Antimicrobial and Cytotoxicity Potential of Acetamido, Amino and Nitrochalcones

Authors: T. C. Tristão[1], F. Campos-Buzzi[1, 2], R. Corrêa[1, 2], R. C. B. Cruz[2], V. Cechinel Filho[1, 2], A. Bella Cruz[1, 2]

Affiliations: [1] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade do Vale do Itajaí, Itajaí, Brazil
[2] Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí, Itajaí, Brazil

Key words
- antibacterial
- antifungal
- Artemia salina
- QSAR
- chalcone

Abstract

Background: Chalcones constitute one of the major classes of natural products belonging to the flavonoid family, and they have been reported as having a range of important therapeutic activities, including some chalcones are effective as antimicrobial agents. Currently, the search for new structures with antimicrobial activity has been intensified due to the emergence of many strains resistant to antibiotics currently used to treat infectious diseases.

Method: 3 chalcone series (amino, acetamido and nitrochalcones) were prepared (23 compounds) and evaluated for their antimicrobial and cytotoxic potential. The effects of substituents on their respective activities also was evaluated.

Results & Conclusion: The results showed that 4 aminochalcones (**2**, **4**, **8, 9**), 3 acetoamidochalcones (**10**, **14**, **18**) and 3 nitrochalcones (**20**, **22**, **23**), exhibited antifungal effects. The aminochalcones were more toxic than the acetamidochalcones, while the nitrochalcones did not present any toxic effect. It was verified that there seems to be structure-activity correlation in some electron-donating and withdrawing substituents groups in rings A and B of the synthetized chalcone analogues and its antifungal and cytotoxic activity.

received 10.04.2012
accepted 18.09.2012

Bibliography
DOI http://dx.doi.org/10.1055/s-0032-1327610
Published online: October 19, 2012
Arzneimittelforschung 2012; 62: 590–594

ISSN 0004-4172

Correspondence
Alexandre Bella Cruz (PhD)
Centro de Ciências da Saúde
Universidade do Vale do Itajaí (UNIVALI)
Rua Uruguai 458
Itajaí 88302-202, SC
Brazil
Tel.: +55/47/3341 7540
Fax: +55/47/3341 7501
bellacruz@univali.br

Introduction

The chalcone class has a common structural framework of 1,3-diphenyl-2-propen-1-one, with 2 aromatic rings joined by a 3-carbon α,β-unsaturated carbonyl system, and constitutes one of the major classes of natural products belonging to the flavonoid family [1].

Chalcones have been reported as having a range of important therapeutic activities, such as antihypertensive and cardiovascular activity, antiprotozoal, anti-inflammatory, antidiabetic, nitric oxide inhibitory effect, anticancer, antiparasitary and antimicrobial activities [2–6].

The antimicrobial activity of chalcones has been investigated by a number of researchers. Sato et al. [7] report growth inhibitory properties of hydroxyl chalcones to *Candida spp* and Nowakowska [5] review the antimicrobial and anti-inflammatory activity of chalcones. Tomar et al. [8] report the synthesis and antimicrobial activity of chalcones containing the piperazine or 2,5-dichlorothiophene moiety. Recently Lahtchev et al. [9] reported a mechanistic study on chalcones using various yeast strains, as well as, Nowakowska et al. [10] and López et al. [11] reported the antibacterial and antifungal activities of these compounds.

Since chalcones are promising from a medical perspective, the aim of this study was to synthesize chalcone analogues, i.e., amino, acetamido and nitrochalcones, and evaluate their antibacterial and antifungal activity against a panel of human pathogens, by determining the minimum inhibitory concentration (MIC) and, preliminary cytotoxicity of the compounds. Also the structure-activity relationship (QSAR) was evaluated.

Materials and methods

Synthesis of acetamido, amino and nitrochalcones

4-Nitroacetofenone and 4-aminoacetofenone were acquired from commercial source, (Aldrich reagents). N-(4-acetylphenyl)acetamide was prepared from a solution of *p*-aminoacetophenone (2.2 g, 16.27 mmol) in water and stirred at room temperature, followed by the addition of acetic anhydride (4.1 mL, 43.43 mmol). The reaction

mixture was then heated at reflux for 2 h. The solution was cooled in an ice bath and the resulting crystals were washed with cold water and filtered under vacuum, giving a yield of 85 % of the titer compound.

All the chalcones were synthesized by addition of 50 % w/v aq. NaOH solution (1.5 mL) to a well stirred solution of appropriate substituted acetofenone (1.7 mmol) and the appropriate substituted benzaldehyde (1.7 mmol) in methanol (50 mL). The reaction mixture was stirred overnight at room temperature, according to the methodology described previously [12]. The mixture was then neutralized with 1 N HCl and the product filtered and extracted with chloroform. The combined organic layers was dried (Na_2SO_4), filtered and evaporated. The products were purified by column chromatography or recrystallization from ethyl alcohol or ethyl alcohol/water.

Chemistry – General experimental procedures

Melting points were determined with a Microquimica AP-300 apparatus (Florianópolis, Brazil) and were uncorrected. IR spectra were recorded with a Bomem 100 (Québec, Canadian) on KBr disks. The ^{13}C and ^{1}H-NMR spectra were recorded on a Brucker 200 MHz (Karlsruhe, Germany). Elemental analysis was determined with a Perkin Elmer 2400 (Norwalk, USA). The percentages of C and H were in agreement with the product formula (within ± 0.4 % of theoretical values). The compounds were dissolved in deuterated solvents from commercial sources with TMS as the internal standard. The purity of the synthesized substances was monitored by thin-layer chromatography (TLC) using Sigma (St. Louis, USA) silica pre-coated plastic plates of 200 µm in thickness with several solvent systems of different polarities. Spots were visualized by short-wave UV light and iodine vapor. Spectral data (IR, ^{1}H- and ^{13}C- NMR) and elemental analysis were in good agreement with the structures. The *log P* values were obtained from the on-line JME molecular editor program Molinspiration, courtesy of Peter Ertl of Novartis, available free on the web site: http://www.molinspiration.com/cgi-bin/properties.

Biological assay – Microorganisms

For the antimicrobial evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Rosario, Argentina, and Control Lab (CL), Rio de Janeiro, Brazil, were used; Bacteria used were *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 23858, *Enterobacter cloacae* ATCC 35030, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 25933, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus saprophyticus* ATCC 35552 and *Streptococcus agalactie* ATCC 13813, and the fungi used were *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Aspergillus niger* ATCC 9092, *Rhizopus* sp CL 35, *Microsporum canis* C112, *Microsporum gypseum* C115, *Trichophyton mentagrophytes* ATCC 9972, *Trichophyton rubrum* C137, *Cryptococcus neoformans* ATCC 32264, *Candida albicans* ATCC 10231 and *Candida krusei* ATCC 6582.

Media and inocula

The bacteria used were cultivated on Mueller-Hinton agar (MHA – Difco) at 35 °C for 24 h. Cell suspension in saline (0.86 %) was adjusted to give a final concentration of 1.5×10^8 cell/mL, standardized with 0.5 on the McFarland scale (λ = 530 nm) [13]. The fungi were cultivated on Sabouraud dextrose agar (SDA – Difco). For the filamentous fungi, the suspensions were obtained according to the reported procedures, and were adjusted to between 1.0×10^6 and 5.0×10^6 spores/mL by microscopic enumeration using a hemocytometer [14]. The yeasts were prepared according to Pfaller et al. [15], adjusting the suspension to give a final concentration of between 1.0×10^6 and 5.0×10^6 cell/mL, also standardized with 0.5 on the McFarland scale (λ = 530 nm).

Antimicrobial evaluation

The minimum inhibitory concentration (MIC) was determined for the organisms by the agar dilution method, which was carried out on slants (1 mL). Stock solutions of each compound in dimethylsulfoxide (DMSO) was diluted to give serial 2-fold dilutions which were added to each medium (MHA for bacteria and SDA for fungi), resulting in 10 different concentrations ranging from 10 to 100 µg/mL. Afterwards, a volume of 1 µL of inoculum suspension, prepared previously, was inoculated with a sterile loop to each slant, with the exception of the sterile control. The antibacterial and antifungal agents, vancomycin (Sigma V2002) and ketoconazole (Sigma K1003), respectively, were included in the assay as positive control. The final concentration of DMSO in the assay did not exceed 2 %. A drug-free saline solution (0.86 %) was used as a blank control. Each assay was repeated 3 times. The slants were incubated at 35 °C for the bacteria and yeasts and at 25 °C for the hialohyphomycete and dermatophyte strains. MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi.

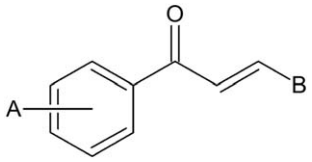
Cytotoxicity activity

The brine shrimp lethality test (BST) (*Artemia salina* Leach) was used for cytotoxicity determination of the compounds. Dried brine shrimp eggs (1 g/L)(Maramar, Arraial do Cabo, RJ, Brazil) were placed in artificial sea water [16]. The pH was adjusted to 9.0 using Na_2CO_3 and incubated at 22–29 °C with strong aeration, under a continuous light regime (2 000 Lux). Approximately 12 h after hatching the phototropic nauplii were collected for the cytotoxicity assay. Serial dilutions were made in the wells of 96-well microplates in triplicate in 100 µL artificial sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10 organisms was added to each well and the plate covered and incubated at 22–29 °C. The toxicity was determined after 24 h of exposure. The numbers of survivors were counted. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation [17].

Statistical analysis

Antimicrobial activity assays were performed in triplicate and repeated twice and the arithmetic means of the MIC were calculated and reported. Cytotoxicity assays were carried out in duplicate, and repeated twice for each compound. Lethal dose (LC_{50}) values and 95 % confidence intervals were determined from 24 h counts using the probit analysis method decribed by Finney [18]. In cases where data were insufficient for this technique, the dose-response data were transformed into a straight line by means of a logic transformation [19].

Table 1 Structure and physical data of chalcone derivatives.



	Code	Substituent in ring		Reaction	Yield	Mp[a]	*log P*
		A	B	time (h)	(%)	(°C)	
Aminochalcones	**1**	4-NH_2	C_6H_5	30	52	102.0–103.0	3.26 ± 0.37
	2	4-NH_2	4-$CH_3OC_6H_4$	11	77	111.5–113.2	3.20 ± 0.38
	3	4-NH_2	4-$CH_3C_6H_4$	10	82	144.5–145.0	3.72 ± 0.38
	4	4-NH_2	4-ClC_6H_4	2.5	77	160.8–162.2	3.78 ± 0.39
	5	4-NH_2	3,4-$Cl_2C_6H_3$	2.5	78	191.1–192.0	4.26 ± 0.41
	6	4-NH_2	4-$NO_2C_6H_4$	10	88	219.3–219.8	3.03 ± 0.41
	7	4-NH_2	4-$N(CH_3)_2C_6H_4$	2.5	53	182.7–184.9	3.76 ± 0.46
	8	4-NH_2	Furan-2-yl	2	79	115.2–115.8	2.88 ± 0.39
	9	4-NH_2	Thiophen-2-yl	10	86	112.5–113.8	3.07 ± 0.56
Acetamidochalcones	**10**	4-$NHCOCH_3$	C_6H_5	28	80	161.7–162.2	3.31 ± 0.37
	11	4-$NHCOCH_3$	4-$CH_3OC_6H_4$	4	84	206.5–207.0	3.26 ± 0.38
	12	4-$NHCOCH_3$	4-$CH_3C_6H_4$	5	79	197.5–199.0	3.77 ± 0.38
	13	4-$NHCOCH_3$	4-ClC_6H_4	2	83	215.0–215.7	3.84 ± 0.39
	14	4-$NHCOCH_3$	3,4-$Cl_2C_6H_3$	4.5	95	210.4–211.0	4.32 ± 0.41
	15	4-$NHCOCH_3$	4-$NO_2C_6H_4$	4.5	89	239.7–241.0	3.09 ± 0.41
	16	4-$NHCOCH_3$	4-$N(CH_3)_2C_6H_4$	4	72	150.9–151.7	3.82 ± 0.46
	17	4-$NHCOCH_3$	Thiophen-2-yl	1	95	118.1–119.0	2.93 ± 0.39
	18	4-$NHCOCH_3$	Furan-2-yl	1	90	147.0–149.3	3.13 ± 0.56
Nitrochalcones	**19**	H	4-$NO_2C_6H_4$	2	93	166.3–166.8	3.79 ± 0.40
	20	4-CH_3O	4-$NO_2C_6H_4$	4	91	174.4–174.9	3.96 ± 0.41
	21	4-CH_3	4-$NO_2C_6H_4$	10	65	163.7–165.9	4.25 ± 0.40
	22	3,4-Cl_2	4-$NO_2C_6H_4$	10	82	189.6–190.2	5.23 ± 0.42
	23	4-F	4-$NO_2C_6H_4$	3.5	80	171.7–172.4	4.01 ± 0.47

[a] Melting points were uncorrected

Results

Chalcone Derivatives

3 chalcone series were prepared (9 aminochalcones, 9 acetamidochalcones and 5 nitrochalcones), resulting in 23 compounds, as shown in **Table 1**. All the compounds were obtained in good yields (53–95%), and were characterized by melting point and conventional spectral data. Inspection of the ^{1}H-NMR spectra suggested that the chalcones were geometrically pure and presented trans configurations (J = 15–16 Hz).

Antimicrobial activities

All the compounds were tested for their growth inhibitory activity against bacteria, yeast and filamentous fungi. The results, shown in **Table 2**, show that antifungal effects were obtained for the compounds of the 3 series against filamentous fungi. In contrast, none of the compounds tested was active against the bacteria and yeasts.

Cytotoxicity activities

The evaluation of the cytotoxicity of the studied chalcones was carried out through the BST. Such screening was used to obtain some idea of their possible effects, since this assay has been considered a useful tool for preliminary assessment of toxicity potential [20].

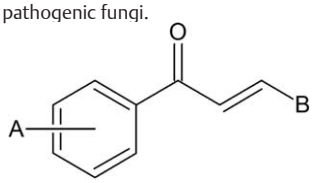
The results showed that the majority of the compounds evaluated were not toxic against *Artemia salina* up to a maximum of LC_{50} 1 000 µg/mL. **Table 3** shows the compounds with high cytotoxicity, like those with values of LC_{50} 31.25 µg/mL (**1**, **2**, **3**), and LC_{50} 62.94 µg/mL (**8**, **9**). The aminochalcones, therefore, presented greater toxic potential than the acetamidochalcones, and the nitrochalcones, which did not present any toxic effect.

Discussion

As can be observed from **Table 2**, the compounds **8**, **9**, **14**, **22** and **23** presented considerable activity against *A. niger*. This fungi is considered the most recalcitrant fungus of the filamentous pathogen group. On the other hand, *Rhizopus* sp was the most resistant filamentous fungus to the chalcones tested, whereas compounds **2**, **4**, **10**, **18**, **20** and **22**, showed activity against one or more dermatophytes, and the acetamidochalcone **10** being the most active, with an MIC value of 20 µg/mL (74 µM). Dermatophytes are considered the main organisms responsible for onychomicosis [21]. Furthermore, despite the availability of new systemic antifungal therapies, nail infections are difficult to eradicate, and recurrence is reported in up to 25–40% of cases [22]. It should also be noted that they are difficult to eradicate due to the invasion of the tissues with hyphal elements which, if not killed, have the ability to regenerate.

Although the synthesis of the amino, acetamido and nitrochalcones present electron-withdrawing and electron-donating substituent groups, as well as different degrees of lipophilicity (*log P*) (**Table 1**), the results show that it is not possible to establish an effective structure-activity relationship with the parameters studied.

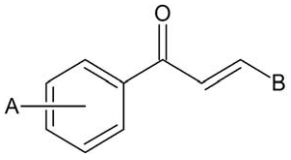
Table 2 Antifungal activity of chalcone compounds against pathogenic fungi.



	Code	Substituent		Minimum Inhibitory Concentration (µg/mL)				
		A	B	*A. nig*	*M. can*	*M. gyp*	*T. men*	*T. rub*
Aminochalcones	**2**	4-NH_2	4-$CH_3OC_6H_4$	>100	>100	>100	40	>100
	4	4-NH_2	4-ClC_6H_4	>100	>100	100	20	60
	8	4-NH_2	Furan-2-yl	40	>100	>100	>100	>100
	9	4-NH_2	Thiophen-2-yl	80	>100	>100	>100	>100
Acetamido-chalcones	**10**	4-$NHCOCH_3$	C_6H_5	>100	20	>100	20	20
	14	4-$NHCOCH_3$	3,4-ClC_6H_3	80	>100	>100	>100	>100
	18	4-$NHCOCH_3$	furanyl	>100	40	>100	>100	>100
Nitro-chalcones	**20**	4-CH_3O	4-$NO_2C_6H_4$	>100	>100	>100	>100	40
	22	3,4-Cl	4-$NO_2C_6H_4$	80	>100	>100	>100	100
	23	4-F	4-$NO_2C_6H_4$	80	>100	>100	>100	>100
	Keto	–	–	4	8	6	8	3

Ketoconazole (Keto); Aspergillus niger (A. nig); Microporum canis (M. can); Microporum gypseum (M. gyp); Trichophyton mentagrophytes (T. men); Trichophyton rubrum (T. rub)

Table 3 Cytotoxic activity of chalcone compounds on *Artemia salina*.



	Code	Substituent		LC_{50} (µg/mL)
		A	B	(LCL-UCL)[a]
Aminochalcones	**1**	4-NH_2	C_6H_5	31.25 (12.65–49.35)
	2	4-NH_2	4-CH_3O C_6H_4	31.25 (12.65–49.35)
	3	4-NH_2	4-$CH_3C_6H_4$	31.25 (12.65–49.35)
	8	4-NH_2	Furan-2-yl	62.94 (20.85–82.85)
	9	4-NH_2	Thiophen-2-yl	62.94 (20.85–82.85)
Acetamido-chalcones	**10**	4-$NHCOCH_3$	C_6H_5	98.28 (61.04–143.26)
	17	4-$NHCOCH_3$	Thiophen-2-yl	795.49 (153.14–1437.85)
	18	4-$NHCOCH_3$	Furan-2-yl	888.09 (349.86–1417.69)

Lethal concentration 50% (LC_{50}); [a] 95% confidence limits; Lower confidence limit (LCL); Upper confidence limit (UCL)

Compound **10**, acetamidochalcone with a phenyl ring in position B, proved to be more active, while its analogous compound **1**, aminochalcone, with the same ring in position B, showed no activity, suggesting that the amide group in ring A may be contributing to the antifungal effect against *M. canis*, *T. mentagrophytes* and *T. rubrum*.

On the other hand, regarding the influence of the substitute on ring A, it was verified that the addition of the amino group (**1–9**) or acetamido group (**10–18**), resulted in reduction of activity, while these molecules are comparable with the chalcones without substituent in the ring A. Our results corroborate previous studies by López et al. [11], which reported an interesting structure-activity correlation in which electron-donating groups diminished the antifungal activity.

Evaluating the nitrochalcones (**19**–**23**), it was verified that compounds **22** and **23** with 3,4-chloro, and 4-fluoro in ring A, respectively, presented MIC values of 80µg/mL (248 and 294 µM) against *A. niger*, suggesting the influence of an electron-withdrawing group. Compound 20, with a 4-methoxy group (electron donating) however, was inactive against *A. niger* but it was active against other fungi species (*T. mentagrophytes*).

Regarding the cytotoxic activity, the evaluation of the physical and chemical parameters of the active compounds shows that practically all the aminochalcones with an electron-donating group in the *p*-position in the phenyl substituent of position B, as well as the one without a substituent, are highly toxic. Regarding the influence of the substituents on ring A, it was observed that aminochalcones exhibited a greater degree of toxicity, when compared with the other substituents.

In this series, some influence is noted of electronegative groups in the phenyl substituent of position B, contributing to the decrease in cytotoxicity. Since the displacement of electron density of this system coupled to substituent group, in the position B, decreases the reactivity of the amino group, in ring A, thus likely decreasing toxicity.

Regarding the nitrochalcones, it is interesting to note that the nitro group may suggest a possible toxic [23], and mutagenic actions [24], but in our results, no toxicity against *Artemia salina*

was observed (CL_{50} >1000µg/mL). It is important to mention that some clinically used drugs contain a nitroaromatic moiety, e.g. nimesulide, flutamide, tolcapone, nitrofurantoin and others. Our results seem to be consistent with those published by Dimmock et al. [25] which also evaluated chalcones **1**, **3**, **4**, **5** and **6**, and found cytotoxic effects for these compounds.

These results are interesting from a medical point of view, as previous studies report a correlation between the cytotoxicity activity detected on BST and anticancer activity [23,26]. We found this cytotoxic activity for compounds **1**, **2**, **3**, **8**, **9** and **10**, which requires further detailed bioassays in the future, to determine its specific pharmacologic activities.

In conclusion, 10 chalcone derivatives exhibited antifungal effects. By contrast, none of the tested compounds was active against bacteria and yeasts. Also, 5 aminochalcones were toxic on BST, while only one acetamidochalcone was toxic, and another 2 presented lightly toxic effects. This activity seems to be related to the electron-donating group substituent at the *p*-position in the phenyl substituent of position B. The nitrochalcones did not present any toxic effect.

Acknowledgments

This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC) and Universidade do Vale do Itajaí (UNIVALI).

Conflict of Interest

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

References

1 *Di Carlo G*, *Mascolo N*, *Izzo AA et al.* Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci 1999; 65: 337–353
2 *Ni L*, *Meng CQ*, *Sikorski JA.* Recent advances in therapeutic chalcones. Expert Opin Ther Patents 2004; 14: 1669–1691
3 *Boeck P*, *Falcão CAB*, *Leal PC et al.* Synthesis of chalcone analogues with increased antileishmanial activity. Bioorg Med Chem 2006; 14: 1538–1545
4 *Campos-Buzzi F*, *Padaratz P*, *Meira AV et al.* 4'-Acetamidochalcone derivatives as potential antinociceptive agents. Molecules 2007; 12: 896–906
5 *Nowakowska Z.* A review of anti-infective and anti-inflammatory chalcones. Eur J Med Chem 2007; 42: 125–137
6 *Bag S*, *Ramar S*, *Degani MS.* Synthesis and biological evaluation of α, β-unsaturated ketone as potential antifungal agents. Med Chem Res 2009; 18: 309–316
7 *Sato M*, *Tsuchiya H*, *Akagiri M et al.* Growth inhibitory properties of chalcones to Candida. Lett Appl Microbiol 1994; 18: 53–55
8 *Tomar V*, *Bhattacharjee G* Kamaluddina *et al.* Synthesis and antimicrobial evaluation of new chalcones containing piperazine or 2, 5-dichlorothiophene moiety. Bioorg Med Chem Lett 2007; 17: 5321–5324
9 *Lahtchev KL*, *Batovska DI*, *Parushev SP et al.* Antifungal activity of chalcones: A mechanistic study using various yeast strains. Eur J Med Chem 2008; 43: 2220–2228
10 *Nowakowska Z*, *Kedzia B*, *Schroeder G.* Synthesis, physicochemical properties and antimicrobial evaluation of new (E)-chalcones. Eur J Med Chem 2008; 43: 707–713
11 *López SN*, *Castelli MV*, *Zacchino SA et al.* In vitro antifungal evaluation and structure-activity relationships of a new series of chalcone derivatives and synthetic analogues, with inhibitory properties against polymers of the fungal cell wall. Bioorg Med Chem 2001; 9: 1999–2013
12 *Satyanarayana M*, *Tiwari P*, *Tripathi BK et al.* Synthesis and antihyperglycemic activity of chalcone based aryloxypropanolamines. Bioorg Med Chem 2004; 12: 883–889
13 *Clinical and Laboratory Standards Institute.* Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard M07-A8. Wayne, PA: CLSI, 2009
14 *Petrikkou E*, *Rodríguez-Tudela JL*, *Cuenca-Estrella AG et al.* Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J Clin Microbiol 2001; 39: 1345–1347
15 *Pfaller MA*, *Burmeister L*, *Bartlett MS et al.* Multicenter evaluation of four methods of yeast inoculum preparation. J Clin Microbiol 1988; 26: 1437–1441
16 *Lewan L*, *Andersson M*, *Morales Gomez P.* The use of Artemia salina in toxicity testing. Altern Lab Anim 1992; 20: 297–301
17 *Solis PN*, *Wright CW*, *Anderson MM et al.* A microwell cytotoxicity assay using Artemia salina (brine shrimp). Planta Med 1993; 59: 250–252
18 *Finney DJ.* Probit Analysis. Cambridge: Cambridge University Press, 1971
19 *Hafner E*, *Heiner E*, *Noack E.* 1977; Mathematical analysis of concentration-response relationship. Arzneim Forsch 1977; 27 1871–1873
20 *Colegate SM*, *Molyneux RJ.* Bioactive natural products: detection, isolation and structural determination. Boca Raton: CRC Press, 2008
21 *Sarifakioglu E*, *Seçkin D*, *Demirbilek M et al.* In vitro anti-fungal susceptibility patterns of dermatophyte strains causing tinea unguium. Clin Exp Dermatol 2007; 32: 675–679
22 *Hay RJ.* The future of onychomycosis therapy may involve a combination of approaches. Br J Dermatol. 2001; 145, S60 3–8
23 *Boelsterli UA*, *Ho HK*, *Zhou S et al.* Bioactivation and hepatotoxicity of nitroaromatic drugs. Curr Drug Metab 2006; 7: 715–727
24 *Rashid KA*, *Mullin CA*, *Mumma RO.* Structure-mutagenicity relationships of chalcones and their oxides in the Salmonella assay. Mutat Res 1986; 169: 71–79
25 *Dimmock JR*, *Zello GA*, *Allen TM et al.* Cytotoxic 4´-aminochalcones and related compounds. Pharmazie 2003; 58: 227–232
26 *Carballo JL*, *Hernández-Inda ZL*, *Pérez P et al.* A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. BMC Biotechnol [Internet] 2002 [Accessed 2012 Mar 27] Avaliable from: http://www.biomedcentral.com/1472-6750/2/17