^{-1}$. While, against *C. neoformans*, six compounds (**12-17**) produced an inhibition \geq 50% at 250 $\mu\text{g mL}^{-1}$ and five of them (**12**, **14-17**) produced the same inhibition at 125 $\mu\text{g mL}^{-1}$ (Figure 2b).

To see the influence of the methylation of the 3-OH group (on the B ring) on the growth of *C. neoformans*, the anticryptococcal activity of **18** (with 3,4-diOH on the B ring) was compared with **17** (with 3-OMe, 4-OH on the B ring),

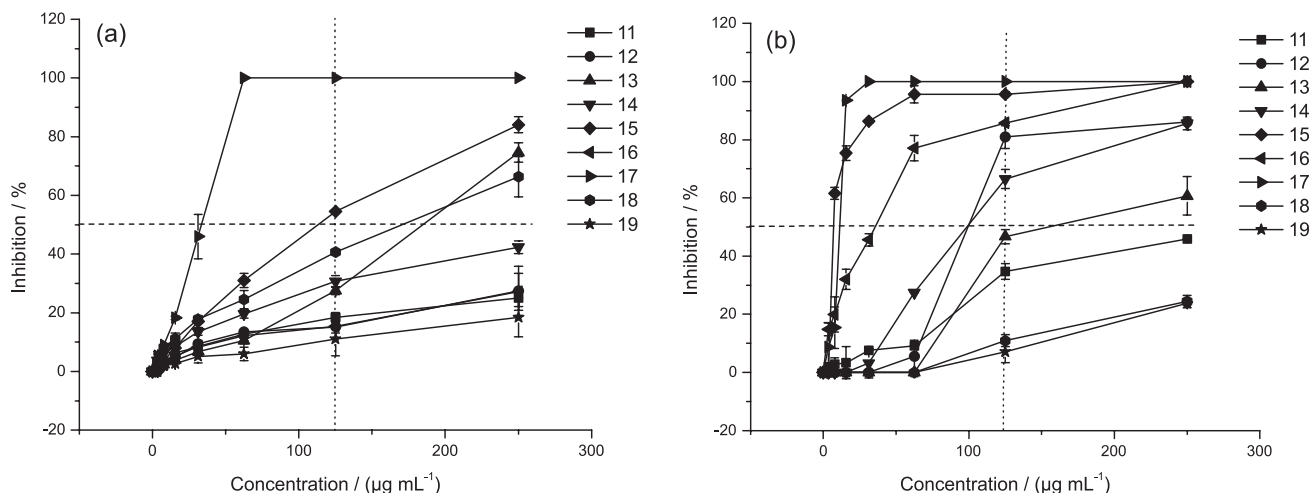


Figure 2. Comparative antifungal activities of chalcones **11-19** against (a) *C. albicans*; (b) *C. neoformans*. The horizontal dotted line was drawn to clearly see the number of compounds that displayed $\geq 50\%$ inhibition of fungal growth. The vertical dotted lines were drawn to clearly see the effects of the different compounds at $125 \mu\text{g mL}^{-1}$.

both with an un-substituted ring A (Figure 3a). With the same purpose, the anticryptococcal activity of **11** (with 3,4-diOH on the B ring) was compared with **13** (with 3-OMe, 4-OH on the B ring), both with three methoxyl groups on the A ring (Figure 3b). Results clearly show that the methylation of the 3-OH increased the activity in different degrees on the A ring unsubstituted and substituted chalcones. However, in the compounds with an unsubstituted A ring the difference in inhibition between **17** and **18** (Figure 3a) was much greater than that observed between **11** and **13** (Figure 3b), showing that the substitution of ring A could play an important role in the activity.

To see the influence of different substituents on ring A on the anticryptococcal activity, all compounds possessing 3-OMe, 4-OH substituents on the B ring (**12-14**, **16**, **17**) were compared in Figure 4.

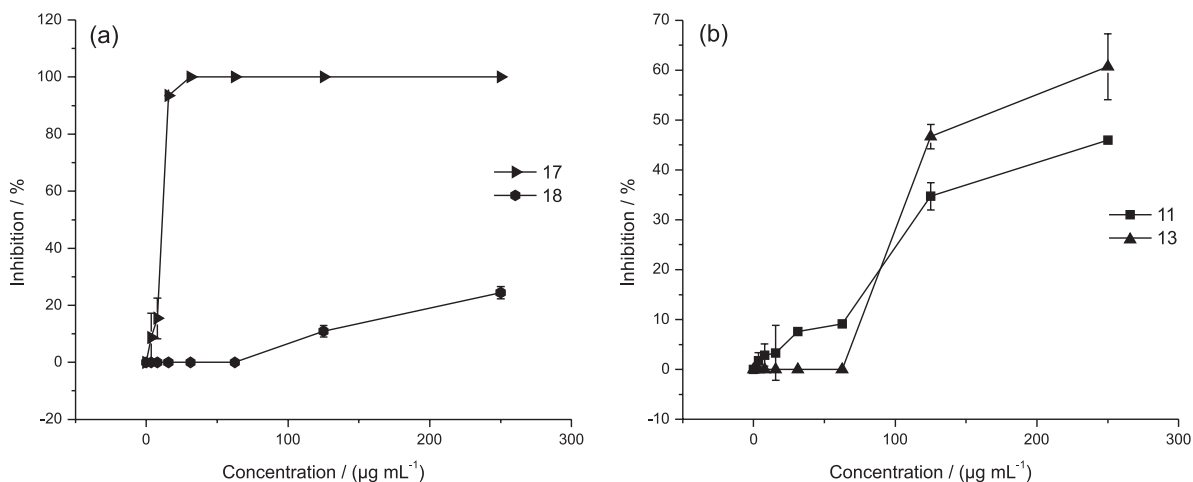


Figure 3. Influence of the methylation of the 3-OH group on ring B on growth of *C. neoformans*, when ring A is (a) unsubstituted (chalcones **17** and **18**) or (b) trimethoxysubstituted (chalcones **11** and **13**). Chalcones **18** and **13** possess 3,4-diOH as substituents on the B ring and chalcones **17** and **11** possess 3-OMe, 4-OH substituents on the B ring.

From Figure 4, it is clear that the chalcone with 3-OMe, 4-OH substituents and unsubstituted-ring A chalcone (**17**) displayed the best activity reaching the highest inhibition (100%) at $7.8 \mu\text{g mL}^{-1}$. The activity decreases in the following order: **17** (unsubstituted-ring A) > **16** (3',4'-(MeO)₂ A ring) > **12** (2',5'-(MeO)₂ A ring) > **14** (2',4'-(MeO)₂ A ring) > **13** (3',4',5'-(MeO)₃ A ring).

In addition, the (MeO)₂ derivatives possessing one of the methoxyl groups at position 2' (**12** and **14**) displayed lower activities than **16** (3',4'-(MeO)₂ derivative), probably due to steric hindrance of the OMe at position 2'.

Our results showed that these chalcones were more active against *C. neoformans* than against *C. albicans*. Regarding the relation between structure and anticryptococcal activity, the methylation of the 3-OH of the B-ring led to an improved activity. In turn, the pattern of substitution of

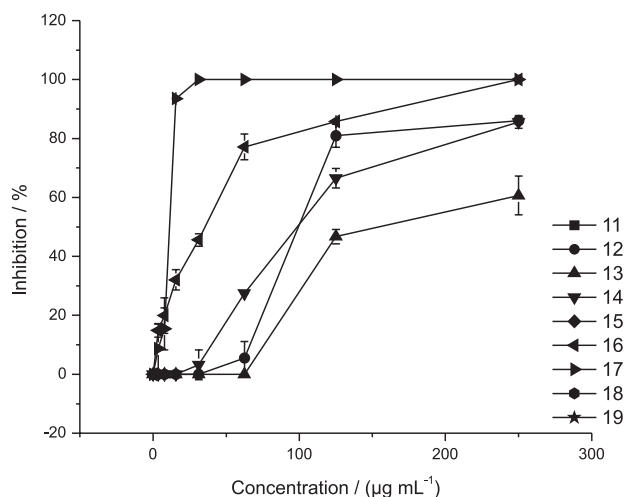


Figure 4. Comparative anticryptococcal activity of 3-OMe, 4-OH chalcones with different substituents on ring A [unsubstituted (**17**); 3',4',5'-(OMe)₃ (**13**); 2',4'-(OMe)₂ (**14**); 2',5'-(OMe)₂ (**12**); 3',4'-(OMe)₂ (**16**)].

the A ring appears to play some role in the anticryptococcal activity.

In vitro antibacterial effects on bacteria pathogenic to plants

Results of the antibacterial assays (Table 4) indicate an outstanding antibacterial activity of compound **12** on the growth of *P. syringae* with a low IC₅₀ value (8 µM or 2.5 µg mL⁻¹). This effect is similar to that observed with streptomycin whose IC₅₀ value is close to 5 µM (2.9 µg mL⁻¹). Although the best inhibitory effect was observed in the previous case, the remaining compounds of the list do not show activity or show a moderate effect (i.e., **19** shows an IC₅₀ = 191 µM and **17**, IC₅₀ = 343 µM) only against *P. syringae*. The other two phytopathogenic bacteria

Table 4. IC₅₀ values of chalcones towards phytopathogenic bacteria

Compound	IC ₅₀ / µM		
	<i>A. tumefaciens</i>	<i>E. carotovora</i>	<i>P. syringae</i>
11	> 500	500	457
12	> 500	> 500	8
13	> 500	> 500	> 500
14	> 500	> 500	> 500
15	> 500	> 500	> 500
16	392	> 500	494
17	> 500	> 500	500
18	> 500	> 500	343
19	500	442	191

Antibacterial activities are shown as IC₅₀ values in µM concentrations; IC₅₀ values were calculated from the first kinetic assay.

were less sensitive to the compounds. However, some sensitivity was observed in the presence of compound **16** for *A. tumefaciens*, and in the presence of the compound **19** against *E. carotovora* and *P. syringae*.

Chalcones **19** and **12** showed an interesting effect on *P. syringae* that is a Gram-negative bacterium, which is classified as pathovars according to its host specificity. Infection with this pathogen produces important economic losses that may affect seriously the net production as well as the quality of the product obtained.

In vitro fungicidal effects on fungi pathogenic to plants

The results of the antifungal activity assay using phytopathogens (Table 5) indicate a high sensitivity to the chalcones, with growth inhibition percentages higher than 51%, at all concentrations tested. Although the positive controls, based on commercial antifungal agents (pure active ingredients), showed strong inhibition (100%) at all concentrations tested in the assays, some chalcones showed inhibition values similar or slightly lower on the growth of the oomycete *P. cinnamomi* (for the case of compound **15**) and *G. fujikuroi* (for the case of compounds **14**, **15** and **17**). This difference was statistically significant with respect to the negative control. The foregoing indicates that this type of compound may have great potential for the treatment of these pathogens. Compound **15** was the most active of this series inhibiting the growth of the two fungi (*B. cinerea* and *G. fujikuroi*) and the oomycete (*P. cinnamomi*). *P. cinnamomi* was the pathogen most sensitive to **15**, registering 100% growth inhibition with the lowest concentration tested (250 µM), achieving effects similar to those observed with metalaxyl.

Additionally, an interesting sensitivity of the fungus *G. fujikuroi* was observed compared to the complete series of chalcones, with percentages of inhibition higher than 75.8%.

In vitro 5-hLOX enzyme assay

Three chalcones displayed an interesting activity as human 5-hLOX inhibitors (Table 6). The analysis of the relationship between structure and activity showed that the most potent inhibitors **11**, **18**, and **19** possess free hydroxyl groups on the A ring (**19**) or catechol groups on the B ring (**11** and **18**) in the structure. The docking studies (Figure 5) revealed that the catechol group present in chalcone **18** was oriented towards the metal, which agrees with a competitive mechanism found in a kinetics experiment (Figure 6). Compound **11**, which was the best inhibitor found, presented a mixed mechanism and was unable to orient the catechol

Table 5. Percentage of growth inhibition of phytopathogenic fungi

Compound	Mycelial growth inhibition of fungal plant pathogens / %								
	<i>P. cinnamomi</i>			<i>B. cinerea</i>			<i>G. fujikuroi</i>		
	250 μ M	500 μ M	1000 μ M	250 μ M	500 μ M	1000 μ M	250 μ M	500 μ M	1000 μ M
11	60.14 \pm 1.26	66.67 \pm 1.26	68.84 \pm 1.26	56.65 \pm 0.97	66.28 \pm 0.63	55.96 \pm 0.0	77.11 \pm 1.45	83.28 \pm 1.21	85.30 \pm 2.35
12	64.13 \pm 1.09	67.75 \pm 1.66	73.55 \pm 0.63	51.83 \pm 1.95	69.27 \pm 0.79	50.46 \pm 0.0	79.54 \pm 1.37	81.09 \pm 0.65	83.29 \pm 0.61
13	61.23 \pm 0.63	64.13 \pm 1.09	69.20 \pm 2.26	60.09 \pm 1.95	67.66 \pm 0.97	71.56 \pm 0.79	78.36 \pm 0.69	81.39 \pm 2.91	83.89 \pm 3.23
14	69.57 \pm 1.09	82.97 \pm 0.77	100 \pm 0.0	50.92 \pm 0.79	72.94 \pm 0.79	100 \pm 0.0	84.64 \pm 2.04	87.58 \pm 0.91	85.96 \pm 2.19
15	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0	69.27 \pm 0.79	72.02 \pm 0.79	76.15 \pm 0.79	85.02 \pm 0.57	86.97 \pm 0.54	88.20 \pm 0.52
16	65.58 \pm 1.66	74.64 \pm 0.63	81.16 \pm 1.26	60.09 \pm 1.95	53.26 \pm 0.07	73.39 \pm 1.59	77.11 \pm 1.45	82.21 \pm 0.63	84.34 \pm 0.59
17	63.77 \pm 1.26	73.55 \pm 0.63	75.36 \pm 1.26	57.34 \pm 1.95	74.77 \pm 0.79	74.77 \pm 5.55	89.06 \pm 0.98	91.38 \pm 0.54	82.44 \pm 3.65
18	59.06 \pm 1.66	67.03 \pm 1.66	74.28 \pm 1.66	51.38 \pm 1.59	59.63 \pm 1.56	67.89 \pm 1.59	79.92 \pm 1.79	80.71 \pm 0.65	81.46 \pm 1.22
19	60.51 \pm 0.63	67.39 \pm 2.17	76.09 \pm 2.17	54.13 \pm 1.59	57.80 \pm 1.56	74.31 \pm 1.59	75.83 \pm 1.94	81.46 \pm 1.12	85.02 \pm 0.57
C+	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 0.0 ^b	100 \pm 0.0 ^b	100 \pm 0.0 ^b	100 \pm 0.0 ^b	100 \pm 0.0 ^b	100 \pm 0.0 ^b
C-	0	0	0	0	0	0	0	0	0

The percentage of mycelial growth inhibition is based on mycelium area measurements after 72 h of incubation. Each point represents the mean of three independent experiments. C+: positive control (^ametalaxyl; ^bcaptan); C-: negative control (stock solution 1% ethanol/water).

group towards the iron, generating an interaction between Asn425 and this group. Compound **19**, that showed the worst IC₅₀ value and had a competitive mechanism, only oriented a hydroxyl group towards the metal.

It is known that flavonoids containing *ortho*-diOH groups have inhibitory activities against LOXs,^{61,62} although it is not the main requirement. We show that there are differences in the type and potency of the inhibition between **11** and **18**, so the presence of a catechol would not be an absolute structural requirement for this activity. Moreover, the most active compound (**11**) has three additional methoxy groups in the 3',4',5' positions.

DPPH radical scavenging activity

The results obtained from the antioxidant activity assays are shown in Table 7. All the compounds were

compared with Trolox. Compounds **11** and **18** showed more potent antioxidant activity than Trolox. The presence of the catechol group resulted in a significant increase of the antioxidant activity with respect to the compounds without this group. Hydroxy groups in *meta* positions (i.e., **19**) and methoxy groups in *meta* and *ortho* positions of the A ring (i.e., **14** and **16**) and methoxy groups in position 3 of the B ring (i.e., **13** and **17**) decrease the antioxidant activity. Hydroxy and methoxy groups on the B and A rings, respectively, increase the antioxidant activity.

In vitro cytotoxic activity of **18**

One of the most effective synthesized chalcone as antioxidant and 5-hLOX inhibitory capacity was **18**. Given that both biological activities are related to proliferation

Table 6. Results of 5-hLOX activity assays in presence of chalcones **11-19**

Compound	Inhibition ^a / %	IC ₅₀ / μ M	K _i / μ M	Type
16	W.A	N.D	N.D	N.D
17	W.A	N.D	N.D	N.D
18	88.36 \pm 2.18	0.023 \pm 0.004	0.01592	competitive
19	58.60 \pm 5.42	0.707 \pm 0.037	1.962	competitive
11	96.30 \pm 1.47	0.011 \pm 0.004	0.006326	mixed
12	W.A	N.D	N.D	N.D
13	W.A	N.D	N.D	N.D
14	41.35 \pm 4.33	N.D	N.D	N.D
15	11.67 \pm 13.13	N.D	N.D	N.D
NDGA	93.42 \pm 5.87	N.D	N.D	N.D

^aChalcone at 10 μ M; IC₅₀: half maximal inhibitory concentration; K_i: inhibitory constant; W.A: without activity; N.D: not determined; NDGA: nordihydroguaiaretic acid.

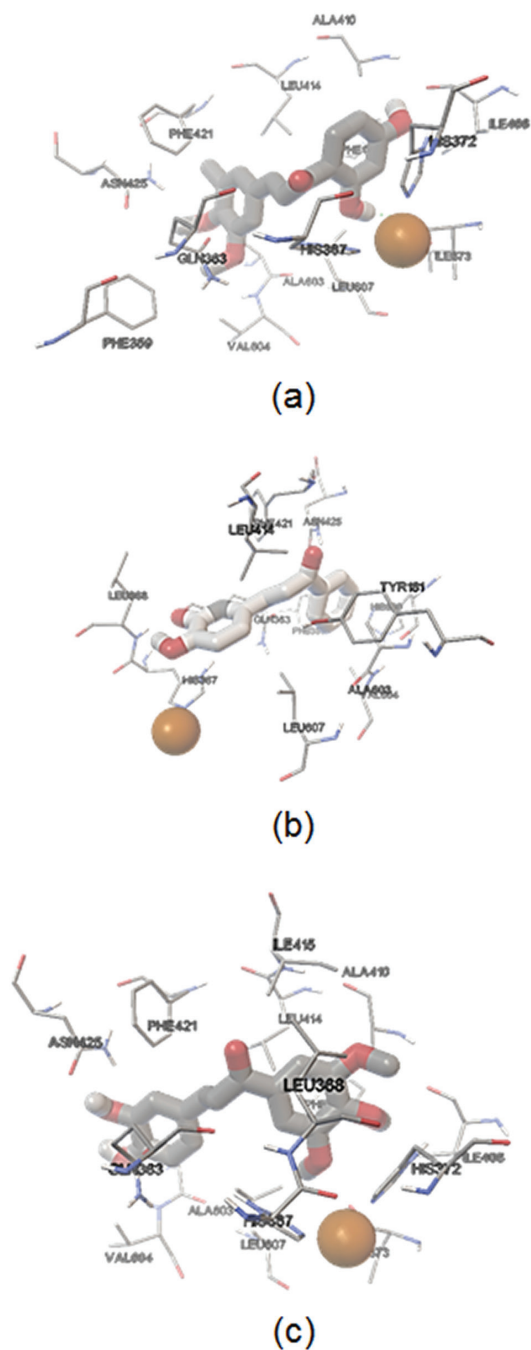


Figure 5. Docking results for chalcones with 5-hLOX. (a) **19**, with a $\Delta G_b = -3.68$ kcal mol⁻¹ and an experimental $k_i = 1.96$ μ M; (b) **18**, with a $\Delta G_b = -4.9$ kcal mol⁻¹ and an experimental $k_i = 0.016$ μ M; (c) **11**, with a $\Delta G_b = -4.25$ kcal mol⁻¹ and an experimental $k_i = 0.06$ μ M. The orange ball represents the iron atom, located in the active site.

and viability of cancer cells, we measured **18**-induced cytotoxic activity against MDA-MB-231 (human breast adenocarcinoma cell line), B16-F10 (mouse melanoma cells) and MEF (primary mouse embryonic fibroblast). Our results in the Table 8 show that **18** exhibits a 4-fold higher selectivity upon B16-F10 compared to the effect on non-cancerous cells (MEF). **18** also exhibited

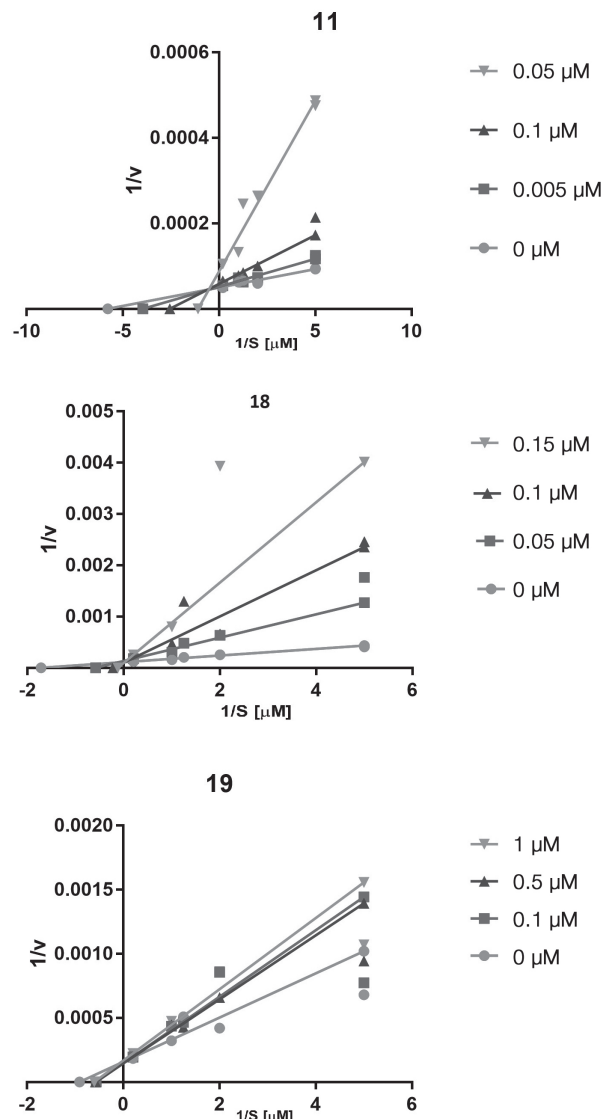


Figure 6. The Lineweaver-Burk plot of the inhibition of 5-hLOX by three chalcones (**11**, **18** and **19**).

Table 7. Screening results of DPPH radical scavenging activity of chalcones **11-19**

Compound	IC ₅₀ ± SD / μ M
11	11.75 ± 0.21
12	33.50 ± 1.52
13	49.04 ± 1.25
14	54.40 ± 5.82
15	NA
16	73.03 ± 7.53
17	104.26 ± 5.44
18	12.45 ± 0.39
19	NA
Trolox	22.54 ± 0.61

Antioxidant activity is shown as IC₅₀ (half maximal inhibitory concentration) values in μ M concentrations. NA: no activity. All compounds were analyzed in triplicate and the results expressed as average ± standard deviation (SD).

cytotoxic activity against MDA-MB-231, but with no selectivity over MEF. When the cytotoxic effect of **18** against B16-F10 was compared with commonly used chemotherapeutic drugs, it could be verified that **18** showed 29- and 8-fold lesser activity than taxol and etoposide, respectively. Furthermore, **18** exhibited 300- and 50-fold lesser cytotoxic activity against MDA-MB231 cell line. It is noteworthy that **18** exhibited antioxidant activity and 5-LOX inhibition at lower concentrations than the needed to exert cytotoxic activity.

Table 8. Cytotoxic activities of **18** against cancer and non-cancerous cells

Compound	IC ₅₀ ± SD / μM		
	MDA-MB-231	B16-F10	MEF
18	67.58 ± 5.76	13.96 ± 2.47	60.66 ± 4.42
Taxol	0.21 ± 0.05	0.48 ± 0.08	0.40 ± 0.07
Etoposide	1.33 ± 0.28	1.74 ± 0.32	1.03 ± 0.17

SD: standard deviation; MDA-MB-231: human breast adenocarcinoma cell line; B16-F10: mouse melanoma cells; MEF: primary mouse embryonic fibroblast.

Conclusions

Apparently a 3,4-diOH substitution on the chalcone skeleton plays a key role in the potentiation capacity of the β-lactam antibiotic methicillin. For this reason, the chalcone **18** was subjected to cytotoxicity studies reinforcing a possible use in the control of resistant pathogens *in vivo*. These studies showed a low cytotoxicity against MEF cell line. This clearly suggests that **18** could be useful for the treatment against resistant bacteria, although further studies are needed to confirm this possibility. Although chalcones with 3,4-*O*-di-hydroxy groups are sometimes considered interferers of all assays,⁶³ it appears not to be applied here because the most active chalcones against phytopathogens and human pathogenic fungi (**12**, **15** and **17**) do not have this substitution pattern.

In both cases, bacteria and fungi, the sensitivity for each molecule can vary, not only by cellular structural characteristics such as wall and plasma membrane, but also by differences in their mechanisms of tolerance to stress and detoxification.⁶⁴

Due to the versatility of biological properties showed here to this kind of compounds, simple and safe polyoxygenated chalcones not only have potential uses in different biological areas, whose specificity or action target depends largely on their functional groups, but also they would help us to fight resistant pathogens to commercial antibiotics through the specific re-sensitization.

Experimental

Chemistry

General data

All purchased chemical reagents (Merck or Sigma-Aldrich) were of the highest available purity and were used without previous purification. Melting points (mp in °C) were measured on a Stuart-Scientific SMP3 melting point apparatus (Stone, United Kingdom) and are uncorrected. Infrared (IR) spectra were recorded as a thin film (**14** and **15**) or in KBr disks (**11**, **12**, **13**, **16**, **17**, **18** and **19**) in a Thermo Scientific Nicolet 6700 FT-IR spectrometer (Massachusetts, USA), and wave numbers are reported in cm⁻¹. Low resolution mass spectra (MS) were recorded on a Thermo Scientific, Trace GC Ultra, ISQ mass spectrometer (Austin, USA) at 70 eV ionizing voltage and are given as *m/z* (rel. int., in percentage). High-resolution mass spectra (HRMS) were recorded on an Exactive™ Plus Orbitrap spectrometer (Thermo Scientific, Bremen, Germany) by infusion, applying a voltage of 5 kV in the positive ionization mode. The spectra were recorded using full scan mode, covering a mass range from *m/z* 140 to 430. The resolution was set to 140000, and maximum loading time for the ion cyclotron resonance (ICR) cell was set to 200 ms. ¹H and ¹³C nuclear magnetic resonance (NMR), 2D heteronuclear single quantum correlation (HSQC) and 2D heteronuclear multiple bond correlation (HMBC) spectra were recorded in CDCl₃ or DMSO-*d*₆ solutions and referenced to the residual solvent peaks at δ 7.26 and 2.50 ppm for ¹H and δ 77.0 and 39.5 ppm for ¹³C, respectively, on a Bruker Avance 400 NMR spectrometer (Rheinstetten, Germany) operating at 400.1 MHz for ¹H and 100.6 MHz for ¹³C. Chemical shifts are reported as δ (ppm), and coupling constants (*J*) are given in Hz. Silica gel (200-400 mesh, Merck, Darmstadt, Germany) was used for column chromatography and HF-254 silica gel plates for thin layer chromatography (TLC). TLC spots were detected both under a UV lamp and by heating after drenching in 10% H₂SO₄ in H₂O. Antioxidant effects were determined in a Thermo Scientific Multiskan GO 96-well plate photometer (Vantaa, Finland).

General experimental procedure

General procedure in alkaline condition

To a solution of acetophenone (1 mol equiv.) and appropriate benzaldehyde (1 mol equiv.) in methanol (5 mL), KOH (4 mol equiv.) was added in one portion. The mixture was warmed to 50 °C and stirred until completion of reaction (monitored by TLC). Then the solvent was removed under reduced pressure, poured into ice water and

neutralized with aqueous 10% HCl solution. The residue was extracted with ethyl acetate (15 mL \times 3 times). The combined organic layer was washed with brine, dried over anhydrous sodium sulfate and filtered; the solvent was evaporated under reduced pressure. The mixture was subjected to silica gel flash column chromatography (ethyl acetate/hexanes as the mobile phase) to yield the products. The fractions of the product were dried and recrystallized from ethanol to obtain pure chalcones.

General procedure in acid condition

To a stirred mixture of acetophenone (1 mol equiv.) and benzaldehyde (1 mol equiv) in absolute ethanol (5 mL), thionyl chloride (0.5 mL, 3 mol equiv.) was added dropwise and the reaction was monitored by TLC. After standing for 16 h, ice water (30 g approximately) was added to the reaction mixture in order to decompose the excess of SOCl₂. Then, ethyl acetate (30 mL) was added and the organic layer was separated, and a new extraction with ethyl acetate was performed. The pooled organic solutions were dried over anhydrous sodium sulfate and filtered; the solvent was evaporated under reduced pressure. The mixture was subjected to silica gel flash column chromatography (ethyl acetate/hexanes as the mobile phase) to yield the products. Solid chalcones were recrystallized from ethanol.

All structures were confirmed by IR and NMR spectra as discussed below.

Physical data of chalcones

1-(3,4,5-Trimethoxyphenyl)-3-(3,4-dihydroxyphenyl)-prop-2-en-1-one (**11**)

It was obtained from 1-(3',4',5'-trimethoxyphenyl) ethanone (**1**, 0.5 g, 2.4 mmol) and 3,4-dihydroxybenzaldehyde (**7**, 0.327 g, 2.4 mmol) in 5 mL of ethanol and 0.5 mL of SOCl₂ (0.82 g, 6.9 mmol), stirring for 16 h as described above. mp 151-153 °C (lit.⁶⁵ 158-159 °C); IR (KBr) ν / cm⁻¹ 3331, 2942, 1645, 1586, 1558; HRMS *m/z*, observed: 331.1178; C₁₈H₁₉O₆ [M + H]⁺ requires: 331.1182; ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, 1H, *J* 15.5 Hz, 2-CH=3-CH), 7.32 (d, 1H, *J* 15.5 Hz, 2-CH=3-CH), 7.27 (s, 1H, 3-ArH-2), 7.25 (s, 2H, 1-ArH-2,6), 7.14 (dd, 1H, *J* 8.2, 1.8 Hz, 3-ArH-6), 6.94 (d, 1H, *J* 8.2 Hz, 3-ArH-5), 6.81 (s, 1H, ArOH-3), 6.59 (s, 1H, ArOH-4), 3.96 (s, 3H, 1-ArOCH₃-4), 3.94 (s, 6H, 1-ArOCH₃-3,5); ¹³C NMR (100 MHz, CDCl₃) δ 56.4 (1-ArOCH₃-3,5), 61.0 (1-ArOCH₃-4), 106.1 (1-ArCH-2,6), 115.0 (3-ArC-2), 115.6 (3-ArCH-5), 119.3 (2-CH=3-CH), 122.8 (3-ArCH-6), 127.7 (3-ArC-1), 133.5 (1-ArC-1), 142.5 (1-ArC-4), 144.2 (3-ArC-3), 145.9 (2-CH=3-CH), 147.1 (3-ArC-4), 153.1 (1-ArC-3,5), 190.3 (1-C=O).

1-(2,5-Dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-one (**12**)

It was obtained from 1-(2,5-dimethoxyphenyl) ethanone (**2**, 0.5 g, 2.78 mmol) and 4-hydroxy-3-methoxybenzaldehyde (**8**, 0.426 g, 2.8 mmol) in 10 mL of methanol and KOH (0.83 g, 14.8 mmol), stirring for 2 h as described above. mp 96-98 °C; IR (KBr) ν / cm⁻¹ 3391, 3067, 3002, 2941, 2836, 1650, 1577, 1513; MS *m/z* (%): 314 (100), 299 (25), 283 (19), 177 (95), 137 (83); HRMS *m/z*, observed: 315.1227; C₁₈H₁₉O₅ [M + H]⁺ requires: 315.1232; ¹H NMR (400 MHz, CDCl₃) δ 7.55 (d, 1H, *J* 15.8 Hz, 2-CH=3-CH), 7.24 (d, 1H, *J* 15.8 Hz, 2-CH=3-CH), 7.18-7.16 (m, 2H, 1-ArH-6', 3-ArH-6), 7.10 (d, 1H, *J* 1.6 Hz, 3-ArH-2), 7.04 (dd, 1H, *J* 8.9, 3.1 Hz, 1-ArH-4), 6.97-6.94 (m, 2H, 1-ArH-3, 3-ArH-5), 5.96 (s, 1H, 3-ArOH-4), 3.95 (s, 3H, ArOCH₃), 3.87 (s, 3H, ArOCH₃), 3.83 (s, 3H, ArOCH₃); ¹³C NMR (100 MHz, CDCl₃) δ 55.8 (ArOCH₃), 55.9 (ArOCH₃), 56.6 (ArOCH₃), 110.0 (3-ArCH-2), 113.4 (1-ArCH-3'), 114.4 (3-ArCH-6), 114.8 (3-ArCH-5), 118.6 (1-ArCH-4), 123.2 (1-ArCH-6), 124.7 (2-CH=3-CH), 127.6 (1-ArC-1), 130.0 (3-ArC-1), 144.1 (2-CH=3-CH), 146.7 (3-ArC-3), 148.1 (3-ArC-4), 152.3 (1-ArC-2), 153.6 (1-ArC-5), 192.8 (1-C=O).

1-(3,4,5-Trimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-one (**13**)

It was obtained from 1-(3',4',5'-trimethoxyphenyl) ethanone (**1**, 0.5 g, 2.4 mmol) and 4-hydroxy-3-methoxybenzaldehyde (**8**, 0.361 g, 2.4 mmol) in 5 mL of ethanol and 0.5 mL of SOCl₂ (0.82 g, 6.9 mmol), stirring for 16 h as described above. mp 88-91 °C (lit.⁴⁵ 75-76 °C); IR (KBr) ν / cm⁻¹ 3512, 3429, 3086, 2994, 2963, 2941, 2839, 1647, 1586, 1561, 1516; MS *m/z* (%): 344 (100), 329 (65), 313 (29), 145 (28); ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, 1H, *J* 15.6 Hz, 2-CH=3-CH), 7.32 (d, 1H, *J* 15.6 Hz, 2-CH=3-CH), 7.30 (s, 2H, 1-ArH-2,6), 7.25 (dd, 1H, *J* 9.6, 1.5 Hz, 3-ArH-6), 7.12 (d, 1H, *J* 1.5 Hz, 3-ArH-2), 6.97 (d, 1H, *J* 8.2 Hz, 3-ArH-5), 5.97 (s, 1H, 3-Ar-OH-4), 3.96 (s, 3H, 3-ArOCH₃-3), 3.95 (s, 6H, 1-ArOCH₃-3,5), 3.94 (s, 3H, 1-ArOCH₃-4); ¹³C NMR (100 MHz, CDCl₃) δ 56.0 (ArOCH₃), 56.4 (1-ArOCH₃-3,5), 61.0 (ArOCH₃), 106.1 (1-ArCH-2,6), 110.4 (3-ArCH-2), 114.9 (3-ArCH-5), 119.6 (2-CH=3-CH), 123.0 (3-ArCH-6), 127.5 (3-ArC-1), 133.8 (1-ArC-1), 142.3 (1-ArC-4), 145.2 (2-CH=3-CH), 146.8 (3-ArC-3), 148.3 (3-ArC-4), 153.1 (1-ArC-3,5), 189.5 (1-C=O).

1-(2,4-Dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-one (**14**)

It was obtained from 1-(2,4-dimethoxyphenyl)ethanone (**3**, 1.0 g, 5.6 mmol) and 4-hydroxy-3-methoxybenzaldehyde

(**8**, 0.93 g, 6.1 mmol) in 5 mL of ethanol and 0.81 mL of SOCl_2 (1.33 g, 11.1 mmol), stirring for 1 h as described above. Pale yellow oil (lit.⁶⁶ sticky-solid); IR (film) ν / cm^{-1} 3370, 2940, 2840, 1648, 1601, 1512; MS m/z (%): 314 (100), 299 (49), 178 (90), 165 (98), 137 (70); ^1H NMR (400 MHz, CDCl_3) δ 7.71 (d, 1H, J 8.7 Hz, 1-ArH-6), 7.60 (d, 1H, J 15.7 Hz, 2-CH=3-CH), 7.33 (d, 1H, J 15.7 Hz, 2-CH=3-CH), 7.15 (dd, 1H, J 8.2, 1.6 Hz, 3-ArH-6), 7.06 (d, 1H, J 1.6 Hz, 3-ArH-2), 6.92 (d, 1H, J 8.2 Hz, 3-ArH-5), 6.54 (dd, 1H, J 8.6, 2.1 Hz, 1-ArH-5), 6.48 (d, 1H, J 2.1 Hz, 1-ArH-3), 6.22 (s, 1H, 3-ArOH-4), 3.90 (s, 3H, 3-ArOCH₃-3), 3.88 (s, 3H, 1-ArOCH₃), 3.85 (s, 3H, 1-ArOCH₃); ^{13}C NMR (100 MHz, CDCl_3) δ 55.5 (ArOCH₃), 55.7 (ArOCH₃), 55.8 (ArOCH₃), 98.6 (1-ArCH-3), 105.0 (1-ArCH-5), 110.2 (3-ArCH-2), 114.8 (3-ArCH-5), 122.2 (1-ArC-1), 122.7 (3-ArCH-6), 124.8 (2-CH=3-CH), 127.8 (3-ArC-1), 132.6 (1-ArCH-6), 142.8 (2-CH=3-CH), 146.7 (3-ArC-3), 147.8 (3-ArC-4), 160.1 (1-ArC-OCH₃), 163.9 (1-ArC-OCH₃), 190.9 (1-C=O).

1-(2,5-Dimethoxyphenyl)-3-phenyl-prop-2-en-1-one (**15**)

It was obtained from 1-(2',5'-dimethoxyphenyl)ethanone (**2**, 2.0 g, 11.1 mmol) and benzaldehyde (**9**, 1.3 g, 12.2 mmol) in 10 mL of methanol and KOH (2.5 g, 44.4 mmol), stirring for 2 h as described above. Yellow oil (lit.⁶⁷ yellow oil); IR (film) ν / cm^{-1} 3060, 3001, 2942, 2911, 2835, 1658, 1594, 1575; MS m/z (%): 268 (100), 253 (15), 237 (25), 177 (60), 165 (70), 151 (61), 103 (65); ^1H NMR (400 MHz, CDCl_3) δ 7.65 (d, 1H, J 15.8 Hz, 2-CH=3-CH), 7.61-7.59 (m, 2H, 3-ArH-2,6), 7.42 (d, 1H, J 15.8 Hz, 2-CH=3-CH), 7.40-7.38 (m, 3H, 3-ArH-3,4,5), 7.19 (d, 1H, J 3.1 Hz, 1-ArH-6), 7.03 (dd, 1H, J 9.0, 3.1 Hz, 1-ArH-4), 6.94 (d, 1H, J 9.0 Hz, 1-ArH-3), 3.86 (s, 3H, ArOCH₃), 3.81 (s, 3H, ArOCH₃); ^{13}C NMR (100 MHz, CDCl_3) δ 55.8 (ArOCH₃), 56.5 (ArOCH₃), 113.4 (1-ArCH-6), 114.4 (1-ArC-3), 119.2 (2-CH=3-CH), 126.9 (1-ArCH-4), 128.4 (3-ArCH-2,6), 128.8 (3-ArCH-3,4,5), 129.6 (3-ArC-1), 130.2 (1-ArC-OCH₃), 135.1 (1-ArC-OCH₃), 143.2 (2-CH=3-CH), 152.6 (1-ArC-2), 153.6 (1-ArC-5), 192.4 (1-C=O).

1-(3,4-Dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-one (**16**)

It was obtained from 1-(3',4'-dimethoxyphenyl)ethanone (**4**, 0.5 g, 2.8 mmol) and 4-hydroxy-3-methoxybenzaldehyde (**8**, 0.42 g, 2.8 mmol) in 5 mL of ethanol and 0.41 mL of SOCl_2 (0.67 g, 5.6 mmol), stirring for 2 h as described above. mp 127-128 °C (lit.⁶⁸ 128-129 °C); IR (KBr) ν / cm^{-1} 3341, 3078, 2992, 2930, 2835, 1643, 1585, 1557, 1510, 1438, 1418, 1267; MS m/z (%): 314 (100), 283 (65), 207 (90), 165 (58), 145 (39); ^1H NMR

(400 MHz, CDCl_3) δ 7.73 (d, 1H, J 15.5 Hz, 2-CH=3-CH), 7.68 (dd, 1H, J 8.4, 1.8 Hz, 1-ArH-6), 7.62 (d, 1H, J 1.7 Hz, 3-ArH-6), 7.40 (d, 1H, J 15.5 Hz, 2-CH=3-CH), 7.23 (dd, 1H, J 8.2, 1.6 Hz, 3-ArH-6), 7.12 (d, 1H, J 1.5 Hz, 3-ArH-2), 6.95-6.92 (m, 2H, 1-ArH-5, 3-ArH-5), 5.95 (s, 1H, 3-ArOH-4), 3.97 (m, 9H, ArOCH₃); ^{13}C NMR (100 MHz, CDCl_3) δ 56.0-56.1 (ArOCH₃), 109.9 (1-ArCH-5), 110.2 (3-ArCH-2), 110.8 (1-ArCH-2), 114.8 (3-ArCH-5), 119.4 (2-CH=3-CH), 122.8 (1-ArC-6), 123.0 (3-ArC-6), 127.6 (1-ArC-1), 131.5 (3-ArC-1), 144.3 (2-CH=3-CH), 146.8 (3-ArC-4), 148.1 (3-ArC-3), 149.2 (1-ArC-3), 153.1 (1-ArC-4), 188.7 (1-C=O).

1-Phenyl-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-one (**17**)

It was obtained from acetophenone (**5**, 0.48 g, 4 mmol) and 4-hydroxy-3-methoxybenzaldehyde (**8**, 0.502 g, 3.3 mmol) in 8 mL of ethanol and 0.24 mL of SOCl_2 (0.39 g, 3.3 mmol), stirring for 6 h as described above. mp 91-92 °C (lit.⁶⁹ 93 °C); IR (KBr) ν / cm^{-1} 3322, 3001, 2938, 2835, 1655, 1583, 1564, 1525, 1454, 1420, 1369, 1248; MS m/z (%): 254 (100), 237 (22), 223 (20), 165 (35), 145 (48), 124 (60); ^1H NMR (400 MHz, CDCl_3) δ 8.01 (d, 2H, J 7.3 Hz, 1-ArH-2,6), 7.75 (d, 1H, J 15.6 Hz, 2-CH=3-CH), 7.60-7.56 (m, 1H, 1-ArCH-4), 7.52-7.48 (m, 2H, 1-ArH-3,5), 7.38 (d, 1H, J 15.6 Hz, 2-CH=3-CH), 7.22 (dd, 1H, J 8.2, 1.4 Hz, 3-ArH-6), 7.13 (d, 1H, J 1.4 Hz, 3-ArH-2), 6.96 (d, 1H, J 8.2 Hz, 3-ArH-5), 5.97 (s, 1H, 3-ArOH-4), 3.96 (s, 3H, 3-ArOCH₃-3); ^{13}C NMR (100 MHz, CDCl_3) δ 56.0 (3-ArOCH₃-3), 110.0 (3-ArCH-2), 114.9 (3-ArCH-5), 119.8 (2-CH=3-CH), 123.4 (3-ArCH-6), 127.5 (3-ArC-1), 128.4 (1-ArCH-3,5), 128.6 (1-ArCH-2,6), 132.6 (1-ArCH-4), 138.5 (1-ArC-1), 145.2 (2-CH=3-CH), 146.8 (3-ArC-3), 148.3 (3-ArC-4), 190.7 (1-C=O).

1-Phenyl-3-(3,4-dihydroxyphenyl)-prop-2-en-1-one (**18**)

It was obtained from acetophenone (**5**, 0.5 g, 4.2 mmol) and 3,4-dihydroxybenzaldehyde (**7**, 0.58 g, 4.2 mmol) in 5 mL of ethanol and 0.31 mL of SOCl_2 (0.5 g, 4.2 mmol), stirring for 16 h as described above. mp 198-200 °C dec. (lit.⁷⁰ 200-201 °C); IR (KBr) ν / cm^{-1} 3482, 3304, 1647, 1582, 1563, 1441, 1365, 1365, 1284; MS m/z (%): 240 (100), 223 (25), 163 (14), 110 (21), 77 (23); HRMS m/z , observed: 241.0861; $\text{C}_{15}\text{H}_{13}\text{O}_3$ [$\text{M} + \text{H}$]⁺ requires: 241.0865; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.42 (br s, 2H, 3-ArOH-3,4), 8.09 (d, 2H, J 7.5 Hz, 1-ArH-3,5), 7.65-7.62 (m, 1H, 1-ArH-4), 7.60 (s, 2H, 2-CH=3-CH), 7.56-7.52 (m, 2H, 1-ArH-2,6), 7.25 (s, 1H, 3-ArH-2), 7.18 (d, 1H, J 8.1 Hz, 3-ArH-6), 6.80 (d, 1H, J 8.1 Hz, 3-ArH-5); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 115.5 (3-ArCH-2), 115.7 (3-ArCH-5), 118.4 (2-CH=3-CH), 122.2 (3-ArCH-6), 126.2

(3-ArC-1), 128.3 (1-ArCH-3,5), 128.7 (1-ArCH-2,6), 132.7 (1-ArCH-4), 138.0 (1-ArC-1), 145.0 (2-CH=3-CH), 145.6 (3-ArC-3), 148.8 (3-ArC-4), 189.0 (1-C=O).

1-(2,4-Dihydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)-prop-2-en-1-one (**19**)

It was obtained from 1-(2',4'-dimethoxyphenyl)ethanone (**3**, 0.5 g, 3.3 mmol) and 3,4,5-trimethoxybenzaldehyde (**10**, 0.65 g, 3.3 mmol) in 6 mL of methanol and KOH (2 g, 35.6 mmol), stirring for 2 h as described above. mp 181-183 °C (lit.⁷¹ 188-190 °C); IR (KBr) ν / cm^{-1} 3329, 3016, 2978, 2943, 2833, 1623, 1583, 1506, 1434, 1375, 1333, 1303; MS m/z (%): 330 (48), 194 (37), 181 (100); ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.51 (s, 1H, 1-ArOH-2), 10.75 (s, 1H, 1-ArOH-4), 8.23 (d, 1H, *J* 9.0 Hz, 1-ArH-6), 7.91 (d, 1H, *J* 15.4 Hz, 2-CH=3-CH), 7.75 (d, 1H, *J* 15.3 Hz, 2-CH=3-CH), 7.23 (s, 2H, 3-ArCH-2,6), 6.44 (dd, 1H, *J* 8.8, 2.3 Hz, 1-ArH-5), 6.29 (d, 1H, *J* 2.3 Hz, 1-ArH-3), 3.86 (s, 6H, 3-ArOCH₃-3,5), 3.71 (s, 3H, 3-ArOCH₃-4); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 56.1 (3-ArOCH₃-3,5), 60.1 (3-ArOCH₃-4), 102.6 (1-ArCH-3), 106.7 (3-ArCH-2,6), 108.2 (1-ArCH-5), 113.0 (1-ArC-1), 120.2 (2-CH=3-CH), 130.1 (3-ArC-1), 133.2 (1-ArCH-6), 139.8 (3-ArC-4), 144.2 (2-CH=3-CH), 153.1 (3-ArC-3,5), 165.2 (1-ArC-4), 165.9 (1-ArC-2), 191.5 (1-C=O).

Cytotoxicity assays

In vitro antibacterial activity assays, human pathogens

Minimum inhibitory concentration assay

Two clinical isolates of methicillin resistant *S. Aureus* (622-4 and 97-7), and one clinical isolate of *E. coli* 33.1, kindly donated by Dr Marcela Wilkens from Universidad de Santiago de Chile (Chile) were used, and as control strains *S. aureus* (NCTC8325-4) and *E. coli* (ATCC25922) were used. The antimicrobial activities of chalcones against strains *E. coli* (ATCC25922), *E. coli* multi-resistant (33.1), *S. aureus* (NCTC8325-4) and methicillin-resistant *S. aureus* 97-7, were determined using the microdilution method established by CLSI.⁷² Briefly, stock solutions (5 mg mL⁻¹) of compounds in DMSO were diluted in Mueller-Hinton broth (MHB) to the different two-fold assay concentrations. The final concentration of DMSO was $\leq 2.5\%$ and does not affect the microbial growth. The obtained solution was then added to MHB, and serially two-fold diluted (in a 96-well microplate). 100 μL of inoculum 1.5×10^6 colony forming unit (CFU) mL⁻¹, prepared in MHB, was then added. The plates were sealed with a tight-fitting plastic cover, then incubated at 37 °C for 18 h. The assay was repeated three times. Wells containing MHB, 100 μL of inoculum and

DMSO (final concentration of $\leq 2.5\%$) served as negative controls. The MIC was defined as the lowest concentration of compounds resulting in the complete inhibition of visible growth.

Checkerboard dilution test

The type of interaction of the combination of chalcones **11** and **18** with methicillin was investigated with the checkerboard method.⁷³ Briefly, the uppermost row (A) of a 96-well microtiter plate contained substance X in a concentration of about four times the expected MIC of the microorganism examined. Each following row (B-H) contained half the concentration of the previous one. The same procedure was carried out along the columns (1-12) with substance Y. So, each well contained a unique combination of the two substances (X and Y). At last 100 μL of Mueller-Hinton broth containing about 10^5 CFU mL⁻¹ were added to the wells and incubated at 37 °C for 24 h. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the fractional inhibitory concentration index (FICI) as follows: FICI = fractional inhibitory concentration of A (FIC_A) + fractional inhibitory concentration of B (FIC_B) = (MIC of drug A in combination / MIC of drug A alone) + (MIC of drug B in combination / MIC of drug B alone). FICI was interpreted as follows: synergy, < 0.5 ; partial synergy, 0.5-0.75; additive effect, 0.76-1.0; indifference, 1.0-4.0; and antagonism, > 4.0 .⁷⁴

In vitro antifungal activity assays against human pathogens

Microorganisms and media

For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC, Rockville, MD, USA), *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264, were used. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula were obtained according to reported procedures⁵⁹ and adjusted to $1-5 \times 10^3$ CFU mL⁻¹.

Fungal growth inhibition

The fungal growth inhibition percentage was determined with broth microdilution techniques performed in 96-well microplates according to the CLSI reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard M27-A3.⁵⁹ For the assay, compound-test wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration 1%), diluted with Roswell Park Memorial Institute (RPMI)-1640

medium, to final concentrations range 250-3.9 $\mu\text{g mL}^{-1}$. An inoculum suspension (100 μL) was added to each well (final volume in the well: 200 μL). A growth control well (GCW) (containing medium, inoculum, and the same amount of DMSO used in a CTW, but compound-free) and a sterility control well (SCW) (sample, medium, and sterile water instead of inoculum) were included for each fungus tested. Microtiter trays were incubated in a moist, dark chamber at 30 °C for 48 h for both yeasts. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B (Sigma-Aldrich, St. Louis, USA) was used as positive control. Tests were performed in triplicate. Reduction of growth for each compound concentration was calculated as follows: $\text{inhibition}(\%) = 100 (\text{OD}_{405} \text{ CTW} - \text{OD}_{405} \text{ SCW}) / (\text{OD}_{405} \text{ GCW} - \text{OD}_{405} \text{ SCW})$, where OD_{405} is the optical density at 405 nm. The concentration of each compound that produces 50% fungal growth reduction (IC_{50}) was taken as the MIC endpoint.

In vitro antibacterial activity assays, phytopathogens

Liquid-dilution methods were used to evaluate the effects of the compounds on the growth of *E. carotovora* (NCPBP 312), *A. tumefaciens* (strain C58C1) and *P. syringae* (NCPBP 281). Bacteria were grown in sterile tubes with 10 mL of Mueller-Hinton (MH) medium and incubated at 27 °C for 12 h with shaking, to produce an initial culture. The antimicrobial activity was evaluated by observing the growth response of both microorganisms in samples with different concentrations of the compounds.⁷⁵⁻⁷⁷ All assays were performed on sterile 96-well microplates with a final volume of 200 μL containing MH broth inoculated with 1 μL aliquots of bacterial suspension (10^5 - 10^6 CFU mL^{-1} , initial culture) in the presence of different concentrations of test compounds (3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250 μM). MH was used as the negative control (C-), and MH with streptomycin⁷⁸ was used as the positive control (C+). The plates were incubated for 7 h at 27 °C. Bacterial growth was monitored by measuring the optical density at 595 nm every hour with a microplate reader. All tests were performed in ten repetitions for each microorganism evaluated. Bacterial growth was shown as the arithmetic mean expressed in terms of the negative control (100% growth). The lowest concentration of the compound preventing the appearance of turbidity was considered to be the MIC.

The first experiment (first kinetic assay) in which the compound was exposed to the bacterial cultures in MH was carried out over a period of 6 h. Subsequently, to determine the minimal bactericidal concentration (MBC), a second

experiment (second kinetic assay) was conducted; this experiment involved taking inoculum from the first kinetic assay and adding it to MH in a new 96-well microplate, which was then cultured for 7 h. The aim of this second culture (second kinetic assay) was to observe and quantify the ability of bacterial cultures to recover from the cytotoxic effect caused by the compounds, which is evidenced by the presence or absence of bacterial growth. This second experiment allows a more accurate conclusion to be reached: if the bacterial culture's growth declines with respect to the culture of the first kinetic assay, then it is related to a bactericidal property. If, on the other hand, the growth continues in line with that of the culture of first kinetic assay, then it is related to a bacteriostatic property.

In vitro fungicidal activity assays, phytopathogens

A virulent isolate of *B. cinerea* obtained from naturally infected grape berries⁷⁹ was prepared and maintained by placing it on potato dextrose agar (PDA) at 5 °C. *P. cinnamomi* was kindly provided by the National Institute of Agricultural Research (INIA, La Platina, Santiago, Chile). The fungus *G. fujikuroi* was kindly provided by Dr B. Fraga, Natural Products and Agrobiology Institute, CSIC, Canary Islands, Spain. The agar dilution technique was used to evaluate the effect of the chalcones on the growth of *P. cinnamomi*,^{80,81} *G. fujikuroi*⁸² and *B. cinerea*.^{83,84} Fungicidal activity assays of these compounds were performed in microcultures by growing the fungi in sterile Petri dishes (35 mm) at a final volume of 2 mL medium containing different compound concentrations. Clarified V8 (Campbell Soup) medium containing metalaxyl⁸⁵ for *P. cinnamomi* or PDA medium for *B. cinerea* and *G. fujikuroi* with captan^{86,87} was used as the positive control (C+), and without fungicide as the negative control (C-). The medium in each slot was then inoculated with a small block (4 mm) of clarified V8 or PDA medium containing fungal hyphae excised from the edge of an actively growing culture. The fungal growth was monitored at different times (24-72 h). To determine the growth of mycelium in the agar plate, the surface of the mycelium was measured using Sigma Scan Pro 5 software.⁸⁸ The final results are expressed as percent growth inhibition, calculated according to the control without fungicide. Each treatment was independently performed in triplicate.

Statistics

A one-way analysis of variance (ANOVA) was performed to identify significant differences among the

treatment and control groups. Tukey's honest significant difference test was applied to compare the means of every treatment against the control and simultaneously establish their significance ($p < 0.05$). All data are presented as mean \pm standard deviation (S.D.).

In vitro 5-LOX enzyme assay

The commercially available enzyme 5-hLOX by Cayman Chemicals Inc. (Ann Arbor, MI, USA) was diluted (1:500) in the assay buffer 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylenediamine tetraacetic acid (EDTA), 10 μ M adenosine triphosphate (ATP) and 10 μ M CaCl₂ at pH 7.5) and mixed with 10 μ M of H₂DCFDA dye the reaction mixture and incubated for 15 min in the assay plate. Later, 280 μ L of buffer and 10 μ M of inhibitor were added *per well*, and finally incubated for 30 min at 37 °C. Finally, the reaction was started by adding a suitable concentration of arachidonic acid (0.5 μ M) and the fluorescence was read in a multimode detector Synergy™ HT Multi-Mode Microplate Reader (Biotek, Bad Friedrichshall, Germany) at 480 nm excitation/520 nm emission after an incubation time of 1 h at room temperature. The IC₅₀ values were obtained for analysis of oxidation of H₂DCFDA dye to the highly fluorescent 2',7'-dichloro-fluorescein (DCF) product and obtained by using the non-linear curve-fitting program. The kinetic assays to determine the mechanism of inhibition were done with the best inhibitors, using different concentrations of the inhibitor (0-1 μ M) and substrate (0.2-5 μ M). Experimental conditions were carried out as described previously. All data were collected in duplicate and the assays were performed on different days to ensure reproducibility of the method. The graphics for IC₅₀ and kinetics studies were performed by GraphPad Prism Demo V.7.⁸⁹

Docking of chalcones with human 5-LOX

The chalcone structures were built with the Molecular Operating Environment software.⁹⁰ ChelpG charges were obtained at the B3LYP/6-31G** level theory, employing the Gaussian 09 package.⁹¹ Docking of all inhibitors into the active site of the crystal structure of 5-hLOX (PDB code: 3O8Y, 2.39 Å resolution) was performed with the AutoDock4 package,⁹² using a Lamarckian algorithm and assuming total flexibility of the inhibitors. The grid maps were made up to 60 \times 60 \times 60 points, with a grid-point spacing of 0.375 Å and the Fe³⁺ as the center of the grid map. The AutoTors option was used to define the ligand torsions, and the docking results were then analyzed by a

ranked cluster analysis, resulting in conformations with the highest overall binding energy (most negative Gibbs free energy binding value, $-\Delta G$).

General procedure to determine the DPPH radical scavenging activity

The radical scavenging activity of the chalcones towards the DPPH radical was measured as described,⁹³ adapted to a screen of 96-well plates. Briefly, stock solutions of each compound were prepared in methanol at 1 mM concentration (10 mL). Dilutions (1-200 μ M) were prepared from the stock solutions. Methanol (90 μ L), each dilution (150 μ L), and DPPH (60 μ L, Sigma-Aldrich, St. Louis, USA) in methanol (0.5 mM), resulting in a final concentration of 0.1 mM DPPH, were added in a 96-well plate. Methanol was used as the blank sample. The mixtures were left for 30 min at room temperature, and the absorbances were then measured at 517 nm. Trolox was used as the standard antioxidant. The radical scavenging activity was calculated as follows: Inhibition(%) = [(blank absorbance – sample absorbance) / blank absorbance] \times 100. The mean of three IC₅₀ (concentration causing 50% inhibition) values for each compound was determined graphically.

In vitro cytotoxic activity of 18

The cell lines used in this work included MDA-MB-231 human breast adenocarcinoma cells, B16-F10 mouse metastatic melanoma cells and MEF primary mouse embryonic fibroblast. Cells were maintained in DMEM high glucose medium (Mediatech, Manassas, VA, USA) supplemented with 10% (MDA-MB-231 and B16-F10) or 15% (MEF) heat-inactivated fetal bovine serum (HyClone Laboratories, South Logan, USA), 100 IU mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin and maintained at 37 °C in a 5% CO₂ humidified atmosphere. Cell viability was measured using CyQuant® Direct Cell Proliferation Assay Kit (Life Technologies, Grand Island, NY, USA) following the manufacturer's instruction. Briefly, 5000 cells well⁻¹ were seeded onto a flat-bottomed 96-well plate in 200 μ L final volume. Six hours after seeding, the culture medium was replaced with the medium containing the tested compounds at concentrations ranging from 0 up to 100 μ M dissolved in DMSO (0.1% final concentration) during 72 h. The concentrations used to calculate the IC₅₀ values were: 100.0, 30.0, 10.0, 3.0, 1.0, 0.3, 0.1, 0.01 and 0.0 μ M. Untreated cells (medium containing 0.1% DMSO) were used as controls. At the end of the incubation, 100 μ L of culture medium was removed from each experimental well and replaced by detection reagent solution provided by the

kit (which is a 1:5 mix of the reagent CyQUANT® direct nucleic acid stain and the reagent labeled as CyQUANT® direct background suppressor I). Cells were incubated for 1 h and fluorescence emission was measured at 535 nm with excitation at 480 nm in a microplate reader (Infinite 200 PRO, Tecan, Grödig, Austria). At least four independent experiments were performed for each concentration. The results from each experiment were transformed to percentage of controls and the IC₅₀ values were graphically obtained from the dose-response curves. The IC₅₀ value was obtained adjusting the dose-response curve to sigmoidal curves (variable slope), generated using GraphPad Prisma 6.0 software.⁹⁴

Supplementary Information

Supplementary information is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

Acknowledgments

This work was supported by FONDECYT (grant No. 1130924), Dirección General de Investigación, Innovación y Postgrado (DGIIP) of the Universidad Técnica Federico Santa María, DICYT-USACH (grant No. 021601VM) and (grant No. 021641MC) of the Universidad de Santiago de Chile. S. Z. and M. R. thank the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), grant PICT 2014-1170.

References

1. Aksöz, B. E.; Ertan, R.; *FABAD J. Pharm. Sci.* **2011**, *36*, 223.
2. Sebtı, S.; Solhy, A.; Tahir, R.; Abdelatif, S.; Boulaajaj, S.; Mayoral, J. A.; García, J. I.; Fraile, J. M.; Kossir, A.; Oumimoun, H.; *J. Catal.* **2003**, *213*, 1.
3. Kumar, S.; Lamba, M. S.; Makrandi, J. K.; *Green Chem. Lett. Rev.* **2008**, *1*, 123.
4. Ndoile, M. M.; van Heerden, F. R.; *Beilstein J. Org. Chem.* **2013**, *9*, 1346.
5. Sheng, R.; Lin, X.; Zhang, J.; Chol, K. S.; Huang, W.; Yang, B.; He Q.; Hu, Y.; *Bioorg. Med. Chem.* **2009**, *17*, 6692.
6. Syam, S.; Abdelwahab, S. I.; Al-Mamary, M. A.; Mohan, S.; *Molecules* **2012**, *17*, 6179.
7. Nagaraju, M.; Deepthi, E. G.; Ashwini, C.; Vishnuvardhan, M. V. P. S.; Nayak, V. L.; Chandra, R.; Ramakrishna, S.; Gawali, B. B.; *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4314.
8. Daskiewicz, J. B.; Comte, G.; Barron, D.; Di Pietro, A.; Thomasson, F.; *Tetrahedron Lett.* **1999**, *40*, 7095.
9. Gall, E. L.; Texier-Boulet, F.; Hamelin, J.; *Synth. Commun.* **1999**, *29*, 3651.
10. Dong, X.; Zhang, Y.; He, J.-L.; Zhang, S.; Zeng, M.-M.; Chen, J.; Zheng, Z.-P.; *Food Chem.* **2016**, *197*, 589.
11. Nam, N.-H.; Kim, Y.; You, Y.-J.; Hong, D.-H.; Kim, H.-M.; Ahn, B.-Z.; *Eur. J. Med. Chem.* **2003**, *38*, 179.
12. Dong, F.; Jian, C.; Zhenghao, F.; Kai, G.; Zuliang, L.; *Catal. Commun.* **2008**, *9*, 1924.
13. Narender, T.; Reddy, K. P.; *Tetrahedron Lett.* **2007**, *48*, 3177.
14. Calloway, N. O.; Green, L. D.; *J. Am. Chem. Soc.* **1937**, *59*, 809.
15. Iranpoor, N.; Kazemi, F.; *Tetrahedron* **1998**, *54*, 9475.
16. Ren, Y.-M.; Zhang, Z.; Jin, S.; *Synth. Commun.* **2016**, *46*, 528.
17. Kakati, D.; Sarma, R. K.; Saikia, R.; Barua, N. C.; Sarma, J. C.; *Steroids* **2013**, *78*, 321.
18. Li, J.-T.; Yang, W.-Z.; Wang, S.-X.; Li, S.-H.; Li, T.-S.; *Ultrason. Sonochem.* **2002**, *9*, 237.
19. Rateb, N. M.; Zohdi, H. F.; *Synth. Commun.* **2009**, *39*, 2789.
20. Petrov, O.; Ivanova, Y.; Gerova, M.; *Catal. Commun.* **2008**, *9*, 315.
21. Singh, P.; Anand, A.; Kumar, V.; *Eur. J. Med. Chem.* **2014**, *85*, 758.
22. Vandeputte, P.; Ferrari, S.; Coste, A. T.; *Int. J. Microbiol.* **2012**, *2012*, article ID 713687.
23. Mahapatra, D. K.; Bharti, S. K.; Asati, V.; *Eur. J. Med. Chem.* **2015**, *101*, 496.
24. Williamson, B.; Tudzynski, B.; Tudzynski, P.; Van Kan, J. A. L.; *Mol. Plant Pathol.* **2007**, *8*, 561.
25. Wulff, E. G.; Sørensen, J. L.; Lübeck, M.; Nielsen, K. F.; Thrane, U.; Jan Torp, J.; *Environ. Microbiol.* **2010**, *12*, 649.
26. Sepúlveda-Chavera, G.; Salvatierra-Martínez, R.; Bilbao-Apata, C.; Sepúlveda-Ramírez, P.; Allende-Castro, M.; Alache-González, J.; *Idesia* **2013**, *31*, 41.
27. Gallo, L.; Siverio, F.; Rodríguez-Pérez, A.-M.; *Ann. Appl. Biol.* **2007**, *150*, 65.
28. Jiménez, E.; Dorta, F.; Medina, C.; Ramírez, A.; Ramírez, I.; Peña-Cortés, H.; *Mar. Drugs* **2011**, *9*, 739.
29. El Khoury, W.; Makkouk, K.; *J. Plant Pathol.* **2010**, *92*, S4.35.
30. Yang, C.; Hamel, Ch.; Vujanovic, V.; Gan, Y.; *ISRNEcol.* **2011**, *2011*, article ID 130289.
31. Díaz-Tielas, C.; Graña, E.; Reigosa, M. J.; Sánchez-Moreiras, A. M.; *Planta Daninha* **2016**, *34*, 607.
32. Guo, M.; Block, A.; Bryan, C. D.; Becker, D. F.; Alfano, J. R.; *J. Bacteriol.* **2012**, *194*, 5054.
33. Prajapat, R.; Marwal, A.; Jha, P. N.; *Int. J. Curr. Microbiol. Appl. Sci.* **2013**, *2*, 83.
34. Moore, L. W.; Pscheidt, J. W.; *Pacific Northwest Plant Disease Management Handbook*; Pscheidt, J. W.; Ocamb, C. M., eds.; Oregon State University: Corvallis, OR, USA, 2018. Available at <https://pnwhandbooks.org/node/408/print>, accessed in September 2018.
35. Lucera, A.; Costa, C.; Conte, A.; Del Nobile, M. A.; *Front. Microbiol.* **2012**, *3*, article 287.

36. <http://www.fao.org/fao-who-codexalimentarius/codex-texts/maximum-residue-limits/en/>, accessed on September 10, 2018.
37. Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E.; *J. Agric. Food Chem.* **2016**, *64*, 1046.
38. Chand, K.; Rajeshwari; Hiremathad, A.; Singh, M.; Santos, M. A.; Keri, R. S.; *Pharmacol. Rep.* **2017**, *69*, 281.
39. Karthikeyan, C.; Moorthy, N. S.; Ramasamy, S.; Vanam, U.; Manivannan, E.; Karunakaran, D.; Trivedi, P.; *Recent Pat. Anti-Cancer Drug Discovery* **2015**, *10*, 97.
40. Ghosh, J.; Myers, C. E.; *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 13182.
41. Steinhilber, D.; Fischer, A. S.; Metzner, J.; Steinbrink, S. D.; Roos, J.; Ruthardt, M.; Maier, T. J.; *Front. Pharmacol.* **2010**, *1*, article 143.
42. Chen, Y.; Zhang, H.; Zhou, H. J.; Ji, W.; Min, W.; *Cancers* **2016**, *8*, article 40.
43. Reddy, N. P.; Aparoy, P.; Reddy, T. C. M.; Achari, C.; *Bioorg. Med. Chem.* **2010**, *18*, 5807.
44. Ball, A. K.; Beilstein, K.; Wittmann, S.; Sürin, D.; Saul, M. J.; Schnütgen, F.; Flamand, N.; Capelo, R.; Kahnt, A. S.; Frey, H.; Schaefer, L.; Marschalek, R.; Häfner, A.-K.; Steinhilber, D.; *Biochim. Biophys. Acta* **2017**, *1862*, 561.
45. Batovska, D.; Parushev, S.; Stamboliyska, B.; Tsvetkova, I.; Ninova, M.; Najdenski, H.; *Eur. J. Med. Chem.* **2009**, *44*, 2211.
46. Abreu, A. C.; McBain, A. J.; Simões, M.; *Nat. Prod. Rep.* **2012**, *29*, 1007.
47. Butts, A.; Krysan, D. J.; *PLoS Pathog.* **2012**, *8*, e1002870.
48. Delattin, N.; De Brucker, K.; Vandamme, K.; Meert, E.; Marchand, A.; Chaltin, P.; Cammue, B. P. A.; Thevissen, K.; *J. Antimicrob. Chemother.* **2014**, *69*, 1035.
49. Tran, T. D.; Do, T. H.; Tran, N. C.; Ngo, T. D.; Huynh, T. N. P.; Tran, C. D.; Thai, K. M.; *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4555.
50. Pfaller, M. A.; Diekema, D. J.; *Clin. Microbiol. Rev.* **2007**, *20*, 133.
51. Sato, M.; Tsuchiya, H.; Akagiri, M.; Fujiwara, S.; Fujii, T.; Takagi, N.; Matsuura, N.; Iinuma, M.; *Lett. Appl. Microbiol.* **1994**, *18*, 53.
52. Batovska, D.; Parushev, St. P.; Slavova, A.; Bankova, V.; Tsvetkova, I.; Ninova, M.; Najdenski, H.; *Eur. J. Med. Chem.* **2007**, *42*, 87.
53. Lathchev, K. L.; Batovska, D.; Parushev, St. P.; Ubiyovk, V. M.; Sibiry, A. A.; *Eur. J. Med. Chem.* **2008**, *43*, 2220.
54. Gupta, D.; Jain, D.; *J. Adv. Pharm. Technol. Res.* **2015**, *6*, 114.
55. Mishra, P.; Sarma, B.; Singhai, P.; Singh, U.; *Mycobiology* **2007**, *35*, 72.
56. Jayasinghe, L.; Balasooriya, B. A.; Padmini, W. C.; Hara, N.; Fujimoto, Y.; *Phytochemistry* **2004**, *65*, 1287.
57. Nowakowska, Z.; *Eur. J. Med. Chem.* **2007**, *42*, 125.
58. López, S. N.; Castelli, M. V.; Zacchino, S. A.; Domínguez, J. N.; Lobo, G.; Charris-Charris, J.; Cortés, J. C.; Ribas, J. C.; Devia, C.; Rodríguez, A. M.; Enriz, R. D.; *Bioorg. Med. Chem.* **2001**, *9*, 1999.
59. Clinical and Laboratory Standards Institute (CLSI); *CLSI Document M27-A3: Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*, 3rd ed.; CLSI: Wayne, PA, 2008.
60. Swinne, D.; Wattle, M.; Nolard, N.; *Rev. Iberoam. Micol.* **2005**, *22*, 24.
61. Mascayano, C.; Espinosa, V.; Sepúlveda-Boza, S.; Hoobler, E. K.; Perry, S.; *Chem. Biol. Drug Des.* **2013**, *82*, 317.
62. Mascayano, C.; Espinosa, V.; Sepúlveda-Boza, S.; Hoobler, E. K.; Perry, S.; Diaz, G.; Holman, T. R.; *Chem. Biol. Drug Des.* **2015**, *86*, 114.
63. Aldrich, C.; Bertozzi, C.; Georg, G. I.; Kiessling, L.; Lindsley, C.; Liotta, D.; Merz Jr., K. M.; Schepartz, A.; Wang, S.; *ACS Cent. Sci.* **2017**, *3*, 143.
64. Haruta, S.; Kanno, N.; *Microbes Environ.* **2015**, *30*, 123.
65. Rao, Y. K.; Fang, S.-H.; Tzeng, Y.-M.; *Bioorg. Med. Chem.* **2009**, *17*, 7909.
66. Goswami, P.; Talukdar, M.; Bora, T. C.; Phukan, P.; Sarma, J. C.; *C. R. Chim.* **2013**, *16*, 442.
67. Kumar, V.; Kumar, S.; Hassan, M.; Wu, H.; Thimmulappa, R. K.; Kumar, A.; Sharma, S. K.; Parmar, V. S.; Biswal, S.; Malhotra, S. V.; *J. Med. Chem.* **2011**, *54*, 4147.
68. Pathak, V.; Ahmad, I.; Kahlon, A. K.; Hasanain, M.; Sharma, S.; Srivastava, K. K.; Sarkar, J.; Shankar, K.; Sharma, A.; Gupta, A.; *RSC Adv.* **2014**, *4*, 35171.
69. Mohamed, N.; Hamada, M.; Yousef, N.; Abdo, M.; *Molecules* **2015**, *20*, 10468.
70. Kakati, D.; Sarma, J. C.; *Chem. Cent. J.* **2011**, *5*, article 8.
71. Zhang, J.; Fu, X.-L.; Yang, N.; Wang, Q.-A.; *Sci. World J.* **2013**, article ID 649485.
72. Clinical and Laboratory Standards Institute (CLSI); *CLSI Document M26-A: Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline*; CLSI: Wayne, PA, USA, 1999.
73. Iten, F.; Saller, R.; Abel, G.; Reichling, J.; *Planta Med.* **2009**, *75*, 1231.
74. Doern, C. D.; *J. Clin. Microbiol.* **2014**, *52*, 4124.
75. Cole, M. D.; *Biochem. Syst. Ecol.* **1994**, *22*, 837.
76. Zampini, I. C.; Vattuone, M. A.; Isla, M. I.; *J. Ethnopharmacol.* **2005**, *102*, 450.
77. Cos, P.; Vlietinck, A. J.; Berghe, D. V.; Maes, L.; *J. Ethnopharmacol.* **2006**, *106*, 290.
78. McManus, P. S.; Stockwell, V. O.; Sundin, G. W.; Jones, A. L.; *Ann. Rev. Phytopathol.* **2002**, *40*, 443.
79. Riggoti, S.; Gindro, K.; Richter, H.; Viret, O.; *FEMS Microbiol. Lett.* **2002**, *209*, 169.
80. Bekker, T.; Labuschagne, N.; Kaiser, C.; *S. Afr. Avocado Grow. Assoc. Yearb.* **2005**, *28*, 60.

81. Horta, M.; Sousa, N.; Coelho, A. C.; Neves, D.; Cravador, A.; *Physiol. Mol. Plant Pathol.* **2008**, *73*, 48.
82. Azor, M.; Gené, J.; Cano, J.; Sutton, D. A.; Fothergill, A. W.; Rinaldi, M. G.; Guarro, J.; *Antimicrob. Agents Chemother.* **2008**, *52*, 2228.
83. Salzman, R. A.; Tikhonova, I.; Bordelon, B. P.; Hasegawa, P. M.; Bressan, R. A.; *Plant Physiol.* **1998**, *117*, 465.
84. Kirubakaran, S. I.; Begum, S. M.; Ulaganathan, K.; Sakthivel, N.; *Plant Physiol. Biochem.* **2008**, *46*, 918.
85. Zhu, G.; Huang, F.; Feng, L.; Qin, B.; Yang, Y.; Hen, Y.; Lu, X.; *Agric. Sci. China* **2008**, *7*, 831.
86. Meepagala, K. M.; Sturtz, G.; Wedge, D. E.; *J. Agric. Food Chem.* **2002**, *50*, 6989.
87. Alizadeh, H. R.; Sharifi-Tehrani, A.; Hedjaroude, G. A.; *Commun. Agric. Appl. Biol. Sci.* **2007**, *72*, 795.
88. *Sigma Scan Pro V. 5.0*; Systat Software Inc., Chicago, Illinois, USA, 2008.
89. *GraphPad Prism Demo V.7*; GraphPad Software Inc., La Jolla California, USA, 2017.
90. *Molecular Operating Environment (MOE), 2013.08*; Chemical Computing Group ULC, Montreal, QC, Canada, 2018.
91. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams-Young, D.; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Keith, T.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.J.; Ochterski, W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J.; *Gaussian 09, Revision A.02*; Gaussian, Inc., Wallingford, CT, 2016.
92. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J.; *J. Comput. Chem.* **2009**, *16*, 2785.
93. Osorio, M.; Quiroz, K.; Carvajal, M.; Vergara, A.; Sánchez, E.; González, C.; Catalán, K.; *J. Chil. Chem. Soc.* **2016**, *61*, 3095.
94. *GraphPad Prism, version 6.00 for Mac OS X*; GraphPad Software, La Jolla, California, USA, 2015.

Submitted: July 9, 2018

Published online: September 13, 2018