

Cyclosporine A decreases the fluconazole minimum inhibitory concentration of *Candida albicans* clinical isolates but not biofilm formation and cell growth

Wibawa, T.^{1,2*}, Nurrokhman¹, Baly, I.¹, Daeli, P.R.¹, Kartasmita, G.² and Wijayanti, N.^{2,3}

¹Department of Microbiology, Faculty of Medicine; Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

²Research Center for Biotechnology; Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

³Laboratory of Animal Physiology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

*Corresponding author email address: twibawa@ugm.ac.id

Received 29 June 2014; received in revised form 20 August 2014; accepted 26 August 2014

Abstract. Among the genus *Candida*, *Candida albicans* is the most abundant species in humans. One of the virulent factors of *C. albicans* is its ability to develop biofilm. Biofilm forming microbes are characterized by decreasing of its susceptibility to antibiotics and antifungal. The fungicidal effect of fluconazole may be enhanced by cyclosporine A in laboratory engineered *C. albicans* strains. The aim of this work is to analyze the synergistic effect of cyclosporine A with fluconazole in *C. albicans* clinical isolates and the effect of cyclosporine A alone in the biofilm formation. Six fluconazole resistant and six sensitive *C. albicans* clinical isolates were analyzed for its minimum inhibitory concentration (MICs), biofilm formation, and cell growths. A semi-quantitative XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was conducted to measure the biofilm formation. Cyclosporine A has synergistic effect with fluconazole that was shown by decreasing MICs of both fluconazole resistant and sensitive *C. albicans* clinical isolates. However, cyclosporine A alone did not influence the biofilm formation and cell growth of both fluconazole resistant and sensitive *C. albicans* clinical isolates. These results indicated that cyclosporine A might be a promising candidate of adjuvant therapy for fluconazole against both fluconazole resistant and sensitive *C. albicans* clinical isolates.

INTRODUCTION

Candida sp are endogenous microbes in several human systems. While these fungi are commonly considered non pathogenic, they are responsible for some opportunistic infections. *Candida sp* are frequently isolated from cardiac devices, joint prostheses, urinary, parenteral nutrition, peritoneal, and vascular catheters (Cuéllar-Cruz *et al.*, 2012). Among this genus, *Candida albicans* is the most abundant species in humans (Eggimann *et al.*, 2003). *Candida albicans* is the primary species and is the etiological agent of invasive candidiasis in immunocompromised and hospitalized patients (Cuéllar-Cruz *et al.*, 2012).

One of the virulent factors of *C. albicans* is its ability to form biofilm (Ronsani *et al.*, 2011) on device surfaces and mucosal tissues (Douglas, 2003; Harriott & Noverr, 2011). Biofilm is a microbial community that structurally attaches to surfaces, and is supported by an extracellular matrix (Tournu & Van Dijck, 2012). The biofilm is known to reduce the susceptibility of microbes to antibiotics and antifungal. This may lead to difficulties to treat such microbes.

Azoles have fungistatic activity against *C. albicans*, but tolerance to this class of treatments may develop. Fluconazole tolerance is regulated by calcineurin, a Ca²⁺-calmodulin-activated serine/threonine-specific protein phosphatase

which is inhibited by cyclophilin-cyclosporine A complex (Sanglard *et al.*, 2003). Cyclosporine A has been reported to have synergistic activity with fluconazole. The fungicidal effect of fluconazole may be enhanced by cyclosporine A (Marchetti *et al.*, 2000a; 2000b; Onyewu *et al.*, 2003; Li *et al.*, 2008). Calcineurin has an important role in mediating fluconazole resistance of *C. albicans* biofilms. Further, synergistic activity of this adjuvant therapy has been shown to inhibit *C. albicans* biofilm formation (Uppuluri *et al.*, 2008; Shinde *et al.*, 2012).

Biofilm formations by resistant and sensitive fluconazole *C. albicans* clinical isolates were inhibited by the presence of fluconazole alone (Bruzual *et al.*, 2007; Kaneko *et al.*, 2013). However, little is known about effect of cyclosporine A alone to biofilm formations of resistant and sensitive fluconazole *C. albicans* clinical isolates. This study examined the effect of cyclosporine A on biofilm formation of resistant and sensitive fluconazole *C. albicans* clinical isolates. This experiment showed that pre-treatment of cyclosporine A to the planktonic state of biofilm forming *C. albicans* did not influence the biofilm formation and cell growth.

MATERIALS AND METHODS

***Candida albicans* culture and identification**

The clinical isolates used in this study were collected from clinical specimens submitted to the clinical microbiology laboratory of Faculty of Medicine, Universitas Gadjah Mada. We used six fluconazole resistant which were consecutively collected and six sensitive *C. albicans* clinical isolates which were randomly chosen in this study. *Candida albicans* clinical isolates were cultured on Sabouraud's Dextrose Agar (SDA) at room temperature for 48 hours. *Candida albicans* were identified based on the macroscopic and microscopic examination with lactophenol cotton blue (LPCB) staining, and also germ tubes test.

Fluconazole susceptibility test

Candida albicans susceptibility against fluconazole was performed with a broth microdilution method to measure the minimal inhibitory concentration (MIC) as recommended by CLSI's (formerly NCCLS) microdilution protocol M27-A. Briefly, a serial dilution of fluconazole was made in the RPMI 1640 medium on 96 well polystyrene plates. *Candida albicans* were inoculated into each concentration of fluconazole to reach 10^3 cells/ml final concentrations and incubated at 35°C for 48 hours. The MIC was determined by a lack of growth in the well at certain fluconazole concentrations.

Biofilm formation

All *C. albicans* clinical isolates were cultured in the 96 well polystyrene plates with RPMI 1640 medium. Before inoculating into the wells, the amount of the *C. albicans* were counted and adjusted into 10^5 cells/ml concentration. The plates were incubated at room temperature for 48 hours without agitation. Then, a semi-quantitative XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was conducted to measure the biofilm formation of fluconazole resistant and sensitive *C. albicans* clinical isolates as recommended by Ramage *et al.* (2001). Briefly, 0.5g/l XTT (Sigma) solution was prepared in Ringer's lactate and sterilized by using 0.22 µm filter. Menadione (Sigma) was added to the XTT solution up to 1mM final concentration. A 100 ml of XTT-Menadione solution was added to each well. The plates were then wrapped to protect from light and incubated in the dark for 2 hours at 37°C. The absorbance was then measured with a microtiter plate reader at 490 nm. A colorimetric change was correlated with the metabolic activity of the biofilm.

Effect of cyclosporine A to the biofilm formation

A few colonies of *C. albicans* clinical isolates from the SDA were inoculated into the RPMI 1640 medium. Three dosages of cyclosporine A (0.6µg/ml, 2.5µg/ml and 10µg/ml) were

added, while control tubes free of Cyclosporine A. The tubes were then incubated at room temperature for 24 hours. The *C. albicans* were centrifuged, followed by pellet washing with PBS for two consecutive times. The cell amount was adjusted to 10^5 cells/ml and cultured in the 96 well polystyrene plates for 48 hours at room temperature. XTT reduction assay was then conducted on all *C. albicans* clinical isolates.

Effect of cyclosporine A to *Candida albicans* growth

A few colonies of *C. albicans* clinical isolates from the SDA agar were inoculated into the RPMI 1640 medium. Several dosages of cyclosporine A (0.6µg/ml, 2.5µg/ml and 10µg/ml) were added, whereas control tubes were free of cyclosporine A. Then, it was incubated at room temperature for 24 hours. The *C. albicans* was centrifuged and then the pellet was washed with PBS for two consecutive times. The cell amount was adjusted to 10^5 cells/ml and cultured in the

96 well polystyrene plates for 48 hours at room temperature. After 48 hours incubation, the *C. albicans* was disrupted and the cells amount was counted using hemocytometer.

Statistical analysis

The difference between treated and control groups was analyzed by using ANOVA and Tukey's HSD test.

RESULTS

All fluconazole resistant *C. albicans* clinical isolates have minimum inhibitory concentration (MIC) more than 64 µg/ml (Figure 1), whereas the fluconazole sensitive showed significantly lower MIC ($p < 0.001$).

Cyclosporine A decreases the fluconazole MIC in both sensitive and resistant *C. albicans* clinical isolates ($p < 0.001$). However, the effect of cyclosporine A does not show dose dependency in both groups (Figure 1).

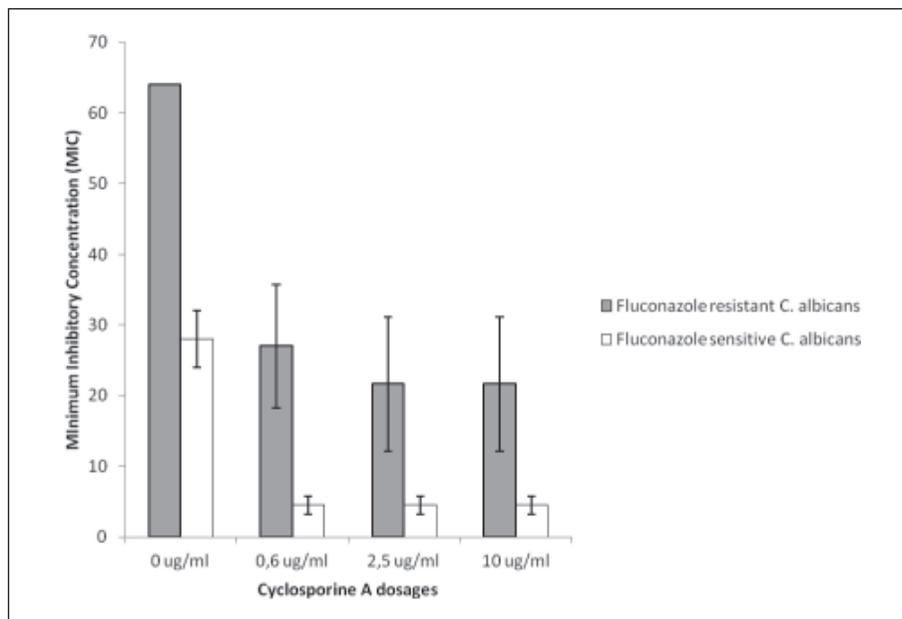


Figure 1. Effect of Cyclosporine A to the minimum inhibitory concentration (MIC) of Fluconazole-Sensitive and Resistant *C. albicans*. The MIC of Fluconazole-resistant and Fluconazole sensitive *C. albicans* were significantly decreased in the presence of Cyclosporine A (< 0.001). The effect of cyclosporine A did not show dose dependency. All fluconazole resistant *C. albicans* clinical isolates have MICs > 64 µg/ml. The error bars in the y-axis were represented for \pm SEM.

The biofilm formation between fluconazole resistant and sensitive *C. albicans* clinical isolates was not significant ($p=0.439$). The cyclosporine A treatment has no effect to the biofilm formation of both *C. albicans* groups ($p=0.323$) (Figure 2).

There is a significant difference of cell growth between fluconazole resistant and sensitive *C. albicans* clinical isolates, fluconazole sensitive *C. albicans* cells count was higher compared to the resistant ($p<0.001$) after 48 hours cultivation. There seems to be difference of dosage effect between fluconazole resistant and sensitive groups. Higher dosage of cyclosporine A treatment associated with lower cell count in fluconazole-resistant *C. albicans*, while in fluconazole sensitive higher dosages was associated with higher cell counts. However, none of the trend was statistically significant (resistant groups $p=0.212$; sensitive groups $p=0.537$) (Figure 3).

DISCUSSION

Our study shows that cyclosporine A decrease the fluconazole minimum inhibitory concentrations (MICs) of fluconazole resistant and sensitive *C. albicans* clinical isolates. Previous report showed synergistic interaction between cyclosporine A and fluconazole resulted to the decreasing of MICs (Marchetti *et al.*, 2003; Butalova & Darwish, 2008). We used clinical isolates rather than laboratory standard and mutants isolates. This may give opportunity to study the magnitude of the cyclosporine A effects that apparently had not difference between the fluconazole resistant and sensitive *C. albicans* clinical isolates.

Li *et al.* (2008) further showed a synergistic effect of cyclosporine A with fluconazole against fluconazole resistant and sensitive *C. albicans* clinical isolates. The MICs of fluconazole sensitive *C. albicans*

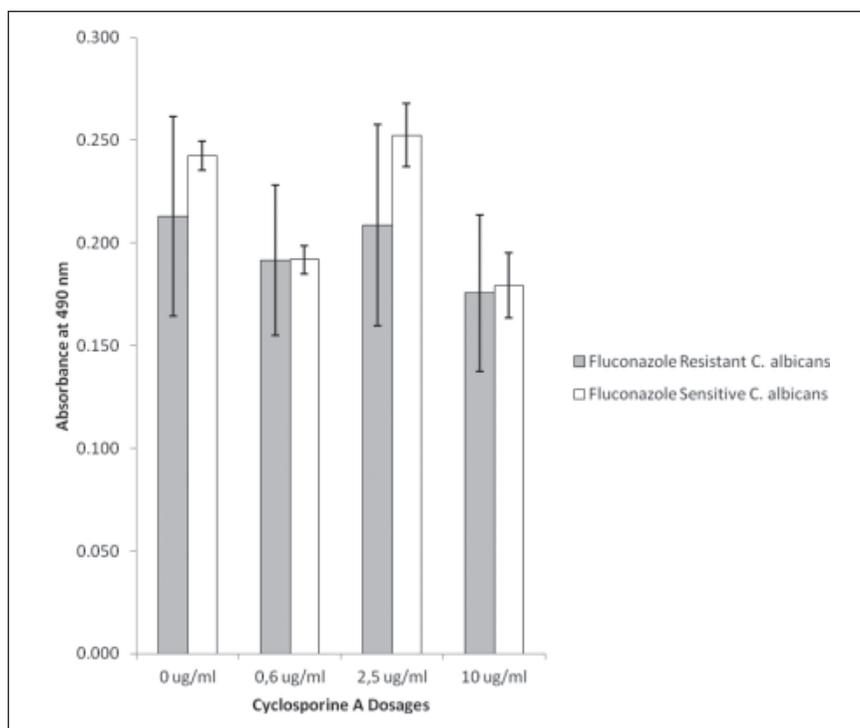


Figure 2. Biofilm formation of Fluconazole-Sensitive and Resistant *C. albicans* were not difference and were not affected by cyclosporine A. Biofilm formation was observed using XTT methods. The absorbance at 490 nm represents biofilm formation of *C. albicans*. The data shown in this figure was obtained by triplicate independence experiments for each *C. albicans* clinical isolates. The error bars in the y-axis were represented for \pm SEM.

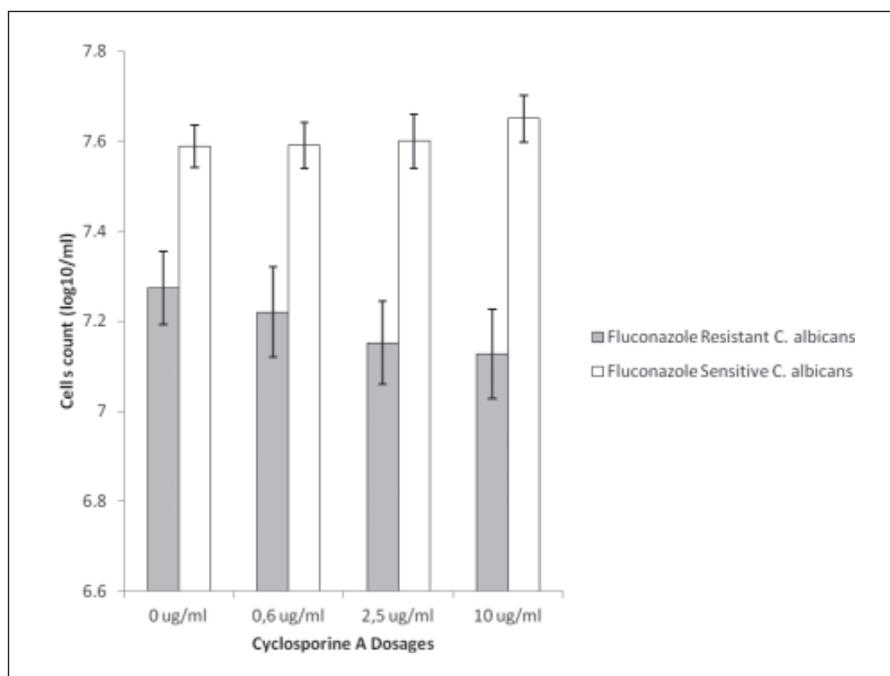


Figure 3. Cell growth of Fluconazole-Sensitive and Resistant *C. albicans* clinical isolates. There is a significant difference of cell growth between two groups ($p < 0.001$). The cell growth was not affected by Cyclosporine A treatment. The data shown in this figure was obtained by triplicate independence experiments for each *C. albicans* clinical isolates. The error bars in the y-axis were represented for \pm SEM.

were moderately decreased and the fluconazole resistant clinical isolates were strongly decreased. Our data showed similar results, the MICs of fluconazole resistant and sensitive *C. albicans* were strongly decreased.

There was no significant difference of biofilm formation among fluconazole resistant and sensitive *C. albicans* clinical isolates. This result was in agreement with previous work of Bruzual *et al.* (2007). This may indicate both groups of *C. albicans* clinical isolates which were used in this work are similar. This data was also supported by our data that according to the partial 18S RNA gene sequences, there were identical sequence among 12 fluconazole resistant and sensitive *C. albicans* clinical isolates (data not shown). However, Vavala *et al.* (2013) reported that the dry weight of fluconazole resistant *C. albicans* clinical isolates biofilm were higher compared to the fluconazole sensitive and this could be due to the difference in gene expression between the

fluconazole resistant and sensitive *C. albicans* during biofilm formation process. The first 24 hours of biofilm formation is important because of the fact that the dry weight increase rapidly during this initial period.

Cyclosporine A pre-treatment did not affect the biofilm formation of fluconazole resistant and sensitive *C. albicans* clinical isolates. This work used a different approach compared to previous report concerning biofilm formation of *C. albicans* (Ramage *et al.*, 2002; Uppuluri *et al.*, 2008;). The presence of cyclosporine A was limited in the 24 hours initial culture of *C. albicans*, which corresponds to the initial stage of biofilm formation. This strategy may facilitate observation of the effect of cyclosporine A alone on the ability of fluconazole sensitive and resistance clinical isolates to develop biofilm.

The fluconazole resistant *C. albicans* clinical isolates showed higher cell growth compared to sensitive *C. albicans*. This

result was different from the previous report (Angiolella *et al.*, 2008) where a prolonged lag phase was observed in the laboratory made fluconazole resistant *C. albicans*, though, the total growth yield after 24 to 48 hours of growth was substantially similar between the laboratory made fluconazole resistant and its parental fluconazole sensitive *C. albicans*. The slower growth of these *C. albicans* clinical isolates may correlate with the fluconazole mechanism of action which is disturbing the ergosterol biosynthesis (Sgherri *et al.*, 2014).

Fluconazole is the most recommended antifungal agent for *C. albicans* infection that may involve local and systemic infection. The rising of fluconazole resistant prevalence will have great consequences in patient care and public health. Our data showed that cyclosporine A may be useful for adjuvant therapy for *C. albicans* infection. The fluconazole MICs reduction in the *C. albicans* clinical isolates may correlate with the therapy effectiveness. Additional knowledge such as the effect of cyclosporine A on the biofilm formation and cell growth may give the new idea further potential benefit of cyclosporine A for *C. albicans* therapy. However, our data was obtained by *in vitro* experiment involving limited amount of *C. albicans* clinical isolates. Further study is needed to gain more data for generalization of this finding to the clinical setting and population.

Acknowledgements: This work was partly funded by a research grant from Faculty of Medicine UGM. The authors thanks to Dr. Agus Salim of Latrobe University, Melbourne, Australia for critically reading the manuscript and performed the statistical analysis.

REFERENCES

- Angiolella, L., Stringaro, A.R., De Bernardis, F., Posteraro, B., Bonito, M., Toccaceli, L., Torosantucci, A., Colone, M., Sanguinetti, M., Cassone, A. & Palamara, A.T. (2008). Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **52**: 927-936.
- Bruzual, I., Riggle, P., Hadley, S. & Kumamoto, C.A. (2007). Biofilm formation by fluconazole-resistant *Candida albicans* strains is inhibited by fluconazole. *Journal of Antimicrobial Chemotherapy* **59**: 441-450.
- Butalova, N.R. & Darwish, R.M. (2008). Effect of Chemosensitizers on minimum inhibitory concentrations of fluconazole in *Candida albicans*. *Medical Principles and Practice* **17**: 117-121.
- Cuéllar-Cruz, M., Vega-González, A., Mendoza-Novelo, B., López-Romero, E., Ruiz-Baca, E., Quintanar-Escorza, M.A. & Villagómez-Castro, J.C. (2012). The effect of biomaterials and antifungals on biofilm formation by *Candida* species: A review. *European Journal of Clinical Microbiology & Infectious Diseases* **31**: 2513-2527.
- Douglas, L.J. (2003). *Candida* biofilms and their role in infection. *Trends in Microbiology* **11**: 30-36.
- Eggimann, P., Garbino, J. & Pittet, D. (2003). Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infectious Disease* **3**: 685-702.
- Harriott, M.M. & Noverr, M.C. (2011). Importance of *Candida*-bacterial polymicrobial biofilms in disease. *Trends in Microbiology* **19**: 557-563.
- Kaneko, Y., Miyagawa, S., Takeda, O., Hakariya, M., Matsumoto, S., Ohno, H. & Miyazaki, Y. (2013). Real-time microscopic observation of *Candida* biofilm development and effects due to micafungin and fluconazole. *Antimicrobial Agents and Chemotherapy* **57**: 226-230.
- Li, Y., Sun, S., Guo, Q., Ma, L., Shi, C., Su, L. & Li, H. (2008). In vitro interaction between azoles and cyclosporin A against clinical isolates of *Candida albicans* determined by the checkerboard method and time-kill curves. *Journal of Antimicrobial Chemotherapy* **61**: 577-585.

- Marchetti, O., Entenza, J.M., Sanglard, D., Bille, J., Glauser, M.P. & Moreillon, P. (2000a). Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **44**: 2932-2938.
- Marchetti, O., Moreillon, P., Glauser, M.P., Bille, J. & Sanglard, D. (2000b). Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **44**: 2373-2381.
- Marchetti, O., Philippe, M., Entenza, J.M., Vouillamoz, J., Glauser, M.P., Bille, J. & Sanglard, D. (2003). Fungicidal synergism of fluconazole and cyclosporine in *Candida albicans* is not dependent on multidrug efflux transporters encoded by the CDR1, CDR2, CaMDR1, and FLU1 genes. *Antimicrobial Agents and Chemotherapy* **47**: 1565-1570.
- Onyewu, C., Blankenship, J.R., Del Poeta, M. & Heitman, J. (2003). Ergosterol biosynthesis inhibitors become fungicidal when combined with Calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. *Antimicrobial Agents and Chemotherapy* **47**: 956-964.
- Ramage, G., Vande Walle, K., Wickes, B.L. & Lopez-Ribot, J.L. (2001). Standardized method for *In Vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy* **45**: 2475-2479.
- Ramage, G., Bachmann, S., Patterson, T.F., Wickes, B.L. & López-Ribot, J.L. (2002). Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *Journal of Antimicrobial Chemotherapy* **49**: 973-980.
- Ronsani, M.M., Rymovicz, A.U.M., Meira, T.M., Trindade Grégio, A.M., Guariza Filho, O., Tanaka, O.M. & Ribeiro Rosa, E.A. (2011). Virulence modulation of *Candida albicans* biofilms by metal ions commonly released from orthodontic devices. *Microbial Pathogenesis* **51**: 421-425.
- Sanglard, D., Ishcer, F., Marchetti, O., Entenza, J. & Bille, J. (2003). Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Molecular Microbiology* **48**: 959-976.
- Sgherri, C., Porta, A., Castellano, S., Pinzino, C., Quartacci, M.F. & Calucci, L. (2014). Effects of azole treatments on the physical properties of *Candida albicans* plasma membrane: A spin probe EPR study. *Biochimica et Biophysica Acta* **1836**: 465-473.
- Shinde, R.B., Chauhan, N.M., Raut, J.S. & Karuppayil, S.M. (2012). Sensitization of *Candida albicans* biofilms to various antifungal drugs by cyclosporine A. *Annals of Clinical Microbiology and Antimicrobials* **11**: 27-34.
- Tournu, H. & Van Dijck, P. (2012). *Candida* Biofilms and the host: Models and new concepts for eradication. *International Journal of Microbiology* **1**: 1-16.
- Uppuluri, P., Nett, J., Heitman, J. & Andes, D. (2008). Synergistic effect of Calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy* **52**: 1127-1132.
- Vavala, E., Colone, M., Passariello, C., Celestino, I., Toccaceli, L., Stringaro, A. & Angiolella, L. (2013). Characterization of biofilms in drug-sensitive and drug-resistant strains of *Candida albicans*. *Journal of Chemotherapy* **25**: 87-95.