Chem Biol Drug Des 2014; **83:** 344–349

Research Article

Synthesis of Isosteric Triterpenoid Derivatives and Antifungal Activity

Adrine Innocente[1], Bruna B. Casanova[1], Fernanda Klein[2], Aline D. Lana[2], Dariane Pereira[2], Mauro N. Muniz[1], Pascal Sonnet[3], Grace Gosmann[1], Alexandre M. Fuentefria[2] and Simone C. B. Gnoatto[1,*]

[1]Laboratório de Fitoquímica e Síntese Orgânica, Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, 90610-000 RS, Brazil
[2]Laboratório de Micologia Aplicada, Departamento de Análises, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, 90610-000 RS, Brazil
[3]Laboratoire des Glucides, FRE CNRS 3517, UFR de Pharmacie Université de Picardie Jules Verne1, Rue des Louvels, 80037 Amiens Cedex 1, France
**Corresponding author: Simone C. B. Gnoatto, simone.gnoatto@ufrgs.br*

Dermatomycoses are among the most widespread and common superficial and cutaneous fungal infections in humans. There is an urgent need to develop efficient and non-toxic antimycotic agents with a specific spectrum of activity. Triterpenes have been demonstrated to exhibit a wide range of biological activities, including antifungal activities. In this study, through hemisynthesis, we aimed to obtain triterpene-isosteric molecules from betulinic and ursolic acids to improve the antifungal activity and spectrum of action of these compounds. Six compounds were resynthesized and tested against eleven mucocutaneous and cutaneous mycotic agents. The results of the susceptibility assays were expressed as the minimal inhibitory concentration (MIC). The MIC values of the piperazinyl derivatives of ursolic and betulinic acids that were active against pathogenic yeasts were in the range of 16–32 μg/mL and 4–16 μg/mL, respectively, whereas fungicidal effects were observed at concentrations ranging from 16 to 128 μg/mL and 8 to 128 μg/mL, respectively. The piperazinyl derivative of betulinic acid exhibited an antifungal profile similar to that of terbinafine and was the most effective derivative against dermatophytes. This strategy led to a promising candidate for the development of a new antifungal agent.

Key words: antifungal activity, betulinic acid, mucocutaneous and cutaneous mycosis agents, synthesis, triterpenes, ursolic acid

Received 5 June 2013, revised 4 September 2013 and accepted for publication 15 October 2013

The risk of fungal infections has increased over the last decades, predominantly among individuals who are immunocompromised due to malignancies, AIDS, and the use of intensive chemotherapy and immunosuppressive drugs (1–3). Mycoses are not always successfully treated because the available antifungal drugs may be ineffective, produce drug-related toxicity, or lead to the development of resistance (4). The development of resistant fungal strains in response to the extensive use of antifungal drugs in the past decades is likely to cause serious problems in the future. Consequently, there is an urgent need to develop efficient and non-toxic antimycotic agents with a specific spectrum of activity (5).

Cutaneous mycoses are among the most common fungal infections and are typically caused by dermatophytes, filamentous keratinophilic fungi that use keratin as a nutrient when they infect skin, hair, and nails (6). Mucocutaneous mycosis is characterized by recurrent or persistent infections of the nails or oral and genital mucosae by *Candida albicans*, non-*albicans Candida,* and other emerging yeast species (7). The azole group of antifungal drugs has been used for the treatment of various fungal infections, especially dermatomycosis and candidiasis (8); however, the widespread use of these drugs has resulted in the development of clinically resistant strains of *Candida* spp., *Microsporum canis*, *M. gypseum*, *Epidermophyton floccosum*, *Trichophyton rubrum,* and *T. mentagrophytes* (9,10).

Triterpenes, including the pentacyclic triterpenes ursolic acid (**1**) and betulinic acid (**2**), are natural products that have been demonstrated to exhibit a wide range of biological activities (11,12). Ursolic acid was identified as one of the active components in *Rosmarinus officinalis* (13), which inhibits the growth of some food-associated bacteria and yeasts (14). Furthermore, ursolic acid was tested against seven species of fungi by Shai *et al.*, yielding minimal inhibitory concentration (MIC) values of 8–63 μg/mL. Additionally, Shai *et al.* (15) reported the excellent antifungal activity of betulinic acid against *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *M. canis,* and *C. albicans,* with MIC values of 12–250 μg/mL.

 doi: 10.1111/cbdd.12251

Our research group have been looking for semisynthetic compounds derived from natural sources, which have potential to be use in the treatment of the protozoal and fungal diseases through a simple and cheap strategy. This approach is important considering a large of protozoal and fungal disease like a *neglected* tropical diseases (NTDs). In this sense, we aim to obtain triterpene-derived molecules from ursolic and betulinic acids to improve the previously reported antifungal activity of these aglycones. Moreover, we expect to propose a preliminary structure–activity relationship that could be used in the development of new drug candidates. In this work, we continue the pharmacological investigation on new piperazinyl derivatives at C-28 of acetyl betulinic acid and acetyl ursolic acid previously synthesized by us (16,18) focusing herein to the antifungal activity considering the reported potential activity of this class of compounds.

Methods and Materials

Plant materials

The bark of *Platanus acerifolia* found on the ground was collected, and a voucher specimen, No. 182537, was deposited in the Botany Department Herbarium of Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Peels of *Malus domestica* (apple) were obtained as a byproduct from the juice industry. Bark was dried in an air-circulating stove at 40 °C for 1 week and crushed, resulting in a fine powder that was submitted to extraction.

Powdered peel materials of *M. domestica* (150 g) were submitted to extraction with ethanol by reflux for 2 h. Most of the ethanol was evaporated to dryness under vacuum, and the resulting precipitate was submitted to crystallization using AcOEt:MeOH (70:30), resulting in 2.6 g of ursolic acid **1**, which was identified by TLC and spectroscopic methods.

Powdered bark material (150 g) of *P. acerifolia* was subjected to reflux with dichloromethane for 2 h. The extract was filtered and evaporated to dryness under vacuum. The corresponding residue was submitted to crystallization using MeOH, resulting in 2.3 g of betulinic acid **2**, which was identified by TLC and spectroscopic methods.

Chemistry

All of the solvents were dried and distilled prior to use. Column chromatography (CC) was carried out on silica gel (Merck, São Paulo, Brazil, 60–230 mesh) using dichloromethane/methanol gradient eluent mixtures (CH_2Cl_2/MeOH).

IR analysis was performed with a PerkinElmer FT-IR System Spectrum BX (São Paulo, Brazil). ^1H and ^{13}C NMR spectra were recorded with a Varian Inova 300 NMR spectrometer and a Varian VNMRS 300 spectrometer. Chemical shifts are shown in parts per million (δ) with tetramethylsilane (TMS) as a reference. HR-EI-MS spectra were recorded with an UltrOTOF (Bruker Daltonics, São Paulo, Brazil) mass spectrometer. Melting points were determined using a Koffler instrument.

Synthesis

The synthetic route is presented in Scheme 1. The full details of the chemical and structural elucidation of the ursolic acid and betulinic acid derivatives have been described previously (16–18).

Antifungal activity

Isolates

The set of isolates included mucocutaneous and cutaneous mycosis agents: *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, *Candida glabrata* HCCGL01, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019, *Epidermophyton floccosum* EPF32, *Trichophyton mentagrophytes* TME22, *Trichophyton rubrum* TRU31, *Microsporum canis* MCA02, *Microsporum gypseum* MGY09, and *Scytalidium dimidiatum* SCY04. These isolates were obtained from the culture collections of the Laboratory of Applied Mycological Research, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Antifungal activity screening assay

Antifungal activity screening was performed using the disk diffusion agar method and confirmed with the broth microdilution technique. Disk diffusion testing was performed essentially as described by the Clinical Laboratory and Standards Institute (CLSI) document M44-A (19,20) using Sabouraud dextrose agar (SDA; Difco, Detroit, MI, USA). Blank disks were pretreated with 10 μL of a 512 μg/mL solution and incubated at 35 °C for 24 h. Confirmatory screening using the broth microdilution technique was performed in 96-well flat-bottom microplates, with each well containing appropriate test samples: approximately 10^4 spores or 10^3 yeast cells in the test compound at a range of concentrations (0.5–256 μg/mL) in Sabouraud broth (Difco). The plates were incubated at 35 °C for 24 h for yeast and 48–72 h for dermatophytes.

Minimal inhibitory concentration

Inocula were prepared according to the documents M27-A3 (yeast) and M38-A2 (filamentous fungi) of the Clinical Laboratory and Standards Institute (CLSI) (21,22). Minimal inhibitory concentration (MIC) values were determined by broth microdilution using the twofold dilution method according to the CLSI guidelines (21,22) with RPMI-MOPS (RPMI 1640 medium containing L-glutamine and without sodium bicarbonate (Sigma-Aldrich Co., St Louis, MO, USA) buffered to pH 7.0 with 0.165 M MOPS buffer). The sample concentrations tested ranged from 0.5 to 256 μg/mL.

The MIC was defined as the lowest concentration of sample at which the micro-organism tested did not demonstrate visible growth. A total of 64 μg/mL fluconazole (Sigma-Aldrich Co.) was used as a positive inhibition control.

Minimal fungicidal concentration

The minimal fungicidal concentration (MFC) was determined by subculturing 10-μL aliquots of all wells without visible growth in SDA with chloramphenicol (Difco) and incubating at 35 °C for 48 h for yeast and 7 days for dermatophytes. MFC values represent the lowest concentration of a compound that produces a 99.9% end-point reduction.

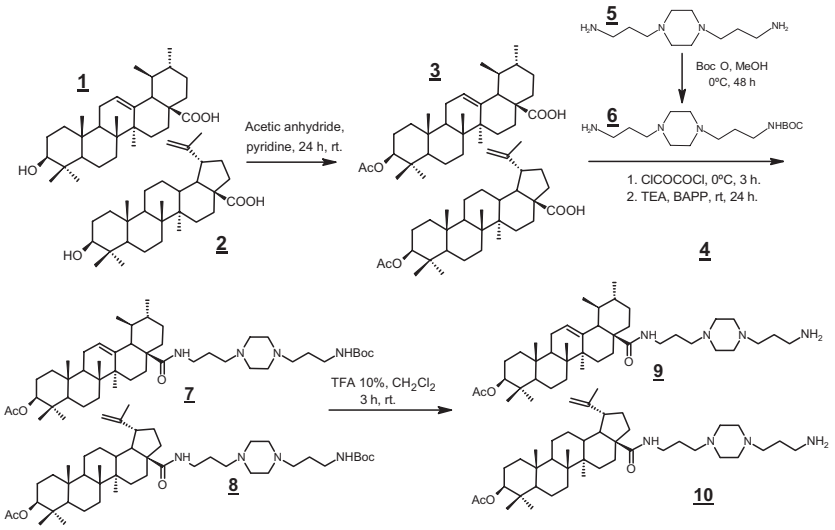
Results and Discussion

In our previous studies, we produced a series of new ursolic acid derivatives with a piperazinyl side chain that showed good antimalarial and aromatase inhibition activities (16–18). Because of the antifungal activity reported for ursolic and betulinic acids (13–15), they were also selected for a medicinal chemistry research program with the aim of identifying new triterpene series with antifungal activity. The rational design was based on the physicochemical properties and structural similarities between ursolic (**1**) and betulinic (**2**) acids (Scheme 1), considering that ursolic acid has an ursane skeleton and betulinic acid has a lupane skeleton. In the present investigation, the lupane skeleton was used as an isostere of the lead structure to develop the compound **10**.

In this work, six pentacyclic derivatives were synthesised using ursolic acid **1** and betulinic acid **2**, as shown in Scheme 1. The derivatives obtained from ursolic acid and betulinic acid were previously reported (16–18) as 3-acetylursolic acid **3**, *N*-{3-[4-(3-aminopropyl)piperazinopropyl] terbutylcarbamate}-3-acetylursolamide **7**, and *N*-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-acetylursolamide **9**. The derivatives of betulinic acid were 3-acetylbetulinic acid **4**, *N*-{3-[4-(3-aminopropyl) piperazinopropyl]terbutylcarbamate}-3-acetylbetulinamide **8**, and *N*-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-acetylbetulinamide **10** (18). All of the compounds were tested for antifungal activity against eleven mucocutaneous and cutaneous mycosis agents.

The results of the susceptibility assays are expressed as MICs (Table 1) and showed that **10** had a broad spectrum of activity and was active against both yeast and filamentous fungi. However, **9** showed antifungal activity against only yeast. Neither the aglycones alone (**1** and **2**) nor their derivatives (**3**, **4**, **7** and **8**) showed antifungal activity in the range of 5.5–256 μg/mL. Because derivatives **9** and **10** were the most active among the tested compounds, the piperazinyl substituent C-28 appears to be important for the antifungal activity of these compounds. To exclude the possibility that the activity could be due to the piperazinyl group alone, **5** was tested and showed no antifungal properties.

The MICs of **9** and **10** for pathogenic yeasts ranged from 16 to 32 μg/mL and from 4 to 16 μg/mL, respectively. Fungicidal effects were observed at concentrations ranging from 16 to 128 μg/mL and 8 to 128 μg/mL, respectively. Based on both the MIC and minimal fungicidal concentration (MFC) parameters, *C. glabrata* and *C. tropicalis* were more susceptible than other tested species. Systemic terbinafine is the treatment of choice for onychomycosis, not topical treatment (19). *In vitro* tests have shown that its MIC is much lower than those of azoles, and it is not only fungistatic but fungicidal (19). Our results showed that compound **10** presented a profile response than terbina-



Scheme 1: Synthesis of ursolic acid and betulinic acid derivatives.

Table 1: MIC and MFC values of compounds **9** (ursolic acid derivative) and **10** (betulinic acid derivative) against yeast and dermatophytes

	9 (μg/mL)		10 (μg/mL)		Antifungal drugs MIC (μg/mL)		
	MIC	MFC	MIC	MFC	AmpB[a]	Mzl[b]	Tbn[c]
Candida albicans	16	128	4	32	0.25	0.5	1
Candida krusei	32	32	16	128	0.25	0.5	8
Candida glabrata	16	16	4	8	0.25	0.25	16
Candida tropicalis	16	128	4	8	0.25	2	16
Candida parapsilosis	16	32	4	32	0.25	0.25	4
Epidermophyton floccosum	>256	NT	32	128	0.25	8	0.25
Trichophyton mentagrophytes	>256	NT	16	128	0.25	0.5	4
Trichophyton rubrum	>256	NT	64	256	0.25	0.5	0.25
Microsporum canis	>256	NT	64	256	0.25	0.5	0.25
Microsporum gypseum	>256	NT	32	256	0.25	0.5	4
Scytalidium dimidiatum	>256	NT	64	256	0.25	2	0.25

NT, not tested.
Compounds **1** to **8** did not exhibit activity against any of the tested species in the range of 0.5–256 μg/mL.
[a]Amphotericin.
[b]Miconazole.
[c]Terbinafine.

fine, as **10**'s MIC of 4 μg/mL against *C. albicans, C. glabrata*, *C. tropicalis,* and *C. parapsilosis* was better than the MIC of terbinafine against the three latter strains.

These results are important because the number of cases of sepsis involving fungi has grown in recent years, with *Candida* spp. being the most common in hospital infections.

Cutaneous mycoses are also an important cause of morbidity, especially in immunocompromised patients. Minor effectiveness of **9** against dermatophytes was observed, but compound **10** showed good activity against dermatophytes, with MICs ranging from 16 to 64 μg/mL and MFCs ranging from 64 to 256 μg/mL.

A preliminary structure–activity relationship (SAR) could be proposed:

- The piperazine chain might be a pharmacophore involved in the antifungal activities of these series of compounds.

- Through rational design based on the isosteric replacement of ursane with lupane, the importance of the lupane skeleton for antifungal activity was demonstrated.

- Compound **9** and **10** differ with respect to topological space (Figure 1). Betulinic acid and compound **10** have a *cis* junction between the A and B rings and lack a double bond in ring C compared with compound **9**, which is derived of ursolic acid. Compound **10** was effective against dermatophytes, suggesting that topological parameters could be responsible for the activity of this compound.

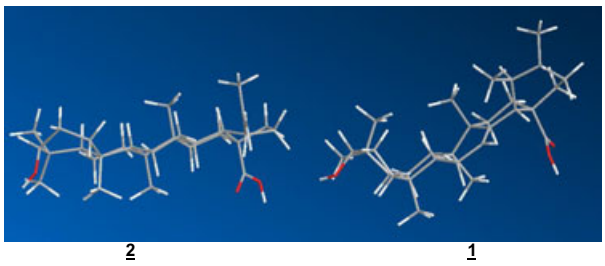


Figure 1: 3D steric images of ursolic acid (**1**) and betulinic acid (**2**). (MM2/ChemBio3D Ultra 11.0 – ChemBioOffice2008).

Finally, to determine the mode of action of the compound **10** on the integrity of the fungal cell wall, the *Sorbitol Protection Assay* was performed (23,24,26). In this assay, MIC determinations were conducted in parallel with and without 0.8 M sorbitol. Sorbitol causes a slight stress to cells, and in the presence of some weak non-specific cell wall inhibitors, cell growth can be inhibited. Results showed that MIC of the **10** did not vary in the presence of sorbitol after 7 days of incubation suggesting that **10** would not act by inhibiting the mechanisms controlling cell wall synthesis. In contrast, a 125-fold increase in MIC was observed for the positive control drug anidulafungin. *Cellular Leakage Assay* was performed to assess whether compound **10** produces fungal membrane damage (23–25). In this assay, cellular components that absorb at 260 nm represent on class of leakage components, especially nucleotides. SDS was used as reference compound, which produces 100% cellular leakage, compound **10** caused much less leakage, about 20%. To determine whether damages the fungal membrane are due binding to the ergosterol, the *Ergosterol Effect Assay* was performed.

This test is based on offering exogen ergosterol to a compound which, when possessing affinity with it, will rapidly form a complex, thus preventing the complexation with the membrane's ergosterol. As a consequence, an enhancement of MIC is observed (23,24). Ours results showed that MIC of 10 against *C. albicans* cells remains unchanged in the presence of different concentrations (50–250 μg/mL) of exogenous ergosterol, therefore suggesting that this compound did not act by binding to ergosterol. In contrast, a fourfold increase in MIC was observed for the positive control drug amphotericin B, whose interaction with ergosterol is well known.

Conclusion and Future Directions

This is the first report of the *in vitro* antifungal activity of piperazinyl derivatives of betulinic and ursolic acids against human mycosis agents. Through rational design based on the isosteric replacement of ursane with a lupane skeleton, we demonstrated that this strategy led to a substantial increase in antifungal activity. Compound **10** exhibited growth inhibitory and fungicidal activities in the range of 4–64 μg/mL against clinical yeast and dermatophyte strains. Regarding the mechanism of action, this triterpene derivative produces a weak damage in fungal membrane and did not act on the ergosterol assay. Further assays are required to elucidate the pathways, whereby this antifungal compound exerts their effects. This study may contribute to the development of new pharmaceutical prototypes derived from natural sources for treating these diseases.

Acknowledgments

This work was supported by grants and financial support from Instituto Nacional de Ciência e Tecnologia para Inovação Farmacêutica (INCT-if), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ-Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil). We also thank Prof. Dr. N. P. Lopes (USP-RP, Brazil) for the HRMS analysis, Prof. Dr. J. Duppont (UFRGS, Brazil) for the NMR analysis, and Tecnovin do Brasil for the apple material.

References

1. Pappas P.G. (2010) Opportunistic fungi: a view to the future. Am J Med Sci;340:253–257.
2. Vinh D.C., Sugui J.A., Hsu A.P., Freeman A.F., Holland S.M. (2010) Invasive fungal disease in autosomal-dominant hyper-IgE syndrome. J Allergy Clin Immunol;125:1389–1390.
3. Romani L. (2011) Immunity to fungal infections. Nat Rev Immunol;11:275–288.
4. Sheehan D.J., Hitchcock C.A., Sibley C.M. (1999) Current and emerging azole antifungal agents. Clin Microbiol Rev;12:40–79.
5. Ostrosky-Zeichner L., Casadevall A., Galgiani J.N., Odds F.C., Rex J.H. (2010) An insight into the antifungal pipeline: selected new molecules and beyond. Nat Rev Drug Discov;9:719–727.
6. Türel Ö. (2011) Newer antifungal agents. Expert Rev Anti Infect Ther;9:325–338.
7. Glocker E., Grimbacher B. (2010) Chronic mucocutaneous candidiasis and congenital susceptibility to Candida. Curr Opin Allergy Clin Immunol;10:542–550.
8. Pozzatti P., Loreto E.S., Lopes P.G.M., Athayde M.L., Santurio J.M., Alves S.H. (2008) Comparison of the susceptibilities of clinical isolates of *Candida albicans* and *Candida dubliniensis* to essential oils. Mycoses;53:12–15.
9. Gupta A.K., Cooper E.A. (2008) Update in antifungal therapy of dermatophytosis. Mycopathologia;166:353–367.
10. Law D., Moore C.B., Wardle H.M., Ganguli L.A., Keaney M.G., Denning D.W. (1994) High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS. J Antimicrob Chemother;34:659–368.
11. Liu J.J. (1995) Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol;49:57–68.
12. Liu J.J. (2005) Oleanolic acid and ursolic acid: research perspectives. J Ethnopharmacol;100:92–94.
13. Collins M.A., Charles H.P. (1987) Antimicrobial os carnosol and ursolic acid: two anti-oxidant constituents of *Rosmarinus officinalis* L. Food Microbiol;4:311–315.
14. Becker H., Scher J.M., Speakman J., Zapp J. (2005) Bioactivity guided isolation of antimicrobial compounds from *Lythrum salicaria*. Fitoterapia;76:580–584.
15. Shai L.J., McGaw L.J., Aderoga M.A., Mdee L.K., Eloff J.N. (2008) Four pentacyclic triterpenoids with antifungal and bacterial activity from curtisia dentata (Burm.f) C.A. Sm Leaves. J Etnopharmacol;119:238–244.
16. Gnoatto S.C.B., Susplugas S., Dalla Vechia L., Ferreira T.B., Dassonville-Klimpt A., Zimmer K.R., Demailly C., Nascimento S.D., Guillon J., Grellier P., Verli H., Gosmann G., Sonnet P. (2008) Pharmacomodulation on the 3-acetylursolic acid skeleton: design, synthesis, and biological evaluation of novel *N*-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-*O*-acetylursolamide derivatives as antimalarial agents. Bioorg Med Chem;16:771–782.
17. Gnoatto S.C.B., Dassonville-Klimpt A., Da Nascimento S., Galerá P., Boumediene K., Gossman G., Sonnet P., Moslemi S. (2008) Evaluation of ursolic acid isolated from Ilex paraguaiensis and derivatives on aromatase inhibition. Eur J Med Chem;43:1865–1877.
18. Innocente A.M., Silva G.N.S., Cruz L.N., Moraes M.S., Nakabashi M., Sonnet P., Gosmann G., Garcia C.R.S., Gnoatto S.C.B. (2012) Synthesis and antiplasmodial activity of betulinic acid and ursolic acid analogues. Molecules;17:12003–12014.

19. Elewski B., Tavakkol A. (2005) Safety and tolerability of oral antifungal agents in the treatment of fungal nail disease: a proven reality. Ther Clin Risk Manage;1:299–306.
20. Clinical Laboratory Standards Institute (2004) Methods for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline M44-A. Wayne, PA: Clinical Laboratory Standards Institute.
21. Clinical Laboratory Standards Institute (2003) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard, 3nd edn. CLSI Document M27-A3. Villanova, PA: Clinical Laboratory Standards Institute.
22. Clinical Laboratory Standards Institute (2002) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved standard. CLSI document M38-A. Villanova, PA: Clinical Laboratory Standards Institute.
23. Escalante A., Gattuso M., Pérez P., Zacchino S. (2008) Evidence for the mechanism of action of the antifungal phytolaccoside B isolated from *Phytolacca tetramera* Hauman. J Nat Prod;71:1720–1725.
24. Carrasco H., Raimondi M., Svetaz L., Di Liberto M., Rodriguez M.V., Espinoza L., Madrid A., Zacchino S. (2012) Antifungal activity of eugenol analogues. Influence of different substituents and studies on mechanism of action. Molecules;17:1002–1024.
25. Lunde C., Kubo I. (2000) Effect of polygodial on the mitochondrial ATPase of *Saccharomyces cerevisiae*. Antimicrob Agents Chemother;44:1943–1953.
26. Frost D., Brandt K., Cugier D., Goldman R. (1995) A whole-cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. J Antibiot;48:306–310.