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Evaluation of antibacterial properties of *Laurencia obtusa* and *Cystoseira barbata* against some bacterial fish pathogens

Adel S. Mekhaimar [1,2], Amel M. El Asely[1], Samar Negm[3], Adel A. Shaheen[1]

[1] *Department. of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Benha University*
[2]*National Services Project Organization, Armed forces, Egypt .*
[3] *Fish Biology and Ecology Department, Central Lab for Aquaculture Research Center, Giza, Egypt*

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ABSTRACT

The present study aimed to evaluate the antibacterial efficacy of the ethanolic extracts derived from two marine seaweeds, namely *Laurencia obtusa* (LOEE) and *Cystoseira barbata* (CBEE), using an agar disc diffusion test. The seaweeds were extracted using 95% ethanol and then analyzed using gas chromatography-mass spectrometry (GC-MS). A total of 97 and 86 compounds were found in LOEE, and CBEE, respectively. The predominant metabolites recorded in both seaweeds were palmitic acid, palmitic acid ethyl ester, butyl glycol, heptadecane, tetradecane, valeric acid, diethyl phthalate, hexanoic acid ethyl ester, ethylene glycol, eicosane, and ethyl oleate. These metabolites are known for their antibacterial properties against various fish pathogens. To assess the antimicrobial effects of the seaweed extract, three bacterial strains that cause diseases in fish were selected; *Streptococcus iniae* (*S. iniae*), *Aeromonas hydrophila* (*A. hydrophila)*, and *Aeromonas veronii (A. veronii)*, and one non-pathogenic bacterium for fish, *Staphylococcus epidermidis* (*Staph. epidermidis), was used* as a reference. The recorded data revealed that LOEE exhibited superior antibacterial properties over CBEE. The LOEE exhibited the largest zone of inhibition, measuring 19.67 ± 0.64 mm, against *Staph. epidermidis*. The agar plates streaked with *S. iniae* had the smallest inhibitory zones, measuring (8.27± 0.14 mm) and (8.37± 0.14 mm) for LOEE and CBEE, respectively. The activity index (AI) showed that LOEE exhibited the maximum inhibition of 0.84 mm and 0.65 mm against *S. epidermidis* and *A. hydrophila*, respectively. Overall, LOEE was effective in suppressing the growth of the majority of the examined bacteria *in vitro*, as compared to CBEE.

1. INTRODUCTION

The estimated cost for disease outbreaks in the aquaculture industry worldwide exceeded US$ 6 billion annually (Cantrell, 2023). Disease management is one of the largest problems facing aquaculture (Naylor et al., 2023). The traditional approach to disease prevention incorporates antibiotics like oxytetracycline, enrofloxacin, and florfenicol into fish feed to prevent bacterial infections. This practice results in the disposal of large quantities of antibiotics and poses an environmental hazard (Sapkota et al., 2008; Assefa and Abunna, 2018), accompanied by the antibiotic resistance of the bacteria found in the aquatic sources.

Society, scientists, and consumers are actively seeking alternatives to antibiotic treatment in aquaculture to mitigate disease effects without affecting fish health or quality (Carbone and Faggio, 2016). Using phytobiotic materials or extracts in the aquaculture industry to stimulate fish immune responses is an active technique to support sustainable aquaculture (Cristea et al., 2012; Elumalai et al., 2020) .

Seaweeds and their secondary metabolites include a variety of compounds such as chlorellin derivatives, halogenated polysaccharides, sulfate polysaccharides, peptides, proteins, vitamins, haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones, sterols, ketones, and cyclic polysulfides. These compounds possess numerous antimicrobial properties (Cunha and Grenha, 2016). Red and brown seaweeds contain short-chain fatty acids (SCFA) like propionate, butyrate, and formate. These SCFAs have been extensively studied and implemented as feed additives (Dittoe et al., 2018). Seaweed typically contains saponin, a compound that affects the integrity of bacterial cell membranes by decreasing surface tension and impairing membrane permeability (Madduluri et al., 2013). Red seaweeds are a significant source of non-animal sulfated polysaccharides, which have high levels of total soluble carbohydrate (TSCC) content. These polysaccharides have anti-tumor, antioxidant, antiviral, and antibacterial properties (Al-Saif et al., 2014). The study conducted by Thanigaivel et al. (2015) suggested that ethanolic and aqueous extracts of Gracilaria folifera and Sargassum longifolium seaweeds have the potential to be used as treatments for Aeromonas salmonicida infection in *Oreochromis mossambicus*. The disc diffusion method was employed to assess the inhibitory effects of algae extracts from *Turbinaria ornata*, *Cystosiera myrica*, and *Padina pavonica* on *Staph. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Madkour et al., 2019). In the present investigation, the antibacterial activity of the ethanolic

* Correspondence to: elsalam.clinic@gmail.com

extracts of *Laurencia obtusa* (LOEE) and *Cystoseira barbata* (CBEE) was studied against pathogenic *S. iniae*, *A. hydrophila*, and *A. veronii* and one non-pathogenic bacterium for fish Staph. epidermidis using agar gel diffusion test.

2. MATERIALS AND METHODS

2.1. Collecting and preparation of seaweeds

Two species of seaweeds (*Laurencia obtusa*; *L. obtusa*; and *Cystoseira barbata*; *C. barbata*) (Fig. 1.a and 1.b) were collected from the intertidal zone at the Red Sea shore near Hurghada city, using a knife from all over the substrate (rock, plant, gravel). Then it was washed thoroughly with seawater to remove the epiphytes, sands, and other extraneous matters, then sent to the Central Laboratory for Aquaculture Research in Abbassa-Sharqiyah to be washed with a 1% potassium permanganate solution, according to Sivakumar et al. (2014). The taxonomic identification of species was done by experts in this field (Botany Department, Faculty of Science, Al-Azhar University). Seaweeds were then oven-dried at 60 °C for 48 h. Dried seaweed material was ground through a Wiley mill to a particle size of 0.5 mm in diameter and then stored in a desiccator (Granado and Caballero, 1994) .

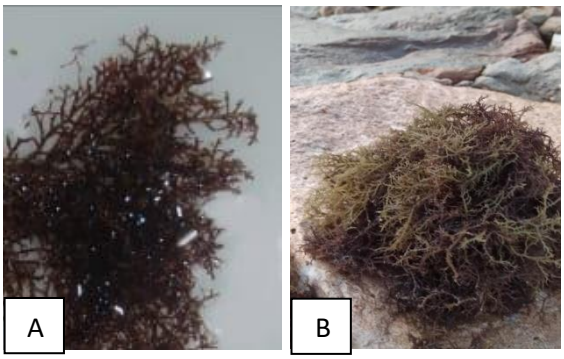

Figure (1) a- Fresh *Laurencia obtusa* seaweed. B- Fresh *Cystoseira barbata* seaweed

2.2. Solvent extraction of seaweeds

Seaweed powder was extracted using 95% ethyl alcohol at a rate of 10 ml of ethyl alcohol per g of seaweed powder) and then mixed in a shaker incubated at 30 °C for 96 h at 50 rpm. Then the extract was filtered by Whatman filter paper No. 1, dried using a rotary evaporator (30 °C) under vacuum, and stored in opaque glass bottles at 4 °C for further use, according to Sivakumar and Kannappan (2013) with some modifications.

2.3. Determination of seaweed's active metabolites using GC-MS

Gas chromatography-mass spectrometry (GC-MS) analysis for both seaweed extracts was performed in the water analysis department of the main chemical warfare laboratories - Egy Army. Using Agilent GC-MS-5975C with the Triple-Axis Detector equipped with an autosampler. The GC column used was fused with a silica capillary column (length 30 m × diameter 0.25 mm × film thickness 0.25 µm) with helium at 1 ml/min as a carrier gas. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 40–700 m/z. The split ratio was adjusted to 1:10, and the injection volume was 1 µl. The injector temperature was 250 °C; the oven temperature was kept at 40°C for 3 minutes and rose to 300 °C for 4 minutes. (total run time: 62 minutes).

Full mass data was recorded between 50-400 Dalton per second at a scan speed of 2000. Peak identification of crude *L. obtusa* and *C. barbata* extracts was performed by comparison with retention times of standards, and the mass spectra obtained were compared with those available in the NIST (National Institute of Standards and Technology) libraries (NIST 14-Mass Spectral Library 2014 version), according to Musharraf et al.(2012) .

2.4. Determination of the antibacterial activity

The crude ethanolic extracts of *L. obtusa* and *C. barbata* were tested against different bacterial pathogens for their antibacterial activity using agar disc diffusion. The tested bacterial strains were *A. hydrophila*, A. veronii, *S. iniae*, and Staph. epidermidis which were identified and verified by the Department of Aquatic Animal Medicine, Benha University and stored at -80 ºC until use. Bacterial strains were activated using nutrient broth and incubated at 30 ºC for 24 h. The suspensions of organisms were initially adjusted with sterile saline (0.7%) to a density equivalent to the 0.5 McFarland standards.

2.4.1. Agar disc diffusion method

The test was conducted in the Al-Fayrouz Farm microbiology laboratory, Port Said Governorate, Egypt. The agar disc diffusion method was followed for the antibacterial susceptibility test (CLSI, 2021). Mueller-Hinton agar MHA (Oxoid, UK) (pH 7.4 ± 0.2). was used for bacterial growth; approximately 20 ml of the autoclaved medium were dispensed into sterile plates and allowed to solidify under aseptic conditions using a laminar flow hood, then bacterial strains were inoculated and spread with a sterile swab on the surface of the agar plates. Approximately 0.20 ml of a 24-hour-broth culture was spread on the surface of sterile MHA plates. 100 µl of tested seaweed crude extracts were transferred to a sterile Whatman No. 1 filter paper disc of 6-mm diameter. After air drying, the discs were placed on the MHA plates and inoculated with each of the previously mentioned microorganisms in triplicate. A disc soaked with a similar quantity of standard oxytetracycline 20% solution was used as the control positive, and another soaked with PBS (pH = 7) was used as the control negative. After the incubation period (30 ºC for 24 h), the inhibition zones were measured. and the mean of the zones of these replicates was recorded. The activity index (AI) was applied to compare the antibacterial activity of each tested seaweed extract against all bacterial pathogens with that obtained from standard antibiotics, according to Shobier and El Ashry.(2021) ,

$$AI = \frac{\text{Mean of extract inhibition zone diameter}}{\text{Mean of the standard antibiotic inhibition zone diameter}}$$

2.5. Ethical approval

The experiment was conducted following the ethics of animal welfare; reviewed and approved by the Faculty of Veterinary Medicine, Benha University committee of Animal ethics (BUFVTM 04-02-24).

2.6. Statistical analysis

Results are presented as the mean ± standard error (SE) of three replicates. The statistical analyses were carried out to determine the degree of significance using one-way analysis of variance ANOVA (Version, 22) at probability level $P \leqslant 0.05$. Duncan post hoc test was used to assess differences among sample means.

3. RESULTS

III.1. Active metabolites in *L. obtusa* and *C. barbata*

GC-MS analysis of both seaweed crude extracts illustrated many chemical constituents according to their retention time, peak area (%), and molecular weight, which were then recognized by comparing their mass spectral fragmentation patterns to those of the known compounds described by the NIST library-14 software, as shown in Figures 2(a and b). LOEE analysis elucidated 83% of the peaks as the main chemical-constituent was an organic compound: 1-Propanol, 2-methyl- (Isobutanol) with (Rt = 6.796 min) with an area (40.39%) followed by 1-Propanol with (Rt = 6.516 min) and of 15.32% area, 1-Butanol, 3-methyl (Isoamyl alcohol) with (Rt = 7.710 min) and an area of about 19.93%, 1-Butanol, 3-methyl- (Isoamyl acetate) with (Rt = 10.259 min) and area scored (8.98%), and Ethanol, 2-butoxy- with (Rt = 11.026 min) with (3.76%) area. While CBEE was found to have a total of 68 peaks with predominant organic constituents as 1-Butanol, 3-methyl (isoamyl alcohol) with Rt = 7.705 min and an area value (34.93%), followed by 1-Butanol, 3-methyl-acetate with (Rt = 10.416 min) and an area, while n-Hexadecanoic acid scored (Rt = 42.792 min) and (7.04%) area, and Hexadecanoic acid, ethyl ester attained (Rt = 43.440 min) and an area (3.47%). Along with the main chemicals found in both seaweed extracts, LOEE also has smaller amounts of other chemicals that are known to kill microbes. These include butyl glycol (an organic ether), palmitic acid (a long chain fatty acid), palmitic acid ethyl ester (a fatty acid ester), heptadecane (an unsaponifiable hydrocarbon), tetradecane (an alkane hydrocarbon), and valeric acid (a long chain fatty acid). CBEE contained diethyl phthalate (phthalate ester), hexanoic acid ethyl ester (aldehydes), ethylene glycol (organic compound), neophyte diene (diterpene), palmitic acid (long chain fatty acid), eicosane (hydrocarbon), ethyl oleate (fatty acid ester), and butyl glycol (organic ether) (table 1).

3.2. Antibacterial inhibition in the agar disc diffusion test

The results revealed that bacterial strains responded differently to both seaweed extracts, with all strains demonstrating sensitivity to commercial antibiotics such as oxytetracycline (20%), which served as the positive control. The negative control (PBS) did not have any effect on any bacterial strain. The crude extract of *L. obtusa* has higher antibacterial activity than that of *C. barbata*. LOEE had significant antibacterial activity against all tested bacterial strains. The highest antibacterial activity was obtained in LOEE against Staph. epidermedis with an inhibition zone (19.67 ± 0.64 mm), followed by LOEE with *A. hydrophila* (15.27 ± 0.32 mm), while LOEE with *A. veronii* showed a partial zone of inhibition (13.2 ± 0.57 mm). While CBEE showed less antibacterial activity than LOEE against all bacterial strains recorded (15.5 ± 0.5, 12.53 ± 0.64, 10.23 ± 0.47, and 8.37 ± 0.14 mm) for Staph. epidermedis, *A. hydrophila*, A. veronii, and *S. iniae*, respectively,

It was observed that *S. iniae* was mostly resistant to both seaweeds (Figure 3 and Table 2).

3.3. Activity Index

As illustrated in Figure 4, the activity index (AI) of the seaweed extracts revealed that L.obtusa ethanolic extract (LOEE) recorded the highest AI (0.84 and 0.65) against S. epidermidis and *A. hydrophila* respectively. This confirms the previous results that indicated LOEE had superior activity against the tested bacteria compared to CBEE.

Figure (2a). Showing GC-MS curve reading illustrating the active metabolites in CBEE which recorded (68) peaks.

Figure (2b). showing GC-MS curve reading illustrating the active metabolites in LOEE which recorded (83) peaks.

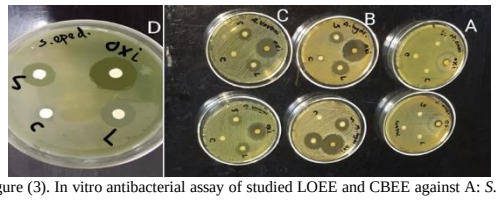

Figure (3). In vitro antibacterial assay of studied LOEE and CBEE against A: *S. iniae*, B: *A. hydrophila*, C: *A. veronii* and D: *Staph. Epedermidis*, where letters OXI = oxytetracycline (control +ve), L= LOEE, S= CBEE and C = (control-ve).

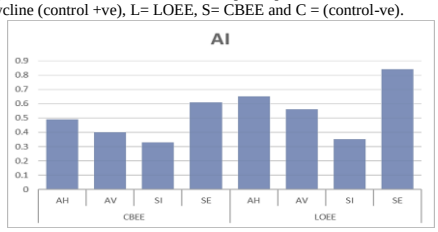

Figure (4). showing Activity Index (AI) of LOEE and CBEE against *A. hydrophila*, *A. veronii*, *S.iniae* and *Staph. epidermidis.*

Table (1). Showed GC-MS analysis of LOEE and CBEE illustrated many chemical constituents according to their retention time, peak area (%), and molecular weight then recognized by comparing their mass spectral fragmentation patterns to that of the known compounds described by the NIST library-14 software and marked with (+), (++), (+++), (++++) and (+++++) according to its abundance.

	LOEE		**CBEE**	
1	Propanol	++++	Diethyl Phthalate	+++++
2	Butanol	++	2,4-Bis (dimethylbenzyl) phenol	++++
3	Formic acid	++	Ethyl palmitate	++++
4	Valeric acid	+++++	Ethylene glycol	+++++
5	Isopentyl alcohol	++++	Isopentyl alcohol	++++
6	Isobutyraldehyde	++++	Ethyl carbamate	+++
7	Butyl glycol	+++++	Butyl glycol	+++++
8	diethyl acetal	+++	Tetramethylenediamine	+++
9	3,4-Dimethylbenzoic acid	++	Orthoformic acid	+++
10	Ethyl hexanoate	++++	Propyl isopropyl ketone	+++
11	Cyclotetrasilane, octamethyl	+++	Propyl ketone	+++
12	Diglycolamine	++	Valeric acid, ethyl ester	++++
13	Pentadecane	++++	Pentadecane	++++
14	Heptadecane	+++++	Heptadecane	+++
15	Icosane	++++	Eicosane	+++++
16	Tricosane	++++	Octadecane	++++
17	Tetracosane	++++	Tetracosane	++++
18	Elaidic acid, ethyl ester	++++	Tetramethylenediamine	++
19	Heptadecane	++++	Neophytadiene	+++++
20	Palmitic acid ethyl ester	+++++	Nonadecane	++++
21	Palmitic acid	+++++	Palmitic acid	+++++
22	Ethyl elaidate	++++	myristic acid	+++
23	Ethyl Oleate	++++	Ethyl Oleate	+++++
24	Pentanoic acid, ethyl ester	++	Docosane	++++
25	Tetradecane	+++++	Ethylhexyl-phthalate	+++
			Malonaldehyde tetraethyl acetal	+++
			isopentanal diethyl acetal	+++

Table (2). Showing inhibition zone represented as means and SE of LOEE, CBEE and Oxytetracycline 20% (control +ve) against *A. hydrophila*, *A. veroni*, *S.iniae* and *Staph. epidermidis.*

Seaweed ethanolic extract	A.hydrophila	A.veroni	S.iniae	Staph. epidermidis	Oxytetracycline 20%
LOEE	15.27± 0.32^{c}	13.2± 0.57^{d}	8.27± 0.14^{e}	19.67± 0.64^{b}	23.43± 0.71^{a}
CBEE	12.53± 0.64^{c}	10.23± 0.47^{d}	8.37± 0.14^{e}	15.5 ± 0.5^{b}	25.23± 0.76^{a}

4. DISCUSSION

Organic acids, long-chain fatty acids, alkaloids, and diterpenes are reported to have antibacterial activities (Ozdemir et al., 2004; Akin-Osanaiye et al., 2011; Singh et al., 2012; Janu and Jaynthy, 2014). Both terrestrial and marine plants commonly recognize fatty acids as essential elements (Saravanakumar et al., 2008). GC-MS analysis of both seaweeds showed a vast variety of chemicals that elucidated antibacterial activities. The obtained results were nearly similar to those reported by Cvitkovic´ et al. (2021) and EL-Sheekh et al .(2022) .

Nearly the same results were obtained by Čagalj et al. (2022), who found that Cystoseira compressa during the whole season GC-MS analysis contained fatty acids, among which oleic acid, palmitoleic acid, and palmitic acid were dominant compounds in the samples. De Alencar et al. (2018) investigated the organic active metabolites in GC-MS analysis of extracts from the marine red algae Pterocladiella capillacea, which possess many fatty acids such as palmitic acid, oleic acid, and arachidonic acid. In the same respect, Krish and Das (2014) reported that the crude methanol, ethanol, and ethyl acetate extracts of Cladophora rupestris using the agar diffusion method showed good antimicrobial activity against Vibrio harveyii, V. parahaemolyticus and V. alginolyticus which may be attributed to the presence of many fatty acids such as palmitic, myristic, oleic, alpha-linolenic, palmitoleic and linoleic acids. In agreement with our results, Cvitkovic´ et al. (2021) found that palmitic, oleic, and linolenic fatty acids are the dominant fatty acids in Cystoseira compressa, *Cystoseira barbata*, Fucus virsoides, and Codium bursa seaweeds when they investigated the extraction of their lipid fractions .

On the other hand, Ozdemir et al., (2004) found that the volatile components of Spirulena platensis consisted of heptadecane (39.70%) and tetradecane (34.61%) as major components, which showed antibacterial activities against many Gram-positive bacteria, including S. epidermidis. Vennila et al., (2020) mentioned that oleic acid, octadecenoic acid, methyl ester, and n-hexadecanoic acid were the most prominent active metabolites in different extracts of Sargassum plagiophyllum, Padina gymnospora and Turbinaria conoides seaweeds.

Regarding the antibacterial activity of seaweeds, the highest antibacterial activity was obtained in LOEE against S. epidermedis followed by LOEE with *A. hydrophila*, while LOEE with *A. veronii* showed a partial zone of inhibition, while CBEE gave lesser antibacterial activity than LOEE against all bacterial strains recorded; Shanab, (2007) recorded that the antimicrobial activity of red seaweeds (Laurencia papillosa and Jania corniculata) might be attributed to the presence of saturated or non-saturated fatty acids. Nearly similar results were applied by Ravi et al. (2019), where the antibacterial activity of the ethanolic extract of red seaweed Jania rubins was the highest (22.66 ± 0.5mm) between different seaweeds against *A. hydrophila*. Kasmiati et al., (2022) stated that methanolic and hexanolic extracts of red seaweed Halymenia durvillei had the highest activity among different extracts against *A. hydrophila*, with inhibition zones of 21.0 mm. Other results showed that red seaweed Asparagopsis taxiformis' water-soluble metabolites have limited potential for inhibiting the growth of *S. iniae* as the inhibition zone was 9–11 mm of the clear zone (Mata et al., 2013). Taskin et al. (2007) reported that the methanolic extract of *C. barbata* recorded moderate inhibition in an agar disc diffusion experiment against all tested pathogens: S. aureus, Micrococcus luteus, Enterococcus faecalis, Enterobacter aerogenes, and Escherichia coli .

Regarding the activity index (AI), it showed that L.obtusa ethanolic extract (LOEE) recorded the highest AI against Staph. epidermidis and *A. hydrophila*, respectively. Nearly similar results were identified by Shobier et al. (2023), who investigated the antibacterial and antifungal activity of five seaweeds belonging to Chlorophyta, Phaeophyta, and Rhodophyta against pathogenic bacteria (Saureus, Escherichia coli, *Pseudomonas aeruginosa*, Enterococcus faecium, Klebsiella pneumoniae, Candida albicans, Fusarium solani) The ethanolic extracts showed reasonable antimicrobial activity against the tested pathogens symbolized by the ethanolic extract of Ulva linza with an activity index of 1.30, followed by the ethanolic extract of Colpmenia sinuosa which exhibited an activity index of 1.29.

5. CONCLUSIONS

The study findings indicate that the extracts derived from red seaweed *Laurencia obtusa* have substantial antibacterial efficacy against the tested bacterial fish pathogens. Consequently, these extracts can serve as potential natural substitutes for antibiotics in the field of aquaculture. Nevertheless, additional research is required to determine the specific active chemicals that are responsible for the antibacterial properties, as well as to assess their safety and effectiveness in vivo.

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