

ORIGINAL ARTICLE

Evaluation of the Anti-Candida effect of Garlic, Shallot and Onion to inhibit the Quorum Sensing Activity

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ABSTRACT

Recently, the incidence of systemic candidiasis which is caused by *Candida albicans* has been increased. Usually, for treatment of systemic fungal infections, azoles such as fluconazole are used. Despite of this, overuse of drugs has caused an extra increasing of the drugs resistance. One of the best strategies to decrease the resistance phenomenon is finding the new derivatives originated from plants instead of chemical antifungals. In the present study the potential of Garlic, Shallot and Onion for growth inhibition of *Candida albicans* were evaluated by use of inhibition zone determination (Disk Diffusion Agar test) and relative MIC (Broth Microdilution test). Time kill study was also performed to indicate the rate of anti-Candida activity of plant-extracts tested in different time intervals. Finally, the expression of selected gene involved in Quorum Sensing such as *TUP1* was evaluated by semi-quantitative RT-PCR. The result have shown significant *in vitro* potential of those plants to inhibit *Candida albicans* growth, which is need further investigations to find its main mechanisms. In addition, the extracts were not able to demonstrate their ability to significant decrease of the selected gene expression. Therefore, further investigation will be needed to find the probable targets of garlic, onion and shallot on the Quorum Sensing phenomenon of *C. albicans*.

Key words: MIC, Time kill, *Candida albicans*, Shallot, Garlic, Onion.

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INTRODUCTION

The dimorphic yeast, *Candida albicans*, is a commensal organism which is commonly found in oral cavity, gastrointestinal tract, female's genital tract and occasionally on the surface of skin and mucous membrane [1]. *Candida albicans* can be isolated from approximately 70% of the healthy population. It is the fourth leading cause of nosocomial bloodstream infections, with mortality rate of 37–44% in severely immune-compromised patients. *Candida* spp. is the most common cause of opportunistic mycoses and its infection generally referred to as candidiasis, which can be further classified into superficial candidiasis, mucocutaneous candidiasis and systemic or invasive candidiasis [2]. Superficial candidiasis is relatively common as compared with systemic candidiasis due to its serious infection that can be fatal. Superficial infections of *C. albicans* could worsen into invasive form and disseminate elsewhere in the body [3]. Virulent factors that contribute to candidiasis are mainly undefined and under investigation. The virulent factors include secreted protease, phospholipase and the ability to change morphology from budding yeast cells into hyphal, filamentous or even mycelial cells form, adhere to the host cells and subsequently penetrate host tissues remains an important virulent determinant for invasive infection. Morphogenesis in *C. albicans* has been extensively investigated *in vitro* [4]. Although, a number of transcriptional regulators have been characterized as main factor in regulating the yeast-to-hyphal shift, many of the target genes involved in morphogenesis have not been identified. Recently, quorum sensing (QS) has been described as a phenomenon contributing to morphogenic control in *C. albicans*. One of the conditions which define the yeast-to-hyphal shift *in vitro* is dependence on cell density to determine morphogenesis; this has been called "inoculum effect" in which this term has been associated with regulation by QS. The

"inoculum effect" is seen when yeast cells are diluted to concentrations less than 10^6 cells ml^{-1} in culture medium to germinate into the hyphal form under certain conditions [5]. However, cells which are inoculated at higher concentrations ($>10^6$ cells ml^{-1}), predominantly remain in a yeast state. Candidiasis is normally treated with antimycotics, which are the common used antifungal drugs in hospitals. For non-severe clinical condition, the used antifungal drugs are including topical clotrimazole, topical nystatin, fluconazole and topical ketonazole, while for severe infection, amphotericin B, caspofungin or variconazole may be used. Moreover, these antifungal drugs often have side effects, and more efforts warranted to find newer treatment regimens for safer and more effective treatment. Also, Azoles disrupt ergosterol biosynthesis in fungi resulting in the formation of cell membrane with altered structure and function [6].

It has been demonstrated that the antimicrobial activity of plants are related to both the allyl and sulfur groups in thiosulfinates, peptides, alkaloid and etc. With an emphasis on above information, plant extracts and their compounds can be used as a potential cheap, affordable antifungal agent with fewer side effects [7,8,9]. So, the aim of this study is to evaluate antifungal activity of Garlic, Shallot and Onion on growth of *Candida albicans* isolates, with effect on genes involved in quorum sensing.

Material and Methods

10 clinical isolates of *Candida albicans* were obtained from microbiological laboratories of Madaran and Labafinezhad Hospital. All samples were isolated from patient's with systemic candidiasis. For control strains, *C. albicans* ATCC 10231 was employed. Each sample was maintained as sterile 20% (v/v) glycerol stocks and subcultured on Sabouraud dextrose agar at 35–37°C for 24–48 h to ensure viability and purity prior to testing.

Plants extract preparation

The fresh plants were washed with distilled water, sliced and dried in the oven for at least two days and then powdered, according to researcher (10). A total weight of 1g of fresh plants powder was added in 10 ml of distilled water to prepare a stock solution of 100 mg ml^{-1} (w / v). The extract was allowed to stand for 30 min in room temperature and then centrifuged at 5000 rpm for 10 min. The supernatant fluid was passed through a sterile 0.22 μm filter (Millipore, UK). The required concentration was prepared by serial dilution of the stock solutions.

Determination of antimicrobial activity

According to the Clinical and Laboratory Standards Institute (CLSI) for yeast cell with some slight modification, paper disks of 6 mm in diameter were prepared using Whatman filter paper for disc diffusion method. The paper disks were autoclaved at 121°C for 20 min before use. The stock solutions of 100 mg/ml of extracts were prepared by dissolving 100 mg of extract into 1 ml solvent. Serial ten-fold dilution was carried out to make different concentrations of plant extracts. Amphotericin B (10 μg), Nistatin (50 μg) and ketoconazole (15 μg) were also used as a standard control antifungal drug. Eventually, 5 μl of each concentration of plant extracts were loaded separately on paper disks and were allowed to dry completely for 30 min. Clinical *Candida albicans* and ATCC(10231) were grown on separate Sabouraud's dextrose agar (SDA) and then passage three times to ensure its viability and activity. Inoculum were prepared by picking five colonies of ≥ 1 mm in diameter from 24 h culture of *Candida* using 5 ml of PBS buffer. Following, the inoculum were centrifuged and the supernatant was removed and the cell pellet was washed with PBS buffer and again centrifuged for 5 min. These steps were repeated at least three times. The cell density was adjusted from 1×10^6 to 5×10^6 cfu/ml using spectrophotometric method at 530 nm to achieve the turbidity equivalent to 0.5 McFarland standards. Working suspensions were prepared by the stock solution with ratio 1:100 with PBS followed by 1:20 dilution with same solution to produce 5×10^2 – 2.5×10^3 yeast cells/ml. The diluted cultures were spread on SDA using a sterile cotton swab. The culture plates were kept at room temperature for 15 min to dry. Subsequently, the plant extract disks were applied on the agar and kept again at room temperature for 15 min and then incubated at 37°C for 24 h. At last, the diameter of zone of inhibition was measured.

The plant extracts were examined in terms of antifungal activities through the determination of MIC, according to CLSI documents with slight modifications. 100 μl of the two fold dilution of the antifungal agents which dissolved in Sabouraud's dextrose broth (SDB) (Fluka, Germany), were inoculated with 100 μl of inoculum containing between 5×10^2 to 2.5×10^3 yeast cfu/ml using U-bottom 96-well microplates (Brand 781660, Wertheim, Germany). The microplates, including plant extracts and cells, were incubated at 35°C for 24h MICs were measured at 530 nm using an EMaxs micro-plate reader. Time-kill methods as detailed above were utilized with the following modifications. The inoculum for each isolate were studied against onion, shallot, and garlic 1×10^6 cell/ml. Antifungals were tested at concentrations equal to $2 \times$ MIC for each isolate. Test samples were incubated at 35°C with agitation. Aliquots were removed from

each test solution for colony count determination at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 h following inoculation. The plating procedures described above were followed for high- and medium-inoculum samples; however, the limit of fungal quantitation was lowered to approximately 30 CFU/ml for each isolate in the low-inoculum group. This was accomplished by plating 100 µl of the test sample directly onto an RPMI 1640 agar plate without dilution. Plates were incubated at 35°C for 24-48 h prior to determination of colony counts. All experiments were conducted in duplicate. Simultaneously, along this stage, microscopic investigation at the time of 0, 24 and 48 hours with extracts effects performed and as control, all of these processes had been done without extracts [11].

Preparation of proper condition for quorum sensing in *Candida albicans* ATCC 10231:

Yeast cells were washed in phosphate buffer saline and adjusted to a cell density of 1×10^6 cells/ml in RPMI-1640. L-glutamine (Sigma), 10% fetal bovine serum (FBS) and 100 U ml⁻¹ penicillin-streptomycin (both from Gibco, Invitrogen) were added to the mixture. The cells were incubated at 37 °C with 5% CO₂.

RNA extraction and cDNA synthesis

A suspension containing different concentrations of antifungal agents and 1×10^6 cells/ml of *C. albicans* ATCC 10231 were prepared. Subsequently, the mixture was centrifuged at 3000 rpm for 10 min and the supernatant was removed. The cells were washed with approximately 2 ml of PBS, and then centrifuged at 3000 rpm for 10 min and the supernatant were removed. The washing process was repeated at least three times. Subsequently, total RNA was extracted using RNeasy mini kit (Qiagen, Germany) for yeast and treated with 1U DNase I (Promega, UK). RNA quality was checked by formaldehyde-denaturing agarose gel electrophoresis at 70 V for 45 min and also the concentration and absorption ratio of RNA was measured for purity estimation using the Nanodrop ND-1000 spectrophotometer. According to manufacturer's protocol, single stranded cDNA was synthesized (approximately 0.5–1 µg) from RNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA). The reverse transcription reactions were performed at least in triplicates.

Semi-quantitative RT-PCR

Candida albicans *TUP1* gene was amplified from the synthesized cDNA. In this study, the primers used were established by other investigators (Table 1). Moreover, beta actin was established as a house-keeping gene and internal control to normalize the dissimilar RNA concentrations during RNA extraction. Furthermore, for each sample an internal negative control (without M-MuLV reverse transcriptase) was performed to ensure that the PCR products were not originated from genomic DNA. PCR products were performed by gel electrophoresis and visualized via the AlphaImager HP imaging system. The PCR products were quantitated in terms of intensity of bands by comparing to known molecular weight DNA markers (Fermentas, USA) using AlphaImager software. The mathematical calculation method of relative quantification was determined as follows:

$$\text{Fold change in target gene expression} = \frac{\text{Ratio of target gene expression (experiment/untreated control)}}{\text{Ratio of reference gene expression (experiment/untreated control)}}$$

Table 1: Oligonucleotide primers used for PCR.

Name of Primer	Sequence	Size of Product	Reference
<i>TUP1</i> Sense	5'AGAAGATTATGACTCAAAGTACCAAC 3'	313 bp	(12)
<i>TUP1</i> Antisense	5'AATGTATTGTGACTTGTCGATAACC 3'		
Beta- <i>ACT</i> Sense	ACCGAAGCTCCAATGAATCCAAAATCC 3"5	516 bp	(13)
Beta- <i>ACT</i> Antisense	GTTTGGTCAATACCAGCAGCTTCCAAA 3" 5		

RESULT

Disk diffusion assay is a simple and reliable preliminary screening test to investigate the antifungal activity of extracts. The plants investigated in this study have been used in traditional medicine for their antimicrobial and detoxification properties. However the result of preliminary test using the disk diffusion method demonstrated that shallot, garlic and onion could be able to show the strong antifungal properties against all *Candida albicans*, but shallot extract antifungal effect was stronger than onion and garlic extract. The diameter of inhibition zone for shallot, garlic and onion extracts with concentration of 100mg/ml, were 25, 17 and 14 mm against *Candida albicans* respectively. Also the diameter of inhibition zone for the antifungal chemical agent Amphotericin B, ketoconazole and nystatin were 16, 33 and 25 mm against *Candida albicans* respectively. Evaluation of plant extracts effect on *Candida albicans* ATCC 10231 indicated, as the time past, colonies of the microorganism shows growth reduction in the presence of

different concentration of aqueous extracts of shallot, garlic and onions. However, in the absence of these extracts excellent growth was observed.

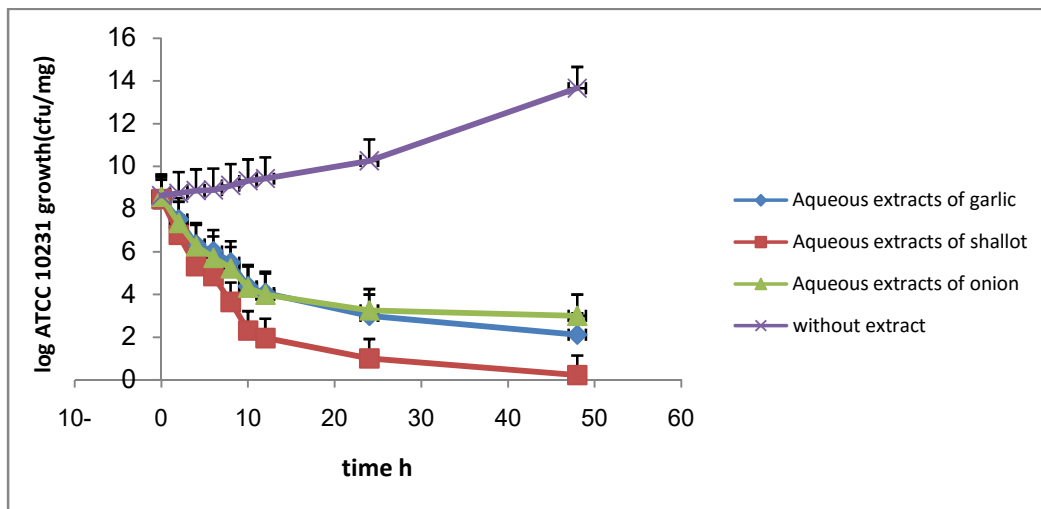


Fig 1. Evaluation of the growth of *Candida albicans* ATCC 10231 in the absence and presence of aqueous extracts of garlic, shallots and onions.

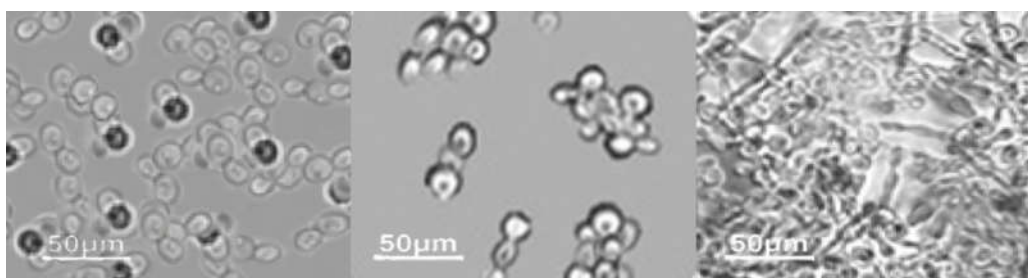


Fig 2. The growth of the yeast *Candida albicans* ATCC 10231 at different times without the presence of plant extracts. Magnification $\times 40$, Bar = $50\mu\text{m}$
The pictures from the left randomly show the time on 0, 12 and 24 hours.

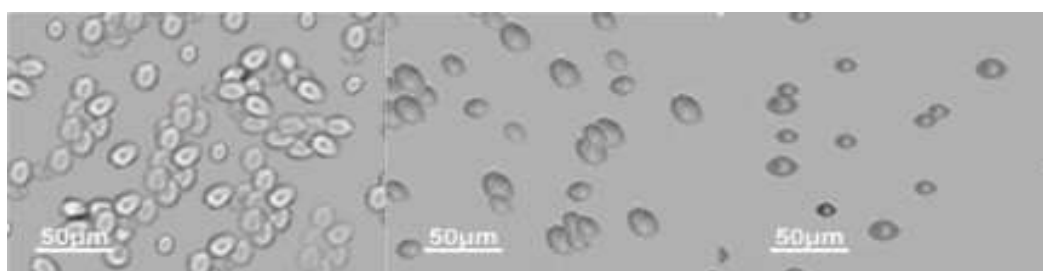


Fig 3. The growth of the yeast *Candida albicans* ATCC 10231 at various times in the presence of aqueous extracts of shallot. Magnification $\times 40$, Bar = $50\mu\text{m}$
The pictures from the left randomly show the time on 0, 12 and 24 hours.

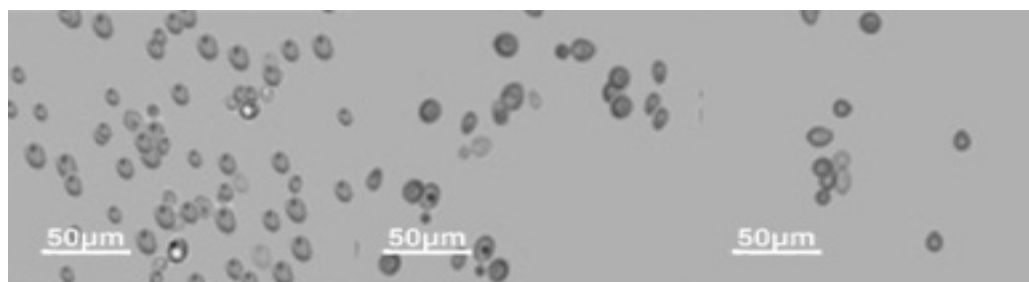


Fig 4. The growth of the yeast *Candida albicans* ATCC 10231 at various times in the presence of aqueous extracts of garlic. Magnification $\times 40$, Bar = $50\mu\text{m}$

The pictures from the left randomly show the time on 0,12 and 24 hours.

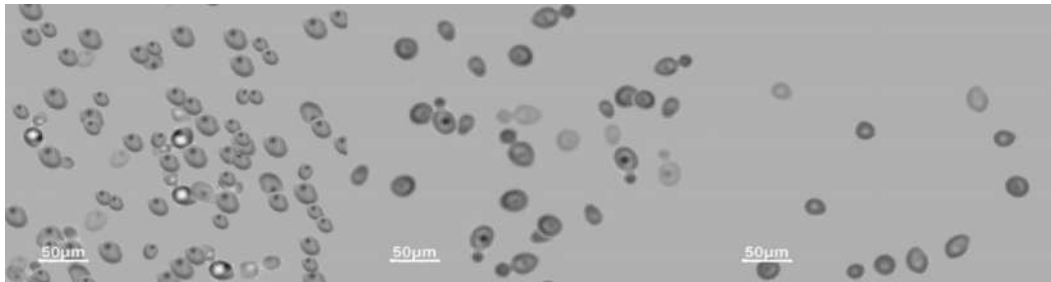


Fig 5.The growth of the yeast *Candida albicans* ATCC 10231 at various times in the presence of aqueous extracts of onion . Magnification × 40, Bar = 50 µm.

The pictures from the left randomly show the time on 0.12 and 24 hours.

According to the Microscopic observations, partly inhibition of quorum sensing due to prevent the formation of hyphae was observed, since the phenomenon of quorum sensing in *C. albicans* formed hyphae direct connection.

Table 2.The MIC and MFC for various species of *Candida albicans* in the presence of aqueous extracts of shallot, garlic and onion.

Isolates	Aqueous extracts of shallot			Aqueous extracts of garlic			Aqueous extracts of onion		
	MIC50	MIC90	MFC	MIC50	MIC90	MFC	MIC50	MIC90	MFC
<i>C.albicans</i> ATCC 10231	6.27	11.28	12.54	12.54	22.57	25.08	12.89	23.20	25.78
<i>C.albicans</i> CI-1*	6.25	11.26	12.51	12.95	23.31	25.90	12.95	23.32	25.91
<i>C.albicans</i> CI-2*	6.80	12.24	13.60	12.90	23.22	25.80	12.80	23.04	25.60
<i>C.albicans</i> CI-3*	6.82	12.27	13.64	12.92	23.25	25.84	12.95	23.31	25.90
<i>C.albicans</i> CI-4*	6.57	11.82	13.14	12.90	23.22	25.80	12.90	23.22	25.80
<i>C.albicans</i> CI-5*	6.55	11.80	13.10	12.80	23.04	25.60	12.90	23.22	25.80
<i>C.albicans</i> CI-6*	6.36	11.44	12.72	12.75	22.95	25.50	12.85	23.13	25.70
<i>C.albicans</i> CI-7*	6.80	12.24	13.60	12.95	23.31	25.90	12.90	23.22	25.80
<i>C.albicans</i> CI-8*	6.42	11.55	12.84	12.96	23.32	25.92	12.79	23.02	25.58
<i>C.albicans</i> CI-9*	6.75	12.15	13.50	12.84	23.11	25.68	12.86	23.14	25.72
<i>C.albicans</i> CI-10*	6.57	11.82	13.14	12.82	23.07	25.64	12.95	23.31	25.90

Clinical Isolates.=*CI: MIC and MFC are measured according to milligram/milliliter.

RT-PCR products indicated ($p \geq 0.05$) *TUP1* gene expression without significant changes under the influence of 2MIC, MIC, ½ MIC plant extracts concentrations. The beta-actin (516bp) and *TUP1*(313bp) genes, which is close to the size of the markers, showed slight differences in the gene expression in control and other samples. The results showed no significant changes ($p \geq 0.05$) for expression of *tup1* gene of *Candida albicans* ATCC 10231 in the presence of plant extract (Fig 6, 7, 8).

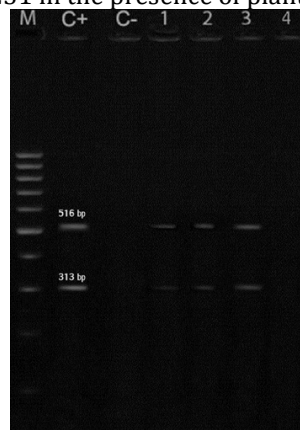


Fig 6. *TUP1* and beta-actin gene expression in *Candida albicans* ATCC 10231 in the presence of different concentrations of Shallot extracts.

C⁺: control positive; C⁻: control negative ; Concentration of Shallot extracts 2MIC1: Concentration of Shallot extracts MIC2: Concentration of Shallot extracts 1/2MIC3: 4: Internal control RT-PCR

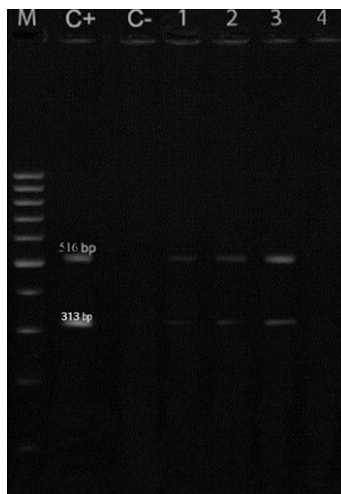
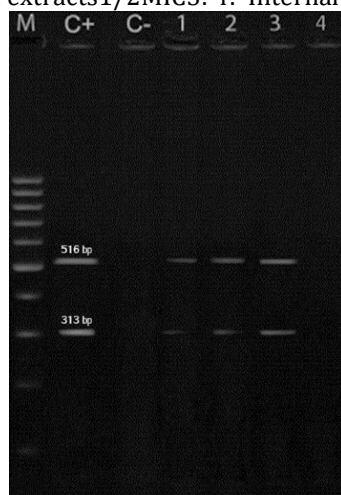


Fig 7 .*TUP1* and beta-actin gene expression in *Candida albicans* ATCC 10231 in the presence of different concentrations of Garlic extracts.

C⁺ : control positive; C⁻: control negative; Concentration of Garlic extracts 2MIC1: Concentration of Garlic extracts MIC2: Concentration of Garlic extracts 1/2MIC3: 4: Internal control RT-PCR



C⁺ : control positive; C⁻: control negative; Concentration of Onion extracts 2MIC1: Concentration of Onion extracts MIC2: Concentration of Onion extracts 1/2MIC3: 4: Internal control RT-PCR

Fig 8.*TUP1* and beta-actin gene expression in *Candida albicans* ATCC 10231 in the presence of different concentrations of Onion extracts.

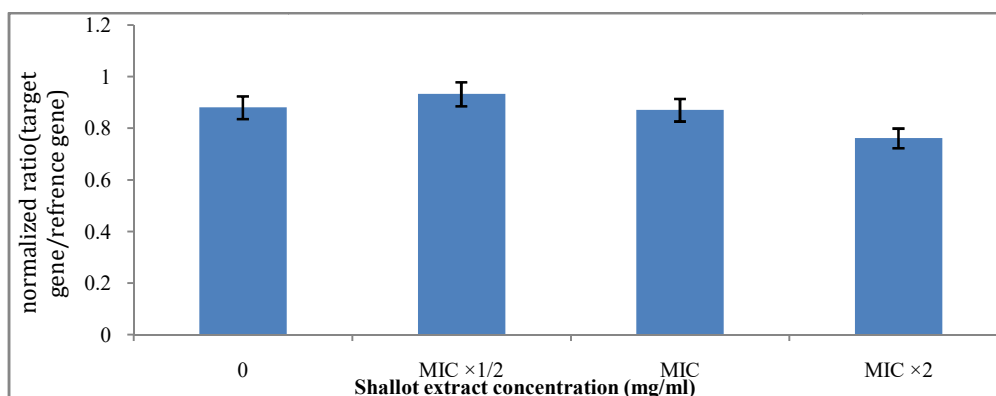


Fig 9.*TUP1* and beta- actin gene expression of *Candida albicans* ATCC 10231 at concentrations of 2 MIC, MIC, 1/2 MIC shallot extract.

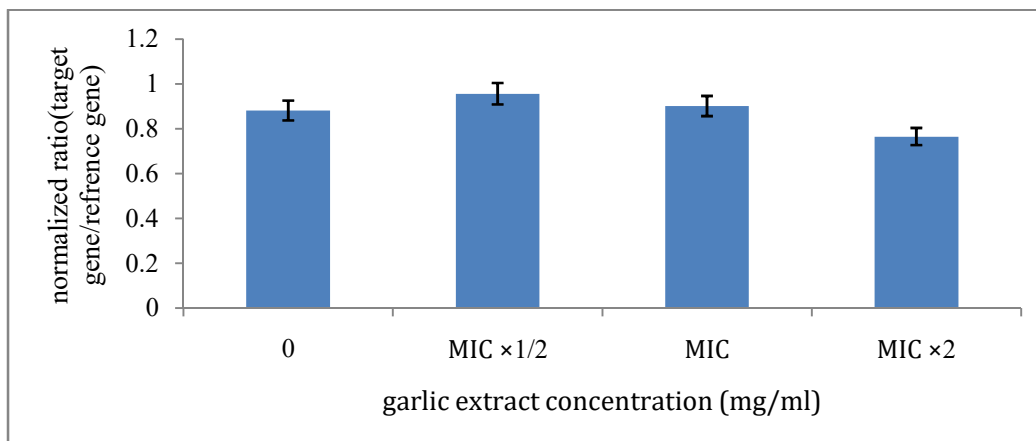


Fig 10. *TUP1* and beta- actin gene expression of *Candida albicans* ATCC 10231 at concentrations of 2 MIC, MIC, ½ MIC garlic extract.

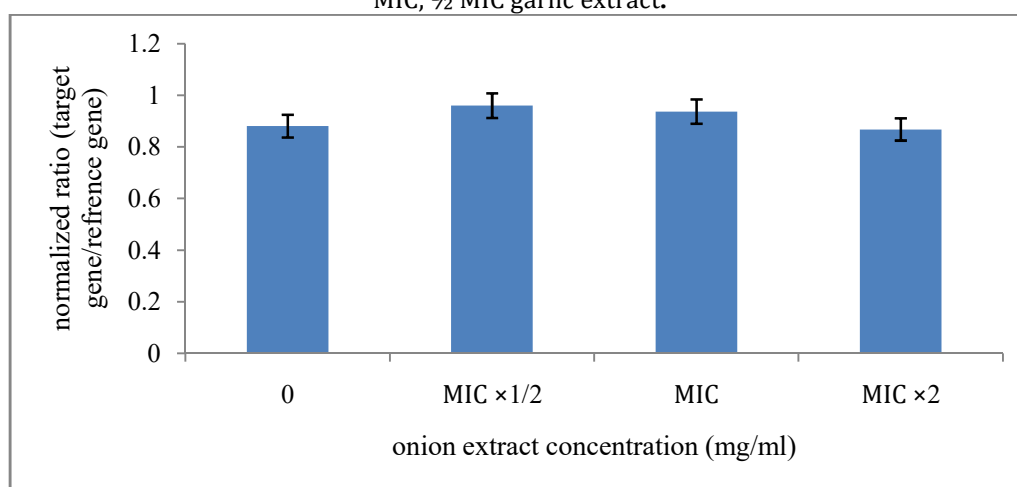


Fig 11. *TUP1* and beta- actin gene expression *Candida albicans* ATCC 10231 at concentrations of 2 MIC, MIC, ½ MIC onion extract.

DISCUSSION

Medicinal, insecticidal, anti-bacterial and anti-fungal properties have been attributed to plant extract. In addition, interest in plant extract as an anti-fungal agent has been renewed recently. Plant extract was demonstrated to be fungicidal against pathogenic yeasts, especially *C. albicans*. Various studies have proved that due to dermatophytes resistance to chemical drugs and their effects, herbal extracts are used instead [14]. In present study, extracts of Shallot, Garlic and Onion were used for their sulfide group effect on cell wall and physiological structure of *candida albicans*. Recent studies showed that herbs such as Shallot in comparison to fluconazole have fewer side effects on dermatophytes, *Candida* and non-pathogenic mold, which makes the herbal medicine more remarkable than chemical drugs. Some researchers have evaluated standard anti-fungal drug compounds against antifungal agents of plant origin. Allicin effect alone and in combination with fluconazole was examined against *Candida* species and also a synergistic effect of them was studied. The results showed that the drug combination work well in anti-fungal properties [11]. On the other hand, some of the important virulence attributes in *Candida* species are include hyphae production, adhesion, phenotypic switching and formation of some extracellular hydrolytic enzymes such as proteinases. Colonization of *Candida* on the surface of tissue is a primary step of infection [15]. Moreover, quorum sensing is a natural obstacle to treatment with some antifungal agents which may result in drug resistance. It is demonstrated that the ability to form quorum sensing and degree of pathogenicity could be collaborative.

CONCLUSION

In present study, the fungicidal concentration of the aqueous Shallot extract against *C. albicans* was higher than Garlic and Onion extracts. In fact, the use of aqueous extracts of Shallot, Garlic and Onion (especially shallot) at different time intervals represent more reduction than in the control group (no extracts shallot, garlic and onion) ($p \leq 0.05$) on yeast cells, so that the cells reach almost to zero after 48 hours of

incubation. According to our results, reduce gene expression by RT-PCR technique has been approved. Molecular studies showed that impact of aqueous extracts of shallot; garlic and onion reduce *TUP1* gene expression in *Candida albicans*. However, in contrast to previous, our findings showed no inhibition effect of these extracts on quorum sensing through changes in *TUP1* gene expression. It is possible that expression of other genes involved in this phenomenon is affected by these extracts. Therefore, consideration of each gene expression involved in this phenomenon in the presence of anti-fungal extracts is critical. The techniques were used in this study may not accurate enough to Measure expression level, so that more appropriate techniques such as Real time RT-PCR is required.

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