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Anti-candidal activity and synergetic interaction of antifungal drugs with differential extract of brown algae *Stocheospermum marginatum*

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ABSTRACT

Candidiasis and candidemia is an emerging condition due to the overgrowth of *Candida albicans* and some of the non-albicans species present in the different mucosal rich sites of healthy individuals. Although there are numerous studies describing the efficiency of phyto-compounds towards candidiasis. This study was evaluated the combinational effects of fluconazole against *Candida albicans* and some non-albicans species, synergistic with different extract of *Stocheospermum marginatum* explored through *in vitro* methods. The average significant zone of inhibition (mm) against all the tested isolates was varied from 0 to 14 mm with the maximum for ethyl acetate extract against *C. parapsilosis*. The maximum and minimum MICs of *S. marginatum* extracts for candida strains were recorded between 0.19 and 50 μg/ml against *C. krusei* and all other candida strains. Fluconazole combined with ethyl acetate extract of *S. marginatum* shown more potential synergetic action against *C. albicans, C. kefyr* and *C. tropicalis* with FITC values lesser than 1. The metabolic footprinting shown that the present of some biologically important components in a different kind of extracted prepared in this study. The present study represents an untapped source of anti-candidal metabolites of *S. marginatum* that could be acted as a phyto skeleton of developing of new therapeutic natural products.

1. Introduction

Candida albicans is the commensal organism, living in the mucous surface such as genital, gastric, respiratory tracts of the healthy individuals, mostly the haemostasis regulated by several species of *Lactobacillus* (Ferrer, 2000; Dignani et al., 2009) and some of the immune responses (Richardson and Moyes, 2015; Qin et al., 2016). The multi drug resistance candidiasis is an emerging condition reports that worldwide considerable mortality. Since 2009, the hospital acquired candida infection has been reported among the five continents (Cortegiani et al., 2018; Hirano et al., 2015). Also, it is estimated that recurrent vaginal candidiasis accounts 158 million women globally per year (Denning et al., 2018) and bloodstream associated candida infection target 8–10% (Pfaller and Diekema, 2007). Uncertainty, due to the alteration of this haemostasis leads to the condition of Candidiasis, thus commensal candida becomes opportunistic in nature (Ascioglu et al., 2002). It is the most common type of nosocomial infection attributing to disturb the millions of women population every year in worldwide mainly by *C. albicans* and some non albicans species. Candida colonisation is mainly attributed by host immune response, the condition that includes immune compressed individuals, during the infection of HIV, administration of immune modulators and extensive chemotherapy (Anaisse et al., 1989). Interleukin 12 Receptor β1 Deficiency was one among the most common condition of 25% of patients displaying mucocutaneous candidiasis (Ouederni et al., 2014). In recent decades the increased incidences of candidiasis reflect to the considerable amount of direct or indirect risk factors including diabetics, hypertension, and intense chemotherapy. The women carrying candida is higher than the man especially in the lower reproductive tract. Disturbance of vaginal lactobacilli and the host defence system took forward the candida to be systematic to the individual.

Azoles are the foremost agent for candida prophylaxis in mucous medicated infections; however, the class of drug like polyene and echinocandin also to be the drug of choice for systemic infection. Due to owing their selective inhibition, extended activity, prolonged action of these drugs forced to use the wild spread for cutaneous to systemic candidiasis (Cornely et al., 2012). Polyene e.g. amphotericin B remains to lesser resistance rate rather than other antifungal agents (Rex et al., 1997; Kanafani and Perfect, 2008; Vincent et al., 2013). During the earlier 1950 amphotericin B was introduced as the first agent for systemic Candidiasis, followed by fluconazole and itraconazole in 1990s. Hence, all three medication were listed as an essential medication by the WHO (Kneale et al., 2016). However, different kinds of adverse effects were reported that including neurotoxicity, nephro-toxicity,

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hypokalaemia, hepatotoxicity and hypomagnesaemia (Mourad and Perfect, 2018; Pasqualotto et al., 2007; Song and Deresinski, 2005; Wazny and Brophy, 2000; Lin et al., 1990; Tucker et al., 1990). There are many research evidenced that extensive, misuse and abuse of powerful antifungal may lead to development of resistance as a consequence of responding external stimuli by an organism. The recent world spread surveillance studies have evidenced that *C. albicans* and some non albicans species showing reduced susceptibility to fluconazole, voriconazole and echinocandins (Robbins et al., 2017; Revie et al., 2018). Along with the clinical resistance, in some cases acquired resistance also reported in most of the candida isolates. Furthermore, the detailed investigations showed drug resistance is due to alteration/over expression of therapeutic targets, development of biofilm, activation of heat shock protein/stress responder and activation of some multidrug transporters (Shapiro, 2011; Revie et al., 2018).

Marine macro algae are a heterogeneous group of organism found in the littoral zone of the oceanic water with attracting attention due to the presence of biologically active molecules (Suganthy et al., 2016; El Gamal, 2009; Garson, 1989). The classes including polysaccharide, small peptides, fatty acids, phlorotannins, terpenes and alkaloids attributing with several biologically important functions (Shannon and Abu-Ghannam, 2016). Even though there are several bio-inhibitory activities of marine algae was described however, to date none of the active constitution from the marine algae has been entered into the clinical trials (Malve, 2016). The brown seaweed *S. marginatum* has shown greater inhibitory activity against different pathogenic microbes (Shanmughapriya et al., 2008). Furthermore, spatane derivatives of *S. marginatum* were presenting with the antioxidant, antimicrobial (Esmaeili and Khakpoor, 2012; Shanmughapriya et al., 2008) and anticancer activity (De Rosa et al., 1999), antiviral property (Adhikari et al., 2006) and cytotoxic activity (Chinnababu et al., 2015). But the specific activity with respect to antibiotic resistance and the interaction of active metabolites with available drugs were not being reported yet. With this concern, the study has aimed to highlight the synergetic activity of the extracts from *S. marginatum* along with fluconazole, against azole resistance isolates.

2. Materials and methods

2.1. Strains and media

In this study there are five different species of candida were procured from NCCPF (National Culture Collection of Pathogenic Fungi). The candida cultures including in this study *C. albicans* (400026), *C. krusei* (440002), *C. kefyr* (410002), *C. parapsilosis* (450001), and *C. tropicalis* (420007). All the strains were grown and maintained in YPD (1% yeast extract; 1% dextrose; 2% peptone) media contains 50 mg of antibiotics to prevent the bacterial contamination. Before starting the experiment the strains were sub cultured in YPD (Yeast Peptone Dextrose) media without addition of any antibiotics. YPD media, agar-agar, fluconazole and the standard antifungal antibiotics disks were obtained from Himedia laboratories. The solvents used in this study were obtained from the lobome chemicals without further filtration. 96 well micro titre plates were obtained from tarsons (941196).

2.2. Collection and preparation of seaweed extract

The samples of *S. marginatum* was collected from the Rameshwaram coast, southeast coast of India (Latitude 9° 17′ 32 N; Longitude 79° 19' 29 E) with the help of local fisherman community. All the samples were washed with the sterile sea water and brought back to the laboratory in sterile polythene bags. A part of algae was subjected to identification using standard key identifiers (Wendy Guiry and Guiry, 2019). To remove the epiphytes and the other debris seaweeds were washed with seawater subsequently three times with distilled water. Shade dried seaweed powder (50 g) was mixed with 150 ml different solvents (Dichloromethane (DCM), ethyl acetate, acetone, methanol) and placed in the rotator shaker. In order to achieve successive extraction of bioactive compounds, the extraction procedure was repeated at least three times. Finally, the solvent contains secondary metabolites were condensed using rotary evaporator. Slim like component obtained and stored at 2 °C until further use (Shanmughapriya et al., 2008; De Rosa et al., 1999; Chinnababu et al., 2015).

2.3. Preliminary anticandidal susceptibility assay

Disk diffusion method was performed to evaluate the effect of the commercially available antifungal antibiotics against Candida isolates (Khan et al., 2018). Briefly, all the test strains were cultivated in YPD medium at 37 °C, care should to taken to maintain the cell load by 10^6 cells/ml using MacFarland standard (Meletiadis et al., 2009). Prepared cells were inoculated on the nutrient agar plates and the standard antifungal disks were individually placed aseptically on the smeared surface. To check the preliminary activity of seaweed extract, the appropriate amount of different extract was added to the 6 mm wells instead of placing disks. Likely, commercially available antibiotic disks were placed on the agar plates instead of pouring in the wells. The results were observed 24 h of past incubation and expressed in terms of zone of inhibition (Khan et al., 2018).

2.4. Interaction of SWE with antifungal antibiotics

Synergetic effects of SWE along with some antibiotics were determined by the disk diffusion method. Herein, commercially available drug loaded disks were soaked in different SWE for 15 mins under the sterile condition and allow them to dry (Toroglu, 2007). After that, the disk diffusion method was performed to determine the combined effects of antifungal activity (Johnson et al., 2004).

2.5. Determination of minimal inhibitory concentration (MIC)

The MIC of different solvent extract of *S. marginatum* was determined by the CLSI (Clinical Laboratory Standards Institute) broth microdilution method (CLSI, 2002). Briefly, over night grown candida culture was diluted with YPD broth at 1×10^6 cells/ml and 100 µl of cell suspension was added to each well of pre-sterilized polystyrene 96 well plates. Additionally, 100 µl of either fluconazole or SWE was diluted in the nutrient broth and distributed through serial dilution. All the plates were incubated at 37 °C for 24 hrs and the minimal dose required for killing the candida cells were identified through the naked eye based on the turbidity of the solution.

2.6. Fractional inhibitory concentration (FIC)

Fluconazole plays a major role in candida prophylaxis, so the combinational effects of fluconazole along with seaweed extracts were determined based on the fractional inhibitory concentration (FIC). FIC was assessed by microdilution chequerboard method with some modifications (Meletiadis et al., 2009). All the assays were performed in the 96-well polypropylene microtiter plates and the FIC were calculated based on the following formula.

$$\text{FIC of seaweed} = (\text{MIC}_{A+B}/\text{MIC}_{A\ \text{alone}} + \text{MIC}_{B+A}/\text{MIC}_{B\ \text{alone}}).$$

A = Fluconazole

B = Seaweed extract

The Criteria for the identification of synergetic effect as follows; synergistic when the FIC value was < 0.5 and antagonistic when the FIC value was > 4. The FIC values from > 0.5 to < 4 will be consider as non informative interaction (Johnson et al., 2004).

2.7. Gas chromatography and mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out using a Shimadzu GC-2010 equipped with the Auto injector (AOC-20i) and Mass library GCMS-QP2020 with a split/splitless injector (250 °C) and flame ionization detector (200 °C). The mass detection ranged from m/z: 50.00 to m/z: 500.00 with an event time of 0.30sec. Helium was used as carrier gas and Pressure was maintained 68.1 kPa, a total flow of 16.2 mL/min and mobile phase was set as 1.20 mL/min. The column temperature was raised constantly form 50 °C–250 °C at 5 °C/min. All the results were compared with mass library provided by NIST and WILEY 8.

2.8. Statistical analysis

All the data of this study has expressed in terms of mean and standard deviation. One-way analysis of variance (ANOVA) was used to compare the significant level of potential activity of seaweed extract with the commonly found anti-fungal agent. Multivariate statistical analysis like cluster analysis and principal component analysis was employed to predict the relationship between the test agents. In all the cases the significant level was consider as 0.05. Origin 8.0 and PAST 3.0 tools were used for statistical analysis of the obtained data.

3. Results

In this study we have investigated the potential effects of different solvent extract of *S. marginatum* against human commensal organism along with the commercially available antifungal agents.

3.1. Susceptibility pattern of test isolates to antifungal drugs

Antifungal resistance pattern of the test isolates was determined by standard disk diffusion methods. There are two broad class of drug were included in this study that is azoles and polyene. Comparing to the all tested azoles itraconazole seems to be more active against the test isolates except *C. krusei*. In opposition to this voriconazole showed no zone of inhibition towards any organism tested (Table 1). Moderate activity was observed for ketoconazole, clotrimazole, miconazole and nystatin. Although amphotericin B and fluconazole showed a unique range of growth inhibition to *C. krusei* and *C. tropicalis* respectively. The multivariate statistical analysis is evidence that the *C. albicans* is more vulnerable than the other test isolates because itraconazole alone to be a drug of choice for treating infection caused reported organism (Fig. 1). Additionally, it was first reported from this study most of the antibiotics have a similar kind of resistance patent towards the tested organisms with respect to the generation of the drugs (Fig. 1). For example the first generation azoles like clotrimazole, miconazole showed a similar level of resistance along with the fluconazole. Furthermore, polyene grouped with ketoconazole and voriconazole. Among the tested drugs itraconazole resulting in the good mode of inhibition to all the tested isolates (Fig. 2).

Table 1
The diameter of zone of inhibition (mm) of different seaweed extracts against different candida species.

Isolates	Acetone	DCM	EA	Methanol	Itr	Flu
C.albicans	10	0	12	< 8	22	0
C.krusei	–	–	–	–	–	0
C. kefyr	–	–	–	–	35	0
C. parapsilosis	12		14	12	21	0
C. tropicalis	–	–	–	–	16	0

EA - ethyl acetate; DCM - Dichloro Methane; Itr – itraconazole; Flu - fluconazole.

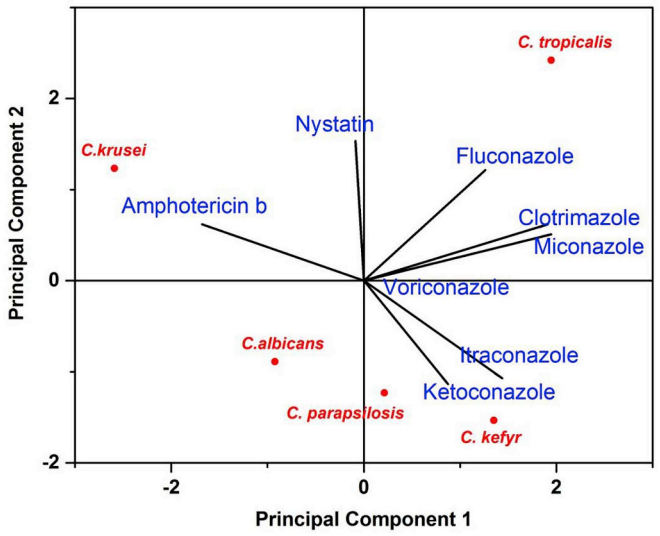

Fig. 1. Multivariate statistical analysis of inter-relationship between antibiotic resistances patterns of the different antibiotics with respect some candida species.

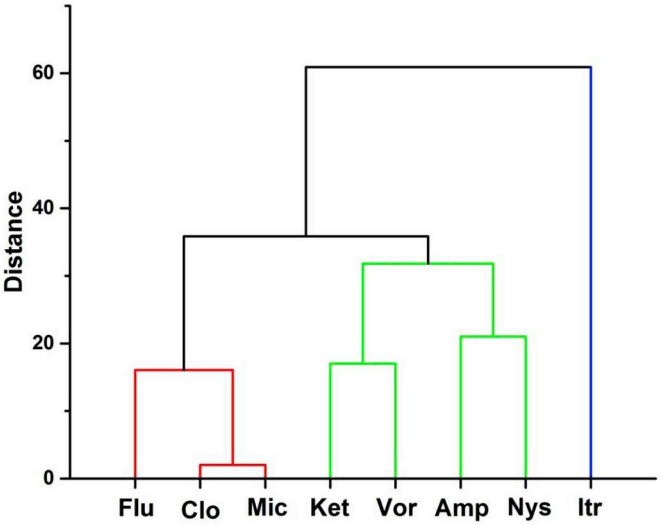

Fig. 2. Antibiotic resistance pattern of different generation of antifungal agents against candida species.

3.2. Primary anticandidal activity

It is necessary to evaluate the potential effect of *S. marginatum* extracts before starting the experiment. As a consequence of this, the preliminary anticandidal activity was performed against *C. albicans, C. parapsilosis, C. tropicalis*, *C. krusei* and *C. kefyr* by agar well diffusion method and shown in Table 1. The results revealed that the extracts showed the activity as follows DCM < methanol < acetone < ethyl acetate. The average significant zone of inhibitory (mm) activity against all test isolates was varied from 0 to 14 mm with the maximum for ethyl acetate extract against *C. parapsilosis*. There is no zone of inhibition was observed for *C. tropicalis*, *C. krusei* and *C. kefyr* by four different extracts. Ethyl acetate and acetone extract showing greater activity against *C. albicans* and *C. parapsilosis.*

3.3. Effect of seaweed extract on the activity of standard antibiotics

After addition of 50 µg/ml of seaweed extract to the commercially available antifungal, the zone of inhibition was slightly varied

Table 2
Zone of inhibition (mm) of commercially available anti-fungal agents along with different seaweed extract. The disk concentration of antifungal as follows; miconazole disc (50 µg), itrconazole (10 µg), clotrimazole (10 µg), voriconazole (1 µg), ketoconazole (10 µg), fluconazole (25 µg), nystatin (100 UI), Amphotericin-B (20 µg).

Seaweed extract		Azole						Polyene	
	Organism	Flu	Itr	Ket	Clo	Vor	Mic	Amp B	Nys
	C. albicans	0	22	–	–	–	–	–	–
	C. krusei	0	–	–	–	–	–	18	17
None	*C. kefyr*	0	35	13	10	–	12	–	–
	C. parapsilosis	0	21	11	< 8	–	< 8	–	–
	C. tropicalis	18	16	–	18	–	18	–	21
	C. albicans	–	13	–	–	–	–	–	–
	C. krusei	14	12	< 8	< 8	–	12	18	12
Ethyl acetate	*C. kefyr*	–	31	14	–	–	–	–	–
	C. parapsilosis	–	20	11	< 8	–	< 8	–	–
	C. tropicalis	12	12	13	13	–	16	18	22
	C. albicans	–	23	–	–	–	–	–	
	C. krusei	20	< 8	< 8	< 8	30	< 8	18	14
Methonol	*C. kefyr*	–	40	13	–	–	–	–	
	C. parapsilosis	–	22	10	< 8	–	< 8	–	
	C. tropicalis	17	17	12	12	–	12	17	15
	C. albicans	–	22	–	–	–	–	–	–
	C. krusei	20	10	< 8	< 8	–	–	19	20
Acetone	*C. kefyr*	–	40	–	–	–	–	–	–
	C. parapsilosis	–	15	< 8	–	–	–	12	–
	C. tropicalis	22	13	13	13	–	14	20	16
	C. albicans	–	15	–	–	–	–	–	–
	C. krusei	21	–	–	12	–	–	16	18
DCM	*C. kefyr*	–	22	–	–	–	–	–	–
	C. parapsilosis	–	19	12	–	–	–	–	–
	C. tropicalis	17	10	–	13	–	–	13	19

Flu – Fluconazole; Ita – Itraconazole; Ket – Ketoconazole; Clo – Clotrimazole.
Vor – Voriconazole; Mic – Miconazole; Amp B – Amphotericin B; Nys - Nystatin.

Table 3
Minimum Inhibitory Concentration (MIC) of seaweed extracts (µg/ml) against different species of opportunistic organism tested through micro dilution test recommended by CLSI (2002).

Isolates	Acetone	DCM	EA	Methanol	Fluconazole
C.albicans	12.5	50	25	25	6.25
C.krusei	0.78	50	0.195	3.12	1.56
C. kefyr	0.78	50	1.56	3.12	0.78
C. parapsilosis	25	50	12.5	25	3.12
C. tropicalis	0.39	50	0.29	1.56	1.56

(Table 2). There is no significant changes in the activity of itraconazole were observed against all the test isolates. But the zone of inhibition against *C. albicans* was changed from 0 mm to maximum of 22 mm against *C. krusei* and *C. tropicalis*. Likely ketoconazole and amphotericin B showed significant increases in the zone of inhibition. Based on this primary evidence the interaction of seaweed metabolites with the drug was confirmed, however in order to understand the behaviour of an interaction of drugs the MIC and FIC were adopted.

Table 4
Interaction of fluconazole along with the seaweed extracts. The mechanism of interaction of crude extract with fluconazole.

Organism	Acetone	Ethyl acetate	Methanol
C.albicans	S (0.01)	S (0.01)	S (0.01)
C. krusei	A (12.02)	A (72.12)	A (12.02)
C. kefyr	A (32.05)	S (0.09)	S (0.08)
C. parapsilosis	NI (2.25)	NI (1.25)	NI (4.51)
C. tropicalis	A (40.06)	S (0.20)	A (32.05)

S = synergy, NI = no interaction, A = antagonism.

3.4. Minimal inhibitory concentration

In this study the MIC of fluconazole in range of 0.78–6.25 µg/ml with greater against *C. albicans*. As presented in Table 3, *C. albicans* alone considers being a resistance to the fluconazole. The growth alterations of different candida sp. were observed in the medium containing minimal of 0.19 µg/ml *S. marginatum* extract. The activity was varied with respect to the different kind of extract as well as the species of an organism. But the MIC of fluconazole ranged between 0.78 and 6.25 µg/ml, it was slightly varied from the MIC of seaweed extracts. The maximum and minimum

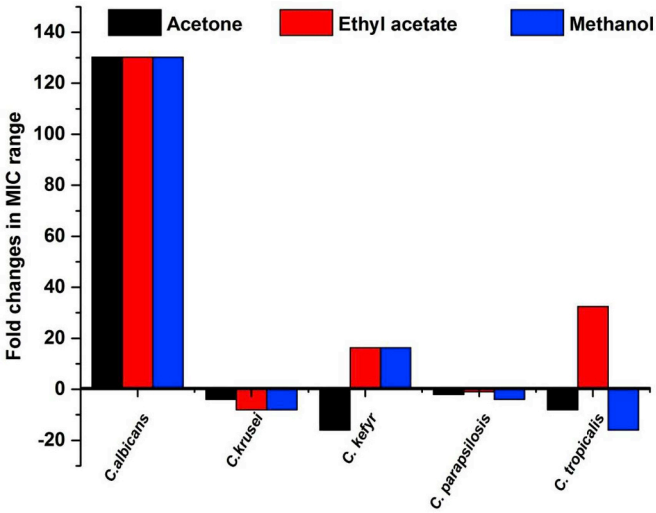

Fig. 3. Variations in the MIC values of fluconazole alone and in combination with seaweed extract against different candida species. The negative values in the X-axis are the indicatives of fold decreases in MIC values after addition of fluconazole.

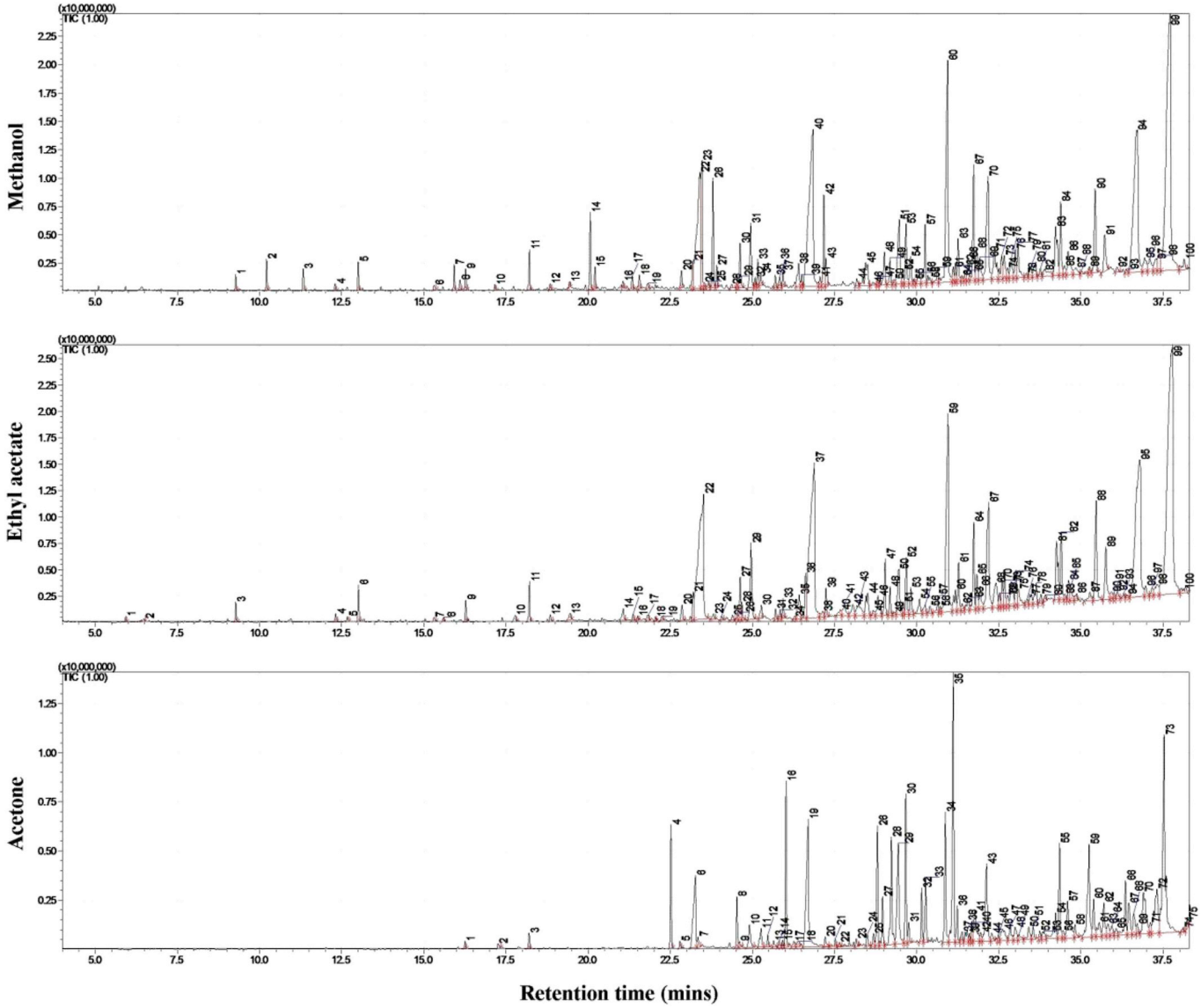

Fig. 4. GC-MS spectra of different solvent extract of *Stocheospermum marginatum.* The spectral images shown that the variation of extracted secondary metabolites with reference to their retention time and the plot was mapped based on the peak length.

MICs of *S. marginatum* extracts for candida strains were recorded between 0.19 and 50 μg/ml against *C. krusei* and all candida strains. This primary screening evidenced that *S. marginatum* extract showed the good inhibitory activity against tested organisms as like as fluconazole except for DCM extract. The DCM extract was the least effective against all the tested strains with the MIC 50 μg/ml. However, the MIC of all organisms was varied differentially. Among them, the ethyl acetate extracts showed greater activity against *C. tropicalis* (0.39 μg/ml) followed by methanol extract against other organism ranged between 0.39 and 25 μg/ml compared to all other extracts. One way ANOVA results indicated that the MIC values of fluconazole were insignificantly ($p > 0.05$) varied among the extract tested but the statistically significant ($p < 0.001$) variation was observed for DCM extract.

3.5. Interaction of fluconazole with different extract of SWE

The results of the interaction behaviour of crude extract along with fluconazole against drug resistance candida species were shown in Table 4. Fluconazole plus ethyl acetate extract shown more potential synergetic action against *C. albicans, C. kefyr* and *C. tropicalis*. Among the tested strains, FIC value of *C. krusei* greater than 4.0 thus defined as antagonism mode of interaction with all prepared extract. Contradict to this none of the extract shown either synergetic or antagonistic effects against *C. parapsilosis.*

3.6. Mass spectrometric metabolic profiling of seaweed extracts

The mass spectral footprints of each extracts were identified with the help of mass library and GC-MS results revealed the presence of different phyto-chemicals (Fig. 4). From the chromatogram peaks, there are different classes of bioactive constituents were identified which includes majorly terpenoids and fatty acids listed in Table 5. There are more than 80 compounds were detected from each of the extracts, however in order to conclude the activity the compounds showing peak height percentage greater than 3.0 alone for further consideration. Among them, the common compounds for each extract were Lupeol, n-Hexadecanoic acid, Cyclolanostan, Azulene and Tetradecanoic acid.

4. Discussion

During the past two decades the majority of antifungal therapeutic failure was observed, the association was directly proven due to the

Table 5
GC-MS analysis of different extracts of *S. marginatum*. The relative abundance common secondary metabolites available in the extracts of *S. marginatum*.

Extract	Peak	Retention time	Height %	Name
Methanol	35	31.12	9.25	Lupeol, trifluoroacetate
	73	37.53	6.8	9,19-Cyclolanostan-3-ol, acetate,
	16	26.02	5.78	Hexadecanoic acid, methyl ester
	30	29.67	5.01	Azulene
	34	30.88	4.48	1,3,6,10-Cyclotetradecatetraene,
	19	26.70	4.45	n-Hexadecanoic acid
	4	22.52	4.3	Methyl tetradecanoate
	26	28.81	4.11	9-octadecenoic acid (Z)-, methyl ester
	28	29.23	3.64	But-3-enal, 2-methyl-4-(2,6,6-trimethyl- 1-cyclohexenyl)-
	29	29.44	3.48	Oleic Acid
	55	34.35	3.21	1,3,6,10-cyclotetradecatetraene
	59	35.25	3.14	Dihydrotachysterol
Acetone	99	37.69	7.62	9,19-Cyclolanostan-3-ol, acetate, (3.beta.)-
	60	30.94	6.63	Lupeol, trifluoroacetate
	40	26.85	4.71	n-Hexadecanoic acid
	94	36.70	4.35	5-(hydroxymethyl)-1,1,4A-trimethyl -6-methylenedecahydro-2-naphthalenol
	23	23.46	3.93	Tetradecanoic ACID
	22	23.40	3.57	6-Hydroxy-4,4,7a-trimethyl-5,6,7, 7a-tetrahydrobenzofuran-2(4H)-one
	67	31.74	3.51	Chrysantenyl 2-methuylbutanoate
	26	23.80	3.39	1-Nonadecene
	70	32.17	3.09	1,3,6,10-Cyclotetradecatetraene,
Ethyl acetate	99	37.77	8.87	9,19-Cyclolanostan-3-ol, acetate, (3.beta.)-
	59	30.96	7.12	Lupeol, trifluoroacetate
	37	26.89	5.64	n-Hexadecanoic acid
	95	36.79	4.97	5-(Hydroxymethyl)-1,1,4A-trimethyl -6-methylenedecahydro-2-naphthalenol
	22	23.51	4.56	Tetradecanoic acid
	67	32.19	3.8	Azulene
	88	35.47	3.61	Allopregnane-3.beta.
	64	31.74	3.14	Chrysantenyl 2-methuylbutanoate

influence of several acquired or intrinsic resistance to the antifungal (Scorzoni et al., 2017; Selmecki et al., 2006; Franz et al., 1998). Frequency of drug resistance has been intended by the mutation in the therapeutic target especially for classes of azoles (Whaley et al., 2017; Jensen, 2016). Hence this is an important and emerging moment to unravel the better antifungal therapy for the candida prophylaxis.

There are number of active metabolites of marine macro algae shown antimicrobial properties either combined or alone. Nishanth Kumar et al. (2014) described the combined effects phenazines from *Pseudomonas aeruginosa* on *Candida* species with clinically used azoles. This study has highlighted the synergy of phenazines with fluconazole by reducing the MIC fourfold but the azoles like itraconazole and clotrimazole shown in different and antagonistic effects respectively. In addition the spatane derivatives of *S. marginatum* shown anti-fungal and anti bacterial activity against clinical pathogens significantly. Our study has evidenced that fluconazole can synergistically combined with phyto-constituents of sea weed, against multidrug resistance candida species, by decreased MIC against *C. albicans* rather than other tested isolates.

Apart from the identification of new potential antifungal drug, it is crucial to overcoming the existing drugs with the possible way to enhance its activity. Hence the natural products paying attention to the scientific community for the managing of drug resistance through the combination therapy. A significant reduction in MIC of fluconazole was noted with addition of eugenol, cinnamaldehyde (Ahmad et al., 2010) and honokiol. We found when the fluconazole is combined with the seaweed extract it enhances the activities of 130 folds against *C. albicans*, 16 folds for *C. kefyr* and 32 folds for *C. tropicalis* were observed compared to the MIC of fluconazole alone (Fig. 3).

As shown in the previous study, the bioactive fractions of *S. marginatum* known to be the antibacterial agent (Shyamali et al., 1982) with the spatane skeleton. Further, it was proven by GC-MS analysis (Esmaeili and Khakpoor, 2012) followed by spectroscopic studies (De Rosa et al., 1999; Chinnababu et al., 2015). The semi synthesized spatane derivatives describing the cytotoxic effects against the human cell lines ranged from 3.62 to 195 μg/ml. The extract of *S. marginatum* was found to be effective to control the growth of multidrug resistance organism *K. pneumonia* and some strains of candida showing greater than 20 mm of zone of inhibition (Shanmugapriya, 2008). The spatane derivatives of *S. marginatum* were more effective against dermatophytic fungi *aspergillus,* the activity comparably equal to ketoconazole and less than cycloheximide (Chinnababu et al., 2015). In previous study, the extract of *S. marginatum* have has reported two to three folds of reduced activity was observed against gram positive and gram negative bacteria in contrast to ciprofloxacin (Esmaeili and Khakpoor, 2012). But the present study, the activity has exceed not more than 14 mm, thus might be happening due the development of antifungal resistance (no zone of inhibition for fluconazole and 1st generation azoles). Based on the scientific literature there is no dataset available on the effects of phyto constituents of *S. marginatum* against drug resistance candida isolates. Hence the present study provides the clear cut evidence of anticandidal effects of *S. marginatum.*

The potential mechanisms were related to down regulation of SAP genes with inhibition of extracellular phospholipase activity. With the fact that a positive correlation existed between these factors and the major virulence properties of Candida, the synergism of the combination may be explained by a decrease in fungal virulence. The results from this study encourage us to consider future use of a combination of an azole and fluoxetine against fungi, and more animal models and in-depth study of the mechanisms are highly warranted.

The bioactive lupeol isolated from the ethanolic extract of *Euclea natalensis* found to significantly effective against *several fungi including Aspergillus* sp (Lall et al., 2006). Lupeol acetate (LA) isolated from the plant latex *Himatanthus drasticus having* Anti-inflammatory activity (Lucetti et al., 2010). The antifungal activity of lupeol medicated by targeting mitochondrial integrity was found to have better activity against *S. cerevisiae* even at the reduced concentration (Haque et al., 2016). Although, n-Hexadecanoic reported in *Pterochadia capillacea*.,

Corallina mediterranea. Jania rubens in red seaweeds (El-Din and El-Ahwany, 2016) and some other terrestrial plants. Nasser et al. (2018) studied that the efficiency of alcoholic and aqueous extracts of vitex having Azulene as one of the components showed that antifungal activity against clinical isolates of *C. albicans* (Nasser et al., 2018). As evidence, due to the presence of bioactive components in the extracts of *S. marginatum* had shown the anticandidal and synergetic activity against the different candida sp.

5. Conclusion

In conclusion, the extract of *S. marginatum* showed the good zone of inhibition and reduced MIC values against most of the fluconazole resistance isolates. Also, fluconazole showed synergism in combination with the different extract of *S. marginatum* against fluconazole resistant *C. albicans*, *C. kefyr* and *C. tropicalis*. The present finding will be used as a primary source to formulating the natural product based drug in the combinational therapy against the drug resistance candidiasis. However, *in vivo* and the clinical studies are needed to confirm the present activity of *S. marginatum*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101145.

References

Adhikari, U., Mateu, C.G., Chattopadhyay, K., Pujol, C.A., Damonte, E.B., Ray, B., 2006. Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. Phytochemistry 67, 2474–2482.
Ahmad, A., Khan, A., Khan, L.A., Manzoor, N., 2010. In vitro synergy of eugenol and methyleugenol with fluconazole against clinical Candida isolates. J. Med. Microbiol. 59, 1178–1184.
Anaisse, E., Bodey, G.P., Kantarjian, H., Ro, J., Vartivarian, S.E., Hopfer, R., Hoy, J., Polston, K., 1989. New spectrum of fungal infections in patients with cancer. Rev. Infect. Dis. 11, 369–378. 3.
Ascioglu, S., Rex, J.H., De Pauw, B., et al., 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin. Infect. Dis. 34, 7–14.
Chinnababu, B., Purushotham Reddy, S., Sankara Rao, P., Loka Reddy, V., Sudheer Kumar, B., Venkateswara Rao, J., Prakasham, R.S., Suresh Babu, K., 2015. Isolation, semi-synthesis and bio-evaluation of spatane derivatives from the brown algae Stoechospermum marginatum. J. Bio. Organic. Med. Chem. Lett. 25 (12), 2479–2483.
Clinical Laboratory Standards Institute, 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. In: NCCLS document M27-A2, 2nd ed. National Committee for Clinical Laboratory Standards, Wayne, PA.
Cornely, O.A., Bassetti, M., Calandra, T., et al., 2012. ESCMID guideline for the diagnosis and management of Candida diseases 2012: nonneutropenic adult patients. Clin. Microbiol. Infect. 18, 19–37.
Cortegiani, A., Misseri, G., Fasciana, T., Giammanco, A., Giarratano, A., Chowdhary, A., 2018. Epidemiology, clinical characteristics, resistance, and treatment of infections by Candida auris. J. Intensive. Care 6 (69).
De Rosa, S., Iodice, C., Khalaghdoust, M., Oryan, S., Rustaiyan, A., 1999. Spatane diterpenoids from the brown alga *Stoechospermum marginatum* (Dictyotaceae). Phytochemistry 51, 1009–1012.
Denning, D.W., Kneale, M., Sobel, J.D., Rautemaa-Richardson, R., 2018. Global burden of recurrent vulvovaginal *candidiasis:* a systematic review. Lancet Infect. Dis. 18, E339–E347.
Dignani, M., Solomkin, J., Anaissie, E., 2009. Candida. In: Anaissie, E., McGinnis, M., Pfaller, M. (Eds.), Clinical Mycology. Elsevier, USA, pp. 197–231.
El Gamal, A.A., 2009. Biological importance of marine algae. Saudi Pharmaceut. J. 18 (1), 1–25.
El-Din, S.M.M., El-Ahwany, A.M.D., 2016. Bioactivity and phytochemical constituents of marine red seaweeds (*Janiarubens, Corallina mediterranea* and *Pterocladiacapillacea*). J. Taibah Univ. Sci. 10, 471–484.
Esmaeili, A., Khakpoor, M., 2012. Biological activities and chemical composition of solvent extracts of *Stoechospermum marginatum* (C. Agardh). Acta Biochim. Pol. 59 (4), 581–585.
Ferrer, J., 2000. Vaginal candidosis: epidemiological and etiological factors. Int. J. Gynaecol. Obstet. 71, 21–27.
Franz, R., Kelly, S.L., Lamb, D.C., Kelly, D.E., Ruhnke, M., Morschhäuser, J., 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical Candida albicans strains. Antimicrob. Agents Chemother. 42 (12), 3065–3072.
Garson, J., 1989. Marine natural products. Nat. Prod. Rep. 6, 143–170.
Guiry, Wendy, Guiry, M.D., Guiry, G.M., 2019. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org, Accessed date: 10 February 2019.
Haque, E., Irfan, S., Kamil, M., et al., 2016. Terpenoids with antifungal activity trigger mitochondrial dysfunction in *Saccharomyces cerevisiae*. Microbiology 85, 436.
Hirano, R., Sakamoto, Y., Kudo, K., Ohnishi, M., 2015. Retrospective analysis of mortality and Candida isolates of 75 patients with candidemia: a single hospital experience. Infect. Drug Resist. 8, 199–205.
Jensen, R.H., 2016. Resistance in human pathogenic yeasts and filamentous fungi: prevalence, underlying molecular mechanisms and link to the use of antifungals in humans and the environment. Dan. Med. J. 63 (10), B5288.
Johnson, M.D., MacDougall, C., Ostrosky-Zeichner, L., Perfect, J.R., Rex, J.H., 2004. Combination antifungal therapy. Antimicrob. Agents Chemother. 48, 693–715.
Kanafani, Z.R., Perfect, J.R., Resistance to antifungal agents: mechanisms and clinical impact. Clin. Infect. Dis.. 46, 120–128.
Khan, M., Ahmed, J., Gul, A., Ikram, A., Lalani, F.K., 2018. Antifungal susceptibility testing of vulvovaginal *Candida* species among women attending antenatal clinic in tertiary care hospitals of Peshawar. Infect. Drug Resist. 11, 447–456.
Kneale, M., Bartholomew, J.S., Davies, E., Denning, D.W., 2016. Global access to antifungal therapy and its variable cost. J. Antimicrob. Chemother. 71 (12), 3599–3606.
Lall, N., Weiganand, O., Hussein, A.A., Meyer, J.J.M., 2006. Antifungal activity of naphthoquinones and triterpenes isolated from the root bark of *Eucleanatalensis*. South Afr. J. Bot. 72 (4), 579–583.
Lin, A.C., Goldwasser, E., Bernard, E.M., 1990. Amphotericin B blunts erythropoietin response to anemia. J. Infect. Dis. 161, 348–351.
Lucetti, D.L., Lucetti, E.C., Bandeira, M.A., et al., 2010. Anti-inflammatory effects and possible mechanism of action of lupeol acetate isolated from *Himatanthu sdrasticus* (Mart.) Plumel. J. Inflamm. 60.
Malve, H., 2016. Exploring the ocean for new drug developments: marine pharmacology. J. Pharm. BioAllied Sci. 8 (2), 83–91.
Meletiadis, J., Pournaras, S., Roilides, E., Walsh, T.,J., 2009. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against *Aspergillus fumigatus*. Antimicrob. Agents Chemother. 54 (2), 602–609.
Mourad, A., Perfect, J.R., 2018. Tolerability profile of the current antifungal armoury. J. Antimicrob. Chemother. 73 (1), i26–i32.
Nasser, K., Mahdieh, S., Seyed, M.M., Mahdiyeh, M., Farshid, K., 2018. Antifungal effects of ethanolic and aqueous extracts of Vitex agnus-castus against vaginal isolates of *Candida albicans*. Curr. Med. Mycol. 4 (1), 1–5.
Nishanth Kumar, S., Nisha, G.V., Sudaresan, A., Venugopal, V.V., Sree Kumar, M.M., Lankalapalli, R.S., Dileep Kumar, B.S., 2014. Synergistic activity of phenazines isolated from *Pseudomonas aeruginosa* in combination with azoles against Candida species. Med. Mycol. 52, 482–490.
Ouederni, M., Sanal, O., Ikinciogullari, A., 2014. Clinical features of Candidiasis in patients with inherited interleukin 12 receptor β1 deficiency. Clin. Infect. Dis. 58 (2), 204–213.
Pasqualotto, A.C., Howard, S.J., Moore, C.B., et al., 2007. Flucytosine therapeutic monitoring: 15 years experience from the UK. J. Antimicrob. Chemother. 59, 791–793.
Pfaller, M.A., Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 133–163.
Qin, Y., Zhang, L., Xu, Z., 2016. Innate immune cell response upon *Candida albicans* infection. Virulence 7 (5), 512–526.
Revie, N.M., Iyer, K.R., Robbins, N., Cowen, L.E., 2018. Antifungal drug resistance: evolution, mechanisms and impact. Curr. Opin. Microbiol. 45, 70–76.
Rex, J.H., Pfaller, M.A., Galgiani, J.N., 1982. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and Candida infections. 21, 944–945.
Richardson, J.P., Moyes, D.L., 2015. Adaptive immune responses to Candida albicans infection. Virulence 6 (4), 327–337.
Robbins, N., Caplan, T., Cowen, L.E., 2017. Molecular evolution of antifungal drug resistance. Annu. Rev. Microbiol. 71, 753–775.
Scorzoni, L., De Paula, E., Silva, A.C., Marcos, C.M., 2017. Antifungal therapy: new advances in the understanding and treatment of mycosis. Front. Microbiol. 8, 36.
Selmecki, A., Forche, A., Berman, J., 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. Science 313 (5785), 367–370.
Shanmughapriya, S., Manilal, A., Sujith, S., et al., 2008. Antimicrobial activity of seaweeds extracts against multiresistant pathogens. Ann. Microbiol. 58, 535–541.
Shannon, E., Abu-Ghannam, N., 2016. Antibacterial derivatives of marine algae: an overview of pharmacological mechanisms and applications. Mar. Drugs 14 (4), 81.
Shapiro, R.S., Robbins, N., Cowen, L.E., 2011. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol. Mol. Biol. Rev. 75, 213–267.
Shyamali, S.M., De SilvaSarath, K.T., Gamage, N., Savitri, K., 1982. Anti-bacterial activity of extracts from the brown seaweed *Stoechospermum marginatum*. Phytochemistry 21 (4), 944–945.
Song, J.C., Deresinski, S., 2005. Hepatotoxicity of antifungal agents. Curr. Opin. Investig. Drugs 6, 170–177.

Suganthy, N., Karutha Pandian, S., Pandima Devi, K., 2010. Neuroprotective effect of seaweeds inhabiting south Indian coastal area (Hare island, Gulf of mannar marine biosphere reserve): cholinesterase inhibitory effect of *Hypnea valentiae* and *Ulva reticulata*. Neurosci. Lett. 468, 216–219.

Toroglu, S., 2007. In vitro antimicrobial activity and antagonistic effect of essential oils from plant species. J. Environ. Biol. 28, 551–559.

Tucker, R.M., Haq, Y., Denning, D.W., et al., 1990. Adverse events associated with itraconazole in 189 patients on chronic therapy. J. Antimicrob. Chemother. 26, 561–566.

Vincent, B.M., Lancaster, A.K., Scherz-Shouval, R., Whitesell, L., Lindquist, S., 2013. Fitness trade-offs restrict the evolution of resistance to amphotericin B. PLoS Biol. 11, e1001692.

Wazny, L.D., Brophy, D.F., 2000. Amiloride for the prevention of amphotericin B-induced hypokalemia and hypomagnesemia. Ann. Pharmacother. 34, 94–97.

Whaley, S.G., Berkow, E.L.,, Rybak, J.M., Nishimoto, A.T., Barker, K.S., Rogers, P.D., 2017. Azole antifungal resistance in *Candida albicans* and emerging non-*albicans Candida* species. Front. Microbiol. 7, 2173.