Antibacterial, cytotoxicity and anticoagulant activities from *Hypnea esperi* and *Caulerpa prolifera* marine algae

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Abstract: Extracts from 2 algal species (*Hypnea esperi* and *Caulerpa prolifera*) from Suez Canal region, Egypt were screened for the production of antibacterial compounds against some pathogenic bacteria. The bacteria tested included *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Aeromonas hydrophila, Bacillus subtilis* and *Staphylococcus aureus*. Algal species displayed antibacterial activity. The methanolic extracts showed variable response by producing various zones of inhibition against studied bacteria. The tested Gram-negative bacteria were less affected by studied algal extracts than Gram-positive bacteria. We determined some biopotentials properties such as cytotoxicity and anticoagulant activity of most potent algal active extracts. The secondary metabolites of only *Hypnea esperi* algal extract effectively prevented the blood clotting to the extent of 120 seconds. Minimum inhibitory concentration (MIC) indicated that all potent tested algal extract C inhibits *Bacillus subtilis* and *Staphylococcus aureus*. Minimum bactericidal concentration (MBC) was between 1 and 1.4mg/ml. The algal isolates from Egypt have been found showing promising results against infectious bacteria instead of some synthetic antibiotics.

Keywords: Antibacterial, cytotoxicity, anticoagulant, MIC/MBC, *Hypnea esperi*, *Caulerpa prolifera.*

INTRODUCTION

Microorganisms like bacteria and fungi have been exploited for almost a century to provide useful drugs, antibiotics and other pharmacologically effective metabolites (Woodruff, 1980). Algae are able to produce a wide variety of pharmacologically active compounds (Ostensivk *et al*., 1998; Pushparaj *et al*., 2014). Discovering new therapeutic compounds is becoming increasingly important as more and more because bacteria become resistant to the usual antibiotics. During second half of the 20th century, algae were screened for their biological activities. Thus, antibacterial effects have been noticed in most of algal classes (Pesando, 1990; Naviner *et al*., 1999; Gonzalo *et al*., 2001; Lima-Filho *et al*., 2002; Schallenberg and Armstrong, 2004; Jung *et al*., 2013). However, most of these antibacterial compound actions have been tested against bacterial human pathogens. The active molecules in algae were rarely purified. More than 150.000 algal species are found in marine and fresh water resource at world but only a few of them have been screened for biological activity (Lima-Filho *et al*., 2002). Primary and secondary metabolites from these organisms may prove to be potential bioactive compounds of interest for the pharmacological industry. Special attention has been reported as active agent against viruses, bacteria and fungi. As an efficient strategy for investigation, organic solvents have been used to extract the possible lipid soluble active principles from algae (Naviner *et al*., 1999; Gonzalo *et al*., 2001; Lima-Filho *et al*., 2002; Selim, 2012). Since Egypt has an extensive coast where algae helping to virtually all groups are present. The goals of the present work include.

Screening *Caulerpa prolifera, Hypnea esperi* marine algae (from Suez Canal region, Egypt) for antibacterial activity.

Selection of potent algal extracts and their purification.
Comparison between the most potent purification active secondary metabolites and antibiotics used for the treatment of bacterial infections.

Determination of some biopotential properties such as cytotoxicity and anticoagulant activity.
Investigation of the antibacterial activities of the potent active algal secondary metabolites against the pathogenic bacteria by following minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and tolerance ratio approaches.

MATERIALS AND METHODS

Algal collection and identification

Samples of marine algae were collected from Suez Canal region, then brought to laboratory in plastic bags containing seawater to prevent evaporation and then washed with water to separate potential contaminants. Marine algae were identified as by Harvey (1988) and found belonging to two families: Rhodophyceae (*Hypnea esperi*) and Chlorophyceae (*Caulerpa prolifera*).

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Methanol extraction

All samples were dried in oven at 40°C then grinded in porcelain mortar. The powder was submitted to lipid-soluble extraction with methanol (50%) using a Soxhlet extractor at 40°C. Samples were then refluxed until saturation (24h) and the respective extracts were dried in an oven at 40°C. Subsequently, the residual extracts were suspended in the respective solvents to a final concentration of 1mg extract /20µl methanol (algal lipid-soluble extract) (Lima-Filho *et al*., 2002; Selim *et al.*, 2013).

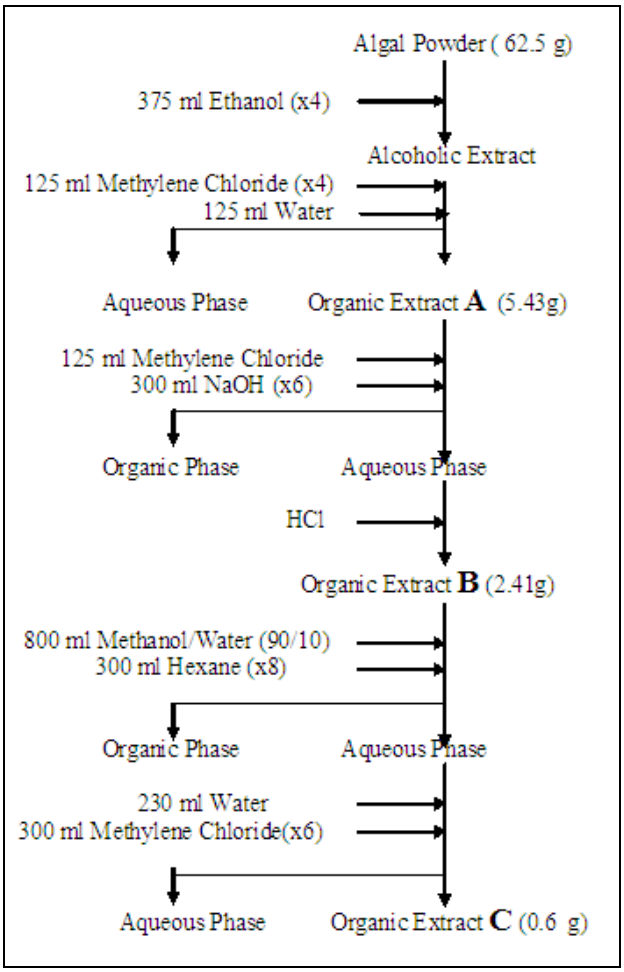

Fig. 1: Process of purification of antibacterial compounds of potent algal extracts (Naviner *et al*., 1999).

Screening for antibacterial activity

Antibacterial activities of the extracts of marine algae were tested against pathogenic Gram-negative strains of *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* isolated by Selim (2011a), *Salmonella typhimurium* (ATCC 19430), and *Aeromonas hydrophila* isolated by Abdel Aziz *et al*. (2004) and Gram-positive strains of *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* isolated by Selim (2011a,b). Single disk method as described by Bauer *et al*. (1966) was used. Bacteria were grown (10^8 cells/ml) in nutrient broth incubated at 37°C for 24h and plated into Petri dishes containing Müeller-Hinton agar (Oxoid Products, UK). Sterile discs of 5 mm diameter were soaked in 20µl of the algal lipid-soluble extracts then placed onto the different bacterial cultures. Antibacterial activity of the algal extracts against the tested bacteria was determined after 24 h by measuring the diameter of the zone of inhibition (mm) around the discs. All experiments were performed in duplicate. Discs without algal extracts soaked in methanol (50%) were used as negative control and, in this case, no antibacterial activity was observed.

Preparation and purification of algal extract

Potent extracts with large inhibition zone were selected for further studies. The powder from the most potent algae were dissolved in ethanol. The methanolic extract was centrifuged at 4300g for 15 min to remove cellular materials. The pellet was re-extracted three and purified according Naviner *et al*., 1999 (fig. 1). For each step, fractions A, B and C were tested against the test bacteria. The antibacterial activity of fraction C was compared to six (Referenced) standard Oxoid antibiotics discs (Tetracycline 10µg, chloramphenicol 30µg, nitrofurantoin 300µg, streptomycin 10µg, vancomycin 30µg and rifampicin 5µg).

Cytotoxicity and anticoagulant activity

Brime shrimp cytotoxicity assay was performed to potent algal extract C with antimicrobial activity of *Hypnea esperi* and *Caulerpa prolifera*. Use the fresh hated free-swimming nauplii of *Artemia salina*. The assay system was prepared with 20 ml of filtered seawater containing concentrations (1, 2, 4 and 6 mg/ml) of extracts C in cavity blocks (Embryo Cup) and 2ml nauplii solution (n=10) each was transferred in experimental, vehicle control and negative control wells. Invariably the concentration of the experimental systems was determined on the bases of exploratory experiments. Based on the percent mortality at 20 and 30°C, the LD_{50} of the tested compound was determined using Rafter cell (Selvin *et al*., 2004). The anticoagulant activity was studied using whole blood clotting time method for potent extracts with anti-microbial activity (Selvin and Lipton, 2004).

Determination of MIC, MBC and tolerance ratio

MIC was defined as the lowest concentration of an antibiotic that inhibited the development of visible bacterial growth in broth (Murray *et al*., 1995). MIC was determined by the broth dilution method. Serial dilutions from 0.1mg to 1mg of potent algal extract C was inoculated into Müller-Hinton broth. Bacterial suspension of 1ml containing approximately 10^5: 5×10^5 cells of *Bacillus subtilis* and *Staphylococcus aureus* were added to each dilution of extract C. Growth of bacteria was checked after overnight incubation at 37°C. MBC defined as the lowest concentration of antibiotic that kills 99.9%

Table 1: The antibacterial activities of algal species on different test bacterial species

Division	Species	Test Microorganisms					
		EC	PA	ST	AH	BS	SA
Rhodophyceae	*Hypnea esperi*	+	-	-	-	+++	+++
Chlorophyceae	*Caulerpa prolifera*	-	++	-	-	+++	+++

The activity is categorized according to the diameter of the inhibition zone around the disc saturated in the sample (+++ ≥ 15mm, ++ < 15mm, + hazy/very hazy inhibition zone, - no activity). Bacterial strains: EC=*Escherichia coli*, PA: *Pseudomonas aeruginosa*, ST: *Salmonella typhimurium,* AH: *Aeromonas hydrophila*, BS: *Bacillus subtilis* and SA: *Staphylococcus aureus.*

Table 2: Inhibition of bacterial growth by 1 mg of algal extract C compared to standard antibiotics.

Algal species and standard antibiotics	EC	PA	ST	AH	BS	SA
Hypnea esperi	+	-	-	-	+++	+++
Caulerpa prolifera	-	++	-	-	+++	+++
Tetracycline 10µg	+++	++	++	++	++	+
Chloramphenicol 30µg	+++	+++	+++	+++	+++	+++
Nitrofurantoin 300µg	+++	+++	+++	-	++	-
Streptomycin 10µg	-	++	-	-	++	-
Vancomycin 30µg	-	++	-	-	++	-
Rifampicin 5µg	+	-	-	+	-	-

Activity is categorized according to the diameter of the inhibition zone around the disc saturated in the sample (+++ ≥ 15mm, ++ <15mm, + hazy/very hazy inhibition zone, - no activity). Bacterial strains: EC= *Escherichia coli*, PA: *Pseudomonas aeruginosa*, ST: *Salmonella typhimurium,* AH: *Aeromonas hydrophila*, BS: *Bacillus subtilis* and SA: *Staphylococcus aureus.*

Table 3: *Artemia* cytotoxicity profile of algal extracts C at 20 and 30^{o}C

Algal Extract C	Concentration (mg/ml)	Mortality (%)	
		20^{o}C	30^{o}C
Hypnea esperi	1	0	10
	2	0	50
	4	20	70
	6	60	95
Caulerpa prolifera	1	0	20
	2	0	60
	4	20	90
	6	40	100

Table 4: MIC, MBC and tolerance ratio of potent algal extracts C on *Bacillus subtilis* and *Staphylococcus aureus*

Bacterial species	Algal extract C	MIC (mg/mL)	MBC (mg/mL)	Tolerance ratio (MBC / MIC)
Bacillus subtilis	*Hypnea esperi*	0.3	0.4	1.3
	Caulerpa prolifera	0.5	0.5	1
Staphylococcus aureus	*Hypnea esperi*	0.5	0.7	1.4
	Caulerpa prolifera	0.6	0.6	1

of the organism. MBC is usually an extension of the MIC, where the organisms are quantitatively subcultured from MIC tubes on antibiotic-free agar medium to indicate the minimum concentration where no viable organism was present in the culture (Collins *et al*. 1998). Degree of tolerance was calculated from MBC/MIC readings (Stokes *et al*. 1993).

RESULTS

Antibacterial activity

Algal Species were screened to potentiality of antibacterial activities (table 1). The tested Gram-negative bacteria were less affected by studied algal extracts than Gram-positive bacteria. The most susceptible bacteria

included *Bacillus subtilis* and *Staphylococcus aureus* while other species responded variably. Except the extract of *Caulerpa prolifer* all potent algal extracts had affect on *E. coli*. The extract C of marine algae was more effective on Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). While extract C showed variable activity on Gram-negative bacteria (table 1). Except chloramphenicol (antibiotic), extract C of all the potent algae was found more effective on *Staphylococcus aureus* than the standard antibiotics used for the treatment of bacterial infections (table 2). In the present work, only the lipophilic phase showed an antibacterial activity while the aqueous extract had no effect.

Cytotoxicity and anticoagulant activity

The secondary metabolites of (only) *Hypnea esperi* algal extract effectively prevented the blood clotting to the extent of 120 seconds (i.e. as an anticoagulant agent) while the control blood clotted within 40 seconds. The secondary metabolites of other extracts did not significantly influence the clotting time. Temperature had a significant influence on the toxicity of the algal extract. The toxicity profile of *Hypnea esperi* and *Caulerpa prolifera* algal extracts considerably increased by a raise in temperature from 20^{o}C to 30^{o}C (table 3). The LD_{50} value of extracts C of *Hypnea esperi* and *Caulerpa prolifera* were recorded 2mg/ml concentration at 30^{o}C and other algal extracts did not show any cytotoxic activities (table 3).

MIC, MBC and tolerance ratio

The MIC and MBC (mg/ml) of the most potent algal extract C against various bacterial spp. Are shown in table 4. MIC indicated that all potent tested algal extract C inhibited *Bacillus subtilis* at concentration above 0.3 mg/ml and in case of *Staphylococcus aureus*, it was found to be 0.5mg/ml. MBC was equal in pattern to MIC in some cases while more in other cases (table 4), (It was between 1 and 1.4 mg/ml).

DISCUSSION

Lipid-soluble extracts from algae have been investigated as a source of substances with pharmacological properties (Lima-Filho *et al*., 2002). Moreover, several different organic solvents have been used for screening algae for antibacterial activity. Olessen *et al*., 1963 recorded antibacterial activity in the organic extracts of some marine algae against *S. aureus*. An increasing inhibitory effect is clearly shown out during this study, extract C appeared to be the most active. *Naviner et al.,* 1999 reported that the aqueous extract of the organic extract (A) by NaOH is equivalent to a saponification as described in lipidic extraction procedures. The second step of purification allows us to discard the non-polar compounds. Thus, the final extract C contains lipophilic compounds moderately polar and saponifiable. However, it is not certain whether or not all these fatty acids, individually or in combination, inhibit the growth of bacteria. Further investigations will permit to determine if it is bacteriostatic or bactericidal effect. Bactericidal activity of unsaturated and saturated long chain fatty acids have been reported by Galbraith and Miller (1973c). They showed that fatty acids of chain length more than 10 carbon atoms induced lysis of bacterial protoplasts. Thus, many authors have found antibacterial activities of microalgae due to fatty acids (Kellam *et al*., 1988; Lima-Filho *et al*., 2002, Ermakova *et al*., 2013).

A lipophilic antibacterial substance named chlorellin produced by *Chlorella vulgaris* was reported to be an auto inhibitor of the bacteria when it was excreted in the culture medium (Pratt, 1948). Other auto inhibitor fatty acid named hydroxyeico sapentaenoic acid has been identified from *Skeletonema costatum* (Imada *et al*., 1992). The results appear play a significant role for algae in the control of infectious diseases and particularly Gram-positive infection. Sastry and Rao (1994) showed antibacterial activity of marine algae against Gram-positive and Gram-negative pathogenic strains after successive extraction with organic solvents. Likewise, Marasneh *et al*., (1995) showed antibiotic activity in organic extracts of six species of marine algae against multi-antibiotic resistant bacteria. These finding were in agreement of the present results. Rossel *et al*., (1987) associated antibiotic activity from 10 marine algae to the presence of unsaturated fatty acids, organic acids and phenol compounds. Selvin and Lipton (2004) reported that *Hypnea musciformis* is an excellent source of anticoagulant, and these finding are in agreement to the present results. Based on the Brime shrimp lethality potential, the medium lethal dose (1.8mg/ml) (Selvin and Lipton, 2004). In the present study, lethality potential value of both *Hypnea esperi* and *Caulerpa prolifera* extracts was calculate to be 2mg/ml. No concentration was tested between 1 and 2mg/ml.

However, further assays need to be done to study the cytotoxic effect of these algae. Concerning *in vivo* experimentation, it would be interesting to test the algae as a food supplement for prevention or therapy of bacterial human diseases (Lima-Filho *et al*., 2002; Katy *et al*., 2014). The potential of these extracts as antimicrobial, antifungal, anti-tuberculosis and anticancer agents needed to study. Using algal extracts as anti-microbial agents is preferred, as they are natural, inexpensive and fulfill the roles of more than one synthetic agent. The studied algae showed good potential sources of bioactive compounds and natural antibacterial agents. Therefore, the potent active extraction of the studied algae, isolated from Egyptian water, recommended to use as antibacterial agents against Gram positive bacteria instead of synthetic antibiotics.

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