Inhibitory effects of carvacrol on the expression of secreted aspartyl proteinases 1-3 in fluconazole-resistant *Candida albicans* isolates

Seyyedeh Sedigh Hosseini1, Mohammad Hossein Yadegari1*, Masoumeh Rajabibazl2*, Ezzat Allah Ghaemi4

1Department of Medical Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2Department of Clinical Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4Department of Microbiology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran

ABSTRACT

**Background and Objectives:** Secreted Aspartyl Proteinase (SAP) is one of the main virulence factors in the pathogenesis of *Candida*. This enzyme is encoded by a family of at least ten genes. Among these genes, the role of *SAP1-3* in mucosal infections is evident. This study aimed to investigate the expression of *SAP1-3* genes of *Candida albicans* isolates after treatment with *Echinophora platyloba* extract, carvacrol and caspofungin drug.

**Materials and Methods:** Vaginal samples of 68 women with suspected vaginitis were obtained and cultured. *Candida albicans* species were identified using phenotypic and genotyping methods. Spectrophotometry was used to investigate the presence of SAP protein in the vaginal samples, and SDS-PAGE was used to confirm its protein composition. Real-time PCR was performed to ascertain the effects of subinhibitory concentrations of *Echinophora platyloba* extract, carvacrol and caspofungin on the expression of *SAP1-3* genes before and after treatment.

**Results:** *C. albicans* was found as the abundant species (59.6%), and different amounts of SAP were present in all vaginal samples, which were higher than *Candida krusei* strain. The protein composition of SAP in *C. albicans* samples was estimated with the approximate molecular weight of 45 kDa. *mRNA* levels of total SAP in FLU-resistant isolates (P=0.01) were more than those of FLU-susceptible isolates (P=0.07). The findings indicated that carvacrol is effective in reduction of *SAP1-3* expression with a particular effect against FLU-resistant isolates.

**Conclusion:** Carvacrol contains an essential oil (carvacrol); therefore, it can be considered as an alternative effective antifungal compound.

**Keywords:** Carvacrol, SAP1-3, *Candida albicans*, Fluconazole-resistant, *Echinophora platyloba*
INTRODUCTION

*Candida albicans* is an opportunistic fungal pathogen that usually lives as an infectious agent in immunocompromised patients, produces allergic reactions, and rarely leads to morbidity and mortality (1). It is also responsible for various infections, ranging from mucosal candidiasis to life-threatening disseminated candidiasis (2). Vaginal candidiasis is one of the most common and important infections in women (3), particularly during the fertile period. It is also responsible for almost 75% of all vaginal yeast infections, and other cases are mainly caused by *C. glabrata* and *C. tropicalis* (4).

Several factors are involved in the pathogenesis of *C. albicans* including adhesion, hyphae production, extracellular hydrolytic enzymes, and phenotype switching (5). Virulence of *Candida* species is directly proportional to the strength of their adhesion to different cells. *C. albicans* has the greatest adhesive ability among the *Candida* species (6).

The proteinase family is differentially regulated and expressed under a variety of laboratory growth conditions during experimental *C. albicans* infections using reconstituted human oral epithelium (RHE) (7) and in vivo (8). Different SAP genes appear to be essential for mucosal (SAPI-SAP3) (9) and systemic (SAP4-SAP6) infections. They are also involved in *C. albicans* adherence, tissue damage (10), and evasion of host immune responses (5). Therefore, inhibition of these proteinases has a protective effect for the host (11).

Fluconazole (FLC) is a member of the azole antifungal class and the most widely used drug for treatment and prevention of candidiasis. It targets essential enzymes such as ERG11 and lanosterol 14- alphademethylase in the ergosterol biosynthetic pathway (12). However, recent studies suggest that its prolonged use may contribute to development of drug resistance in *C. albicans* and other species (13).

Caspofungin is another new and effective antifungal drug that was developed as a potential antifungal and anti-pneumocystis agent (14). *In vitro*, caspofungin is fungicidal against *Candida* species (including azole-resistant species), and fungistatic against *Aspergillus* species. Caspofungin appears to have a slightly higher incidence of side effects and potential for drug-drug interactions. In addition, there is some evidence of decreased susceptibility among some strains of *Candida*, which may lessen its future utility. Comparison of caspofungin with natural drugs could pose as an interesting approach to limit the emergence and spread of these organisms, which are currently difficult to treat. Recently, there has been considerable interest in the study of plant materials as sources of new compounds for processing into therapeutic agents. One approach may be the use of extracts that have been shown to be safe potential agents in treatment of infections (15). In this context, *Echinophora platyloba* and its major phenolic component carvacrol [2-methyl-5-(1-methylthethyl) phenol] (2-isopropyl-5- methylphenol) have been given a lot of attention in recent *in vivo* and *in vitro* studies (16), and are known for their wide spectrum of antimicrobial activity. They possess multiple biological properties such as anti-inflammatory, anti-leishmanial, anti-oxidant and hepatoprotective activities (17).

Given the above, this study aimed to analyse the *in vitro* expression of SAPI-3 genes in *C. albicans* isolates via the simple, fast and sensitive methods of spectrophotometry, phenotypic, SDS-PAGE and Real-time PCR. Additionally, changes in the expression level of SAPI-3 virulence genes were determined by Real-time PCR after the addition of sub-inhibitory concentrations of *Echinophora platyloba* extracts, carvacrol, and caspofungin.

**Sampling, culture and identification of yeasts.**

This study was performed during July to December 2014 on the female patients who were referred to a qualified physician or midwife in Gorgan, Iran. Vaginal samples were obtained from 68 patients (aged 18-46 years) suffering from burning, itching, malodor- ou and cheesy vaginal secretions, with confirmed diagnosis by Dacron swabs. The vaginal swabs were taken from the lateral vaginal wall; for the patients presenting with infection, the swabs were taken directly from the infection areas. One of the vaginal swabs was subjected to direct examination and inoculated on the surface of YPD (yeast extract-peptide-dextrose) medium containing 2% glucose, 2% peptone, and 1% yeast extract and then incubated at 30°C for 48 hours. The purpose of each procedure was to preserve the integrity of RNAs for the subsequent analysis of *SAP* gene expression in the clinical samples. The swab samples were used to determine *Candida* colony-forming unit counts and yeast identification by CHROMagar *Candida*. In order to verify the performance of Chromagar, standard strain of
C. albicans (ATCC10231), which produces a bright green colour, was used.

**Plant material and preparation of extract.** Echinophora Platyloba (Iranian endemic plant) samples were collected from the mountains of Central Zagross, Chaharmahal and Bakhtiari Province during May-September, 2014. Their identity was confirmed. Harvested flowering aerial parts (leaves and flowers) were dried at room temperature for one week. The percolation method was used to obtain crude extract by stirring 100 mg of ground samples with 30 ml of pure ethanol (analytical grade; Merck, Germany) for 30 min. The samples were filtered by a Whatman no. 4 filter paper (18).

**Antifungal susceptibility test.** The standard powders of FLC (F8929, Sigma-Aldrich) and caspofungin (SML0425, Sigma-Aldrich) were prepared in 1ml of sterile Dimethyl Sulfoxide, and carvacrol essential oil (W224502, Sigma-Aldrich) was prepared in 1ml of ethanol-98%. The susceptibility of C. albicans strains to antifungal agents was determined by the broth micro-dilution version (19) of M27-A3 method according to the guidelines of CLSI (Clinical and Laboratory Standard Institute). After 48 hours of incubation at 30°C, MIC (minimum inhibitory concentration) was determined visually by comparing its turbidity with the drug-free growth control well. For the fluconazole, caspofungin, Echinophora extract and carvacrol the MIC values were defined as the lowest drug concentration for which the well was optically clear. The MIC was defined as the lowest concentration exhibiting >90% inhibition of visible growth compared to the growth of the control.

**Determination of total SAP by spectrophotometry and SDS-PAGE methods.** C. albicans strain was grown in YEPD in an incubator (Heraeus) for 48 h at 27°C. The induction of C. albicans SAPs was performed as described previously. Briefly, 100 µl of C. albicans suspension was added to 10 ml of Yeast Carbon Base (YCB) (Sigma) contained BSAm1%. The mixture was incubated for 7 days at 27°C in a shaker at 150 r.p.m. Thereafter, titres (c.f.u.) were determined. In brief the yeast cells were removed by centrifugation at 1500×g for 30 min. The supernatants were adjusted to pH 6.5 with NaOH to limit auto-degradation and were kept at -20°C.

A 0.1-ml volume of culture supernatant was mixed with 0.4 ml of 0.1 M citrate buffer containing BSAm1% at pH 3.2 and incubated for 1h at 37°C. The reaction was stopped with 0.5 ml of 5% trichloroacetic acid (TCA) on ice for 15 min, and the mixture was centrifuged at 8-12,000 rpm for 10 min. Then the absorbance was read at 280 nm against distilled water, which was blank (20). Standard strain of C. albicans (ATCC10231) was used as the positive control. SDS-PAGE was done according to the method of Lammeli (21).

**RNA extraction & analysis of SAP gene expression.** In order to analyze the expression of SAP1-3 genes, RNA was extracted from C. albicans isolates before and after treatment with the MIC concentration of each extract using Trizol reagent (Invitrogen Co., 15596026) according to the manufacturer’s protocol. Complementary DNA was synthesized using revert aid first strand cDNA synthesis kit followed by DNasel (Thermo Fisher Scientific Co., EN0521) treatment.

The cDNA was synthesized using the iScript cDNA synthesis kit (Fermentase Co., k1622) according to the manufacturer’s protocol. ACT1 primers for Real-time PCR analysis were designed using the PRIMER3 web-based software (http://frodo.wi.mit.edu/primer3). The primers were checked for specificity through the BLAST search available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A primer set for the hemochromatosis gene was designed to be used as an internal control (Table1).

**Real-time PCR.** The RealQ Plus 2x Master Mix Green (Ampliqon) was used to determine the relative level of SAP1–3 mRNA transcripts with actin 1 (ACT1) as a reference housekeeping gene. The PCR process was performed according to an optimized protocol. The relative quantification of SAP1–3 gene expression was performed by Syber green dye. Each test was performed in triplicate, and mean values of the relative expression were determined for each SAP gene.

The expression levels of SAP1–3 were evaluated using the 2-ΔΔCt method; where, C1 is the average threshold of cycles from three independent experiments (7). Data were presented as the fold change in gene expression normalized to the 18SrRNA gene as a control.
Table 1. Primers used for Real time-PCR analysis of the SAP and control genes’ expression

| Accession number | Sequence(5’–3’) | Primer | Product size (bp) |
|------------------|-----------------|--------|------------------|
| XM-712960.1      | GGGTTTTTGGTGTTGGCTTC | SAP1F   | 200              |
|                  | GCACAGTGAGGAGACTGAG | SAP1R   | 192              |
| XM-705969.1      | TGGTGATTGAGTACCACCA | SAP2F   | 231              |
|                  | GCAATCGGAAGCTGGA  | SAP2R   |                  |
| XM-718117.1      | TGGTGTTTCTACGTTTCC | SAP3F   |                  |
|                  | CCAATCCCTAAAATCCCTTG | SAP3R |                  |
| XM-717232.1      | CCAGCTTTTCTACGTTC | ACT1R   | 209              |
|                  | CTGTAACCACGTTCAGAC | ACT1F   |                  |

Statistical analysis. The obtained data were analysed using SPSS-18 statistical software and the Kruskal-Wallis test in groups. Mann-Whitney’s U-test was done to analyse the inter-group relationships. All analyses were performed at 95% confidence level. The REST (Relative Expression Software Tools) software (2009) was also employed to calculate the ratio between the amount of target molecule and reference molecule within the same sample. In this model, the target gene expression was normalized by ACT1 expression, which is a non-regulated reference gene. The normalized value was then applied to compare differential gene expression in different samples.

RESULTS

42 out of the 68 direct smeared samples were culture positive with 5 different yeast strains; 47.8% of which were C. albicans. Other species including C. parapsilosis (24%), C. glabrata (17.8%), C. tropicalis (8.4%) and C. krusei (2%) were detected by the phenotypic methods and RFLP-PCR (data not shown).

Determination of total SAP by spectrophotometry. The activation of SAP enzymes based on the amount of substrate BSA decomposition was investigated by spectrophotometry and light absorption at 280 (8).

In the test tube containing SAP enzyme and the substrate, these enzymes caused to break down of BSA proteins, and small peptides were achieved. In the control tube, standard strain of C. albicans was considered as a strains that has SAP enzyme and Candida krusei was considered as low value of SAP enzyme level; so with the addition of TCA to the samples, substrate and enzyme precipitated and only small peptides derived from proteolysis of the enzyme can be studied by spectrophotometer. The results showed that the enzyme had high proteinase activity in comparison with the negative control, and this case with a positive control strain of standard strains of C. albicans was confirmed (Table 2).

Confirmation of total SAP by SDS-PAGE. Comparison of the standard strain as positive control with the isolates was done by the SDS-PAGE method. In this study, protein markers with wide range of molecular weight from 14.4 to 116 kDa was used. The results showed one protein band in the SAP extract with a molecular weight of almost 45KD (Fig. 1).

Table 2. Determination of SAP activity by spectrophotometry in C. albicans isolates

| Strain            | Mean SAP level (µg/ml) |
|-------------------|------------------------|
| C. albicans*      | 6.82±1.60              |
| (ATCC10231)       |                        |
| FLU-resistant(n=7)| 9.05±1.10              |
| FLU-susceptible(n=13)| 2.82±1.68          |
| C. krusei*        | 0.01±0.08              |
| (ATCC6285)        |                        |

SAPs average level in the four tested groups: Significant differences within the groups were determined by Kruskal-Wallis test (n = 20, P < 0.05). Significance of differences between the groups was assessed by Mann-Whitney’s U-test, (n = 20, P < 0.05).

*ATCC 10231 C. albicans* = Positive control
*ATCC6285 C. krusei = Negative control
The SDS-PAGE results demonstrated that the SAP level of the 35% isolates increased compared with the standard strain C. albicans as a positive control. The rest of C. albicans isolates showed significantly different SAP expression compared with the standard strain C. krusei as a negative control (P<0.05).

**In vitro susceptibility analysis.** In order to assess the antifungal effect of the Echinophora, carvacrol, and caspofungin, C. albicans isolates were exposed to different concentrations of each extract and drug. The lowest MIC of the clinical isolates of C. albicans to the extract of Echinophora platyloba and carvacrol was 64mg/ml and 0.25 µg, respectively.

Evaluation of the MIC values showed that carvacrol was active against all the tested strains. The highest level of activity was observed against 13 FLU-susceptible isolates with a MIC value of 0.25–1.23 µg/ml (Table 2).

The MIC values of Echinophora ethanolic extract ranged from 64mg/ml to 512 mg/ml against the FLU-resistant isolates. Other Echinophora MIC values ranged from 1mg/ml to 64mg/ml against the FLU-susceptible C. albicans isolates. Carvacrol was found as the active constituent of Echinophora, with MIC values ranging from 0.03 to 23µg/ml (Table 2). Both Echinophora and carvacrol showed a broad spectrum of activity against a variety of pathogenic yeasts including fungi with decreased susceptibility to fluconazole (Table 3). Nevertheless, carvacrol proved to be more active against candida isolates, as did Echinophora platyloba.

**SAP 1-3 expression in C. albicans isolates.** SAP1–SAP3 expression by C. albicans in both FLU-susceptible and FLU-resistant isolates are shown in Table 3. FLU-susceptible C. albicans isolates showed lower expression for the target genes while FLU-resistant isolates had a significant increase in SAP1-3 gene expression. Higher SAP1-3 expressions were found in strains 14-20 (FLU-resistant) compared with strains 1-13 (FLU-susceptible). The mean of the targeted gene’s expression level between the resistant and susceptible strains was significantly different (P <0.008) (Table 4).

**Effect of Echinophora Platyloba, carvacrol, and caspofungin drug on expression of SAP1-3.** C. albicans isolates (susceptible and resistant) were tested extensively for SAP activity in the presence and absence of sub-MIC concentrations of Echinophora platyloba, carvacrol, caspofungin, and fluconazole. Expression of SAP1 in susceptible and resistant isolates decreased after treatment with Echinophora by about 3.6 and 2 fold, respectively (P<0.02) (Fig. 2(a, d)). Expression of SAP1 significantly decreased after treatment with carvacrol inhibitors in both isolates (P<0.001) (Fig. 2(a, d)). Expression of SAP1 in susceptible and resistant strains decreased by about 4.7 and 4 fold, respectively.

### Table 3. In-vitro susceptibility testing of Fluconazole, Caspofungin, Echinophora platyloba and Carvacrol against clinical isolates of C. albicans (n = 20) by microbroth dilution assay (µg/ml) (CLSI method)

| Species (No. of isolates) | Antifungal agent | Range      | 50% | 90% | Number of resistance (%) |
|---------------------------|------------------|------------|-----|-----|--------------------------|
| C. albicans (20)          | Fluconazole      | 0.031-128  | 8   | 16  | 7(35%)                   |
|                           | Echinophora      | 31-512000  | 16000| 32000| 9(45%)                   |
|                           | Carvacrol        | 0.03-8     | 1.23| 2.46| 3(15%)                   |
|                           | Caspofungin      | 0.03-12    | 1   | 2.3 | 6(30%)                   |

Resistance is defined as the following MIC in Fluconazole ≥ 64; Echinophora > 32000; Carvacrol ≥ 3.0 and Caspofungin ≥ 2.0.

---

**Fig. 1.** SAP protein isolates of C. albicans in the BSA medium. 1. Standard strain of C. albicans, 2- 4. C. albicans isolates, K- Standard strain of C. krusei stained with coomassie blue.
Table 4. SAP1-3 genes expression in C. albicans isolates

| Subject group                  | Strain number | Expression of gene (%) |       |       |
|--------------------------------|---------------|------------------------|-------|-------|
|                                |               | SAP1       | SAP2       | SAP3       |
| FLU-susceptible isolate        | 13            | 90         | 89         | 53         |
| FLU-resistant isolate          | 7             | 89         | 79         | 68         |
| C. albicans ATCC10231          | Positive control | 100       | 82         | 51         |

**NOTE.** Data are no of subjects (%) who were positive for the expression of a particular gene. Detection of SAP1-3 mRNA expression in the susceptible and resistant C. albicans isolates. The total percentage of subjects expressing each SAP gene is illustrated in each group: Susceptible (n=13) and Resistant (n=7). Data are shown as means ± SD from at least three experiments.

**Fig. 2.** Real time PCR for each gene was performed using 1 µg of total RNA. Gene expression is indicated as a fold-increase relative to control (black bars), carvacrol (gray bars), Echinophora (dark gray bars) and caspofungin (light gray bars). The results were normalized against ACT1 housekeeping gene expression, which was assigned a value of 1 (P<0.05).
tively after treatment with caspofungin (P<0.01) (Fig. 2(a, d)). Expression of SAP2 in the resistant and susceptible isolates decreased by about 2 and 1.6-fold after treatment with Echinophora (P<0.03) (Fig. 2(b, e)).

Expression of SAP2 significantly decreased following treatment with carvacrol inhibitors in both strains relative to the control (p<0.034) (Fig. 2(b, e)). Expression of SAP3 in susceptible and resistant isolates decreased by about 3.2 and 2.8 fold, respectively after treatment with caspofungin (P<0.03) (Fig. 2(c, f)).

Expression of SAP1-3 was significantly altered when C. albicans isolates were challenged with sub-MIC concentration of different antifungal agents (Fig. 2; P<0.05). Carvacrol down-regulate SAP1-3 expression more than Echinophora and caspofungin, and the difference was statistically significant (P<0.03). Of all the agents tested, carvacrol caused the highest down-regulation of SAP1-3 expression (Fig. 2).

DISCUSSION

Incidence of Candida species has shown an increasing trend in recent years, mostly due to rising number of immunosuppressed patients and widespread use of broad-spectrum antibiotics that leads to the increase of drug-resistant Candida strains (1).

This study aimed to determine the in vitro activity of SAP1-3 in several isolates of C. albicans from vaginal samples, cultured in the YEP medium and ascertain the effects of subinhibitory concentrations of Echinophora platyloba extract, carvacrol and caspofungin on the expression of secreted aspartyl proteinases 1-3 before and after treatment. All the tested isolates (20) were found positive for production of SAP (Table 2). The results showed that 40 out of 68 patients were infected by yeast, 47.8% of which had C. albicans. After SAP confirmation in the vaginal samples by spectrophotometry (22), the protein composition of SAP was analysed by SDS-PAGE, and a band with the weight of almost 45 kDa (Fig. 1) (23) was observed, which corresponds to the study of Zaugg and et al (24). The average level of C. albicans SAP was significantly more in the FLU-resistant isolate than in the FLU-susceptible isolate.

The study findings also showed increased gene expression of SAP1-3 in FLU-resistant C. albicans isolates compared to the susceptible isolates. This indicates a clear link between the expression of SAP 1-3 exposure to antifungal agents and drug resistance.

The results of the present study imply that the majority of SAPs important for pathogenicity during vaginal infections with C. albicans are SAP1-3. We found that the expression of SAP1 gene of C. albicans isolates was more than that of SAP2 and SAP3 (Table 4). The observation of high-density SAP1 and SAP3 increased further in FLU-resistant C. albicans isolates (Table 4) (25). However, the expression levels of SAP1 and SAP3 were significantly greater in the FLU-resistant isolates than FLU-susceptible isolates. Therefore, they play a major role in adhesion, and colonization mucosal surface conferred drug resistance of C. albicans isolates (26). Also we found that the expression level of SAP2 was higher in the FLU-susceptible isolates than in FLU-resistant isolates; this shows the role of SAP2 in the development of C. albicans pathogenicity (Table 3) (27-28). Therefore, it seems necessary to find new drug agents having the least toxicity, and side effects in the treatment of Candida infections.

Echinophora platyloba, carvacrol, FLU and caspofungin are used to inhibit SAP expression with wide-spectrum of antifungal activities. Evaluation of MIC values showed that Echinophora was active against all the tested strains. The highest level of activity was observed against FLU-resistant isolates, with a MIC value of 64mg/ml (Table 3).

Essential oils and their components have been timely honored for their pharmaceutical properties (30). Carvacrol containing essential oil has been reported to have antiseptic, antibacterial, antiviral and antifungal activities (16).

Echinophoral and carvacrol showed a broad spectrum of activity against a variety of pathogenic yeasts, including fungi with decreased susceptibility to FLU (Table 3). Carvacrol has been proved to be more active against Candida strains, and the fungistatic and fungicidal properties of essential oil probably associated with its high carvacrol content (29-31).Thirteen of 20 isolates of C. albicans grown under SAP-inducing conditions responded in a dose-dependent manner to partial growth inhibition by caspofungin and FLU by decreasing their extracellular SAP activity. This finding shows that carvacrol downregulates the gene expression of SAP2 activity in Flu-susceptible and Flu-resistant isolates comparing to Echinophora and caspofungin (Fig. 2) (32). Also downregulation of SAP1 and SAP3 expression was observed in both FLU-resistant and susceptible isolates. Therefore, carvacrol is effective in reduction of SAP1-3 expression, with a particular effect against FLU-resistant
isolates. This confirms a general relationship between FLU-susceptibility and relative expression and activity of SAP.

Considering the above findings, particularly the possible mechanisms of action, which may induce side effects in humans, these antifungals need further investigations. Toxicity studies, improved formulations, determination of optimal concentrations for clinical applications and comparative studies on the therapeutic efficacy of carvacrol with drugs currently in use are recommended to further control of fungal infections.

ACKNOWLEDGEMENT

The authors would like to thank the Department of Medical Mycology, Tarbiat Modares University, and Faculty of Medicine, Golestan University of Medical Sciences, Iran for their help and scientific assistance during this research.

REFERENCES

1. Wisplinghoff H, Bischoff T, Tallent SM, Seift H, Wenzel R, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clini Infect Dis 2004;39:309-317.
2. Arendrup MC, Rodriguez-Tudela JL, Park S, Garcia-Effron G, Delmas G, Cuenca-Estrella M, et al. Echinocandin susceptibility testing of Candida spp. using EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time, and drug lots. Antimicrob Agents Chemother 2011; 55:1580-1587.
3. Consolaro L, Albertoni A, Yoshida C, Mazucheli J. Correlation of Candida species and symptoms among patients with vulvovaginal candidiasis in Maringa. Rev Iberoam Micol 2004; 21:202-205.
4. Jaeger M, Plantinga TS, Joosten LA, Kullberg BJ, Netea MG. Genetic basis for recurrent vulvo-vaginal candidiasis. Curr Infect Dis Rep 2013;15: 136-142.
5. Naglik JR, Challacombe SJ, Hube B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 2003;67:400-428.
6. He XY, Meurman JH, Kari K, Rautemaa R, Samaranayake LP. In vitro adhesion of Candida species to denture base materials. Mycoses 2006; 49:80-84.
7. Negri B, Silva N, Lopes L, Henriques M, Azeredo O, Oliveira K. An in vitro evaluation of Candida tropi-calis infectivity using human cell monolayers. J Med Microbiol 2011;60:1270-1275.
8. Staib P, Kretschmar M, Richterlein T, Hof H, Morschhauser J. Transcriptional regulators Cph1p and Efg1p mediate activation of the Candida albicans virulence gene SAP5 during infection. Infect Immun 2002; 70: 921-927.
9. Taylor BN, Staib P, Binder A, Biesemeier A, Sehnal M, Röllinghoff M et al. Profile of Candida albicans secreted aspartic proteinase elicited during vaginal infection. Infect Immun 2005;73:1828-1835.
10. Schaller M, Schafer W, Korting HC, Hube B. Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. Mol Microbiol 1998; 29: 605-615.
11. Hube B, Naglik J. Candida albicans proteinases: resolving the mystery of a gene family. Microbiology 2001;147:1997-2005.
12. Vanden Bossche, H, Marichal P. Molecular mechanisms of drug resistance in fungi. Trends Microbiol 1994; 2:393-400.
13. Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol Microbiol 2003;48:959-976.
14. McCormack PL, Perry CM. Caspofungin: a review of its use in the treatment of fungal infections. Drugs 2005; 65:2049-2068.
15. Pinto E, Vale-Silva L, Cavaleiro C, Salgueiro L. Antifungal activity of the clove essential oil from Syzygium aromaticum on Candida, Aspergillus and dermatophyte species. J Med Microbiol 2009;58:1454-1462.
16. Dorman HJ, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Appl Microbiol 2000; 88: 308-316.
17. Weber FJ, de Bont JA. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 1996; 1286:225-245.
18. Aslani P, Yadegari M, Rajabibazl M. Investigation the effect of Echinophora platyloba and Satureja bachtiarica on MDR1 and ERG11 gene expression in fluconazole resistance clinical isolates Candida albicans using Real-time PCR. Eur J Exp Biol 2014;41:375-379.
19. CLSI (2008). M27A-3, Reference method for broth dilution antifungal susceptibility testing of Yeasts. Approved standard; 3rd edition, Vol. 28 No. 14. Clinical and laboratory Standards Institute, Wayne, PA19087, USA.
20. Rehani S, Rao NN, Rao A, Carmelio S, Ramakrishnaih SH, Prakash PY. Spectrophotometric analysis of the expression of secreted aspartyl proteinases from Candida in leukoplakia and oral squamous cell carcinoma.
21. Laemmli UK. Cleavage of structural proteins during the assembly of head of bacteriophage. *Nature* 1970; 227(5259):680-685.
22. Dostal L, Hamal P, Pavlickova L, Soucek M, Ruml T, Pichova I, et al. Simple method for screening *Candida* species isolates for the presence of secreted proteinases: a tool for the prediction of successful inhibitory treatment. *J Clin Microbiol* 2003; 41:712-716.
23. Cutfield S, Marshall C, Moody P, Sullivan F, Cutfield J. Crystallization of inhibited aspartic proteinase from *Candida albicans*. *J Mol Biol* 1993; 234: 1266-1269.
24. Zaugg C, Borg-von Zepelin M, Richard U, Sanglard D, Monod M. Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* 2001; 69:405-412.
25. Naglik JR, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, Weindl G, et al. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* 2008;154: 3266-3280.
26. Monroy-Ramirez HC, Basurto-Islas G, Mena R, Cisneros B, Binder LI, Avila J, et al. Alterations in the nuclear architecture produced by the overexpression of tau protein in neuroblastoma cells. *J Alzheimers Dis* 2013;36: 503-520.
27. WU T, Samaranayake LP, Leung WK, Sullivan PA. Inhibition of growth and secreted aspartyl proteinase production in *Candida albicans* by lysozyme. *J Med Microbiol* 1999; 48 :721-730.
28. Schaller M, Korting H.C, Borelli C, Hamm G, Hube B. *Candida albicans* secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. *Infect Immun* 2005;73: 2758-2765.
29. Cadicamo CD, Mortier J, Wolber G, Hell M, Heinrich IE, Michel D, et al. Design, synthesis, inhibition studies, and molecular modeling of pepstatin analogues addressing different secreted aspartic proteinases of *Candida albicans*. *Biochem Pharmacol* 2013;85: 881-887.
30. van Alphen LB, Burt SA, Veenendaal AK, Bleumink-Pluym NM, van Putten JP. The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. *PLoS One* 2012;7(9):e45343
31. Manohar V, Ingram C, Gray J, Talpur NA, Echard BW, Bagchi D, Preuss HG. Antifungal activities of origanum oil against *Candida albicans*. *Mol Cell Biochem* 2001;228 (1-2):111-117.
32. Cho O, Shiokama T, Ando Y, Aoki N, Uchara C, Mae-da E, et al. Screening of compounds from an FDA-approved drug library for the ability to inhibit aspartic protease secretion from the pathogenic yeast *Candida albicans*. *Pharmaceut Reg Affairs* 2014; 3: 2167-7689.