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ORIGINAL ARTICLE

Fisetin as a promising antifungal agent against *Cryptococcus neoformans* species complex

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Abstract

Aims: The aim of this study was to investigate the mechanisms of action of fisetin, a flavonol with antifungal activity previously evaluated against the *Cryptococcus neoformans* species complex.
Methods and Results: Ergosterol content and flow cytometry analysis were determined for the *C. neoformans* species complex in the presence of fisetin and ultrastructural analysis of morphology was performed on *Cryptococcus gattii* and *C. neoformans*. Decrease in the total cellular ergosterol content after exposure to fisetin ranged from 25·4% after exposure to 128 μg ml^{-1} to 21·6% after exposure to 64 μg ml^{-1}of fisetin compared with the control (without fisetin). The fisetin effects obtained with flow cytometry showed metabolic impairment, and alterations in its normal morphology caused by fisetin in *C. neoformans* cells were verified using scanning electron microscopy.
Conclusions: Fisetin is a compound that acts in the biosynthesis of ergosterol. Flow cytometry showed that fisetin reduced viability of the metabolically active cells of *C. gattii*, while morphological changes explain the action of fisetin in inhibiting growth of these fungi.
Significance and Impact of the Study: This study supports the idea that fisetin may represent a good starting point for the development of future therapeutic substances for cryptococcosis.

Introduction

Increases in antimicrobial resistance, the number of patients at moderate risk and the side effects of commercially available antifungal drugs have increased the need to develop new and more effective antifungal agents. A large number of medicinal plants contain compounds with antimicrobial activity (Cowan 1999). Among the plant compounds, flavonoids have been considered to be possible sources of new therapeutics for viral, bacterial and fungal infections (Zandi *et al.* 2011; Shafaghat *et al.* 2012).

The *Cryptococcus neoformans* species complex, which is pathogenic to humans, is the causative agent of cryptococcosis and is responsible for over 600 000 deaths per year, especially in patients with AIDS (Bose *et al.* 2003; Zaragoza *et al.* 2010). The yeast agents of cryptococcal meningitis fungal infection are ubiquitous in the environment and most people are exposed to them (Goldman *et al.* 2001). The drugs available to treat cryptococcosis are toxic and require prolonged use, which leads to the development of resistance. Thus, resistance is a problem in the clinical response to treatment (Sanglard *et al.* 2009). Fisetin (3,3′4′7-tetrahydroxyflavone), a flavonol commonly found in the resin of *Hymenaea courbaril* L. (popularly known as jatoba in Brazil), possesses antioxidant and anti-inflammatory antimicrobial activities (Woodman and Chan 2004; Park *et al.* 2007; Touil *et al.* 2011). This compound presents low toxicity, with an IC50 to a Balb/c 3T3-A31 fibroblast cell line greater than the minimal inhibitory concentration detected *in vitro* against *C. neoformans* (Costa *et al.* 2014).

The limited knowledge about the mechanism of action in plants and their compounds has led us to address this issue. In this work, the mechanisms of action of fisetin against the *C. neoformans* species complex were evaluated.

Materials and methods

Flavonoid fisetin

Fisetin, a compound extracted from fresh xylem sap of *H. courbaril* was analyzed and characterized by ^{1}H and ^{13}C NMR [Varian Mercury plus BB spectrometer, operating at 300·059 MHz (^{1}H) and 75·458 MHz (^{13}C) using $CDCl_3$ solutions with TMS as an internal standard] according to Costa *et al.* (2014). This compound was dissolved in dimethylsulfoxide (DMSO) (Sigma, St Louis, MO) to prepare the stock solution (2048 μg ml^{-1}) and stored at −20°C until further use.

Fungal strains

The fungal strains *C. neoformans* (ATCC 28957) and *Cryptococcus gattii* (ATCC 24065), were used in this study. They were maintained on Sabouraud dextrose agar at −70°C (Difco Laboratories, EUA) and subcultured on the same medium for 72 h before testing at 28°C.

Quantification of ergosterol content

Total intracellular sterols were extracted as described by Arthington-Skaggs *et al.* (1999). Briefly, a suspension of cells of one strain of the *C. neoformans* grown on Sabouraud dextrose agar adjusted with a haemocytometer to a final density of 10^6 CFU ml^{-1} was incubated in 50 ml of Sabouraud dextrose broth (Difco® Laboratories, EUA) containing 1× MIC (128 μg ml^{-1}) or ½ MIC (64 μg ml^{-1}) of fisetin; and 1× MIC (0·25 μg ml^{-1}) or ½ MIC (0·125 μg ml^{-1}) of itraconazole along with positive control (without test compound). These MIC values were previously determined by *in vitro* susceptibility method according to Costa *et al.* (2014).

The cultures were maintained at 35°C for 72 h with shaking at 200 rev min^{-1}. The stationary phase cells were harvested by centrifugation at 3500 ***g*** for 5 min and washed with sterile distilled water, and the net wet weight of the cell pellet was determined.

An aliquot (3 ml) of 25% alcoholic potassium hydroxide solution was added to each pellet and vortexed for 1 min, and the cell suspensions were then transferred to sterile tubes, incubated in an 85°C water bath for 4 h and allowed to cool completely to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane, followed by vigorous vortex mixing for 3 min. The heptane layer was transferred to a glass screw-cap tube and stored at −20°C for 24 h. A 1-ml aliquot of sterol extract was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 200 and 350 nm using a Varian Cary 50 Bio spectrophotometer (Mulgrave, Victoria, Australia) that was set to UV-visible in this wavelength range.

The ergosterol content was calculated as a percentage of the wet weight of the cell using the following equations:

$$\%\,\text{ergosterol} + \%24(28)\text{dehydroergosterol (DHE)} = [(A281 \cdot 5/290) \times F]/\text{pellet weight}$$

$$\%\,24(28)\text{DHE} = [(A230/518) \times F]/\text{pellet weight}$$

$$\%\,\text{ergosterol} = [\%\,\text{ergosterol} + \%\,24(28)\text{DHE}) - \%\,24(28)\text{DHE},$$

where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per centimeter) for crystalline ergosterol and 24(28) DHE respectively. Values were shown in terms of mean ± standard error of mean (SEM) of all three respective categories.

Flow cytometry

Flow cytometry analysis using propidium iodide (PI) and FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-[(benzo-1,3-thiazol-2-yl)]methylidene)-1-phenylquinolinium iodide] was performed according to the method of Pina-Vaz *et al.* (2005) and Pinto *et al.* (2009). *Cryptococcoccus neoformans* suspension was prepared in Sabouraud dextrose broth medium and incubated overnight in a water bath at 35°C with agitation at 200 rev min^{-1}. The suspension was centrifuged, washed and resuspended in phosphate-buffered saline solution (PBS) with 2% glucose (pH 7·0) and adjusted with a haemocytometer to a final density of 10^6 CFU ml^{-1}. This suspension was incubated without agitation with fisetin at concentrations of 2× MIC (256 μg ml^{-1}), 1× MIC (128 μg ml^{-1}) and ½ MIC (64 μg ml^{-1}) for 2 h at 35°C. After the incubations, the cells were washed and resuspended in 500 μl of PBS with 2% D-glucose (w/v).

The yeast cells were stained with 1·0 μg of PI/ml (Sigma) in HEPES solution (pH 7·2) supplemented with 2% glucose and incubated in the dark at room temperature for 30 min. Untreated (drug-free control) and killed cells in 70% ethanol were stained and used as controls in every experiment.

In the same manner, the yeast cells were stained with 0·5 μmol l^{-1} FUN-1 in HEPES solution with 2% glucose and incubated in the dark at room temperature for 30 min. Unstained cell suspensions (drug free) were included as autofluorescence controls, and cells treated for 1 h with 1 mmol l^{-1} sodium azide, control of metabolic impairment, were stained under the same

conditions with FUN-1 and used as controls. Flow cytometry was performed using an Accuri C6 (Becton Dickinson Biosciences, Ann Arbor, MI) flow cytometer with a 488-nm blue argon laser emitting at 15mW and the results were analyzed using CFLOW PRO Software (version 1.0.202.1, Becton Dickson Biosciences, Ann Arbor, MI).

Scanning electron microscopy

Samples for scanning electron microscopy (SEM) analysis were prepared according to the method of Faganello *et al.* (2006) with some modifications. The yeasts of the *C. neoformans* species complex (*C. neoformans* ATCC 28957 and *C. gattii* ATCC 24065) were cultured in Sabouraud dextrose agar (SDA) for 72 h at 30°C in the presence of 128 μg of fisetin. Small blocks of agar with culture were fixed overnight at 4°C with 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0·1 mol l^{-1} sodium cacodylate buffer with 3% saccharose at pH 7·2. The samples were then washed in the same buffer three times for 15 min each and postfixed with 1% (w/v) osmium tetroxide for 1 h at 4°C in the dark.

The specimens were then dehydrated in an ethanol series (30, 50, 70 and 90%) for 15 min in each ethanol dilution and finally in absolute ethanol twice for 150 min. The dehydrated specimens were dried in a critical point drier (Autosamdri, 815) with liquid CO_2 and coated with gold in a sputter-coater (Denton Vaduum, Desk V). The material was examined in a Jeol JSM 6610 scanning electron microscope at an accelerating voltage of 20 kV.

Results

Quantification of ergosterol content

Growth of the *C. neoformans* in the presence of an inhibitory or subinhibitory concentration of fisetin altered the sterol pattern of these strains. A dose-dependent decrease in ergosterol production was observed. The degree of sensitivity of the ergosterol biosynthetic pathway to the effects of fisetin was decreased, as occurred for the broth microdilution method. The decrease in the total cellular ergosterol content ranged from 25·4% after exposure to 128 μg ml^{-1} fisetin to 22·8% after exposure to 64 fisetin when compared with the control (without fisetin).

In the same way, the results demonstrated that the ergosterol content (at 282 nm) in the plasma membrane of the *C. neoformans* ATCC 28957 was inhibited by different concentrations of itraconazole. After incubation of yeast with 0·25 and 0·125 μg ml^{-1} traconazole, the percentages of the ergosterol content in the plasma membrane compared with those of the control were reduced by 38·9 and 22·8% respectively. The efficacy of fisetin (128 and 64 μg ml^{-1}) and itraconazole (0·25 and 0·125 μg ml^{-1}) on the ergosterol content in the plasma membrane of yeast is shown in Fig. 1.

Flow cytometry

The results of the flow cytometric studies with the *C. neoformans* (ATCC 28957) treated with fisetin for 2 h showed different outcomes according to the employed fluorescent probe.

The cells stained with PI showed a very low intensity of fluorescence in the cells treated with fisetin at different concentrations, a finding which agrees with the cell viability results (viability was not affected or was scarcely affected in these experimental conditions, even at the highest fisetin concentrations), while ethanol-treated cells showed high fluorescence (Fig. 2).

The yeast cells treated with FUN 1 and fisetin at concentrations of 256, 128 and 64 μg ml^{-1} showed increased fluorescence intensity compared to nontreated cells. A clear dose-dependent effect was observed at the three concentrations (Fig. 3).

Scanning electron microscopy

SEM observations revealed that the *C. neoformans* species complex cells seemed to be normal in appearance when

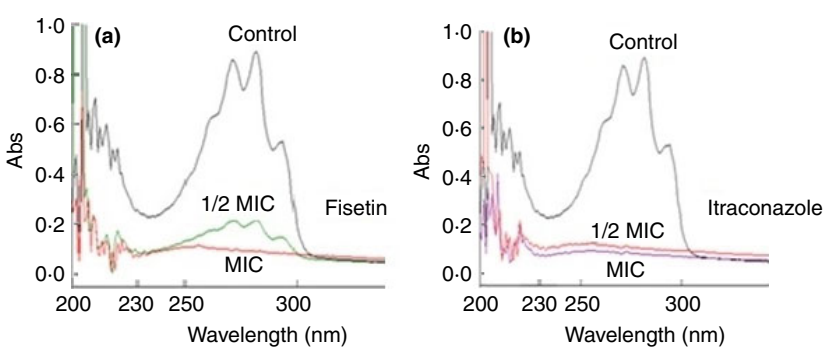

Figure 1 Inhibition of ergosterol biosynthesis in the *Cryptococcoccus neoformans* species complex. (a) UV spectrophotometric sterol profiles of cells treated with fisetin; (b) UV spectrophotometric sterol profiles of cells treated with itraconazole.

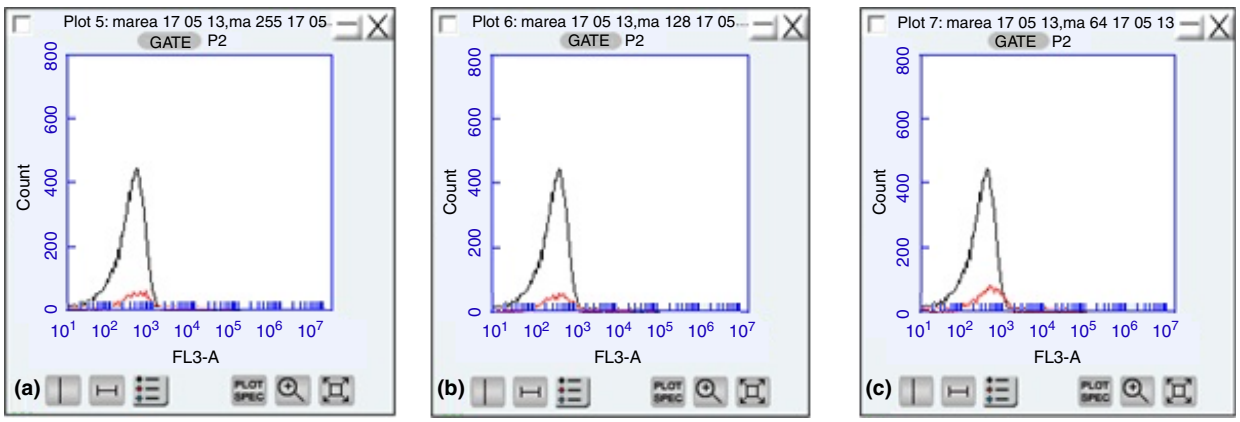

Figure 2 Propidium iodide-stained *Cryptococcus neoformans* species complex cells analyzed by flow cytometry at FL-3 log after treatment with serial concentrations of fisetin. (a) 256 μg ml^{-1}; (b) 128 μg ml^{-1} and (c) 64 μg ml^{-1}.

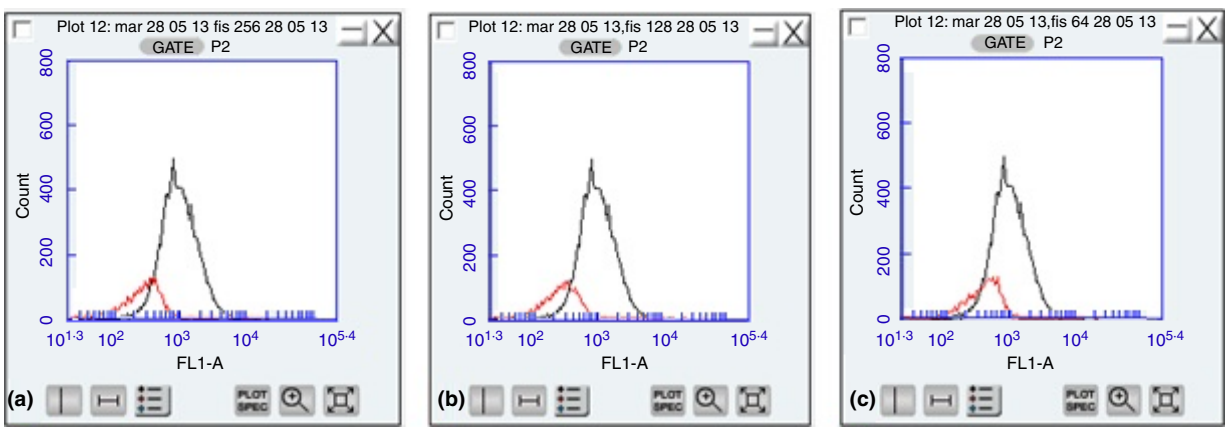

Figure 3 FUN-1-stained *Cryptococcus neoformans* species complex cells analyzed by flow cytometry at FL-1 log after treatment with serial concentrations of fisetin. (a) 256 μg ml^{-1} (b) 128 μg ml^{-1} and (c) 64 μg ml^{-1}.

cultured in SDA agar. *C. neoformans* ATCC 28957 and *C. gattii* ATCC 24065 showed globular cells with a relatively smooth surface except for rare small protrusions and large granules (Fig. 4).

After being exposed to 128 μg of fisetin, the cells changed from a normal morphology and presented a retracted cytoplasm and many small protrusions. The roughness of the surface of the cell was larger in *C. gattii* than in *C. neoformans,* as shown in Fig. 4. The capsular fibrils of yeast cells exposed to fisetin were noticeably shorter than those in control cells (data not shown).

Discussion

Some compounds such as flavonoids are strong candidates for the development of new antimicrobial agents. Fisetin, a flavonoid with antioxidant and anti-inflammatory activities (Ali *et al.* 1998), was the object of our study and proved to be a promising candidate substance for the treatment of cryptococcosis. The toxicities of available drugs, such as polyenes and azoles, can be a cause of treatment failure (Carrillo-Munoz *et al.* 2006). Fisetin exhibited wide-spectrum antifungal activity, as evidenced by MIC values against all of the *C. neoformans* species complex tested isolates in previous experiment (Costa *et al.* 2014). The mechanisms through which fisetin mediates its antimicrobial effects can be explained by its mode of action. Thus, we analyzed whether fisetin inhibited the ergosterol content of *C. neoformans* cells, affected viability and metabolism, and led to altered morphology.

Given that azole antifungal agents act by inhibition of ergosterol synthesis (Sanglard 2002), measurement of alterations in ergosterol content appeared relevant. Ergosterol is specific to fungi and is the major sterol component of the fungal cell membrane (Rodriguez *et al.* 1985).

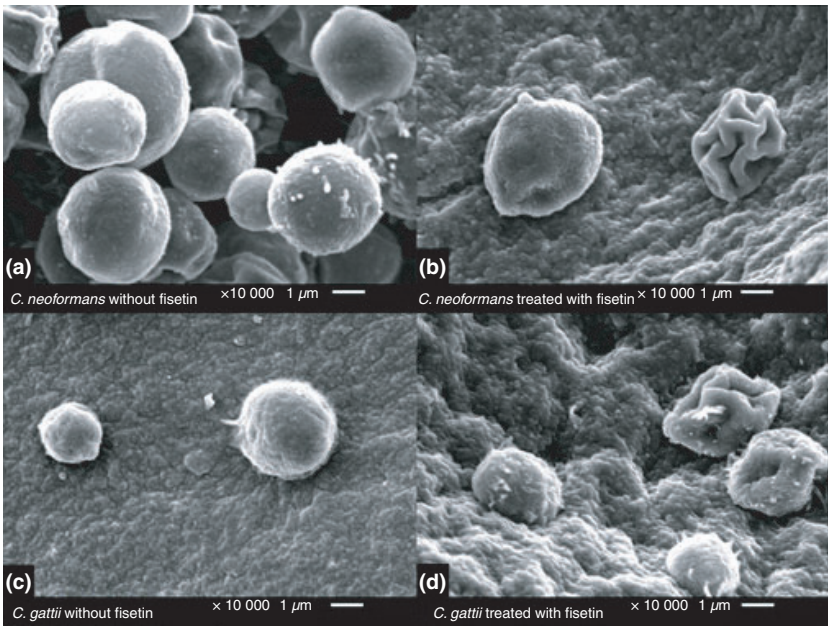

Figure 4 The effect of fisetin on the morphology of the *Cryptococococcus neoformans* species complex by SEM shows retracted cytoplasm and many small protrusions. (a) *C. neoformans* ATCC 28957 without treatment (control); and (b) Treated with fisetin; (c) *Cryptococcus gattii* ATCC 24065 without treatment (control) and (d) Treated with fisetin.

The mechanism of action by which the azole drugs inhibit fungal cell growth occurs by disruption of normal sterol biosynthetic pathways, leading to reduced ergosterol synthesis. The lower quantity of ergosterol observed in *C. neoformans* species complex isolates in the presence of inhibitory (38·9%) and subinhibitory (22·8%) concentrations of fisetin in comparison with the control revealed that this compound considerably impaired the biosynthesis of ergosterol. Similarly, some studies have shown that the essential oils of *Syzygium aromaticum* and *Anethum graveolens* L. can cause a considerable reduction in the quantity of ergosterol (Pinto *et al.* 2009; Tian *et al.* 2012). Thus, ergosterol, which is a component of the cell membrane, could be the target of fisetin.

The analysis of flow cytometry with low penetration of PI in yeast isolates in the presence of fisetin (2× MIC) showed that the structure of the cell membrane was poorly disrupted by this compound. Similar results were found in *Aspergillus fumigatus* isolates, where clove oil did not produce any appreciable PI staining (Pinto *et al.* 2009). In contrast, other reports (Carson *et al.* 2006; Pasqua *et al.* 2007; Bakkali *et al.* 2008) found that some essential oils have an influence on biological membranes. The increase in fluorescence intensity obtained with FUN-1 showed that the cells were impaired in terms of their metabolism, indicating a disorder in the cells metabolic state. In this way, it can be concluded that the effects of fisetin on metabolic impairment were more pronounced than the lesions of the cell membrane. The performance of the PI probe showed that fisetin is fungistatic. This effect was similar to triazoles, which are only fungistatic drugs (Pina-Vaz *et al.* 2001). According to Pina-Vaz *et al.* (2000), some drugs that incorporate PI in a dose-dependent manner in *Candida* cells such as Ibuprofen are considered fungicidal.

In our study, SEM showed that fisetin has an effect on the *C. neoformans* species complex through alterations of its normal morphology, with retracted cytoplasm and the formation of many small protrusions. It is difficult to explain the mechanism of any compound in fungal cell morphogenesis related to growth inhibition. According to Sharma and Tripathi (2008), modifications induced by essential oils may be related to the interference of oil components with the enzymatic reactions of cell wall synthesis, which affects fungal morphogenesis and its growth. In another work, Park *et al.* (2009) showed that the activity of citral, eugenol and α-terpineol might be due to problems of normal metabolism in fungal cells, which results from the destruction of organelles in the cytoplasm and altered morphology of the cell membrane. Although not conclusive, this last explanation seems more consistent with our work, because in flow cytometry, the flavonoid fisetin showed metabolic impairment in *C. neoformans* cells.

In addition to the target effects of a compound, the determination of toxicity is important before consideration of the compound as a potential drug. In the case of fisetin, there was no evidence of either short- or long-term toxicity. Cytotoxicity analysis showed an IC 50 that was greater than the MIC for fisetin against *C. neoformans* cells (Costa *et al.* 2014). Mice fed fisetin at 500 ppm for several months showed no indications of toxicity (Maher *et al.* 2011). Moreover, fisetin seems to have no overt systemic immunosuppressive side effects

(Gelderblom *et al.* 2012). Thus, this study supports the idea that fisetin may represent a good starting point for the development of future therapeutic substances for cryptococcosis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Ali, R.M., Houghton, P.J., Raman, A. and Hoult, J.R.S. (1998) Antimicrobial and antiinflammatory activities of extracts and constituents of *Oroxylum indicum* (L.) Vent. *Phytomedicine* **5**, 375–381.

Arthington-Skaggs, B.A., Jradi, H., Desai, T. and Morrison, C.J. (1999) Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* **37**, 3332–3337.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008) Biological effects of essential oils – a review. *Food Chem Toxicol* **46**, 446–475.

Bose, I., Reese, A.J., Ory, J.J., Janbon, G. and Doering, T.L. (2003) A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot Cell* **2**, 655–663.

Carrillo-Munoz, A.J., Giusiano, G., Ezkurra, P.A. and Quindos, G. (2006) Antifungal agents: mode of action in yeast cells. *Rev Esp Quimioter* **19**, 130–139.

Carson, C.F., Hammer, K.A. and Riley, T.V. (2006) *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clin Microbiol Rev* **19**, 50–62.

Costa, M.P., Bozinis, M.C., Andrade, W.M., Costa, C.R., da Silva, A.L., de Oliveira, C.M.A., Kato, L., Fernandes, O.F.L. *et al.* (2014) Antifungal and cytotoxicity activities of the fresh xylem sap of *Hymenaea courbaril* L. and its major constituent fisetin. *BMC Complement Altern Med* **14**, 245.

Cowan, M.M. (1999) Plant products as antimicrobial agents. *Clin Microbiol Rev* **12**, 564–582.

Faganello, J., Arruda, W., Schrank, A. and Vainstein, M.H. (2006) An alternative method to prepare samples of the pathogenic yeast *Cryptococcus neoformans* for scanning electron microscopy analysis. *J Microbiol Methods* **64**, 416–419.

Gelderblom, M., Leypoldt, F., Lewerenz, J., Birkenmayer, G., Orozco, D., Ludewig, P., Thundyil, J., Arumugam, T.V. *et al.* (2012) The flavonoid fisetin attenuates postischemic immune cell infiltration, activation and infarct size after transient cerebral middle artery occlusion in mice. *J Cerebr Blood F Met* **32**, 835–843.

Goldman, D.L., Khine, H., Abadi, J., Lindenberg, D.J., Pirofski, L.A., Niang, R. and Casadevall, A. (2001) Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics* **107**, e66–.

Maher, P., Dargusch, R., Bodai, L., Gerard, P.E., Purcell, J.M. and Marsh, J.L. (2011) ERK activation by the polyphenols fisetin and resveratrol provides neuroprotection in multiple models of Huntington's disease. *Hum Mol Genet* **20**, 261–270.

Park, H.H., Lee, S., Oh, J.M., Lee, M.S., Yoon, K.H., Park, B.H., Kim, J.W., Song, H. *et al.* (2007) Anti-inflammatory activity of fisetin in human mast cells (HMC-1). *Pharmacol Res* **55**, 31–37.

Park, M.J., Gwak, K.S., Yang, I., Kim, K.W., Jeung, E.B., Chang, J.W. and Choi, I.G. (2009) Effect of citral, eugenol, nerolidol and α-terpineol on the ultrastructural changes of *Trichophyton mentagrophytes*. *Fitoterapia* **80**, 290–296.

Pasqua, R.D., Betts, G., Hoskins, N., Edwards, M., Ercolini, D. and Mauriello, G. (2007) Membrane toxicity of antimicrobial compounds from essential oils. *J Agric Food Chem* **55**, 4863–4870.

Pina-Vaz, C., Rodrigues, A., Sansonetty, F., Martinez-de-Oliveira, J., Fonseca, A.F. and Mardh, P.-A. (2000) Antifungal activity of local anesthetics against *Candida* species. *Infect Dis Obstet Gynecol* **8**, 124–137.

Pina-Vaz, C., Sansonetty, F., Rodrigues, A.G., Costa-de-Oliveira, S., Tavares, C. and Martinez-de-Oliveira, J. (2001) Cytometric approach for a rapid evaluation of susceptibility of *Candida* strains to antifungals. *Clin Microbiol Infect* **7**, 609–618.

Pina-Vaz, C., Costa-de-Oliveira, S., Rodrigues, A.G. and Espinel-Ingroff, A. (2005) Comparison of two probes for testing susceptibilities of pathogenic yeasts to voriconazole, itraconazole, and caspofungin by flow cytometry. *J Clin Microbiol* **43**, 4674–4679.

Pinto, E., Vale-Silva, L., Cavaleiro, C. and Salgueiro, L. (2009) Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* **58**, 1454–1462.

Rodriguez, R.J., Low, C., Bottema, C.D. and Parks, L.W. (1985) Multiple functions for sterols in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **837**, 336–343.

Sanglard, D. (2002) Importancia clínica de los mecanismos de resistencias a los antifúngicos en las levaduras. *Enferm Infecc Microbiol Clin* **20**, 225–234.

Sanglard, D., Coste, A. and Ferrari, S. (2009) Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. *FEMS Yeast Res* **9**, 1029–1050.

Shafaghat, A., Salimi, F. and Mahmoodi, R. (2012) Antioxidant, antimicrobial activity and chemical analysis of the flavonoid from *Chaerophyllum macropodum* (Boiss.). *J Med Plant Res* **6**, 2111–2116.

Sharma, N. and Tripathi, A. (2008) Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiol Res* **163**, 337–344.

Tian, J., Ban, X., Zeng, H., He, J., Chen, Y. and Wang, Y. (2012) The mechanism of antifungal action of essential oil from dill (*Anethum graveolens* L.) on *Aspergillus flavus*. *PLoS ONE* **7**, e30147.

Touil, Y.S., Auzeil, N., Boulinguez, F., Saighi, H., Regazzetti, A., Scherman, D. and Chabot, G.G. (2011) Fisetin disposition and metabolism in mice: identification of geraldol as an active metabolite. *Biochem Pharmacol* **82**, 1731–1739.

Woodman, O.L. and Chan, E.C. (2004) Vascular and anti-oxidant actions of flavonols and flavones. *Clin Exp Pharmacol Physiol* **31**, 786–790.

Zandi, K., Teoh, B.T., Sam, S.S., Wong, P.F., Mustafa, M.R. and Abubakar, S. (2011) *In vitro* antiviral activity of fisetin, rutin and naringenin against dengue virus type-2. *J Med Plant Res* **5**, 5534–5539.

Zaragoza, O., García-Rodas, R., Nosanchuk, J.D., Cuenca-Estrella, M., Rodríguez-Tudela, J.L. and Casadevall, A. (2010) Fungal cell gigantism during mammalian infection. *PLoS Pathog* **6**, e1000945.