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

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Received: October 2014

Accepted: November 2018

## 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<sub>1</sub>), *Nizimudidnia zanardini* concentration (X<sub>2</sub>), *Cystoceria indica* concentration (X<sub>3</sub>) and *Padina australis* concentration (X<sub>4</sub>), 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<sup>-1</sup> *G. arquata* extract, 5 mg ml<sup>-1</sup> *N. zanardini* extract, 4.5 mg ml<sup>-1</sup> *C. indica* extract and 2 mg ml<sup>-1</sup> *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 properties, antibacterial but may synergize with other drugs (Pyun and Shin, 2006; Ushimaru et al., 2012). Combining antifungal drugs ic 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 antifungal activity of seaweed extracts evaluated with a disc diffusion assay by loading 15  $\mu$ g/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  $(5.0 \times 10^2 \text{ to } 2.5 \times 10^3 \text{ cells per})$ ml). Before adjusting the inoculum, 0.1 of various anticandidal ml the 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  $\geq 99.99\%$  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) used. The four independent was variable sets were concentrations of G.  $ml^{-1}$ ,  $X_1$ ), arquata extract (mg Nizimudidnia zanardini extract (mg ml  $^{1}$ , X<sub>2</sub>), Cystoceria indica extract (mg  $ml^{-1}$ , X<sub>3</sub>) and Padina australis extract (mg ml<sup>-1</sup>,  $X_4$ ) (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 -1 0 4 7		level	
	-1	0	+1	
$X_1$ concentrations of <i>Gracilaria arquata</i> extract (mg ml <sup>-1</sup> )	4	7	10	
$X_2$ concentrations of <i>Nizimudidnia zanardini</i> extract (mg ml <sup>-1</sup> )	2.5	5	7.5	
$X_3$ concentrations of <i>Cystoceria indica</i> extract (mg ml <sup>-1</sup> )	2.5	5	7.5	
$X_4$ concentrations of <i>Padina australis</i> extract (mg ml <sup>-1</sup> )	1	2	3	

Where Y is offset term,  $\alpha_0$  is a constant,  $\alpha_i$ ,  $\alpha_{ii}$  and  $\alpha_{ii}$  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 experiments. preliminary 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 use of different random by

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 than 20 mm diameter  $(24\pm0.9, 23\pm0.0)$ and  $21\pm0.7$  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.

	Inhibitory activity against Candida albicans			
species	Inhibition zone (mm)	MIC (mg ml <sup>-1</sup> )	MFC (mg ml <sup>-1</sup> )	
Gracilaria arquata	$21 \pm 0.7$	7.12	8.2	
Nizimudidnia zanardini	$23 \pm 0.0$	4.75	5.25	
Cystoceria indica	$19 \pm 0.6$	5	6	
Padina australis	$24 \pm 0.9$	1.25	1.7	
Nystatin	$26\pm0.1$	25 ( $\mu g m l^{-1}$ )	$30 (\mu g  m l^{-1})$	

The four individual parameters were optimized in the Box–Behnken design  $(4^3 \text{ factorial})$  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= -  $18.35972+4.22778X_{1}+2.97X_{2}+3.23333$   $X_{3}+8.95833X_{4}-0.11X_{1}X_{2}-0.14X_{1}X_{3} 0.18333X_{1}X_{4}+0.208X_{2}X_{3}+0.43X_{2}X_{4} 0.16X_{3}X_{4}-0.13472X_{1}^{2}-0.368X_{2}^{2} 0.284X_{3}^{2}-2.1625X_{4}^{2}$ 

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 <sub>1</sub>	$\mathbf{X}_2$	X <sub>3</sub>	X <sub>4</sub>	_ ` `	
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	

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<sup>2</sup>) 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
$\mathbf{B}^2$	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.

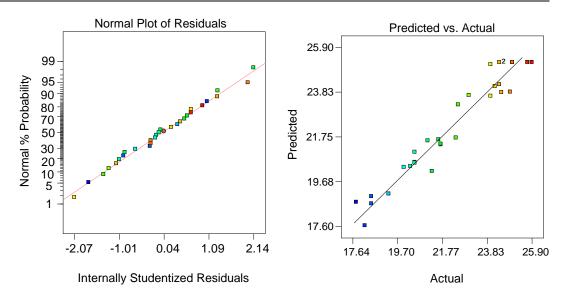


Figure 1: Normal plot of residual and relationship between the observed and predicted values.

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. arguata 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. concentration arquata 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<sup>-1</sup>. 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 increased zone 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<sup>-1</sup> appeared be disadvantaged regarding the to inhibition of the growth. which indicated that the Ν. 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<sup>-1</sup>, and after that decreased.

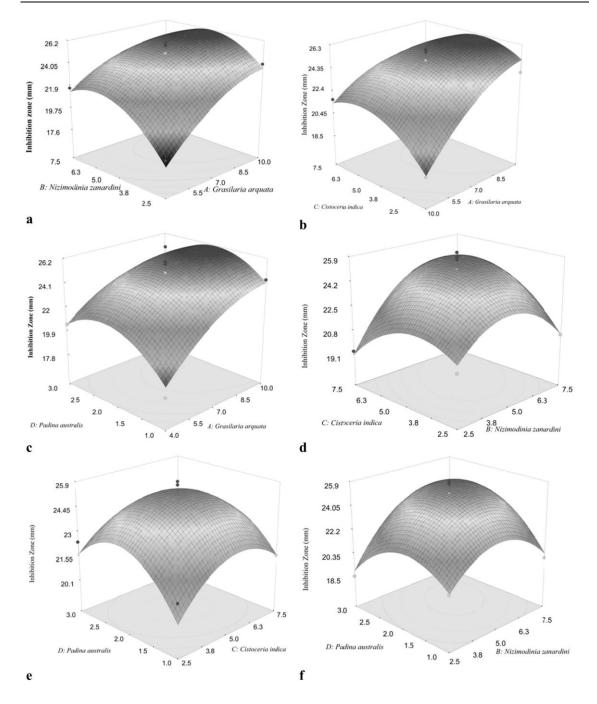


Figure 2: Response surface plots of the effect of seaweed extract concentrations and their mutual interactions on the Inhibition of *Candida albicans* growth.

#### 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). In this study, inhibition zones of C. albicans growth were used as a signal antimicrobial activity. However, of inhibition zone 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 research, past 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), Е. compressa (IZ: 10 mm), P. capillacea (IZ: 15.4 mm), P. pavonica (IZ: 15.4 mm) and Ulva rigida ((IZ: 12 mm) was reported (Ertürk 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 abovementioned research as the inhibition zone was more than 20 mm.

Shobier *et al.* (2016), reported the MIC value of *U. lactuca* (8-16  $\mu$ g ml<sup>-1</sup>) and *U. fasciata* (128  $\mu$ g ml<sup>-1</sup>). The chloroform extract of *P. gymnospora* showed MIC at 16  $\mu$ g ml<sup>-1</sup> against *Candida sp.* (Guedes *et al.*, 2012). Ertürk and Tas (2011) reported more

than 10  $\mu$ g ml<sup>-1</sup> MIC for U. rigida С. extracts against albicans. Anticandidal activities of Brazilian seaweed extracts showed MIC at the range of 65-303 µg ml<sup>-1</sup> (Stein et al., Ethanol 2011). extracts of Н. musciformis demonstrated MIC at 8 µg ml<sup>-1</sup> 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 the active biochemical detect 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 following with the concentrations of different seaweed extracts:

*Gracilaria arquata* extract (10 mg ml<sup>-1</sup>), *Nizimudinia zanardini* extract (5 mg ml<sup>-1</sup>), *Cystoceria indica* extract (4.5 mg ml<sup>-1</sup>), *Padina australis* extract (2 mg ml<sup>-1</sup>).

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

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 different herbs between 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 effective more 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<sup>-1</sup>), *N. zanardini* extract (5 mg ml<sup>-1</sup>), *C. indica* extract (4.5 mg ml<sup>-1</sup>), *P. australis* extract (2 mg ml<sup>-1</sup>) which resulted in a growth inhibition zone of 26.23 mm for *C. albicans*.

### Acknowledgments

The author would like to thank Dr. Ahmad Gharaei, Head of the Hamoon International Wetland Research Institute, for his invaluable help during various stages of this research.

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