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Screening of marine algae (*Padina* sp.) from the Lengeh Port, Persian Gulf for antibacterial and antifungal activities

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ABSTRACT

Objective: To evaluate the antibacterial efficacy of different solvent extracts of *Padina* sp. against selected human pathogenic bacteria and fungi species such as *Escherichia coli*, *Shigella* sp., *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa*, *Aspergillus flavus* and *Candida albicans*.
Methods: Various solvents including methanol, ethyl acetate, chloroform and hexane were used to acquire crude extracts from marine algae *Padina* sp. After crude preparation, antibacterial and antifungal activities were screened against clinically important human pathogenic bacteria using disc and well diffusion methods. For all the bacterial species used in this research, minimum inhibitory concentration was undertaken considering various solvent extracts of *Padina* sp. To ensure the accuracy of experiments, a positive control was also included.
Results: Confirmed that hexane is the best solvent to extract antimicrobial agents from *Padina* sp. Among selected bacteria, *S. aureus* was the most sensitive test microorganism. While, all other microorganisms showed resistance against methanol, ethyl acetate, chloroform extracts. In fact, by increasing concentration of hexane extract, inhibition of *S. aureus* growth or antimicrobial activity was increased. Growth inhibition zone in well method showed better results compared to disc diffusion method. The minimum inhibitory concentration and minimum bactericidal concentration of hexane extract were 15 and 30 mg/mL against *S. aureus*, respectively. All *Padina* sp. extracts did not reveal any antifungal activities against fungi species in this study.
Conclusions: Brown algae extracts showed sufficient antibacterial properties against *S. aureus*. Therefore, *Padina* sp. in this research can be a good candidate to design and manufacture novel antibacterial agents used in pharmaceutical industries.

1. Introduction

To date, infectious diseases are one of the main causes of high mortality in global human societies. Synthetic drugs and medicines ultimately are led to increase antibiotic resistance. Infectious diseases caused by bacteria and fungi have raised awareness to improve public health and protect global economies. Drug resistant infections especially with bacterial involvement are still a major challenge. Therefore, it is essential to discover new antibiotic compounds from alternative sources including oceans and marine organisms. Marine natural sources such as seaweeds and marine algae can be excellent alternative to remedy the situation by screening, developing and manufacturing antibacterial and antifungal compounds from novel bioactive substances. There is an increasing demand for therapeutic drugs from natural products. Recently, the high potential to contribute marine organisms, especially algae to the discovery of new bioactive substances is being increased[1]. The new therapeutic agents should be effective and have a novel mechanism of action regarding resistance behavior[2]. Marine algae are potential renewable resources in the world. Algae are considered as diverse source of secondary metabolites having a broad spectrum of biological activities such as antimicrobial[3], antiviral[4], antifungal[5], anti-allergic[6], anti-cancer[7], anti-fouling[8] and antioxidant[9] that have been used to develop the pharmaceutical industries.

Despite the importance of medicinal properties and pharmaceutical applications of marine algae, few pharmacological researches undertaken to detect novel therapeutic agents for infectious diseases treatments[10]. There are many scientific records with reference

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to a wide spectrum of pathogen inhibitory compounds extracted from marine algae against human bacterial and fungi pathogens worldwide[11]. Fortunately, the Iranian southeast coasts are covered by a wide variety of marine algae. The main objective of the present study was to examine the antimicrobial and antifungal effects of brown algae extracts. For this reason, *Padina* sp. was selected to evaluate bioactive compounds using different solvent systems against four human pathogenic bacteria and two fungus species.

2. Materials and methods

2.1. Algal material

The brown alga, *Padina* sp. were collected from the intertidal regions of the Lengeh Port (latitude 54° 52' N; longitude 26° 32' E) southeast coast of Iran, during low tide. The collected marine algae were first identified morphologically using valid identification keys. Samples rinsed thoroughly with sea water to remove sands and epiphytes and then washed with distilled water. Algae samples were shade dried for 2 weeks and then were crushed by an electric grinder, the obtained powder was stored at –20 ℃ until the extraction process.

2.2. Preparation of algal extracts

The extraction was carried out using a Soxhlet extractor for 10 h. Different solvents were used successively with gradient polarity (methanol, ethyl acetate, chloroform and hexane). The extracts were concentrated under reduced pressure by using rotary vacuum evaporator in 40 ℃ and then stored in refrigerator for further use[12].

2.3. Microorganisms and media

Strains of bacteria [*Staphylococcus aureus* (*S. aureus*) and *Shigella* sp., *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*)] and fungus [*Candida albicans* (*C. albicans*), and *Aspergillus flavus* (*A. flavus*)] were obtained from the Institute of Razi, Iran. The species of bacteria for susceptibility test were grown in Muller-Hinton agar (Merk, New Jersey, United States) and species of fungus were grown in Potato Dextrose agar (Oxoid, Hampshire, United Kingdom). The concentrations of bacterial and fungal suspensions were standardized by matching to the 0.5 McFarland standard. All solvents applied in this research were of high performance liquid chromatography grade.

2.4. Antimicrobial activity

Antimicrobial activity of crude extracts was undertaken by two ways of the agar diffusion method (well and disc). Overnight nutrient broth culture of the test organisms which adjusted to 0.5 McFarland were firmly seeded over the agar plate using sterile cotton swab. The amount of 25 μL of each extract (100, 300 mg/mL) was poured into a 6 mm diameter well or loa ded on paper discs. Standard antibiotic, gentamicin (10 mg/disc) and nistatin (300 mg/disc) were used as positive controls and solvents were used as negative control. Inhibition zone diameters were measured in mm after incubation for 24 h at 37 ℃[13,14]. Statistical analysis for all the data was undertaken.

2.5. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

MIC is the lowest concentration of an antimicrobial agent (extract) which can inhibit the growth of bacteria. The MIC of extract was determined by a macrodilution broth assay which is performed according to standard National Committee for Clinical Laboratory Standards (NCCL, 2006)[15]. Two-fold serial dilution of algal extract was prepared in nutrient broth to obtain 30.00–0.46 mg/mL concentration. Briefly, added 0.5 mL nutrient broth to 1–10 tubes and 2 tubes contain 1 mL nutrient broth. Then added 30 mg/mL extract to 1, 2, 10 tubes. Dilutions were made by transferred 0.5 mL from 2 tubes to the next tube and discarded 0.5 mL from 7th tube. After completing the dilutions, added 0.5 mL of a standardized inoculums (1/500 diluted 0.5 McFarland) to 1–8 tubes. The 8th tube serves as control for growth of the organisms, 9th tube serves as control for medium and 10th tube serves as control of extract. After overnight incubation, each tubes that inhibited visible growth was recorded as MIC. A total of 100 μL of contents of each tubes that showing inhibition of growth was subcultured onto a nutrient agar plate and treatments that killing 99.9% bacteria was recorded as MBC. Statistical analyses for all the data were carried out.

2.6. Antifungal activity of A. flavus

Antifungal susceptibility test was carried out by two technical methods. To determine the antifungal activity of extracts by diffusion method, 6 mm of two-day culture of *A. flavus* was cultured on Potato Dextrose agar and incubated in 28 ℃ until 3 cm diameter of fungus was achieved. Then wells and discs containing extracts were placed with 1 cm distance, separately. After 2 days incubation, antifungal activity of extracts was evaluated and diameter of inhibition zone was recorded.

In the next method, 500 μL of extracts was poured in plates and Potato Dextrose agar medium was added with subsequently spinning in clockwise. After colling, 6 mm *A. flavus* fungus by pipet pasteur was cultured on Potato Dextrose agar plates. Samples without any extract treatment were considered as controls. Plates were incubated at 28 ℃ for 2 days. The results were determined from the presence or absence of growth and size of *A. flavus*.

2.7. Statistical analysis

All tests were performed in triplicate and expressed as mean ± SD. Statistical analysis was carried out using SPSS version 16 software[16]. A One-way ANOVA was used to determine the difference in treatments when significant differences between means were applied by Tukey test.

Table 1

Activity of different extracts of *Padina* sp. against human pathogens in disc and well diffusion assays.

Microorganism	Methanol	Ethyl acetate	Chloroform	Hexane	Gentamicin	Nitroforantuin	Nistatin
S. aureus	-	-	-	9.0 ± 0.5*	23.0 ± 0.5[c]	22.0 ± 0.5[b]	Nt
P. aeruginosa	-	-	-	-[a]	17.0 ± 0.5[b]	-[a]	Nt
E. coli	-	-	-	-[a]	9.3 ± 0.3[a]	19.0 ± 1.1[b]	Nt
Shigella	-	-	-	-[a]	18.0 ± 0.5[b]	21.0 ± 0.5[b]	Nt
C. albicans	-	-	-	-[a]	Nt	Nt	26.6 ± 1.2[a]
A. flavus	-	-	-	-[a]	Nt	Nt	16.3 ± 0.8[b]

Results of well diffusion were quoted in parenthesis. Different letters represent the statistical comparisons between groups by using ANOVA followed *post-hoc* Tukeys test ($P < 0.05$).

-: No activity; Nt: Not tested; *: 300 mg of extracts.

3. Results

The antibacterial activities of different extracts of *Padina* sp. against 4 bacterial and 2 fungal strains are shown in Table 1. Methanol, ethyl acetate and chloroform extracts did not show any antimicrobial effects against the tested microorganisms in both disc and well diffusion assays. However, hexane extract exhibited antibacterial effect against only Gram-positive bacteria, *S. aureus*. As observed, inhibition zone of *S. aureus* were 7.6 ± 0.3 and 8.3 ± 0.3 in disc diffusion assay and were 8.6 ± 0.3 and 9.0 ± 0.5 in well diffusion test in 100 mg/mL and 300 mg/mL concentrations, respectively.

Results of antifungal activity of extracts on *A. flavus* by mixture extracts with Potato Dextrose agar medium method did not reveal any significant inhibitory effects.

4. Discussion

The aim of this research was to evaluate the activity of *Padina* sp. from coastal regions of Lengeh Port in northern area of Persian Gulf to produce novel bioactive agents with potential pharmaceutical applications. In fact, the capability of marine algae to produce bioactive compounds has introduced them with a potential biomedicine interest. Worldwide records proved that, marine algae possess unique structure bioactive compounds with special physiological and metabolic properties[11]. Among marine algae examined in this regard, *Padina* sp. has revealed effective antibacterial activities against most human pathogenic bacteria[11].

Previous studies suggested different results from the impact of effective solvents on algal extracts and antimicrobial substances. Although, a variety of solvents have been applied for extracting antimicrobial compounds of seaweed, it is still uncertain what kind of solvent would be the most effective for extraction. A few researchers used different solvents for extraction of seaweed and mentioned different points. For example, Sangeetha *et al.*[17] reported that methanolic extract was the best solution. Patra *et al.*[18] indicated that chloroform and ethyl acetate were the best solutions for extracting the antibiotic principle. Our present investigation showed that marine algae extract prepared using hexane showed higher activity compared to other extracts. Our results were similar with previous investigations suggesting significant antibacterial activity in hexane extract undertaken by Ozdemir *et al.*[19] in 2006 and Demirel *et al.*[20] in 2009. These findings indicated that extraction method had definite effect on the isolation of bioactive compounds.

Antibacterial activity was exhibited with the presence of hexane extract. Hexane extract of *Padina* sp. showed inhibiting activity against *S. aureus*. Similar to this study Lima-Filho *et al.*[21] in 2002 reported hexane extract of *Ulva fasciata* inhibited the growth of *S. aureus*. However, Ozdemir *et al.*[19] in 2006, reported hexane extract of *Dictyopteris membranaceae* and *Cystoseria barbata* had no antibacterial activity against *S. aureus*.

In the current study, hexane extract was most effective against Gram-positive bacteria compared to Gram-negative bacteria. These results confirmed earlier investigations on antibacterial activity of seaweeds that established by many scientists such as Kandhasamy and Arunachalam[22] in 2008; Kolanjinathan *et al.*[23] in 2013 and Krishnapriya *et al.*[24] in 2013.

The resistance of Gram-negative bacteria towards antibiotic compounds might be due to the hydrophilic surface of outer membrane which is composed of high amounts of lipopolysaccharide molecules. This fact can act as a barrier to the penetration of numerous antibiotic molecules. Therefore, bacterial membrane are capable of breaking down the molecules introduced from outside by the enzymes located in the periplasmic space. Although, the Gram-positive bacteria do not possess such outer membrane and cell wall structures[2].

In other study conducted by Patra *et al.*[25] in 2008, the antibacterial activity of methanol extract of *Sargassum* sp. in different concentrations was evaluated and reported that the high concentrations of extracts have higher antibacterial activity against *S. aureus* and *E.coli* compared to low concentrations. In addition, Demirel *et al.*[20] in 2009, showed antibacterial activity of hexane extract of brown algae is increased with increasing concentrations against *S. aureus*. These results are in accordance with present study and suggest that by increasing concentration of hexane extract, inhibition of *S. aureus* growth or antimicrobial activity is increased. Therefore, increasing the concentration of extract is led to the higher potential of antimicrobial activities.

In this research, MIC and MBC values of hexane extract were obtained 15 and 30 mg/mL, respectively. However, it should be noted that the extracts obtained in this work, were not purified and consisted of a variety of compounds. Hence, high value of purified extract is required for testing MIC and MBC to show exact

correlation between undertaken studies in this regard[26,27].

In this study, none of the examined extracts showed toxicity against *A. flavus*. This result was previously obtained by Padmakumar and Ayyakkannu[28], which confirmed resistance of *A. flavus* against seaweed extracts. Therefore, the results of present study were similar to Tuney *et al.*[29] investigation that evaluated antibacterial and antifungal activities of methanol, acetone and diethyl ether extracts of *Padina pavonica* (*P. pavonica*).

There have been reports on significant antimicrobial activities of marine macroalgae by several research teams. In Vijayabaskar and Shiyamala[30] and Kolanjinathan[23] studies methanol and hexane extracts of macroalgae showed significant antimicrobial activity against *E. coli*, *P. aeruginosa*, *Shigella* and *S. aureus*. Kayalvizhi *et al.*[31] tested methanol and chloroform extracts of *Padina boergessenii* against *C. albicans*, *A. flavus*, *E. coli*, *S. aureus*. The results showed *C. albicans* was resistant against chloroform extract whereas other microorganisms were sensitive against examined extracts.

Variable toxicity of seaweed related to some factors such as habitat and seasonal time of algal collection, different growth stages of algae, variation in algal genus and species, methods of extraction and type of solvents, screening tests and the types of tested bacterial strains.

Vijayabaskar and Shiyamala[30] indicated that the methanol extracts of brown algae, *Sargassum wightii*, *Turbinaria ornata* possess strong antimicrobial activity against tested microorganisms. These differences with our study can be related to the genus and species of algae.

Ben Ali *et al.*[32] evaluated secondary metabolites and antibacterial activity of *P. pavonica* in variable seasons and demonstrated that *P. pavonica* have higher antibacterial activity in summer. The stronger activity in summer may be due to rapid growth of the algae and high rate of photosynthesis. Ktari *et al.*[33], Marti *et al.*[34], and Ibrahim *et al*.[35] revealed variable cytotoxic and antitumor activities of algal in variable seasons and geographic locations. Therefore, depending on the collection of samples carried out in winter, the low antimicrobial activities may be related to the harvesting season.

Geographic location also is an important factor in algal toxicity. In Ktari *et al.*[36] study, *P. pavonica* collected in spring from the eastern coast of Tunisia did not exhibit any effects against *S. aureus* while in Ben Ali *et al.* study, the same algae species collected in spring from northern coast of Tunisia showed a significant antibacterial activity against *S. aureus*.

In some studies carried out on antibacterial activity of seaweed from Oman Sea and Persian Gulf coasts, no antibacterial activity was observed from algal extracts. Movahhedin *et al.*[37] screened methanol, dichloromethane and hexane extracts of marine algae from Oman Sea coasts as antimicrobial substances and reported that the tested bacteria were resistant to extracts of algae. In another study was done by Jassbi *et al.*[38] in the Persian Gulf coast, was also showed resistance of most of the microorganisms to algal extracts. Similar to our study, microorganism were resistance to methanol, ethyl acetate and chloroform extract. This similarity may be due to geographical zone. On the other hand, producing of secondary metabolites levels in seaweed depends on ecological parameters such as salinity, temperature, nutrients, UV radiation[39].

Valgas *et al*.[40] used variable screening methods to determine antibacterial activity of natural products. The well variant of the diffusion method was more sensitive than the disc variant. The disc is composed of cellulose and many free hydroxyl groups are put out on their glucose. Thus, polar cationic antibacterial substances or compounds adsorb to the disc and cannot diffuse into the medium while apolar compounds do not adsorb on the surface of the discs and diffuse into the media. Also, the extracts that possess heavy molecular compounds cannot diffuse in agar and therefore cannot show their activity.

In current study, different extracts were used with gradient polarity (methanol, ethyl acetate, chloroform, and hexane) and only hexane extract exhibited antibacterial activity. This would be the reason that methanolic, ethyl acetatic and chloroformic extracts are able to dissolve more compounds compared to hexane extract.

It is evident from the present study that antibiotic substances of *Padina* sp. may possibly be apolar and hydrophobic compounds. However, in order to exactly clarify the issue, it is necessary to purify the antibiotic compounds and conduct subsequent isolation and purification methods to obtain fractions from *Padina* species algae which already showed a good potential of antimicrobial activities against selective bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

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