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Studies on the Bioactivity of Different Solvents Extracts of Selected Marine Macroalgae Against Fish Pathogens

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Abstract: Selected species of marine benthic algae belonging to the Phaeophyceae and Rhodophyceae, collected from different coastal areas of Alexandria (Egypt), were investigated for their antibacterial and antifungal, activities against fish pathogens. *In vitro* screening of organic solvents extracts from the marine macroalgae, *Laurencia pinnatifida* (Hudson) Stackhouse, *Pterocladia capillaceae* (Gmelin), *Halopteris scoparia* (Linnaeus) Kützing, *Stepopodium zonale* (J.V. Lamouroux) and *Sargassum hystrix* var. *fluitans* Børgesen, showed specific activity in inhibiting the growth of five virulent strains of bacteria pathogenic to fish *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *V. tandara*, *Escherichia coli* and of two fungi *Aspergillus flavus* and *A. niger*. Acetone and ethanol extracts of all test macroalgae exhibited antibacterial activity, while acetone extract of *S. hystrix* exhibited the highest antifungal activity. Macroalgal extracts inhibited bacteria more readily than fungi, besides, the extracts of the Rhodophyceae species showed the greatest activity against current test bacteria rather than fungi. Cluster analysis revealed the general response of the tested pathogens to the action of the different algal extracts. Composition of the most potent algal extracts included acetone extracts of *L. pinnatifida*, *P. capillaceae* and *S. hystrix* and ethanol extract of *P. capillaceae* was determined using GC-MS. The present study provides the potential of red and brown macroalgae extracts for the development of anti-pathogenic agents for use in fish aquaculture.

Key words: Macroalgae, fish pathogens, extracts, antibacterial, antifungal, linkage

INTRODUCTION

Marine algae have proven to be rich source of bioactive compounds with biomedical potential (Fitton, 2006), not only for human but also for veterinary medicine. Competition for space and nutrients led to the evolution of antimicrobial defense strategies in the aquatic environment. The interest in marine organisms as a potential and promising source of pharmaceutical agents has increased in the last years. Many bioactive and pharmacologically active substances have been isolated from microalgae (Cooper *et al*., 1983; Findlay and Patil, 1984; Viso *et al*., 1987; Kellam *et al*., 1988) and mangroves (Premnathan *et al*., 1992, 1996; Changyi *et al*., 1997). Compounds with antibacterial, antifungal and antiviral activities have been detected in green, brown and red algae too (Lindequist and Schweder, 2001; Mayer and Hamann, 2002; Newman *et al*., 2003). Algal bioactive substances have been, extensively studied by Burkholder *et al*. (1960), Ehresmann *et al*. (1977), Moreau *et al*. (1984), Reichelt and Borowitzka (1984), Hornsey and Hide (1985), Vlachos *et al*. (1999), González *et al*. (2001), Nora *et al*. (2003), Ghosh *et al*. (2004), Freile-Pelegrin and Morales (2004) and Salvador *et al*. (2007). Fishes are susceptible to a wide variety of bacterial pathogens (Schmidt *et al*., 2000). Bacterial diseases are responsible for heavy mortality in wild and cultured fish. Therefore,

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numerous investigations concerning the inhibiting activities of macroalgae against fish pathogens were reported by Sridhar and Vidyavathi (1991), Mahasneh *et al.* (1995) and Liao *et al.*, (2003). *Pseudomonas fluorescens* and *Aeromonas hydrophila* have been associated with septicemia and ulcerative condition in a wide range of fish species (Cipriano, 2001). *Pseudomonas septicemia* is a hemorrhagic condition of fish usually associated with stress or improper health management (Saharia and Prasad, 2001). The causative agent of vibriosis is the genus *Vibrio*, which can cause significant mortality (=> 50%) in fish culture. *Vibrio anguillarum*, causes disease in many fish such as the salmon, cod, char, halibut, Japanese eel, rainbow trout, as well as shellfish such as the shrimp (DiSalvo *et al.*, 1978; Ellis, 1989; Wiik *et al.*, 1989; Inglis *et al.*, 1993; Blanch *et al.*, 1997; Benediktsdóttir *et al.*, 1998; Actis *et al.*, 1999; Eguchi *et al.*, 2000; Kent and Poppe, 2002). *Esherichia coli* are a common contaminant of seafood in the tropics and are often encountered in high numbers. Fish probably acquire *E. coli* when they eat food contaminated with feces (Gomez *et al.*, 2008). The benthic fish contains *E. coli* inside their intestines (Rio-Rodriguez *et al.*, 1997). Disease germs like *E. coli* can cause serious, life threatening illness within hours or days. Shellfish such as shrimp, crab, clams and oysters can contain life-threatening bacteria and viruses. *Aspergillus niger* and *A. flavus* were isolated from naturally diseased fish (Abdelhamid, 2007). *Aspergillus flavus* is a fungus associated with the Nile *Tilapia* fish. It is known to produce aflatoxins, a group of acutely toxic and potentially carcinogenic.

The objective of the present study was to elucidate the possible use of different solvents extracts from five-selected macroalgae, for inhibition of some fish pathogens. Acetone, ethanol, methanol and hexane extracts of the three brown macroalgae *S. zonale* (J.V. Lamouroux), *H. scoparia* (Linnaeus) Kützing and *S. hystrix* var. *fluitans* Børgesen and of the two red macroalgae, *L. pinnatifida* (Hudson) Stackhouse and *P. capillaceae* (Gmelin), were screened for their inhibitory activities against the selected fish pathogens. The five pathogenic bacterial strains, *P. fluorescence*, *A. hydrophila*, *V. anguillarum*, *V. tandara*, *E. coli* and the two fungi *A. flavus* and *A. niger* were the test fish pathogens. Cluster analysis was used to show the close linkages between the selected macroalgal species and the response of fish pathogens to the action of the different algal extracts. Compositions of the most potent algal extracts were analyzed, for their chemical constituents, using gas chromatography-mass spectrometry.

MATERIALS AND METHODS

Algal Materials and Preparation of the Extracts

Macroalgae samples were collected during low tide from the Alexandria Mediterranean coast during summer 2008. Ecological damage during harvesting was prevented. All samples were brought to laboratory in plastic bags containing seawater, to prevent evaporation. Then algae were washed with distilled water to separate potential contaminants and epiphytes. Macroalgae were identified. Three brown algae; *S. zonale* (J.V. Lamouroux), *H. scoparia* (Linnaeus) Kützing and *S. hystrix* var. *fluitans* Børgesen and two red macroalgae; *L. pinnatifida* (Hudson) Stackhouse and *P. capillaceae* (Gmelin) were taken for experimental use. Samples were air dried under shade at room temperature. The dry algae were ground, using electric mixer grinder. Known weight of each dried alga (2-3 g) was homogenized in a manual porcelain mortar with different solvents. Then, they were soaked in 10 mL of each of the solvents acetone, ethanol, methanol and hexane. All samples were kept in screw capped glass vials and left in darkness for one week. The extracts with different solvents were centrifuged (at 4°C, 10,000 rpm, 10 min). The supernatants of each treatment were separated and filtered, using cheesecloth and refiltered through a Whatman filter No. 4. The filtrate was concentrated using a rotary evaporator and stored at 4°C in a sterile tube until use for the bioassay experiments.

Antimicrobial Bioassay Tests

Bacterial bioassays comprise different test bacteria, *P. florescence*, *V. anguillarum*, *V. tandara*, *E. coli*, *A. hydrophila* and the fungi *A. flavus* and *A. niger*.

Antimicrobial activity was evaluated using well-cut diffusion technique (El-Masry *et al*., 2000). Wells were punched out using a sterile 0.7 cm cork borer in nutrient agar plates inoculated with the test microorganism and each of the well bottoms was sealed with two drops of sterile water agar. About 100 µL of algal extract were transferred into each well.

Wells loaded with the extracting solvents were used as controls. All plates were incubated at 30°C for 24 h. The inhibition zone was determined for each well and expressed in millimeter.

Chemical Analysis of the Algal Extracts

The gas liquid chromatography coupled with mass spectrometry detection technique allows good qualitative and quantitative analysis of the fractionated extracts with high sensitivity to the smaller amounts of components. Accordingly, identification of the chemical constituents of fractionated extracts, for the selected macroalgae, which showed effective antibacterial and antifungal activities against the test bacteria and fungi, were analyzed. This was by using (Hewlett Packard) HB 5890 gas liquid chromatography (GLC) coupled with 5989 B series mass spectrometer (MS) at, Central Lab Unit in the High Institute of Public Health, Alexandria University, Egypt.

The gas liquid chromatography was equipped with a split less injector at 240°C and a flame ionization detector (FID) held at 300°C using helium as a carrier gas. Samples were separated on a capillary column Hp-5 (Avondale, PA, USA) (30 m long and 0.25 mm internal diameter) of 0.25 µm film thickness, (5% diphenyl, 95% dimethyl polysiloxane). The temperature of the gas chromatograph column was initiated from 80°C and then increased to a maximum final temperature of 300°C at a heating rate of 40°C min^{-1}, holding the maximum final temperature for residence time of 4 min. The temperature of the ion source in the mass spectrometer was held at 300°C. All mass spectra were recorded in the electron impact ionization (EI) at 70 electron volts. The mass spectrometer was scanned from m/z 40 to 410 at a rate of two scans per second. An integrator automatically calculated peaks areas.

Neither internal nor external chemical standards were used in this chromatographic analysis. Interpretation of the resultant mass spectra were made using a computerized library-searching program (Database/Wiley 275.1) and by studying the fragmentation pattern of such compound resulted from mass spectrometry analysis. Concentration of such compound was calculated by the following formula:

$$\text{Compound concentration percentage} = [P1/P2] \times 100$$

where, P1 is the peak area of the compound and P2 is whole peak areas in the fractionated extracts.

Statistical Analysis

Statistical analysis were performed by *STATS*® for windows Version 5, 1985-1995, using the cluster analysis for data upon complete linkage level, to clarify the relations between data. The term cluster analysis actually encompasses a number of different classification algorithms.

RESULTS

Antimicrobial activities of crude extracts of five marine algal species represented by three Phaeophyceae (*S. zonale, H. scoparia* and *S. hystrix)* and two Rhodophyceae (*L. pinnatifida* and *P. capillaceae*), were examined against seven test microorganisms (*P. florescence, V. anguillarum, V. tandara, A. hydrophila, E. coli, A. flavus* and *A. niger*). As shown in Fig. 1 acetone extracts of all algal species showed inhibitory activities against *P. florescence* with inhibition zone diameters ranging from 16 to 20 mm. The extracts of *L. pinnatifida*, *H. scoparia* and

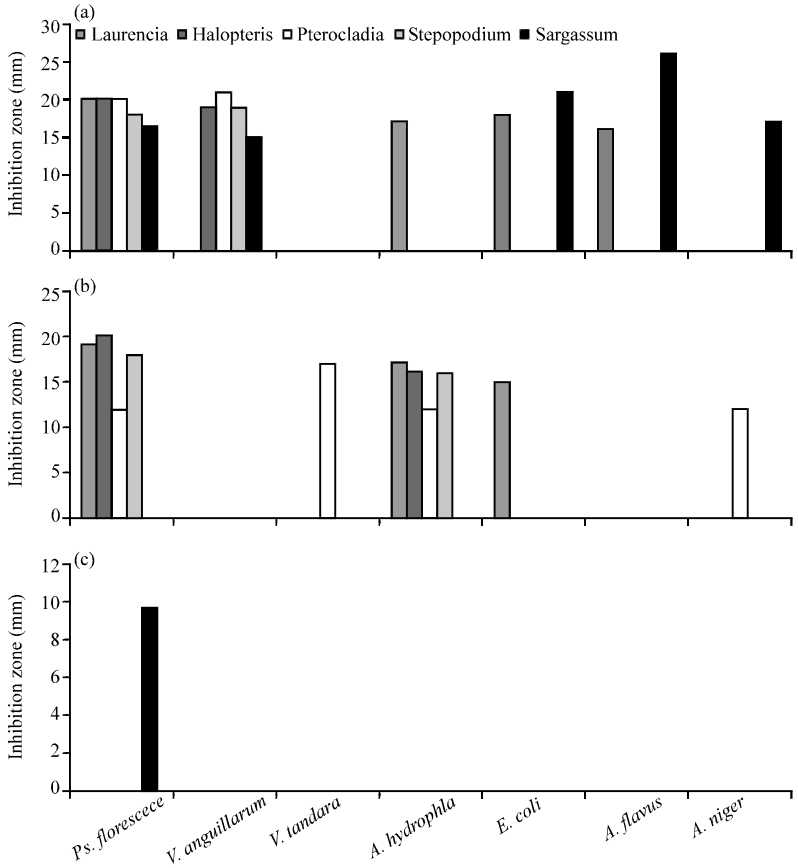

Fig. 1: Inhibition zones of different macroalgal solvents extracts. (a) Acetone; (b) Ethanol and (c) Methanol

P. capillaceae were the most effective against such fish pathogen. *E. coli* was resistant to all algal extracts except the acetone extract of *L. pinnatifida* and *S. hystrix* with halo diameter of 18 and 21 mm, respectively. On the other hand only acetone extracts of *L. pinnatifida*, *H. scoparia* showed negative effect against *V. anguillarum*.

Ethanol extracts of all test algal species except *S. hystrix* showed inhibitory activities against *P. florescence* and *A. hydrophila*, while *E. coli* and *V. tandara* were sensitive only to ethanol extracts of *P. capillaceae* and *L. pinnatifida*.

Methanol extracts of *S. hystrix* showed the lowest inhibitory effect (10 mm) only against *P. florescence*. On the other hand, all bacterial species were resistant to hexane extracts of all algal species.

Antifungal activities of algal extracts were observed for acetone extracts of *L. pinnatifida* and *S. hystrix* with inhibition zone diameter of 16 and 26 mm, respectively. Therefore, these two species showed relatively broad spectrum of activity against the tested pathogens. In addition, anti-*Aspergillus flavus* was detected using ethanol extract of *P. capillaceae* (12 mm), while *A. niger* was inhibited only by acetone extract of *S. hystrix* (17 mm).

The dendrogram (Fig. 2a) showing the cluster analysis, revealed the response of the tested pathogens to the action of the different algal extracts. The analysis differentiated the response into three groups. The first group comprised *A. niger* and *A. flavus* which represented the most closely

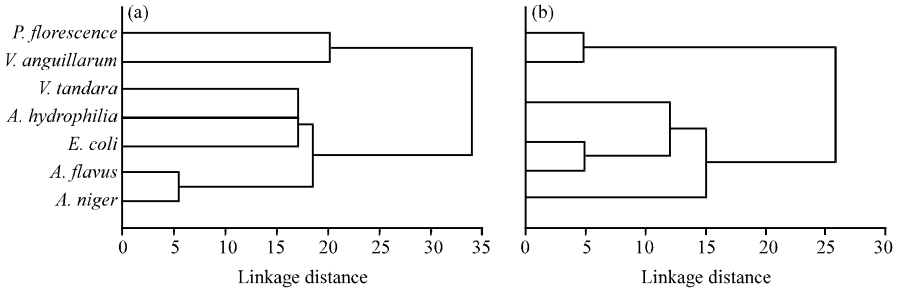

Fig. 2: Dendrogram showing the response of some fish pathogens to different macroalgal extracts. (a) Acetone extracts and (b) Ethanol extracts potency single linkage euelidean distance

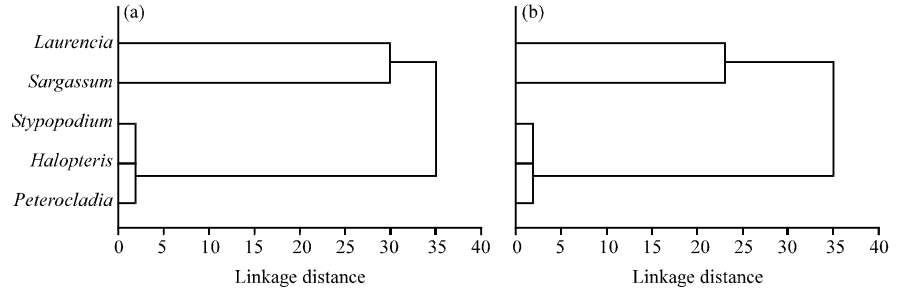

Fig. 3: Cluster analysis showing the close linkage between the different macroalgal extracts using different solvents. Where: a and b are acetone and ethanol extracts, respectively

related pathogens. The close relationships was followed by the second group (*E. coli*, *A. hydrophila* and *V. tandara*) while the third one, including *P. florescence* and *V. anguillarum*, showed the least response to the action of the algal extracts. Extraction using ethanol showed different results (Fig. 2b), where *P. florescence* and *V. anguillarum* were better in response to the action of algal extracts compared with that when using acetone. The same trend was exhibited for *E. coli*. *Aeromonas hydrophila* and *V. tandara* were affected similarly by the algal components extracted by ethanol. On the other hand, *A. niger* showed separate cluster at linkage distance (15).

The design of action of the different macroalgal species was studied also using the cluster analysis as shown in Fig. 3a, b. The antimicrobial action produced by *S. zonale, H. scoparia* and *P. capillacea* was closely linked, followed by *L. pinnatifida and S. hystrix*. The same pattern was sustained using ethanol (Fig. 3b)

Chemical Analysis of the Most Potent Algal Extracts

Chemical composition of the most potent algal extracts included acetone extracts of *L. pinnatifida, P. capillaceae* and *S. hystrix* and ethanol extract of *P. capillaceae* was determined using GC-MS. The chemical constituents in the crude extracts, retention time, chemical formula, molecular weight and the peak area or yield of each component are also shown in Table 1.

Results indicated that, the main common component in the acetone extracts of *L. pinnatifida* and *P. capillacea* is 4-hydroxy-4-methyl-2-pentanone representing 64.38 and 58.60%, respectively and with molecular weights 116.08 and 207 g, respectively.

Table 1: Chemical composition of the selected algal extracts using GC-MS

Components	Retention time	Formula	MW	Area (%)
Acetone extract of *L. pinnatifida*				
4-hydroxy-4-methyl-2-pentanone	2.32	$C_6H_{12}O_2$	116.08	64.38
N,N'-Bis(4-diethylamino-2 ethoxybenzylideneamino) guanidine	7.43	$C_{28}H_{45}N_{70}$	495.37	0.71
6,7-Benzo-phenothiazine-5,5-dioxide	8.26	$C_{16}H_{11}NO_2S$	281.05	8.84
4'methyl-2-phenylindole	8.49	$C_{15}H_{13}N$	207.11	13.16
3,3,4-trimethyl-2,2-diphenoxythietane	8.75	$C_{18}H_{20}O_2S$	300.12	4.33
Tetradecanoic acid, (3,3a,4,6a,7.8)	8.96	$C_{31}H_{48}O_6$	516.35	3.54
Acetone extract of *S. hystrix*				
Chaenorin	5.38	$C_{31}H_{40}N_4O_5$	548.30	12.40
20.xi.-lanosta-7,9(11)-diene-3.beta., 18,20-triol	6.26	$C_{30}H_{50}O_3$	458.38	14.89
Hexadecanoic acid	6.97	$C_{16}H_{32}O_2$	256.24	23.10
1,3-benzenedicarboxylic acid	7.49	$C_8H_6O_4$	166.03	27.46
N-methyl-2-iodo-pyrrole	8.27	C_5H_6IN	206.96	11.71
Benzene, 1,4-bis (trimethylsilyl)	8.54	$C_{12}H_{22}Si_2$	222.13	10.44
Acetone extract of *P. capillaceae*				
4-hydroxy-4-methyl-2-pentanone	2.39	$C_6H_{12}O_2$	116.08	58.60
2,3-dimethyl-5-hexan-3-ol	3.56	$C_8H_{16}O$	28.12	6.86
Heptadecane	6.27	$C_{17}H_{36}$	240.28	6.53
Ethanol extract of *P. capillaceae*				
1,3,6,9b-tetraazaphenalene-4-carbonitrile,7, 9-dibromo-2-(dibromomethyl)	4.52	$C_{11}H_3Br_4N_5$	520.71	10.12
1,3,5,7,9,11-hexavinyl-3,5,9,11	6.27	$C_{28}H_{54}O_{11}Si_6$	734.23	6.28
Hexadecanoic acid	6.87	$C_{16}H_{32}O_2$	256.24	6.17
Tris (trimrthylsilyl)-8,2'-thioanhydroadenosine	6.99	$C_{19}H_{35}N5O_3SSi_3$	497.18	7.84
Corydaldine	7.68	$C_{11}H_{13}NO_3$	207.09	13.01
1,3-dimethyl-4-azaphenanthrene	8.15	$C_{15}H_{13}N$	207.11	15.69
N-cyano-N', N', N'',N''-tetramethyl	8.85	$C_8H_{13}N_7$	207.12	17.02
1,1,1,3,5,5,5-heptamethyltrisiloxane	11.06	$C_7H_{22}O_2Si_3$	222.09	23.87

DISCUSSION

Emergence of microbial disease in aquaculture industries implies serious loses. Usage of commercial antibiotics for fish disease treatment produces undesirable side effects. Marine organisms are a rich source of structurally novel biologically active metabolites (Borowitzka and Borowitzka, 1992). Cell extracts and active constituents of various algae may be potential bioactive compounds of interest in the pharmaceutical industry (Rodrigues *et al*., 2004).

In the current research, acetone was the best solvent for extracting the bioactive compounds, meanwhile it gave the highest antimicrobial activity against the selected pathogens. This was in contrast with those investigated by Tüney *et al*. (2006).

Ethanol extracts ranked the second order sustaining high inhibition zone diameters, but ethanol extract of S. *hystri*x didn't show any sign of antimicrobial activity against the selected pathogens. On the other hand, methanol extracts of all test macroalgae didn't show any activities against the microbial pathogens, except *S. hystri*x, which showed activity against *P. florescence* . This could be probably due to the difference in the solubility of bioactive metabolites in the corresponding solvents. Nevertheless, acetone followed by ethanol extracts of most test algae showed high antimicrobial activities. However, Das *et al*. (2005) examined acetone, ethanol and methanol extracts of other algae and showed moderate to high activity against strains of virulent pathogens *P. florescence*, *A. hydrophila*, *V. anguillarum* and *E. coli*.

Hexane extracts of all algal species had no antimicrobial activity. These results could be related to solubility of the bioactive compounds in acetone, ethanol and even methanol rather than hexane. The second explanation may be due to lysis of the algal cells such as *Sargassum* sp., *Pterocladia* sp. and *Laurenchia* sp. by the organic solvents, which lead to release of membrane-bound vesicles that contain the active metabolites. Such condition might have occurred for all solvents used in the present research, with the exception of hexane as confirmed by De Nys *et al*. (1998).

Screening for antimicrobial activity of the selected algal species showed that the extracts derived from the red macroalgae were more efficient than those obtained from the brown macroalgae in combating bacterial pathogens as reported by Salvador *et al.* (2007). The most preferred species overall was *Laurencia pinnatifida*.

Cluster analysis showed that *A. flavus* and *A. niger* were the most affected pathogens by the acetone extract, followed by *A. flavus* and *E. coli* using the ethanol extract of the different algal species. This could be probably due to that, they might have common defensive substances against the algal extracts.

High linkage was observed between the actions of *S. zonale*, *H. scoparia* and *P. capillaceae* for acetone extract and also for the ethanol extracts, compared with that of *L. pinnatifida* and *S. hystrix*. This might be attributed to that, *S. zonale*, *H. scoparia* and *P. capillaceae* were collected from Abu-Qir beach in Alexandria, which is polluted and subjected to greater exposure to pathogens or to grazers that led these algal species to develop strong chemical allelopathically active substances. However, Levin (1971) showed that the qualitative and quantitative distribution of allelochemicals varied and influenced by growth conditions and physiological state of the algae.

Composition of algal extracts depends on the environmental factors and on the specificity of the organism. Chemical constituents of the most promising algal extracts of *L. pinnatifid*a and *P. capillaceae* had a major and common component; 4-hydroxyl-4-methyl-2-pentanone which was the highest concentration compared with the other components. This compound was previously detected as a volatile oil fraction from Rhodophyceae and Phaeophyceae and had antimicrobial activity (De Rosa *et al*., 2003).

The major components in the acetone extract of *S. hystrix* were, 1, 3-benzenedicarboxylic acid and hexadecanoic acid, the latter compound was also detected in the ethanol extract of *P. capillaceae* with only (6.87%). Hexadecanoic acid was also detected using GC-MS by Hattab *et al*. (2007) and was reported to exhibit antimicrobial activities (Köse *et al*., 2007).

Generally, the common chemical compounds, with different percentage areas that were detected for species belonging to different groups, probably due to the difference in polarity of the used solvents and to habitat environmental factors.

CONCLUSIONS

The extracts derived from the red macroalgae were more efficient than those obtained from the brown macroalgae in combating bacterial pathogens rather than pathogenic fungi. The most preferred species over all was *L. pinnatifida*. On the other hand, acetone extract of the brown alga *S. hystrix* was the most effective over all selected macroalgae in inhibiting significantly, pathogenic test fungi.

The results clearly showed that macroalgae possess antibiotic activity against fish pathogenic bacteria and fungi and that they are a potential source for biologically active compounds. This opens new possibilities for prophylaxis and therapy of fish diseases. It is recommended that selected macroalgae should be used as a feed component for aquaculture in addition to, or instead of, commercial antibiotics. Macroalgae should also be investigated as a prolific resource for purified bioactive compounds, which could be useful as starting structures for drug development.

The potential high economic value of those marine algae could be assayed for aquaculture and for biomedical purposes through further researches and investigations.

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