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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

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ABSTRACT

Candida albicans is an opportunist yeast which changes into fungus infection in humans with immunity system deficiency when the required conditions are present. It may cause fatal infections. The Bakhtiari Summer Savoury and Platyloba are anti-fungus plants that effects on MDR 1 and ERG 11 genes expression. This genes have an important role in resistance against Fluconazole.In this study effect of this plants on level expression evaluated by using real time PCR. The ethanolic extract of Savoury and Platyloba were prepared through drying the plants in Percolation method and then 500, 256, 125, 64, 32, 16, 8, 4, 2 and 1 mg/ml concentrations of the solution cocultured with 1×10^6 clinical isolates of C. albicans after 24 h incubation at 37°C the MIC and MFC were determined. Then C .albicans isolates were treated in MIC concentration of plants. The RNA of isolates were extracted before and after treatment with the plants , then cDNA synthesized and the real time- PCR was investigated. The results revealed that the MIC for the alcoholic essences of Satureja and Platyloba were 256 and 64 mg/ml, respectively in the clinical isolates of C. albicans. Also, their MIC and MFC were 512 and 128 mg/ml, respectively. Moreover, the result of expression level of two genes in real-time PCR reaction showed the Platyloba was able to inhibit the ERG11, while the Savoury was neutral in this regard. nowadays, application of the plants with anti-microbial qualities which have less side effects is increasing; because of drug resistances. With regard the present study's findings, the Platyloba is effective in reduction of ERG11 expression which in turn plays a basic role in resistance against Fluconazole. Therefore, the plant may be used as a suitable alternative for drugs and introduced as an efficient anti-fungus factor.

Keywords: Satureja Bakhtirica, Echynophora Platyloba, Anti-fungus effects, Candida, ERG11, MDR1.

INTRODUCTION

Candida species are the most frequent fungal pathogens in humans which could create a wide spectrum serious infections including oral and vaginal candidiasis (1,2) in immunocompromised populations, including AIDS patients, transplant recipients, and cancer patients.

Candida spp are the fourth most common agent of all hospital-acquired bloodstream infections (BSIs) in the United States; such infections have an attributable mortality of up to 49(3)

In Addition, significant increase resistance to traditional antifungal agents occurred. (4,5) the mechanisms of drug resistance are complicated, One of the possible mechanism for drug resistance in *C.albicans* is over expression of the *Candida* drug resistance (CDR)genes, which encode transporters of the ATP-binding cassette (ABC) family, and the multidrug resistance (MDR)gene, which codes for major facilitator transporter.(6,7)

In a previous investigation mutations of *ERG11* gene were shown to coexist in a collection of clinical *C. albicans* isolates with fluconazole-resistant.(8)

Plant derived ,medicines have been part of traditional health care in most parts of the world for thousands of years, therefore nowadays there is increasing interest in plants as sources of agents against microbial diseases.(9,10)

Several iranian herbs for example: *Satureja bachtiarica*, *Echiophora platyloba*, *Kelussia odoratissima* and *Achillea kellalensis* have been used as customary medicines by the indigenous people of Chaharmahal va Bakhtiari, Southwest Iran (11,12)

For this reason, it is required to establish the therapeutic proceedings of traditional plant medicines as the source for the progress of safe and more effective drugs with the least side effect.

The purpose of this study was to determine the anti-*Candida* activity of the extracts of two plant species that are endemic Iranian plants and their effect in expression of MDR and ERG11 in resistant clinical isolates of *C.albicans* was assessed.

MATERIALS AND METHODS

Fungal strain

The activity of extracts was assayed against clinical isolates of *C. albicans* . In this study, we used 20 clinical isolates of *C.albicans* obtained from vaginal candidiasis. All of strains were resistant to fluconazol that confirmed by using disk diffusion method according CLSI guideline(13). *C. albicans* reference strain ATCC10231, was included in this study.

The isolates grown overnight at 37°C on Sabouraud dextrose agar (Merck, Germany) plates, and inoculums for the assays was organized by diluting scraped cell mass in solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometer reading at 600 nm.

Cell suspensions were finally diluted to 10^6colony forming units (CFU)/ml for use in the assays.

Plant material and Preparation of extract

Satureja bachtiarica and *Echinophora Platyloba* Iranian endemic plants were composed from mountain areas of Central Zagross, Chaharmahal va Bakhtiari district, during May to September, 2012 .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. Briefly the extracts were obtained by stirring 100 mg of ground samples with 30 ml of pure ethanol (analytical grade; Merck, Germany) for 30 min. Samples were filtered by a Whatman no 4. filter paper.(14)

Anti- fungal activity of plants extract

200 microlitres of fungal suspension(10^6 CFU/ml) were added to the media and cocultured with different concentration of each extract nd then incubated at 37°C and examined for 48h.

The 10th tube was made without any extract(control for media) and the 11th tube without a fungal suspension (control for contamination).

The concentration of extract in the tubes was 500, 256, 125, 64, 32, 16, 8, 4, 2 and 1 mg/ml respectively.

Concentration of extract in the first tube, which inhibits the growth of *Candida albicans,* was recorded as the minimal inhibitory concentration.

RNA extraction and Real-time analysis
In order to analyze the expression of *MDR and ERG11* genes, RNA was extracted from *C. albicans* isolates before and after treatment with MIC of each extract of plants using glass beads and the denaturing buffer agents in an RNase-free environment (15). All RNA samples were treated with 1U of DNaseI (Fermentas) per 10µl of RNA at 37°C for 1 h to prevent contamination with genomic DNA . The quality of RNA was checked using a 5 µl of the RNA which were separated using 2% agarose gel electrophoresis, visualized and photographed with an Image Master® Video Documentation System The cDNA was synthesized using 2-step RT-PCR kit (Vivantis, Malaysia) following manufacturer's instructions .

The synthesized cDNA samples were stored at –20 °C and directly used in Real- time assay .The housekeeping gene AC*T1* used as a control.

The PCR primers used to amplify and identify the *C. albicans ERG11*, and *MDR1* genes were shown in table1.

SYBR® Green I(fermentas), a specific DNA-binding dye, was used to detect amplification. The cycling conditions were as follows: 35 cycles of denaturation for 5 min at 95 °C, annealing for 30 s at 65 °C for *ACT1*, *CDR1*, at 55 °C for *ERG11*, and elongation for 10 s at 72 °C, followed by termination in a final cooling step for 30 s at 72 °C.

The expression levels of *MDR1* and *ERG11* were evaluated using the $2–\Delta\Delta Ct$ method, where *Ct* was the average threshold cycle number from three independent experiments.(16) Data were presented as the fold change in gene expression normalized to the *18SrRNA* gene as a control.

Table1: Nucleotide sequence primers used to amplify the study genes in clinical isolates of *C.albicans* using in Real-time PCR

Primer sequence	Primer
5' TTT GGT GGT GGT AGA CATA3'	ERG11(F)
5'GAA CTA TAA TCA GGG TCA GG - 3'	ERG11(R)
5'TTC TTG GGT GGA TTC TTT GC 3'	MDR1(F)
5' GCA TCT AAA CTC CAA GTG GC 3'	MDR1(R)
5' CCA GCT TTC TAC GTT TCC 3'	ACT1(F)
5' CTG TAA CCA CGT TCA GAC 3'	ACT1(R)

Statistical analysis
The data of gene expression were subjected to the Analysis of Variance (One-way ANOVA) in Tukey range. The differences with $P<0.05$ were considered significant.

RESULTS

Effects of Plants on *C.albicans* isolates growth
For assessment the antifungal effect of plants , *C.albicans* isolates were exposed to different concentrations of plants.

In this study, clinical isolates of *Candida albicans* are sensitive to a minimum dilution of 64mg/ml and 256 of ethanolic extract of *Echinophora platyloba* and *Satureja bachtiarica* respectively .

There is an overt growth of yeast in the control tube (10th tube). The concentration of 256 and 64 mg/ml is minimum inhibitory dilution of this study.

Minimum fungicidal concentrations (MFC) of ethanolic extract of *Echinophora platyloba* and *Satureja bachtiarica* is 128mg/ml and 512 mg/ml respectively .

Table2: Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of extracts against *C. albicans* isolates

Candida albicans (ATCC10231)		Clinical isolates of *Candida albicans*		
MFC	MIC	MFC mg/ml	MIC mg/ml	Plants
512	256	512	256	*Satureja bachtiarica*
64	32	128	64	*Echinophora Platyloba*

Effects of *Satureja bachtiarica* and *Echinophora Platyloba* on the expression of MDR1 and ERG11 genes
For evaluating the effects of plants on expression of genes encoding fluconazole resistance , clinical isolates of *C.albicans* were cultured on Sabouraud dextros agar in presence of plants (256 and 64mg/ml) for 48h at 37ºC.

Total RNA was extracted, and the expression of *MDR1* and *ERG11* genes was evaluated by Real- Time PCR. There were no primer dimers or non-specific amplification products according to the melting curves and melting peaks of the two tested genes. A single band of PCR product with the expected length on agarose gel electrophoresis also confirmed the specificity of the PCR reactions (data not shown). The result showed that *Echinophora platyloba* decreased mRNA levels of ERG11gene

In concentration of 64 mg /ml and gene expression was reduced by *Echinophora platyloba.* Whereas ,*Satureja bachtiarica* did not inhibit the expression of *ERG11* gene at concentrations of 64 mg/ml. However, in concentration of 64 and 256 mg/ml, MDR1gene expression was not reduced by *Echinophora platyloba* and *Satureja bachtiarica* respectively, Because of reduction in the expression of MDR1 gene by this plants was not significant.

Based on the statistical analyses, reduction in the expression of ERG11 gene examined in this study was significant only for *Echinophora platyloba* ($P<0.05$).

DISCUSSION

It is obvious that the *CDR1 and ERG11* genes, is observed in some of the resistant clinical *C. albicans* isolates(17) Interestingly, use of the component with potential capacity of inhibitory effect on the expression of this genes is remarkable. So , this study was designed to determine the antifungal activities of *Echinophora platyloba* and *Satureja bachtiarica* against fluconazole resistant clinical isolates of *C.albicans* in comparative to the reduction in the expression of important key genes of fluconazole resistance including MDR1 and ERG11.

Real-time quantitative PCR is a renowned method for the quantification of mRNA levels in cells, tissues and other clinical samples, even for specimens with a very low mRNA level for a specific gene such as the expression of various genes in fungi (especially clinical isolates of *C. albicans*). (18,19)

Antifungal drug resistance has become a main problem in the immunocompromised patients especially in HIV-infected patients who have received long-term antifungal therapy (20,21) further, it was shown some natural component have the benefit effect compared with synthetic drugs.(22,23)

Previous studies were obtained antifungal compounds from plants with inhibitory effect on fungal growth (25و24)

Rohi boroujeni et al were assayed for the in vitro antifungal activity against *Candida albicans* (ATCC10231), using agar dilution methods. *Echinophora platyloba* extracts showed relatively high anti-*Candida* activity against the tested fungi.(26)

In the present study, we showed a powerful inhibition of *C.albicans* growth exposed to the studied plants. Some active ingredient may contribute to the inhibitory effect of this plants. The result of previous studies for identification of phenolic compounds showed that the major components of *S. bachtiarica* were carvacrol and thymol. Some studies declare that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (27,28).

Also according to the Real-time results there was a considerable reduction in the expression of important fluconazole resistance gene i.e. *ERG11* ,in spite of the identified antifungal activities of *Echinophora platyloba*, very little has been established about its effect on fluconazole resistance genes expression level.

We reported for the first time that *Echinophora platyloba* reduces expression of *ERG11* gene in clinical isolates of *C.albicans* due to a notable inhibition in mRNA level of this gene was shown.

Since the *ERG11* was the most affected gene under the test situation, which showed significant reduction at mRNA level.

We believe , these results designate that *Echinophora platyloba* may be employed successfully as a good candidate in inhibition of *C.albicans* growth with a potential source of natural antifungal agents and controlling the fluconazole resistance gene.

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