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Synergistic effects of Iranian seaweed compounds for anticandidal properties and optimization by Response Surface Methodology

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Abstract

An optimization procedure was applied to investigate the anticandidal property of four different Iranian seaweed extract compositions. Response Surface Methodology (RSM) with the four levels- three–factor Box–Behnken design, including *Gracilaria arquata* concentration (X_1), *Nizimudidnia zanardini* concentration (X_2), *Cystoceria indica* concentration (X_3) and *Padina australis* concentration (X_4), was used to screen for anticandidal property screening. Multiple regression analysis of data showed that the coefficient determination (R^2) was appropriate for the second-order model. The anticandidal property of the seaweed extract composition of 10 mg ml^{-1} *G. arquata* extract, 5 mg ml^{-1} *N. zanardini* extract, 4.5 mg ml^{-1} *C. indica* extract and 2 mg ml^{-1} *P. australis* extract was found to be optimal. Under the optimized composition, the *Candida albicans* growth inhibition zone was 26.23 mm. The experimental yield was well matched with the predicted yield.

Keywords: Anticandidal property, Response Surface Methodology (RSM), Seaweed extract, Box-Behnken design, *Candida albicans*

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Introduction

In the past three decades, fungal infections have increased due to the rising immunocompromised population (Black and Baden, 2007; Anaissie, 2008; Meunier and Lukan, 2008). *Candida albicans* (*C. albicans*) is the main commensal fungus living in the human vaginal canal, oral cavity, and gastrointestinal tract (Xu *et al*., 1999). Due to the delay in the early diagnosis of fungal infection and deficit available antifungal drugs, the antifungal resistance is the main problem (Cannon *et al*., 2007). Developing drug resistance is an old phenomenon. Toxic environmental stresses are obligate Microorganisms responding for millennia (Wright, 2007). Thus *candidiasis* remains a significant social and clinical problem (Kustos *et al*., 2006). In recent years, there have been increasing efforts to find new antifungal compositions.

Recently, finding new antifungal drugs from plants has been interested due to the resistant strains and presence a few antifungal classes (Mishra *et al*., 2007; Peres *et al*., 2010). In more recent years, seaweed research has been increased to find new and effective medicines from natural sources (Kumar *et al*., 2013). Many seaweeds natural products are being used, and different antibacterial and antifungal compounds have been reported from algal extracts (Guedes *et al*., 2012).

Drug synergism is a recently reported novel idea (Silverman and Holladay, 2014). The antimicrobial medicinal plants that show synergism with antimicrobial medicines is essential source of antimicrobial compounds for the use in combination therapies (Rakholiya *et al*., 2013). These compounds may have no strong antibacterial properties, but may synergize with other drugs (Pyun and Shin, 2006; Ushimaru *et al*., 2012). Combining antifungal drugs is increasingly thought of as a useful approach (Marr *et al*., 2004; Sobel, 2004); more significant antimicrobial effect than the individual drug effect and possible smaller doses need than with monotherapy are the potential benefits, thus resulting in maximizing the spectrum of antifungal coverage (Sobel, 2004).

This study was conducted to optimize the anticandidal properties of compositions of four different seaweed extracts from Chabahar coast (Iran) using RSM to evaluate the growth inhibition of *C. albicans*.

Material and methods

Algae were collected from the beach of Chabahar (Iran) at a latitude of 25° 16′ N and longitude of 60° 39′ E during the low tide. After washing with distilled water, algae was dried in an oven at 45 ˚C for 3 h and crushed in a lab blender (Guedes *et al*., 2012). 125 g of dried samples macerated in 250 ml of ethanol. The extract was filtered and treated in a Rota evaporator at 40 ˚C. The weight of the dried extract was 4.58 g.

Pure cultures of *C. albicans* procured from the Persian Type Culture Collection (PTCC), Tehran, Iran. First, SDA broth was prepared, inoculated, and incubated at 25 ˚C. Then, the

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antifungal activity of seaweed extracts evaluated with a disc diffusion assay by loading 15 µg/ml of the extract on to the blank disk. Nystatin (Padtan teb, 100 units) used as a positive control.

Minimum inhibition concentrations (MIC) of the seaweed extracts evaluated according to the reference method (Standards, 2002), with some modifications. Briefly, The *C. albicans* was cultured in RPMI 1640 broth medium (5.0×10^2 to 2.5×10^3 cells per ml). Before adjusting the inoculum, 0.1 ml of the various anticandidal concentrations were added to the test tubes based on the ranges mentioned in Table 1 for each algal extract, 0.9 ml of which was placed in each tube in the dilution series and mixed. The tubes incubated at 35˚C for 48 h. Microscope used in order to yeast cells number counting. The lowest concentration of the extracts (colonies by ≥99.99% compared to the control) was taken as the minimum fungicidal concentration (MFC) (Chudzik *et al*., 2013).

To determine the best combination of seaweed extracts by enhanced anticandidal properties, a four-variable, three-level Box-Behnken design (BBD) was used. The four independent variable sets were concentrations of *G. arquata* extract (mg ml^{-1}, X_1), *Nizimudidnia zanardini* extract (mg ml^{-1}, X_2), *Cystoceria indica* extract (mg ml^{-1}, X_3) and *Padina australis* extract (mg ml^{-1}, X_4) (Table 1). The response was the growth inhibition zone. The following equation performed for the experimental data after regression analysis:

$$Y = \alpha_0 + \sum_{i=1}^{4} \alpha_i X_i + \sum_{i=1}^{4} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \alpha_{ij} X_i X_j$$

Table 1: Independent variables and their levels used in the response surface design.

Independent variables	Factor level		
	-1	**0**	**+1**
X_1 concentrations of *Gracilaria arquata* extract (mg ml^{-1})	4	7	10
X_2 concentrations of *Nizimudidnia zanardini* extract (mg ml^{-1})	2.5	5	7.5
X_3 concentrations of *Cystoceria indica* extract (mg ml^{-1})	2.5	5	7.5
X_4 concentrations of *Padina australis* extract (mg ml^{-1})	1	2	3

Where Y is offset term, α_0 is a constant, α_i, α_{ii} and α_{ij} are regression coefficients and X_i and X_j are the levels of the independent variables. The range of independent variables and their levels are presented in Table 1, obtained of preliminary experiments. Design-Expert software (7.0.0 trial, Stat-Ease Inc., Minneapolis, MN, USA) used to analysis of the experimental design. The usefulness of the design evaluated by use of different random combinations of parameters optimized design.

Results

A summary of the anticandidal activity of seaweed extracts against *C. albicans* is shown in Table 2. The ethanolic extract from all species of seaweeds was active against the yeast *C. albicans.* The extract of *Padina australis*, *N. zanardini*, and *G. arquata* showed significant inhibition zone of greater

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than 20 mm diameter (24±0.9, 23±0.0 and 21±0.7 mm, respectively).

The MIC and MFC values of algal extracts against *C. albicans* are summarized in Table 2. The least MIC and MFC values were recorded for *P. australis* crude extract as 1.25 and 1.7, respectively. The maximum concentration of MIC and MFC values was recorded for the crude extract of *Gracilaria arquata.*

Table 2: Inhibitory activity of different Seaweed extracts and Nystatin against *Candida* sp. Individually.

species	Inhibitory activity against *Candida albicans*		
	Inhibition zone (mm)	MIC (mg ml^{-1})	MFC (mg ml^{-1})
Gracilaria arquata	21± 0.7	7.12	8.2
Nizimudidnia zanardini	23± 0.0	4.75	5.25
Cystoceria indica	19± 0.6	5	6
Padina australis	24± 0.9	1.25	1.7
Nystatin	26± 0.1	25 (µg ml^{-1})	30 (µg ml^{-1})

The four individual parameters were optimized in the Box–Behnken design (4^3 factorial) using 29 design experiments (Table 3). The mean values of the responses (*Candida sp.* growth inhibition zone) obtained from the different combinations of seaweed extracts are summarized in Table 4. The application of RSM yields the below regression equation:

$$Y= -18.35972+4.22778X_1+2.97X_2+3.23333X_3+8.95833X_4-0.11X_1X_2-0.14X_1X_3-0.18333X_1X_4+0.208X_2X_3+0.43X_2X_4-0.16X_3X_4-0.13472X_1^2-0.368X_2^2-0.284X_3^2-2.1625X_4^2$$

Table 3: Experimental design used in RSM studies by using four independent variables with five center points showing the observed Inhibition Zone of *Candida albicans* growth.

Run No.	Coded levels of variable				Inhibition Zone (mm)
	X_1	X_2	X_3	X_4	
1	-1	-1	0	0	18.2
2	1	-1	0	0	24
3	-1	1	0	0	21.7
4	1	1	0	0	24.2
5	0	0	-1	-1	21.3
6	0	0	1	-1	21.6
7	0	0	-1	1	22.4
8	0	0	1	1	21.1
9	-1	0	0	-1	17.8
10	1	0	0	-1	24.4
11	-1	0	0	1	20.5
12	1	0	0	1	24.9
13	0	-1	-1	0	20.5
14	0	1	-1	0	20.5
15	0	-1	1	0	19.3
16	0	1	1	0	24.5
17	-1	0	-1	0	18.5
18	1	0	-1	0	24

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Table 3 continued:					
19	-1	0	1	0	21.7
20	1	0	1	0	23
21	0	-1	0	-1	20.3
22	0	1	0	-1	20
23	0	-1	0	1	18.5
24	0	1	0	1	22.5
25	0	0	0	0	25.9
26	0	0	0	0	25
27	0	0	0	0	24.4
28	0	0	0	0	25.7
29	0	0	0	0	25

Based on the ANOVA of the regression, the model was significant. A high F-value (16.32) and a low P-value (p<0.0001) was seen (table 4). Correlation coefficient (R^2) value was 0.9423. The value of the adjusted determination coefficient was 0.8845 and coefficient of variation was a low 3.74%. The statistical analysis has a high significant value (p<0.0001), testifying the fitness of the model for the growth inhibition zone. F-value was non significant (p<0.2288) for the lack of fit.

Table 4: ANOVA results of growth inhibition zone of *Candida albicans* as affected by different concentrations of seaweed extracts during an optimization experiment.

	Sum ofSquares	df	Mean Square	F Value	P Value
Model	156.49	14	11.18	16.32	<0.0001
Linear					
A-G. arcuata	56.77	1	56.77	82.88	<0.0001
B-N. zanardini	13.23	1	13.23	19.32	0.0006
C-C. indica	1.33	1	1.33	1.95	0.1847
D-P. australis	1.69	1	1.69	2.46	0.1388
Interaction					
AB	2.72	1	2.72	3.97	0.0660
AC	4.41	1	4.41	6.44	0.0237
AD	1.21	1	1.21	1.77	0.2051
BC	6.76	1	6.76	9.87	0.0072
BD	4.62	1	4.62	6.75	0.0211
CD	0.64	1	0.64	0.93	0.3501
Quadratic					
A^2	9.54	1	9.54	13.92	0.0022
B^2	34.31	1	34.31	50.10	<0.0001
C^2	20.44	1	20.44	29.84	<0.0001
D^2	30.33	1	30.33	44.29	<0.0001
Residual	9.59	14	0.68		
Lack of Fit	8.13	10	0.81	2.23	0.2288
Pure Error	1.46	4	0.37		
Cor Total	166.08	28			

Residual values distribution in the normal probability plot (Fig.1a) forms a straight line by a normal distribution of residual values on both sides. Further, the observed values was compared with adequate predicted values. The parity plot (Fig.1b) was at an acceptable level.

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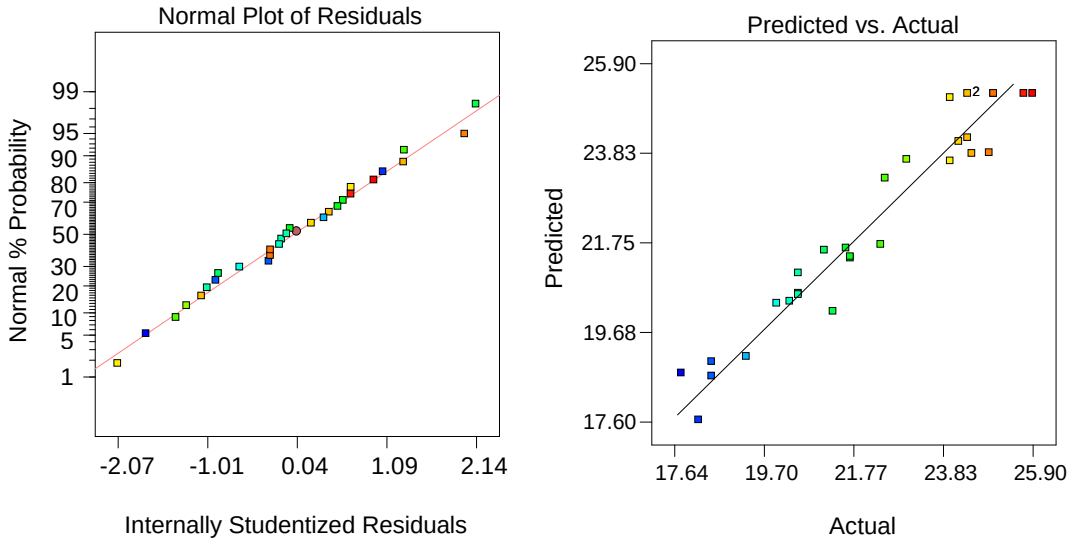

Figure 1: Normal plot of residual and relationship between the observed and predicted values.

The main and interactive effects of the independent variables on the dependent one illustrated by the 3D response surface plots (Fig. 2a-f). Fig. 2a, b and c shows the effects of *G. arquata* concentration (X_1) with each of the three other factors on the inhibition of *C. albicans* growth. Based on the Fig. 1a, b and c, it implies that the *G. arquata* concentration is more significant than the other variables. In all cases there was a clear optimal concentration of *G. arquata* that peaked at 10 mg ml^{-1}. Fig. 2a, d and e show the effects of *N. zanardini* concentration (X_2) with each of the three other factors on the inhibition of of growth of *Candida* sp.. In all situations, the inhibition zone increased with increasing *N. zanardini* concentration from 3.8 to 6.3 mg/ml, while less than 3.5 and more than 6.5 mg ml^{-1} appeared to be disadvantaged regarding the inhibition of the growth, which indicated that the *N. zanardini* concentration has remarkable effects on the response. The effects of *C. indica* concentration (X_3) interaction with each of the other factors on the inhibition zone are shown in Fig. 1b, d and e. Higher concentration resulted in higher inhibition. The inhibition zone of *C. albicans* growth increased by increasing the concentration from 3.6 to 6.3 mg ml^{-1}, and after that decreased.

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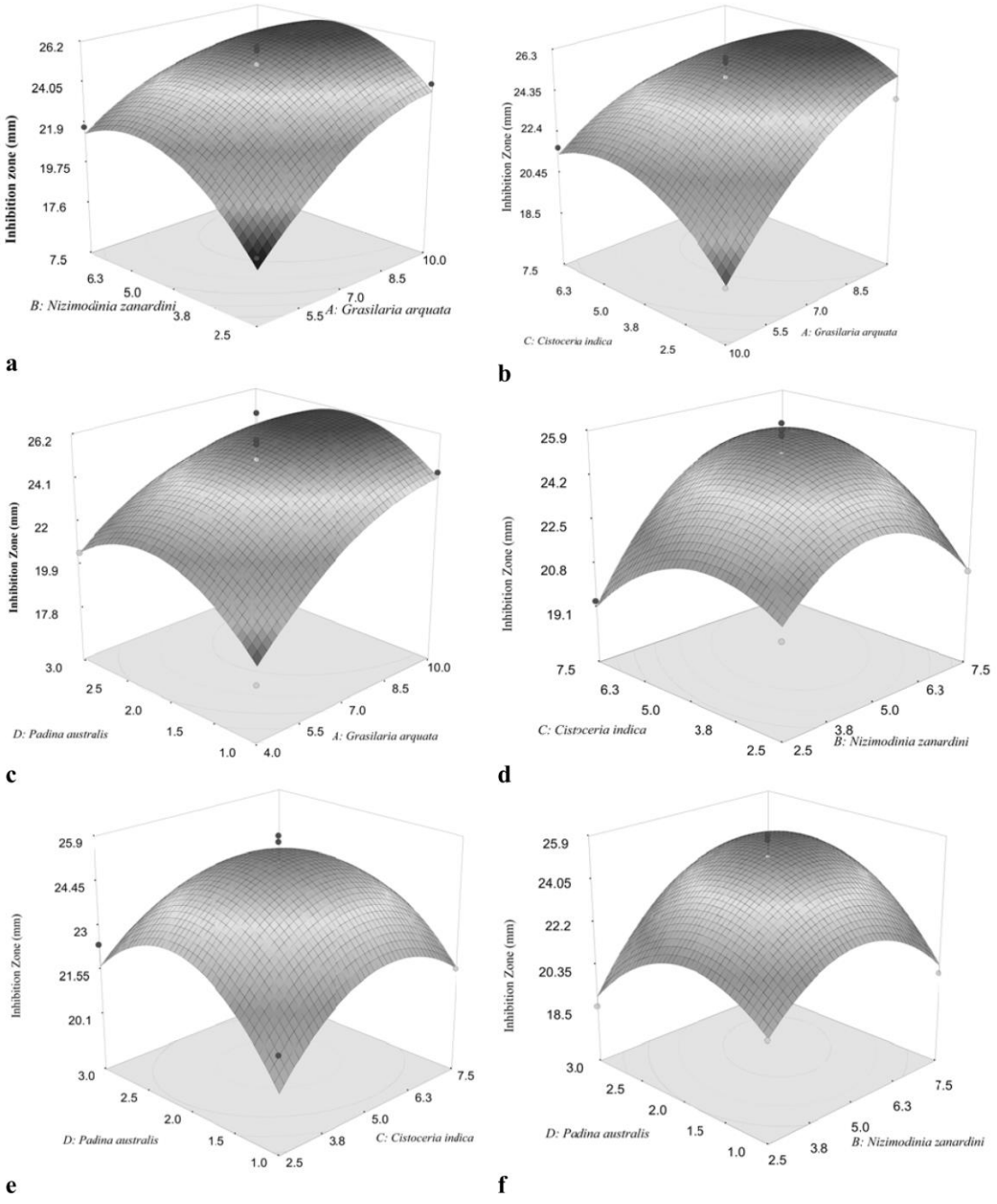

Figure 2: Response surface plots of the effect of seaweed extract concentrations and their mutual interactions on the Inhibition of *Candida albicans* growth.

Discussion

In recent years, fungal infections are an increasing health problem, ranging from external to deeply disseminated (Duraipandiyan and Ignacimuthu, 2011). Seaweeds can produce a different kind of secondary metabolites and is a source of bioactive metabolites. Compounds with cytotoxic and antimicrobial activities have been reported from a range of marine algae (Yuan *et al*., 2005; Bansemir *et al*., 2006; Chew *et al*., 2008).

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In this study, inhibition zones of *C. albicans* growth were used as a signal of antimicrobial activity. However, zone inhibition diameters varied according to the kind of seaweed extracts; but three different seaweed extracts showed an inhibition zone of more than 20 mm. This inhibition zone diameter showed the higher activity of the extracts against the yeast. This high activity may be due to the presence of bioactive metabolites soluble in ethanol (Tuney *et al*., 2006).

Based on past research, the anticandidal activity of the marine algae, *Asparagopsis armata*, (inhibition zone [IZ] of 53.2 mm), was evaluated (Salvador *et al*., 2007). Also the anticandidal activity of *U. lactuca* (IZ: 5.2-23.2 mm), *U. fasciata* (IZ: 6 mm), *E. compressa* (IZ: 10 mm), *P. capillacea* (IZ: 15.4 mm), *P. pavonica* (IZ: 15.4 mm) and *Ulva rigida* ((IZ: 12 mm) was reported (Ertörk and Tas, 2011; Shobier *et al*., 2016). Based on the literature, antifungal effects against *C. albicans* was reported for chloroform and ethyl acetate extracts of brown algae (ranged from 9 to 25 mm), but *U. lactuca* extracts showed no activity (Mhadhebi *et al*., 2012; Guedes *et al*., 2012). The results of the present study were better than most of the above-mentioned research as the inhibition zone was more than 20 mm.

Shobier *et al*. (2016), reported the MIC value of *U. lactuca* (8-16 µg ml^{-1}) and *U. fasciata* (128 µg ml^{-1}). The chloroform extract of *P. gymnospora* showed MIC at 16 µg ml^{-1} against *Candida sp.* (Guedes *et al*., 2012). Ertörk and Tas (2011) reported more than 10 µg ml^{-1} MIC for *U. rigida* extracts against *C. albicans*. Anticandidal activities of Brazilian seaweed extracts showed MIC at the range of 65–303 µg ml^{-1} (Stein *et al*., 2011). Ethanol extracts of *H. musciformis* demonstrated MIC at 8 µg ml^{-1} against *C. albicans*. The MIC and MFC results of the present study were higher than these studies. Maybe the more screening is needed in order to detect the active biochemical substances in the crude extracts.

Correlation coefficient (R^2) value in this study implies that sample variations of 94.23% for growth inhibition zone are attributable to the independent variables. The adjusted determination coefficient also confirm the model significance. A high degree of precision and a good deal of reliability of the experimental values was evaluated by the value of coefficient of variation. The model must exhibits a good fit and checking the model adequacy is essential (Wang *et al*., 2008). F-value for the lack of fit confirming the model validity. The results confirmed that the model is adequate for the prediction of experimental variables. The resulting maximum value of growth inhibition zone of 26.23 mm obtained at 91.7% desirability with the following concentrations of different seaweed extracts:

Gracilaria arquata extract (10 mg ml^{-1}), *Nizimudinia zanardini* extract (5 mg ml^{-1}), *Cystoceria indica* extract (4.5 mg ml^{-1}), *Padina australis* extract (2 mg ml^{-1}).

In the past years, combination therapies study by more than one active

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ingredient more interested at pharma industries in compare to the search for specifically targeted drugs, for example, most Chinese drugs are mixtures of diverse herbal medicines (Qiu, 2007). Inside total extract of an herb, or between different herbs extracts, synergistic interactions could be seen (Williamson, 2001). This synergy may be the reason of isolating failed for single active compound from herbal drugs (Stermitz *et al*., 2000). Maximum therapeutic efficacy with minimal side effects could be resulted in remedial treatment containing multiple drugs with related mechanisms (Wang *et al*., 2008).

Among two agents by synergistic interaction, one of them enhanced the activity of the second one and they could act more effective in combination. An increasing of antimicrobial activity, better treatment at antimicrobial resistance, inhibition of resistance development, use less dosage of toxic agents, and enhanced bactericidal activity compared with monotherapy is the other improved efficacy of combination therapy (Harris and Coote, 2010).

Synergistic effects of antibacterial compounds is a new research category. The combination therapy of herbal extracts and antimicrobial agents can result in the inhibition of microbial pathogens, or it may happen because the combination is as effective as their individual therapeutic potential (Gibbons, 2004).

Based on our results, the combination of seaweed extracts had an additional inhibition effect on the growth of *Candida albicans*, compared with individual extract usage. This could be a new finding, in anticandidal therapy. No literature found on the synergistic effects of marine algae extracts, but synergistic effects of catechins, antimycotics, and copper sulfate in the anticandidal therapy was reported (Anand and Rai, 2017). Also, a combination of menthol with itraconazole and nystatin has been showed the synergistic anticandidal activity (Sharifzadeh *et al*., 2017). The synergistic effect of optimized seaweed extract compounds could be a novel category in the Candidiasis therapy.

In conclusion, the combined extracts of four different seaweeds are useful materials for *C. albicans* growth inhibition versus using this extract individually. The highest inhibition zone of candida growth was found based on the following concentrations: *G. arquata* extract (10 mg ml^{-1}), *N. zanardini* extract (5 mg ml^{-1}), *C. indica* extract (4.5 mg ml^{-1}), *P. australis* extract (2 mg ml^{-1}) which resulted in a growth inhibition zone of 26.23 mm for *C. albicans*.

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