

Antibacterial and antifungal activities of brown alga *Zonaria tournefortii* (J.V. Lamouroux)

A. ISMAIL^{*,1,2}, K. BEL HADJ SALAH², M. AHMED³, M. MASTOURI⁴,
A. BOURAOUI¹ and M. AOUNI²

Laboratoire des Maladies Transmissibles et Substances Biologiquement Actives,
Faculté de Pharmacie Université de Monastir, TUNISIA.
E. Mail: ismail_ml@yahoo.fr

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ABSTRACT

The antibacterial and antifungal activities of brown alga (*Zonaria tournefortii*) against 4-pathogenic fungi [*Candida albicans*, *Cryptococcus neoformans*, *Scytalidium dimidiatum* and *Scopulariopsis brevicaulis*] and several multi-resistant *Staphylococcus aureus* (MRSA) were investigated. Aqueous extract of fresh alga (*Zonaria tournefortii*) and the fractions (obtained after crude extract purification by flash chromatography using C18 column) were tested for antimicrobial activity. The *Z. tournefortii* aqueous extract was highly active against MRSA and two fungi *Candida albicans* and *Cryptococcus neoformans*. On fractionation, 6-fractions (F1-F6) of crude extract were obtained, and amongst them F2 and F3 were highly active against MRSA, and F2 had the strongest activity. Thus *Z. tournefortii* is promising candidate for antimicrobial agents especially against human and animal MRSA, *C. albicans* and *C. neoformans*.

Key words: Antimicrobial activity, aqueous extract, brown alga, *Candida albicans*, *Cryptococcus neoformans*, fungi, MRSA, *Scopulariopsis brevicaulis*, *Scytalidium dimidiatum*, *Staphylococcus aureus*, *Zonaria tournefortii*.

INTRODUCTION

The discovery of novel substances with antibacterial properties that can be incorporated into new medicines is a solution to the global problem posed by antibiotic-resistant bacteria (4). Emergence of multi-resistant *Staphylococcus aureus* (MRSA) strains of *Staphylococcus aureus* has increased the urgent need for new antibiotics (13, 39). Also, the increasing importance of fungal infections, the difficulties encountered in their treatment and the increase in their resistance to antifungal agents, have stimulated the search for antifungal, therapeutic alternatives (32). Marine organisms are an attractive resource of new natural products. In marine ecosystems, several organisms produce bioactive metabolites in response to ecological pressures such as competition for space, feeding and reproduction (23,38). Within these groups, seaweeds which are sessile organisms are prime candidates to possess biologically active metabolites, hence, are prolific source of secondary metabolites and are presumed to contain broad range of

*Correspondence author, ¹Unité de Recherche des Substances Actives Marines, Laboratoire de Pharmacologie, Faculté de Pharmacie, Université de Monastir, Tunisia, ²National Institute of Marine Sciences and Technologies, 28, 2 march street 1934 -2035 Salammbô, Tunisia, ³Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan, ⁴Hospital Universitaire Fattouma Bourguiba Avenue Farhat Hached, 5000. Monastir. Tunisia

biologically active substances with rich pharmacological potential (12,34). Extracts of marine algae exhibits antibacterial activity (7,42,43) and some seaweeds are antifungal (6,7,20). Tunisian coast is rich in diverse macroalgae (10,11) and numerous studies have highlighted the antimicrobial potential of microalgae and seaweeds (15,22). *Z. tournefortii* [(J.V.Lamouroux) Montagne, Phaeophyceae Class] is a marine brown alga common in Mediterranean Sea and in Tunisian coast (11). This alga occurs throughout the year in sciaphilous habitats and is present in subtidal biotopes (40) in warm-water areas (36). It is rich source of bioactive compounds but its antimicrobial potential is still under investigation. This led us to conduct a preliminary study to assess its antibacterial and antifungal potential against MRSA and few pathogenic fungi. Discovering new sources of bioactive compounds against these fungi is necessary, as *Candida albicans* causes nosocomial infections and non-gonococcal urethritis (NGU) (29). Similarly *Cryptococcus neoformans* (pathogen of systemic mycoses) causes cryptococcosis in 15% of AIDS patients. Besides, the *Candida* spp. and *Cryptococcus* spp. causes spoiling of tomato fruits and *Candida* spp spoils the juices (29).

This study aimed to assess the antimicrobial potential of brown alga (*Z. tournefortii*) collected from the Tunisian coast against some human and animal pathogens.

MATERIAL AND METHODS

The brown alga (*Z. tournefortii*) was collected by SCUBA-diving in spring of 2003 from Tabarka (North-West, Tunisia). Samples were washed twice with sterile seawater followed by washing with sterile distilled water to remove salts, epiphytes, sand particles and other matter. The cleaned material was stored frozen at -20°C until use.

The Bacterial strains used in this study were 13-multiresistant *S. aureus* strains (coded: 1, 3, 5, 12, 16, 17, 19, 20, 21, 22, 25, 26 and 27) and were supplied by Hospital Fattouma Bourguiba, Monastir. These cultures were maintained by regular transfers on Muller-Hinton agar medium.

The test fungi were two opportunistic pathogens: [*C. albicans* (C.P. Robin) and *C. neoformans* (San Felice)] and two hyphomycetes [*Scytalidium dimidiatum* (Penz.) and *Scopulariopsis brevicaulis* (Sacc.)]. Pasteur Institut, Paris supplied the *S. dimidiatum* and laboratory of Microbiology, Faculty of Medicine Besancon supplied *C. albicans*, *C. neoformans* and *S. brevicaulis*. Fungi were preserved frozen or subcultured regularly on Sabouraud glucose agar (SGA) medium.

Preparation of aqueous extract

Aqueous extracts of seaweed were prepared by macerating 500 g fresh material in distilled water for 24 h at room temperature (20-25°C). Mixture was stirred several times with a sterile glass rod, filtered through cotton wool and the liquid was lyophilized using a freeze dryer. The dry powder was weighed (21% yield) and stored at -20°C until use as the source of inhibitory material.

Antibacterial assays

Antibacterial tests were done using Agar well diffusion method (31). Twenty five ml of sterilized Muller Hinton agar (28) was transferred to Petri dishes (9 cm dia) and

allowed to solidify for 30 min. The test organisms were cultured in Muller-Hinton broth and incubated at 37°C for 6 h to allow bacteria to grow into the exponential phase. Cultures were then adjusted by serial dilution with Mueller-Hinton broth to 10^6 cfu mL⁻¹. Two hundred µl of this suspension was then spread on each µM-H agar media using a sterile swap stick and allowed to dry for 30 min. A Cork borer (5 mm) was used to bore wells in the agar medium. Lyophilized seaweed extract powder was in sterile distilled water at 5 mg mL⁻¹ concentration and filtered through 0.22 µm filter. Twenty µl of this crude extract was transferred to wells aseptically. Sterile distilled water 20 µl, was used as negative control and Oxacilline 0.1 mg mL⁻¹ was used as positive control. The Petri dishes were then incubated at 4° C for 3 - 4 h to allow diffusion of extracts in wells (24), afterwards they were incubated at 37°C for 18 - 24 h. The inhibition zone around the wells was measured in mm.

Determination of Minimum Inhibitory Concentration (MIC)

For bacteria, the MIC was determined by the broth micro-dilution method using decreasing doses of active extract or fractions to identify the lowest concentration of active substance that can inhibit bacterial growth. A pure culture of single bacterium grown in Mueller-Hinton broth to exponential growth phase was adjusted with serial dilution in Mueller-Hinton broth to about 10^6 cfu mL⁻¹. The antimicrobial substance was serially diluted to 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg mL⁻¹ using the sterile diluents (Mueller-Hinton broth). Hundred µl of standardized inoculums was added to each sterile test tube, and then 100 µl active fraction of different concentration was added. The tubes were then incubated at 37°C for 18- 24 h, thereafter the tubes were examined for bacterial growth as indicated by turbidity. The last tube in the dilution series that did not show growth corresponded to the minimum inhibitory concentration (MIC) of antimicrobial agent. Oxacilline was prepared at 5 to 200 µg mL⁻¹ concentrations and used as the standard positive control. Tubes containing Muller-Hinton broth without extracts served as negative control.

Antifungal assays

Antifungal activities of *Z. tournefortii* aqueous extracts and semi purified fractions were determined by agar incorporation method (9, 18). Briefly, the test was performed in sterile Petri dishes (30 mm dia) containing Sabouraud Glucose Agar (SGA). The crude extract or lyophilized powder suspension were mixed aseptically with 10 mL of SGA to give 0.25, 0.5, 0.75, 1 and 2 mg mL⁻¹ concentrations and allowed to cool. SGA without extract was used as the negative control. After the medium had solidified in Petri dishes (30 mm d), the plates were inoculated with a 3-day culture suspension in sterile distilled water (1 ml) adjusted with spectrophotometer (A595 nm) to 10^5 conidia mL⁻¹ for *S. brevicaulis* and *S. dimidiatum* and a 3-day yeast culture suspended in sterile distilled water and adjusted to 10^5 spores/ml. The plates were then incubated for 7 days at 24°C for *S. dimidiatum* and *S. brevicaulis*, 24 h at 37°C for *C. albicans* and 48 h at 37°C for *C. neoformans*. The lowest concentrations which inhibited the fungal growth were considered as the minimal inhibitory concentration (MIC) of the crude extract or fractions. Fluconazole was used as positive control at 5-200 µg mL⁻¹ concentrations. Tests were carried out in triplicate.

Fractionation of lyophilized crude extract

Fractionation of algal aqueous crude extract was achieved by flash chromatography on a solid support, using 3 ml cartridge, filled with grafted silica (C-18 column 5cm, sep-pak, Supelco) and packed with 5 ml methanol followed by 5 ml of distilled water. One g crude extract was dissolved in methanol in presence of one g silica C-18. The solution was made solvent free by evaporating in a rotating evaporator (Buchi, B-480) at 40°C. The powder obtained was placed in the top of a C-18- Cartridge (Sep-pak, suppelco) for fractionation. The cartridge was placed in the vacuum extractor (SPE Manifold vacuum, Supelco) and eluted with 100 ml lots of 0, 20, 40, 80 and 100 % methanol in water yielding 6-fractions of decreasing polarity (Fig 1). The collected fractions were evaporated under vacuum, recovered in distilled water and lyophilized. Lyophilized fraction powders were stored at -20°C until use.

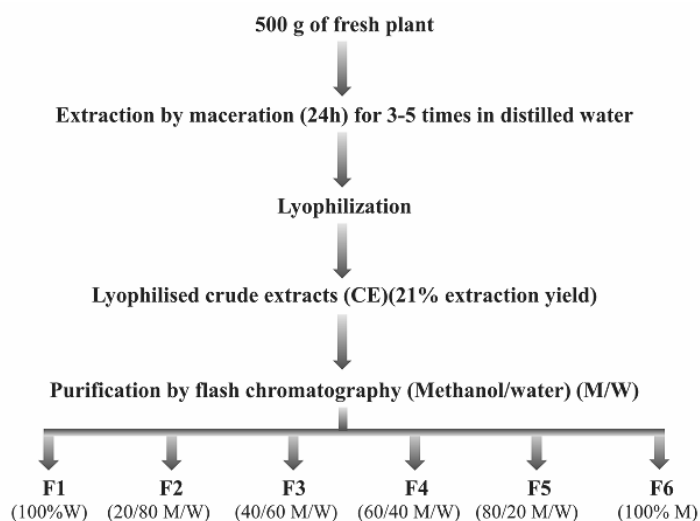


Figure 1. Purification procedure of *Zonaria tournefortii* lyophilized crude extract by flash chromatography using gradient of methanol / water.

Statistical analysis

Data were statistically analyzed using IBM SPSS personal computer statistical package (version 20, SPSS Inc, Chicago). Analysis of variance (ANOVA) was performed to test the significance of the difference in the antibacterial activities of the extracts.

RESULTS AND DISCUSSION

The aqueous crude extract of *Z. tournefortii* was tested at different concentrations against 13-multi-resistant strains of *S. aureus* and also against four fungi *C. albicans*, *C. neoformans*, *S. dimidiatum* and *S. brevicaulis*. Crude extracts of 5 mg/mL concentration, showed strong antibacterial activity against 9-strains of MRSA tested with 9-14 mm zone of inhibition (Table 1).

Table 1. Antibacterial activities of *Zonaria tournefortii* aqueous crude extract applied at 5 mg mL⁻¹

Extract	Concentration (mg mL ⁻¹)			Test strains (MRSA)												
	1	3	5	12	16	17	19	20	21	22	25	26	27			
Crude Extract	14.0	12.0	10.7	-	-	-	13.0	-	9.3	13.0	12.7	11.0	10.7			
Oxacilline	0.1	20	0	18.3	18	18.3	15	16	11	16	18	17.7	17.3	16		

Values are mean of three replicates, -: Not active,

Table 2. Antibacterial activities of *Zonaria tournefortii* semi purified fractions (F1-F6) applied at 5 mg mL⁻¹

Extract	Concentration (mg mL ⁻¹)			Test strains (MRSA)												
	1	3	5	12	16	17	19	20	21	22	25	26	27			
F1	5	-	-	-	-	-	-	-	-	-	-	-	-			
F2	5	25.0	23.7	12.0	-	-	12.7	-	16.7	11.3	22.0	11.7	15.7			
F3	5	14.7	11.7	11.3	-	-	12.7	-	12.0	13.0	14.0	12.0	12.0			
F4	5	-	-	-	-	-	-	-	-	-	-	-	-			
F5	5	-	-	-	-	-	-	-	-	-	-	-	-			
F6	5	-	-	-	-	-	-	-	-	-	-	-	-			
Oxacilline	0.1	20	0	18.3	18	18.3	15	16	11	16	18	17.7	17.3	16		

Values are mean of three replicates, F1-F6: semi-purified fractions, -: not active.

Table 3. MIC of *Zonaria tournefortii* active fractions F2 and F3 against nine MRSA test strains

Fractions	Test strains								
	1	3	5	19	21	22	25	26	27
F2	0.25	0.5	4	3	0.25	2	1	1	0.25
F3	2	2	2	1	1	0.5	0.5	1	1
T+	0.05	0	0.1	0.1	0.1	0.1	0.05	0.1	0.05

F2, F3: semi-purified active fractions, T⁺: Positive control; oxacilline MIC expressed in mg/ml

Similarly, among the 6-semi-purified fractions (F1, F2, F3, F4, F5 and F6) tested at 5 mg mL^{-1} concentration (Lyophilized powders diluted in sterile distilled water) against the pathogens F2 and F3 fractions showed high antibacterial activity. However, fractions F1, F4, F5 and F6 showed no activity against all tested pathogens (Table 2). The F2 fraction obtained with 80/20 water/Methanol elution showed the strongest antibacterial activity especially against MRSA strains 1, 3 and 25 (Fig. 2) with inhibition zone reaching 25 mm and MIC ranging from 0.25 mg mL^{-1} to 1 mg mL^{-1} (Table 3). Diameters of inhibition zones with fraction F2 against MRSA strains 1, 21 and 25 were strongest than positive control (Fig. 2). Fraction F3, less polar than F2 and obtained by (60:40) water/methanol, was very inhibitory to MRSA with MIC ranging from 2 to 0.5 mg mL^{-1} (Fig. 3).

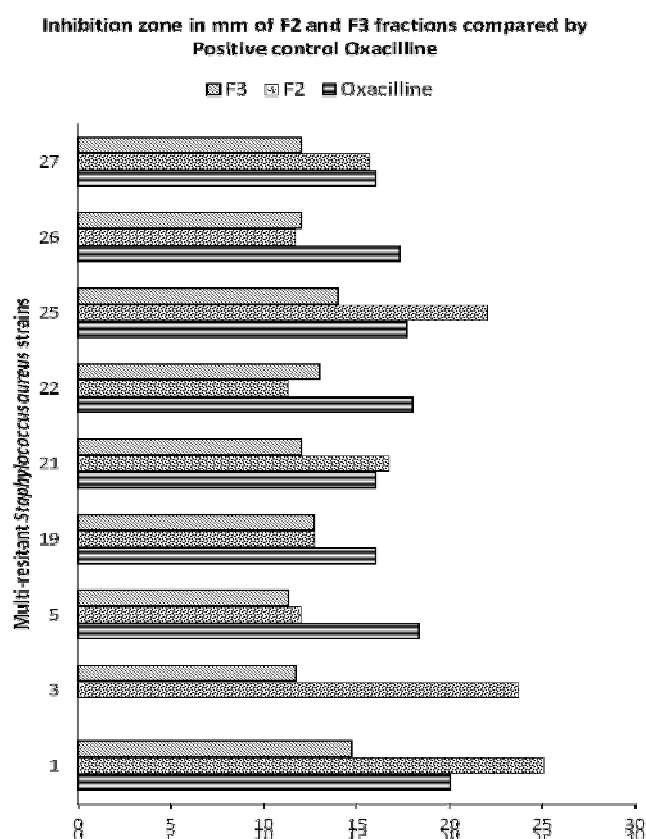
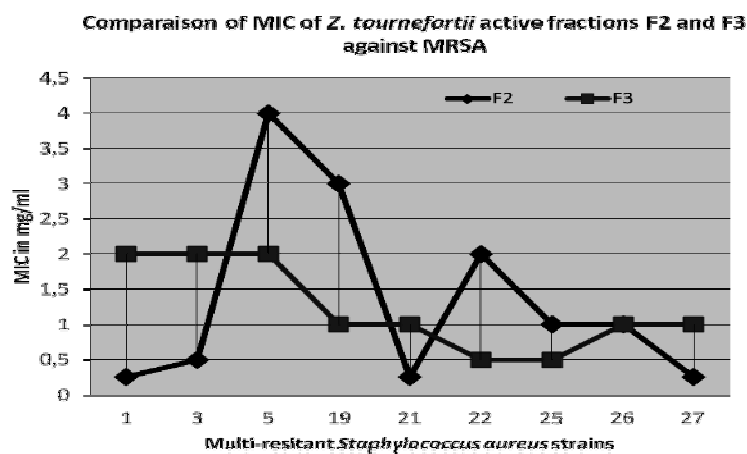


Figure 2. Inhibition zones (mm) of F2 and F3 fractions against MRSA.

Crude extract of *S. tournefortii* was inhibitory to two yeasts, *C. albicans* and *C. neoformans* (Table 4). Similarly, Fraction F2 and F3 were also active against these two yeasts with Fraction F3 having the strongest activity (Table 5.) Fractions F4-F6, did not affect the four tested fungi. The two hyphamycets tested namely *S. dimidiatum* and *S. brevicaulis* were resistant to both algal crude extract and semi purified fractions. The minimum inhibitory concentration (MIC) of F2 and F3 against *C. albicans* and *C. neoformans* (Table 5), were relatively low compared to the positive control MIC value, (1 mg mL^{-1} against *C. albicans* and 0.5 mg mL^{-1} against *C. neoformans*).

Figure 3. Minimal inhibitory concentration (MIC) of *Z. tournefortii* active fractions F2 and F3 against MRSA.Table 4. Antifungal activities of *Z. tournefortii* aqueous crude extract

Extracts	Concentration (mg mL ⁻¹)	Fungi test strains			
		<i>C. albicans</i>	<i>C. neoformans</i>	<i>S. dimidiatum</i>	<i>S. brevicaulis</i>
Crude	2	+++	+++	---	---
Extract	1	+++	+++	---	---
	0.75	---	---	---	---
	0.5	---	---	---	---
	0.25	---	---	---	---
T+	0.1	+++	+++	+++	+++

+: Presence of antifungal activity (test in triplicate +++), -: No activity (---) tested in triplicate, T+: Positive control (Fluconazole)

Table 5. Antifungal activities of *Zonaria tournefortii* semi purified active fractions F2 and F3, against fungi

Extracts	Concentration (mg mL ⁻¹)	Fungi test strains			
		<i>C. albicans</i>	<i>C. neoformans</i>	<i>Scytalidium dimidiatum</i>	<i>Scopulariopsis brevicaulis</i>
F2	2	+++	+++	---	---
	1	+++	+++	---	---
	0.75	---	---	---	---
	0.5	---	---	---	---
	0.25	---	---	---	---
F3	2	+++	+++	---	---
	1	+++	+++	---	---
	0.75	---	+++	---	---
	0.5	---	+++	---	---
	0.25	---	---	---	---
T+	0.2	+++	+++	---	---
	0.1	+++	+++	---	---
	0.05	---	---	---	---
	0.01	---	---	---	---
	0.005	---	---	---	---

F2, F3: active semi-purified fractions, +: Presence of antifungal activity (test in triplicate +++), ---: no activity (test in triplicate), T+: positive control (Fluconazole).

The discovery of novel antibacterial compounds that can be incorporated into new medicines is the solution to global problem posed by antibiotic-resistant bacteria (4). Consequently, many researches are oriented to find novel active products against these pathogens from marine organisms and especially seaweeds which contain a broad range of biological active compounds with rich pharmacological potential. The antibacterial activity of seaweeds such as *Gracilaria ahangii* and *Stichophus badionotus* against MRSA is known (29). Also, Eom *et al.* (16) highlighted the antibacterial activity of brown alga *Phaeophyta ecklonia stolonifera* against MRSA. Antibacterial activity of green alga *Ulva lactuca* against MRSA has also been reported (21).

Our results showed that aqueous extract of *Z. tournefortii* at relatively low concentration (5 mg mL^{-1}) was active against MRSA. Also the two fractions, F2 eluted with 80/20 water/methanol and F3 eluted with 60/40 water/methanol, strongly inhibited the MRSA especially strains 1, 21, 25 and 27. We also noticed that strain 3, which is oxacilline resistant, was strongly inhibited by F2 fraction (Figure 2) with an MIC of 0.5 mg mL^{-1} (Fig 3). This fraction apparently contains a compound in a relatively purified state and is able to inhibit the growth of oxacilline resistant *Staphylococcus aureus*. Chambah-Saidane (14), examined the antibacterial activities of *Z. tournefortii* aqueous extract harvested in 2001 from Tunisian coast and found weak antibacterial inhibition against 3 MRSA strains. However, fractions obtained from crude extract didn't show any antibacterial activity. These findings are contrary to our results of this study. We therefore hypothesize that this difference in antibacterial activity of same alga specie was due to the influence of season of collection and/or geographic location, because in earlier mentioned study, the algae was harvested during different season and locality. This hypothesis is confirmed by several studies of Martí *et al.* (30) and Salvador *et al.* (35) who highlighted the seasonal, physical and biological factors that influenced the antimicrobial secondary metabolites production by seaweeds. Other studies (25,30) have also reported variable cytotoxic and antitumoral activities of the brown alga *Padina pavonica* and 32 Mediterranean seaweed communities depending on location and season.

Ismail-Ben Ali *et al.* (20), earlier reported the seasonal variations in antibacterial activity of brown alga *Padina pavonica* collected monthly from Tunisian coast. Extracts prepared from this alga collected in September were strongly active, while those collected in October were totally inactive against tested bacteria (18 human and fish pathogens). Experimental conditions used may also be important, Chambah-Saidane (14) reported *Z. tournefortii* extracts tested by disc diffusion method revealed very weak inhibitory activities than well diffusion method.

Z. tournefortii methanol/toluene extracts showed no antibacterial, antifungal, antiviral, cytotoxic or antimutagenic potential (4). However, Vlaseos *et al.* (45) reported that *Z. tournefortii*, extracts had strong antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Acinetobacter*. This supports our findings and confirms the importance of *Z. tournefortii* as source of novel antimicrobial agents and that geographic location and other factors influence the production of biological compounds by this alga. From our study, it appeared that algae collected during the spring, contains the best level of antibacterial substances.

Fungi are also gaining importance for increased incidence of chronic, often fatal, mycoses in immune compromised patients (15). Kim *et al.* (27) evaluated the antimicrobial activities of 35 seaweed extracts against pathogenic bacteria and *Candida*

sp.. In our investigation, aqueous extract of *Z. tournefortii* had strong antifungal activity against the two yeasts *C. albicans* and *C. neoformans* and the F3 fraction (40/60 methanol /water) showed the strongest activity followed by fraction F2. We therefore, conclude that the two fractions F2 and F3 contain compounds with both antibacterial and antifungal components. While the antibacterial activity is present in higher concentration in fraction F2 but the antifungal compounds were more in fraction F3. These two semi purified fractions (F2 and F3) are worthy of further analysis to obtain the pure compounds responsible for their activities.

Besides, some work on chemical composition of *Z. tournefortii* and other *Zonaria* species has been reported. Anwar *et al.* (5) reported total synthesis of a polyunsaturated chromone metabolite isolated from *Z. tournefortii*. In addition, a novel acylphloroglucinol was isolated and major lipid component was identified by Amico *et al.* (2, 3). In similar report, two minor lipid components were characterized from *Z. tournefortii* (41). Previous studies on other *Zonaria* species showed interesting results on *Zonaria subarticulata* (44), which demonstrate antimicrobial activities against 12- bacteria, 2- yeasts and 2- moulds. Likewise, Zhang *et al.* (47) isolated a phloroglucinol-derived lipids; Spiralisones A-D from *Zonaria spiralis* with promising kinase inhibitory and antibacterial activity against *B. subtilis*. Furthermore, Wisespongpan and Kuniyoshi (46) isolated three phloroglucinols from *Zonaria diesingiana* with antibacterial and cytotoxic activities. Although the importance of seaweeds of *Zonaria* genus, antimicrobial potentialities of *Z. tournefortii* are still being investigated. Barreto *et al.* (8) showed antifungal activity of the alga organic extract against filamentous fungi. This is the first report which highlighted antimicrobial potentialities of *Zonaria tournefortii* aqueous extract and semi purified fraction at very low MIC (0.5 mg mL^{-1}) against the medically important fungi *Candida albicans* and *Cryptococcus neoformans*, which represent the principal yeasts pathogenic to humans, since *Candida albicans* is responsible for nosocomial infections; they are also most causative agents of nongonococcal urethritis (NGU) and cause candidiasis of the oral cavity and tongue associated with AIDS (33). Similarly *Cryptococcus neoformans* is a pathogen of systemic mycoses causing Cryptococcosis found in 15% AIDS patients. In addition the two fungi *Candida sp.* and *Cryptococcus sp.* causes serious problems to spoil tomato fruits, *Candida* species spoil the juices (33).

Zonaria tournefortii aqueous extract and semi purified fraction (F2 and F3) showed inhibitory activity at very low MIC (0.25 mg mL^{-1}) against *Staphylococcus aureus*, which causes toxic shock syndrome and bacterial (Septic) meningitis. It is most important bacteria that cause diseases in almost every organ of human body especially in hospitals (33).

CONCLUSIONS

The aqueous extract of brown alga *Z. tournefortii* and especially semi purified fractions F2 and F3 were found antimicrobial with very low MIC against MRSA and *C. neoformans*. Thus it is new and promising candidate for novel antifungal and antibiotic drugs, mainly against multi-resistant *S. aureus* and the two fungi *C. albicans* and *C. neoformans*. Further investigations to identify the active compounds are necessary.

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