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Macroalgal activity against multiple drug resistant *Aeromonas hydrophila*: A novel treatment study towards enhancement of fish growth performance

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ABSTRACT

Objective: The aim of this study was to evaluate the efficiency of macroalgal extracts as antibacterial agent against multidrug-resistant (MDR) bacteria isolated from Nile tilapia (*Oreochromis niloticus*) as well as to enhance the fish growth performance by macroalgae diet application.
Methods: A total of 50 swabs were collected from the diseased organs of tilapia fish including gills, skin, spleen, intestine, liver, kidney and muscle. The isolated bacteria were identified and then confirmed by using VITEK 2. Eight macroalgal species were collected from Abu-Qir, Alexandria coast, Egypt. After determination of their biomass, three solvents were used to prepare algal extracts. The antibacterial activities of different macroalgal extracts were measured against MDR *Aeromonas hydrophila* 6 (MDRAH6) using well-diffusion method. The mechanism by which macroalgal extract affects MDR bacteria was conducted by using transmission electron microscope (TEM). To evaluate the safety of the promising algal extract, GC-MS was performed to detect the composition of *S. vulgare* extract. In addition, growth performance was measured as an application of algal extracts into fish feed.
Results: Between eight collected macroalgal species, *Sargassum vulgare* showed the highest biomass production (53.4 g m^{-2}). In addition, its ethanolic extract showed the highest significant antibacterial activity with MIC value of 250 μg ml^{-1}. TEM examination showed distinctive changes in the treated MDRAH6 cells including rupture of the cell wall, leakage of cytoplasmic contents, alterations in the cytoplasm density in addition to totally cell deformation. In addition, GC-MS analysis revealed eleven identified components in *S. vulgare* ethanolic extract, in which 9,12-octadecadienoyl chloride and hexadecanoic acid methyl ester were dominant (46.6 and 19.7 %, respectively). Furthermore, dietary replacement of fish meal with *S. vulgare* ethanolic extract significantly enhanced the growth performance and survival of Nile tilapia with a significant reduction in the total bacterial count.
Conclusion: Ethanol extract of the brown macroalga *S. vulgare* could be a promising antibacterial and a new active agent against MDR *A. hydrophila*, which could be a major causative agent of Nile tilapia fish diseases. In addition, this study recommended *S. vulgare* as a natural and effective source to enhance the growth performance of Nile tilapia. In fact, isolation and examination of the individual antibacterial active compounds of the *S. vulgar* ethanolic extract are under investigation.

1. Introduction

World aquaculture has been revealed as a fastest-growing animal food source for increasing fish supply with an average annual increase of 6 % per year [1]. Reverter et al. [2] reported that fish accounted for 17 % of animal protein and 6.5 % of all protein consumed. However, infectious diseases in world aquaculture production represent tremendous interests worldwide resulting in total or partial loss of production [3,4]. Due to high fish concentrations and lack of sanitary barriers, it facilitates the spread of pathogens and thus producing high mortality levels [5]. In order to avoid such high economic losses in fish, several veterinary drugs are applied in aquaculture as a trial to prevent or treat disease

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outbreaks [6]. However, Seyfried et al. [7] concluded that the extensive use of veterinary drugs is becoming restricted due to their high-risk factors and rapid development of multidrug-resistant bacteria (MDR). The wide use of synthetic antimicrobials as trichlorfon or praziquantel has led to resistance development [8]. Although the potential effect of vaccination in the treatment of aquaculture diseases has also been regarded, it is specific and too expensive for widespread use [9]. Besides, the potential harm of veterinary drug treatments on both human health and environments. Most pathogens associated with fish fitness and health being opportunistic and, thus natural sources should be applied as an alternative solution against microbial fish infections [10,11]. Therefore, the use of natural treatments with the limitation of chemical products in aquaculture could enhance the optimal utilization of aquaculture products [2].

Aeromonas hydrophila, A. salmonicida, *Pseudomonas*, *Vibrio*, and *Flavobacterium* have been reported to cause disease leading to high mortalities in fish [12]. However, *Aeromonas* infections are among the most common fish diseases which have relatively high antibiotic resistance [13]. *Aeromonas hydrophila* causes bacterial hemorrhagic septicemia, the rot of fan/tail and epizootic ulcerative syndrome in fresh water [14] and marine fish [15]. It is Gram-negative motile, straight rods belongs to Aeromonadaceae family and it is common in freshwater and occasionally marine species [16,17]. *Aeromonas hydrophila* pathogenicity has been the focus of many investigations [18–20]. Yambot and Inglis [21] recorded acute mortality with apparent clinical signs included opaqueness in eyes, exophthalmia, and eventual orbit bursting in Nile tilapia (*Oreochromis niloticus*) infected with *A. hydrophila*. Five *Aeromonas* species have been associated with human disease; and more than 85 % of them are attributed to *A. hydrophila* and were determined in infants, gastroenteritis, and sepsis [22,23]. MDR has been recorded for several *A. hydrophila* strains around the world [24,25]. MDR in aeromonad is undoubtedly originated from transferable R-plasmids [26]. Interestingly, exposure to one antibiotic may confer resistance to another one and the emergence of MDR pathogens [27]. Because of the emergence of antibiotic resistance, there is a major problem with the treatment of *A. hydrophila* infections in Nile tilapia. A wide range of antibiotics as sulphonamides, chloramphenicol, streptomycin, tetracycline and ampicillin has been found to have decreasing effectiveness [28,29].

Marine ecosystem provides an important source of chemical compounds which have many therapeutic applications such as antibacterial, antiviral and anticancer activities because of its biodiversity [30]. The ability of seaweeds to produce biologically active secondary metabolites has been widely documented. There are several reports regarding the broad range of biological activity of compounds derived from macroalgae [31–34]. In order to find natural alternative drugs against fish bacterial pathogens in aquacultures, the present study aimed to evaluate the antimicrobial activity of macroalgal extracts collected from Alexandria, Egypt against MDR bacteria isolated from Nile tilapia fish. In addition, the effect of algal extract application on fish growth performance was conducted. Though literature showed diverse studies of bioactivity of marine algae, the present work is comparatively a new concept.

2. Material and methods

This study was conducted on 200 tilapia fish collected from different private fish farms at Kafr El-Sheikh city, Kafr El-Sheikh Governorate, Egypt. Fish were transported alive in large plastic bags filled two-thirds with dechlorinated water and provided with an air pump to restore the oxygen needed and essential care management during transportation from fish farms to the Faculty of Veterinary Medicine, Damanhur University, Egypt. Fish were transferred into glass aquaria (110 × 60 × 60 cm) immediately after reached. Clinical examination was done on a live fish and any abnormal swimming fish behavior, fish activity, and body coloration were observed. Fish with abnormal characters (n= 60) were selected for further studies.

2.1. Isolation, identification, and maintenance of bacterial cultures

Bacterial swabs were isolated from organ's diseased Nile tilapia fish including gills, skin, spleen, intestine, liver, kidney, and muscles. The collected swabs were streaked aseptically on nutrient agar, blood agar and MacConkey's agar (oxoid) [35]. The plates were incubated at 37 °C for 18–24 h. Preliminary identification of bacteria was based on colony characteristics of the tested bacteria such as colony description, hemolytic activity on blood agar and enzyme activities of the tested isolates. For bacterial identification, biochemical tests were performed on colonies. Biochemical tests for identification of Gram-negative bacteria were conducted (oxoid) as follows; Simon's citrate agar, Kliger iron agar, indole, urea, lysine iron agar and motility. In the same context, Gram-positive bacteria were identified based on catalase and coagulase tests. All bacterial isolates were confirmed by using VITEK 2 (Biomérieux, France) and the results were interpreted by using VITEK software (version 06.01). The identified isolates were maintained in Luria-Bertani (LB) medium consists of Tryptone (1 %), yeast extract (0.5 %) and 1 % sodium chloride (1 %) during all the experiments of the study.

2.2. Macroalgae collection and extract preparation

Macroalgae were collected from Abu-Qir, Alexandria coast, Egypt (N 31° 19' E 30° 03', Fig. 1) by a quadrate technique using 50 × 50 cm steel quadrate [36]. Three quadrate samples were taken at the collection site. All algal populations within the quadrate were collected carefully and washed with seawater to remove epiphytes and other marine organisms. The collected macroalgae were transported to the laboratory in sterile polythene bags, identified according to Taylor [37], then dried in oven at 40 °C until constant dry weight. The biomass was determined for each species as gm dry weight per square meter (g m^{-2}). Organic solvents (ethanol, methanol, and acetone) were used for extraction. Each powdered sample (5 g) was soaked overnight in 25 ml of the desired solvent. The resultant crude extracts were filtered and then concentrated in a rotary evaporator at 40 °C. The crude extracts were weighed and were suspended in the dimethyl sulfoxide (DMSO) to a final concentration of 200 mg mL^{-1} and stored in a refrigerator at 4 °C.

2.3. Antimicrobial testing assay

The Susceptibility was performed to pure culture bacterial isolates using twenty-five different antibacterial agents (Oxoid, Basingstoke, UK) by Kirby-Bauer disc diffusion method on Muller-Hinton agar plates [38]. The antimicrobial agents and their potencies (μg) were illustrated in supplementary materials (Table S1). The sizes of inhibition zones were interpreted by referring to Clinical and Laboratory Standards Institute (CLSI) standards [39]. The tested bacterial isolates were grown overnight on nutrient agar. The inoculum turbidity was adjusted equivalent to 0.5 McFarland (approximately at 10^8 CFU/ml). MDR isolates were selected, and the most prevalent drug-resistant isolate was tested against the collected algal extracts. The inocula were spread uniformly over the agar plates surface by a glass rod. To test the antibacterial activity, macroalgal extract was prepared. Briefly, each powdered sample (5 g) was soaked overnight in 25 ml of the organic solvents (ethanol, methanol, and acetone). After resultant crude extract