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Antimicrobial, Antiplasmid and Cytotoxicity Potentials of Marine Algae *Halimeda opuntia* and *Sarconema filiforme* collected from Red Sea Coast

Samy A. Selim

***Abstract*—**The antimicrobial, antiplasmid and cytotoxic activities of marine algae *Halimeda opuntia* and *Sarconema filiforme* were investigated. Antimicrobial bioassay against some human pathogenic bacteria and yeast were conducted using disc diffusion method. *Halimeda* extract exhibited antibacterial activity against six species of microrganisms, with significant inhibition against *Staphylococcus aureus*. While *Sarconema* extract was better potent as antifungal against *Candida albicans*. Comparative antibacterial studies showed that *Halimeda* extract showed equivalent or better activity as compared with commercial antibiotic when tested against *Staphylococcus aureus.* Further tests conducted using dilution method showed both extracts as having bacteriostatic mode of action against the tested microorganisms. Methanol extract of two species showed significant cytotoxicity (LC_{50} <500µg) on brine shrimp. *Halimeda opuntia* showed highest cytotoxic activity (LC_{50} =192.3µg). Also, the present investigation was undertaken to investigate the ability of methanolic extract of the algal extracts to cure R-plasmids from certain clinical *E. coli* isolates. The active fraction of *Halimeda* and *Sarconema* could cure plasmids from *E. coli* at curing efficiencies of approximately 78%. The active fraction mediated plasmid curing resulted in the subsequent loss of antibiotic resistance encoded in the plasmids as revealed by antibiotic resistance profile of cured strains. The screening results confirm the possible use of marine algae *Halimeda opuntia* and *Sarconema filiforme* as a source of pharmacological benefits.

***Keywords*—**Antimicrobial, antiplasmid Cytotoxicity, Marine Algae

I. Introduction

IN the last ten years, the interest on the study of seaweeds as sources of bioactive compounds has increased. Such tendency could be at least partially explained on the basis of their chemical components, which exhibit different beneficial properties for the human health [1]. It is well known that algae contain minerals, polysaccharides, amino acid derivatives, carotenoids and phenolic compounds. These can display antioxidant properties at very low concentrations, which has been exploited on the food industry to protect foods from oxidation process [2]. In addition, epidemiological research has supported the existence of an inverse correlation between the incidence of cardiovascular diseases and consumption of phenolic rich foods [3]. However research towards the use of seaweeds for the treatment of various diseases has received less attention.

S. S is with the [1]Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Al-Jouf University, P.O. 2014, Skaka, Saudi Arabia, [2]Microbiology Section, Botany Department, Faculty of Science, Suez Canal University, P.O. 41522, Ismailia, Egypt (e-mail: sadomm2003@yahoo.com).

In recent years, pharmacological firms have started looking towards seaweeds for new natural products for pharmacological benefits [4].

Different seaweed extracts have received increased attention, due to their potent pharmacological effects, particularly *in vivo* hypolipidemic [5] antioxidant [2], immunological [6] and antitumoral activities [7]. Similarly, *in vitro* studies have confirmed the antioxidant properties of seaweed extracts [8] which has suggested their possible uses as nutraceuticals and/or phytodrugs. Some groups have studied the ability of seaweed extracts to inhibit lipid peroxidation or to scavenge free radicals [9-10]. Different compounds such as carotenoids, mycosporine-related amminoacids and terpenoids together with phenolic compounds as cinnamic acids, phlorotannins, and bromophenols has been identified among the principal entities responsible for these properties in marine algae [11-12]. Chemicals responsible for antibiotic activities are widespread in macroalgae. Interesting substances in particular are the halogenated compounds such as haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones and ketones [13].

A major problem in antimicrobial chemotherapy is the increasing occurrence of resistance to antibiotics, which leads to the insufficiency of antimicrobial treatment. The overuse of antibiotics and consequent antibiotic selection pressure is thought to be the most important factor contributing to the appearance of different kinds of resistant microbes [14]. The genetical basis of resistance is often the R-plasmid, which can be transferred to other bacteria in the environment of the recipient, and these extrachromosomal DNA sequences can be responsible for the emergence of multiple resistance to antibiotics. Plasmid may be lost spontaneously in a very low frequency (10^{-5} to 10^{-7}), but certain effects can increase the probability of plasmid loss, which is the basis of artificial plasmid elimination. In early studies, acridine orange, ethidium bromide and later sodium dodecylsulfate were found to be powerful plasmid eliminators. Their toxicity did not allow their *in vivo* testing for their antiplasmid effect. Molnar et al. investigated the effects of many tricyclic drugs on plasmid replication and found that two drugs applied in everyday practice exerted antiplasmid activity [15]. Chlorpromazine and promethazine eliminated the tetracycline,
chloramphenicol, streptomycin and sulfonamide resistance of an *E. coli* strain. That finding resulted in systematically synthetized phenothiazine and acridine derivatives being studied for their biological activity and a relevant proportion of the tested molecules proved to have an antiplasmid effect [15]. In a previous study, it was shown that metabolites isolated from Egyptian marine algae *Halimeda opuntia* and

Sarconema filiforme could inhibit pathogenic bacteria and have biopotantials effect [16]. Hence, the present study undertakes the extracted in different solvents of major secondary metabolites from samples of *Halimeda opuntia* and *Sarconema filiforme* collected from Red Sea coastal waters of Egypt. Algal extracts compounds were tested for their antimicrobial activities against some human pathogenic bacteria and yeast. Potencies of these compounds were determined by comparing their antimicrobial activities against commercially available antibiotics. Extracts compounds were subjected to antimicrobial testing via dilution method to determine their possible mode of action, MIC (Minimum Inhibition Concentration) and MMC (Minimum Microbicidal Concentration) activities. Also the present report describes the screening of these potential marine algae of Red Sea coast for antiplasmid and cytotoxic activity.

II. Materials and Methods

A. Algal Materials and Preparation of the Material

The fresh samples of marine algae *Halimeda opuntia* and *Sarconema filiforme* were washed with seawater and fresh water to remove salts epiphytes, microorganisms and other suspended materials. The clean algae were frozen and lyophilized. The dry material was stored at − 20 °C.

B. Chemical Extraction of Algae

The extracts were obtained by macerating 30 g of the dried plant in cold methanol for 48 h, with the resultant extract being filtered. Then concentrated to dryness in a rotary evaporator under reduced pressure and after evaporation in vacuum the extracts were stored at − 20 °C until use. Ethanol, chloroform, dimethyformamide, dimethyl sulfoxide, hexane or water extracts were obtained similarly. The extracts were diluted in different solvents for approximately 2.5 mg/ml, and then sterilized by passage through 0.45 µm filter.

C. Antimicrobial Tests

The agar diffusion assay was performed according to modified Kirby-Bauer disc diffusion method [17]. One loopful of each test organism (*Pseudomonas aeruginosa; Escherichia coli; Proteus vulgaris; Serratia marcescens; Staphylococcus aureus; Micrococcus luteus; Enterococcus feacalis; Bacillus subtilis; Bacillus cereus; Bacillus megaterium; Candida albicans* ATCC 44831*; Candida utilis* and *Saccharomyces cerevisiae*) was suspended in 3 ml 0.9% NaCl solution separately. The bacterial strains used in this work (others than ATCC strains) were isolated from human beings and belong to the microbiological laboratory collection of the General and Environmental Microbiology Department, Pecs University, Hungary. Nutrient agar (for bacterial strains) and YEA media (for fungi) were inoculated with this suspension of the respective organism and poured into a sterile petri dish. Paper discs (Whatman, 6 mm) impregnated with 2 mg /disc of the respective pure compounds was placed on the seeded agar plates. The solvent of each extract was used as a negative control. A pre-diffusion for 3 h was guaranteed. Inhibition zones were measured after 18 h incubation at 37 °C. The inhibition zones were measured excepting the 6 mm paper disc. Every experiment was carried out 10 times. Representative halos were those measuring a diameter superior to 10 mm. Similar antibacterial bioassays were also conducted for comparative analysis using *Halimeda* and *Sarconema* methanolic algal extracts and 12 types of Oxoid antibacterial and antifungal susceptibility discs (Table II). Concentration used were according to NCCL levels; 30 µg per disc.

D. Determination of MIC and MMC

MIC was determined by the broth dilution method. Serial dilutions from 0.5 to 5 mg/ml for *Halimeda* and *Sarconema* methanolic algal extracts were added to Müller-Hinton broth. Bacterial suspension of 1 ml containing approximately 1: 5×10^5 cells (*Pseudomonas aeruginosa*; *Escherichia coli; Staphlococcus aureus*; *Enterococcus feacalis*; *Bacillus cereus*; *Candida albicans*; *Candida utilis* and *Saccharomyces cerevisiae*) were added to each dilution of extract. Growth of bacteria was checked after overnight incubation at 37 °C. MMC is usually an extension from the MIC, where the organisms are quantitatively subcultured from MIC tubes on antibiotic free agar medium to indicate the minimum concentration was no viable organism appears in the culture.

E. Antiplasmid testing

The plasmid curing was performed by method described by Deshpande *et al*. [18]. In brief, the culture was grown in presence of a curing agent at specified concentration for 24 h at 37 °C and then plated on Luria agar plates to obtain isolated colonies. Isolated colonies were then replica plated on to Luria agar and Luria agar containing antibiotics. The colonies which grew on Luria agar but failed to grow in presence of antibiotics were considered as putative cured derivatives. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested. The curing agent was tested at 25, 50, 100, 200 and 400 µg per ml concentrations. Dimethyl sulphoxide (DMSO) was used as negative control in plasmid curing experiment. The loss of plasmid DNA in the cured derivatives was further confirmed by agarose gel electrophoresis of plasmid DNA preparation of cured derivatives.

F. Brine shrimp lethality test

Brine shrimp lethality test for larvae nauplii was used to determine the toxicity of ethanol extract of seaweeds [19]. A concentration of 50, 100, 250, 500, 1000 and 2000 µg/ml of methanlic extracts were prepared and 2 ml were transferred in glass vial and left open for 48 hours to evaporate the organic solvent before adding the nauplii. Brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) were hatched in a shallow rectangular container (60 x 30 cm) filled one fourth with sea water at 27-30^{o}C with aeration. A plastic divider with holes was placed in the container to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller

compartment was illuminated. After 48 hours, the phototropic nauplii were collected from the illuminated side. Ten brine shrimp nauplii were transferred in each vial. Two ml of sea water was added in each vial of ethanol extract before adding the nauplii. Observations were recorded after 24 hours and survivors were counted and percent death at each dose level was calculated. LC_{50} were calculated using probit analysis [20].

G.Statistical analysis

Data were subjected to one-way analysis of variance for means of comparison, and significant differences were calculated according to Duncan's multiple range test. Data are reported as means ± standard error of the means. Differences at $P < 0.05$ were considered statistically significant. spss (version 11.0) was used to perform the statistical analysis.

III. Results

Results are described in "Table I". The largest halos were achieved by the methanol and dimethyformamide extracts of marine algae *Halimeda opuntia* and *Sarconema filiforme* against *Pseudomonas aeruginosa, Escherichia coli, Serratia marcescens, Staphylococcus aureus, Enterococcus feacali, Bacillus subtilis, Bacillus cereus, Candida albicans, Candida utilis* and *Saccharomyces cerevisiae.* Other algae ethanol, chloroform, dimethyl sulfoxide, hexane or water extracts achieved halos not superior to 10 mm or were not identified (data not shown). Likewise, solvents used alone (controls) produced no halo (data not shown). Methanol and dimethyformamide extracts of marine algae *Halimeda* and *Sarconema* showed more antimicrobial activity on *Saccharomyces cerevisiaeis* strain versus other tested microorganisms. *Halimeda* extract exhibited antibacterial activity against six species of microorganisms, with significant inhibition against *Staphylococcus aureus*. Results of paper disc diffusion assay of the methanol extracts as compared with commercially available antibiotics are shown in table II. Comparative antibacterial studies showed that *Halimeda* extract showed equivalent or better activity as compared with commercial antibiotic when tested against *Staphylococcus aureus*. On the other hand, *Sarconema* extract was better potent as antifungal against *Candida albicans*. As for the commercial antibiotics, amikacin showed potent inhibition against all studied bacteria. In addition, all studied bacteria were relatively resistant towards the cephalexin "Table II".

MIC was assessed for potent selected plant extracts (*P. harmala* and *S. officinalis*) on the isolated bacterial strains by giving due consideration to bacterial growth inhibition by the respective plant products "Table III".

TABLE I
ANTIMICROBIAL ACTIVITY OF THE INVESTIGATED METHANOL (M) AND DIMETHYFORMAMIDE (DF) EXTRACTS (2 MG/DISC) OF OF MARINE ALGAE HALIMEDA OPUNTIA AND SARCONEMA FILIFORME IN AGAR DIFFUSION ASSAY (INHIBITION ZONE WAS MEASURED WITHOUT PAPER DISC)

		Halimeda		*Sarconema*	
	Strains	**M**	**DF**	**M**	**DF**
Gram Negative	*Pseudomonas aeruginosa*	-	18	20	-
	Escherichia coli	22	-	11	-
	Proteus vulgaris	-	-	-	-
	Serratia marcescens	-	23	-	-
Gram Positive	*Staphylococcus aureus*	27	-	-	-
	Micrococcus luteus	-	-	-	-
	Enterococcus feacalis	13	-	-	-
	Bacillus subtilis	22	-	-	-
	Bacillus cereus	-	17	17	-
	Bacillus megaterium	-	-	-	-
Yeast	*Candida albicans*	10	-	13	-
	Candida utilis	-	25	-	-
	Saccharomyces cerevisiae	13	13	20	11

TABLE II
COMPARATIVE ANTIBACTERIAL ACTIVITY OF HALIMEDA AND SARCONEMA METHANOLIC ALGAL EXTRACTS AND ANTIBIOTICS AGAINST TESTED STRAINS OF BACTERIA AND YEAST

		Halimeda		*Sarconema*	
	Strains	**M**	**DF**	**M**	**DF**
Gram Negative	*Pseudomonas aeruginosa*	-	18	20	-
	Escherichia coli	22	-	11	-
	Proteus vulgaris	-	-	-	-
	Serratia marcescens	-	23	-	-
Gram Positive	*Staphylococcus aureus*	27	-	-	-
	Micrococcus luteus	-	-	-	-
	Enterococcus feacalis	13	-	-	-
	Bacillus subtilis	22	-	-	-
	Bacillus cereus	-	17	17	-
	Bacillus megaterium	-	-	-	-
Yeast	*Candida albicans*	10	-	13	-
	Candida utilis	-	25	-	-
	Saccharomyces cerevisiae	13	13	20	11

TABLE III
MIC AND MMC OF HALIMEDA AND SARCONEMA METHANOLIC ALGAL EXTRACTS AGAINST TESTED STRAINS OF BACTERIA AND YEAST

Strains	**Halimeda**		**Sarconema**	
	MIC	**MMC**	**MIC**	**MMC**
Pseudomonas aeruginosa	0.5	0.5	2	4
Escherichia coli	0.5	0.5	2	4
Staphlococcus aureus	0.5	4	2	4
Enterococcus feacalis	2	2	-	-
Bacillus cereus	0.5	0.5	1	0.5
Candida albicans	-	-	3	3
Saccharomyces cerevisiae	1	2	2.5	3

Accordingly, the MIC of *P. harmala* was 4 mg/ml on *S. aureus* and 0.5 mg/ml on all *Bacillus* species. Likewise, the MIC *S. officinalis* on *E. coli* was 0.5 mg/ml. The MIC of *P. harmala* on *S. aureus* was at the maximum concentration of 4 mg/ml. The MBCs of the extracts were in general significantly higher than the corresponding MIC values "Table III". MBC of 3 mg/ml was reached by the extracts of *P. harmala* and *S. officinalis* against *P. aeruginosa*. Results obtained from disc diffusion method, followed by measurements of MIC values, indicated that *E. coli* is the most sensitive microorganism tested, with the lowest MIC value (0.5 mg/ml) in the presence of the plant extract isolated from *S. officinalis*.Methanolic

extracts were investigated for their antiplasmid effect on 9 *E. coli* clinical bacterial strain "Fig. 1*"*. The results showed that both extracts has considerable antiplasmid activity, which can approximate 78% (7/9 for *Halimeda* and *Sarconema* extracts).

Fig. 1 Plasmid DNA profile of E. coli and cured derivatives by agarose (0.8%) gel electrophoresis for confirming the antiplasmid activity of methanol extract of Halimeda and Sarconema seaweeds

Out of two species, have showed LC_{50} <500 µg in methanolic extracts. Methanolic extracts of seaweed were evaluated for their cytotoxicity at different concentrations and were classified as non-cytotoxic (NCT<50% death), mild cytotoxic (MCT>50% death but <75% death) and highly cytotoxic (HCT>75% death) at 1000 µg/ml based on their lethality to brine shrimp "Table IV". Among these two seaweeds, *Halimeda opuntia* showed the LC_{50}=192.3 µg, while *Sarconema filiforme* was found to be moderately effective (MCT>50% death but <75% death) on nauplii of brine shrimp (LC_{50}= 328.9 µg) "Table IV*"*.

IV. Discussion

The main objective of this study was to evaluate the ability of *Halimeda opuntia* and *Sarconema filiforme* seaweed species from Red Sea coast to inhibit the growth of some pathogenic microbes with the aim to use them in the future as alternatives to common antibiotics. Several solvents were used for the extraction of freeze-dried seaweed powder. Our data revealed that considerable inhibition zones were only observed for the methanol and dimethyformamide extracts. Therefore, the compounds responsible for the antimicrobial activity assume, that the active compounds could be, at least partly, lipophilic

TABLE IV
PERCENT DEATH OF BRIME SHRIMP AND LC_{50} AT DIFFERENT CONCENTRATION OF *HALIMEDA* AND *SARCONEMA* METHANOLIC ALGAL EXTRACTS

Concentration (µg)	*Halimeda opuntia*	*Sarconema filiforme*
50	0	0
100	10	15
250	65	25
500	85	76
1000	95	100
2000	100	100
LC_{50}	**192.3**	**328.9**

halogenated compounds. Halogen-containing terpenoids, acetylens and phenols have been identified in several seaweed species as biologically active compounds [21-23]. Both algae could also be responsible for the observed effects against pathogenic bacteria. Besides halogenated compounds, fatty acids have been identified as antimicrobial substances in algae [24]. Bansemir et al. [25] have investigated the antibacterial activities of the extracts from 26 algae species prepared by dichlorometane, methanol and water against five fish-pathogenic bacteria. The highest activities were obtained by the dichloromethane prepared extracts. They have reported that the most active algal species was *Asparagopsis armata* against all tested bacteria. Ely et al. [26] have shown the methanolic extract of *Cladophora prolifera* had moderate bactericidal activity against *S. aureus* and *Vibrio cholerea*. Freile-Pelegrin and Morales [27] studied ethanolic extracts from different thallus regions (apical, basal and stolon) of *Caulerpa* spp. They indicated that the stolon was the region having the highest antibacterial activity.

In this study, some of the bacterial strains (*Proteus vulgaris*, *Micrococcus luteus* and *Bacillus megaterium*) did not respond to extracts, whereas the purified fractions showed broad-spectrum activity against multiple strains. This might be due to masking of antibacterial activity by the presence of some inhibitory compounds or factors in the extract as observed by Sastry and Rao [28]. The variation of antibacterial activity of our extracts might be due to distribution of antimicrobial substances, which varied from species to species as suggested by Lustigman and Brown [29]. Similar observations were made by Vlachos et al. [30] who found that fractionation of crude extracts tested enhanced their activity against both Gram negative as well as the resistant Gram positive pathogens. MIC values of the extracts are much higher than those of the positive control substance antibiotics. This is not surprisingly because extracts are complex mixtures of many compounds and the portion of active compounds is very low.

Majority of the plasmid curing agents reported earlier such as acridine dyes, ethidium bromide and sodium dodecyl sulphate are unsuitable for therapeutic application due to their toxicity or mutagenic nature [31-32]. Also each of the known curing agents is effective against only a limited number of plasmids in a limited number of hosts. Thus, there is a constant need of identifying novel curing agents that are more effective and non toxic. The present results have offered organic extracts of *Halimeda opuntia* and *Sarconema filiforme* as a new and safe plasmid curing agent. These finding resulted in the possibility of a new type of combination between antibiotics and potential drugs effective against plasmid encoded multiple antibiotic resistance. Identification of a novel curing agent derived from plant is significant, since majority of natural products are non toxic to human and environment.

Previous reports of plant derived curing agents are limited. Plumbagin, 5-hydroxy-2-methyl-1, 4-nepthoquinone isolated from *Plumbago zeylanica* cured R-plasmids in *E. coli* [33]. This was probably the first report in which herbal compound

had cured/ eliminated plasmid encoding antibiotic resistance. In another study [34], the alcoholic extract of *P. zeylanica* cured R plasmid harbouring *E. coli* with 14 per cent. Recently antiplasmid activity of essential oils was reported by Schelz *et al*. [35]. However, in the present investigation, we have shown that the fraction from these seaweeds could effectively eliminate R plasmids from reference strains as well as Gram-positive and Gram-negative strain of clinical isolates. The frequency of spontaneous loss for such plasmids has been known to be less than one in 10^6 cells. In comparison, the antibiotic resistance curing efficiencies observed in present study were extremely high (10^6 times higher). The antibiotic resistance may occur due to mutations. Mutagenic activity of the compound can be harmful especially in clinical applications. It is necessary to ensure that antibiotic resistance curing was due to loss of plasmid-encoded genes and not due to mutations, which was confirmed by the physical loss of plasmid observed in agarose gel electrophoresis. The concentrations of the curing agents used in this study were sub inhibitory, since bacteria were already resistant to these concentrations of compound. It can be assumed that bacteria are less likely to develop any mechanism to counter the plasmid curing property of the methanol extract of seeweeds.

Many of these secondary metabolites biosynthesized by the marine plants are well known for their cytotoxic property [36]. Of the two seaweed extracts examined for brine shrimp cytotoxicity, *Halimeda opuntia* was found effective and showed LC_{50} was 192.3 µg. The genera *Halimeda* is found to contain highly active but unstable sesquiterpenoids and diterpenoids. Some of these diterpenoids exhibited cytotoxic and antimicrobial activities [37]. Seaweeds exhibit a high level of fatty acid diversity and many of which possess potential bioactivity [38]. Several cytotoxic compound such as fucoidans, laminarians, and terpenoids stated to posses anticancer, antitumor and antiproliferative properties are reported to be abundant in seaweeds [39]. These compounds could be further explored as novel leads to cancer chemoprevention and complementary chemotherapy and necessitates further investigation [40].

Finally, before starting the use of seaweeds for therapy of microbial diseases *in vitro* and *in vivo* toxicity studies with seaweeds, fractions and purified compounds have to be done. Only dependent on these results a decision can be made which material would be the most suitable for a possible practical use. The results clearly show that seaweeds are an interesting source for biologically active compounds that may be applied for prophylaxis and therapy of bacterial and fungal diseases additionally or instead of commercial antibiotics. An alternative approach to the use of extracts, fractions or purified compounds from algae as drugs might be to employ. However, prior further investigations regarding toxicity, stability and metabolism of seaweeds and seaweed components must be undertaken.

V. Conclusion

In this study, the *in vitro* antimicrobial, antiplasmid and cytotoxic acitivites of two algal species were collected from Red Sea Coast of Egypt. Differences among the results of the activities of extracts were observed with the studies which were carried out from other countries of the world. However, in this study, without using any seperation and fractionation steps, strong cell inhibition (90%) was obtained by the crude extracts were also found as having reasonable cytotoxic activities against the assessed brine shrimp. Therefore, these two alga species were found as prominent for further antimicrobial, antiplasmid and cytotoxic activity studies.

Compounds responsible for antimicrobial activities are widespread in algae as shown by previous studies. Inhibition zones for antimicrobial activities obtained by the same extracts of algae samples were in the range of moderate to strong. Differences observed by the other studies are contributed by several factors, mainly infraspecific variabilities in the production of secondary metabolites. In the next studies, collecting these studied species in different seasons and using different extraction methods, seperation steps, and their cytotoxic and antimicrobial activities should be monitored.

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