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Bioactivity of crude ethanolic and hexane extracts from *Sargassum siliquosum* JG agardh against fish pathogens

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Abstract

Microorganisms pathogenic to fish lead to morbidity-linked economic losses and pose threat to human and animal health. Organisms that have potent antimicrobial properties against fish pathogens include brown seaweeds. This study determined the antimicrobial activity of the crude extracts of *Sargassum siliquosum* through Disc Diffusion Assay. The ethanol extract of *S. siliquosum* was found to exhibit antibacterial activity against *P. aeruginosa*, *P. vulgaris* and *A. hydrophila* with zones of inhibition of 13.17 ± 0.42 mm and 16.08 ± 1.81 mm and 12.52 ± 2.17 mm, respectively. The hexane extract had a 16.0 ± 0 mm zone of inhibition against *P. vulgaris*. This investigation reveals that *S. siliquosum* is a potential source of bioactive compounds against disease-causing fish pathogens.

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Introduction

The aquaculture industry has become an important industry to ensuring food security in the Philippines as well as becoming a decent source of employment and foreign exchange goods. The practice is quite extensive and makes use of a wide variety of species (Aypa, 1995). A lot of bacterial diseases has been recognized for the past years and bacteria-related mortality has posed a great problem and danger to aquaculture industry (Lee *et al*., 1986).

Many of the waters in which fish are reared in are contaminated with industrial waste. Heavy metals such as copper are also sometimes used to treat phytoplankton blooms. These chemicals are not only toxic for aquatic life, but they are also known to lower the immunity of fishes against harmful pathogens. Species of pathogens of aquaculture fish in fresh or brackish waters in Asia include *Edwardsiella, Flexibacter columnaris* and *Aeromonas hydrophila* (Chen, 1991). Diseases from *Vibrio* and *Microcystis* have been known to cause mortality and widespread of fish-kills in pens (Lio-Po, 1984). The fact that the fish are also reared in large numbers in enclosed spaces promotes the spread of these diseases.

To treat the aforementioned pathogens, substances such as antibiotics, sulphonamides, and furane-derivatives are used. The primary choice of treatment is antibiotics which are pumped into the pens of fishes. Misuse of antibiotics has led to emergence of drug-resistant bacteria limiting therapeutic choice of health care practitioners (Chen, 1991). Antibiotic resistance may also be brought about by horizontal gene transfer between bacteria (Ochman *et al.*, 2000). As such, there is great need to find alternative antibacterial substances to prevent further emergence of drug resistance as well as mortality-linked economic losses due to diseases in fish.

Marine organisms are major sources of novel bioactive compounds that are being utilized in the pharmaceutical industries. Most of the compounds isolated from marine organisms are now being studied for biomedical research and being developed as new pharmaceuticals (Freitas, 2002). With the troublingly increasing resistance of bacteria to antibiotics over the years, the search for novel compounds not based on existing synthetic antimicrobial agents has become more pressing. Marine algae are promising sources of bioactive compounds because they live in complex environments and thus have a number of unique secondary metabolites in order to cope with the extreme conditions they are used to. Additionally, marine algae are also taxonomically diverse, and this amount of biodiversity is certain to yield new bioactive therapeutic compounds (Eom *et al*., 2012).
There has been few researches on the potential of macroalgae to inhibit the activity of harmful pathogens in fish. The objectives of this research is to test the bioactivity of *S. siliquosum* crude extract against fish pathogenic microbes and to find if this extract can serve as a possible antimicrobial compound. The study is significant because aquaculture has become an integral part of Philippine industry and fish reared are indeed being killed by bacterial pathogens. Alternative sources of antibiotics can not only circumvent ever-present diseases but prove to be more useful in the long-run than commercial antibiotics.

Materials and methods

Extraction of crude extract from S. siliquosum

Specimens of *S. siliquosum* were collected from marine waters of Bolinao, Pangasinan with the help of the UP Marine Science Institute (UP MSI) team in May 2013. The samples were then air dried and macerated then soaked in 95% ethanol for three days. The crude extract obtained was subjected to hexane partitioning to acquire the non-polar compounds The filtrate was then concentrated using rotary evaporator. The concentrated extracts were dried and stored at 4°C in tightly sealed vials prior to antibacterial assays.

Preparation of culture media and microorganisms

Microorganisms were obtained from the culture collections of UP Diliman Institute of Biology and UP

Los Banos National Institute of Molecular Biology and Biotechnology. Bacterial cultures were maintained in Nutrient Agar while marine microorganisms were maintained in Marine Agar. Nutrient agar, Mueller Hinton agar, McConkey Agar, Luria Broth and Nutrient Broth were prepared according to manufacturer's instructions. Prepared media were refrigerated prior to use.

Disc Diffusion Assay

The disc diffusion assay is the gold standard procedure for antimicrobial susceptibility testing. Sterile paper discs (6mm in diameter) were prepared and autoclaved for 15 minutes at 121°C and 15 psi. Approximately 20 µl (100 mg/ml) of the *S. siliquosum* crude extract was impregnated in the paper disc and tested against several strains of aquatic pathogens including: *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterobacter aerogenes, Vibrioalginolyticus* and *Aeromonas hydrophila*.

The bacteria were subcultured in nutrient agar then incubated for 24 hours prior to use. Culture medium for marine bacteria was supplemented with 1.5% NaCl.

Each of the test aquatic pathogens was subcultured separately by suspending them in 10-mL peptone water. Turbidity of each solution was standardized using McFarland's standard. The bacteria were then inoculated in Mueller-Hinton Agar (MHA) using sterile cotton swab. Approximately 20 µL of crude ethanolic and hexane extracts (100 mg/mL) and 95% methanol (as negative control)added to sterile filter paper discs and 5 µg Ciprofloxacin disc (as positive control) were placed on inoculated MHA. Zones of inhibition were measured using Vernier caliper after incubation of the cultures at 37°C for 24 h. Each set of testing was carried out in triplicates. Microbial index of the extracts were also calculated using the following formula:

$$\text{Microbial Index} = \frac{\text{Inhibition zone} - \text{Diameter of disc}}{\text{Diameter of disc}}$$

Results and discussion

Previous studies on *Sargassum* species exemplify its physiological and biological activities. Sulfated polysaccharides extracted from *Sargassum siliquosum* were found to exhibit concentration-dependent free radical scavenging activity. Itscytotoxic activity against HepG2 and renal carcinoma cell lines was also revealed (Vasquez *et al.*, 2012). However, there is no published research yet regarding the antimicrobial activity of *S. siliquosum*.

The antibacterial activities of ethanol and hexane extracts of *S. siliquosum* were evaluated by disc diffusion assay. *S. siliquosum* extracts showed varying levels of antimicrobial activities. Crude ethanolic extract was able to inhibit *P. aeruginosa*, *P. vulgaris* and *A. hydrophila* while hexane extract inhibited *P. vulgaris*.

Table 1. Zone of Inhibition (mm) in MHA cultures of pathogenic marine bacteria with *Sargassum siliquosum* ethanol and hexane extract.

Bacteria	Ethanol Extract	Hexane Extract	Ciprofloxacin* (5µg)
Aeromonas hydrophila	12.52 ± 2.17 mm	-	19.00 ± 1.0 mm (I)
Proteus vulgaris	16.08 ± 1.81 mm	16.00 ± 0 mm	21.3 ± 0.99 mm (S)
Pseudomonas aeruginosa	13.17 ± 0.42 mm	-	32.5 ± 4.1 mm (S)

*Ciprofloxacin served as positive control. Zone of inhibition interpretation was based on Clinical and Laboratory Standards Institute 2006 and 2015 guidelines where R- Resistant; I – Intermediate; S – Susceptible.

Ciprofloxacin, a fluoroquinolone that inhibits nucleic acid synthesis was used as positive control. The interpretation of zone of inhibition measurements in millimetres brought about by ciprofloxacin (5µg) according to Clinical and Laboratory Standards Institute 2006 and 2015 guidelines is shown in Table 1. *P. aeruginosa*, *P. vulgaris* and *A. hydrophila* were all susceptible to ciprofloxacin. *E. aerogenes* and *V.*

alginolyticus on the other hand were found both resistant to ciprofloxacin and algal extracts. 95% methanol used as the negative control showed no antimicrobial activity against the microorganisms.

S. siliquosum ethanol and hexane extracts exhibited the highest zone of inhibition against *P. vulgaris* (Fig. 1 and Fig. 2) with microbial indices of 1.82 and 1.81, respectively (Table 2). *P. vulgaris* is the cause of blotch disease in live fish organisms (Bullock *et al.*, 1971), red spot disease (Conroy & Herman, 1970) and tail rot (Thankappan Pillai, 1984).

Table 2. Microbial indices computed from MHA cultures of pathogenic marine bacteria with *Sargassum siliquosum* ethanol and hexane extract.

Organism	Extract	Microbial index
P. aeruginosa	*S. siliquosum* (ethanol)	1.31
P. vulgaris	*S. siliquosum* (ethanol)	1.82
A. hydrophila	*S. siliquosum* (hexane)	1.81

Ethanol extracts of *S. siliquosum* also exhibited antimicrobial activity against *P. aeruginosa* (Fig. 3) and *A. hydrophila* (Fig. 4) with microbial indices of 1.31 and 1.20, respectively (Table 2). *P. aeruginosa* infection of fish is dangerous to fish health. It causes severe damage such as necrosis and disorganization of the hepatic cells (Amosu, 2012). *A. hydrophila* causes the disease known as hemorrhagic septicaemia in fishes (Swann & White, nd).

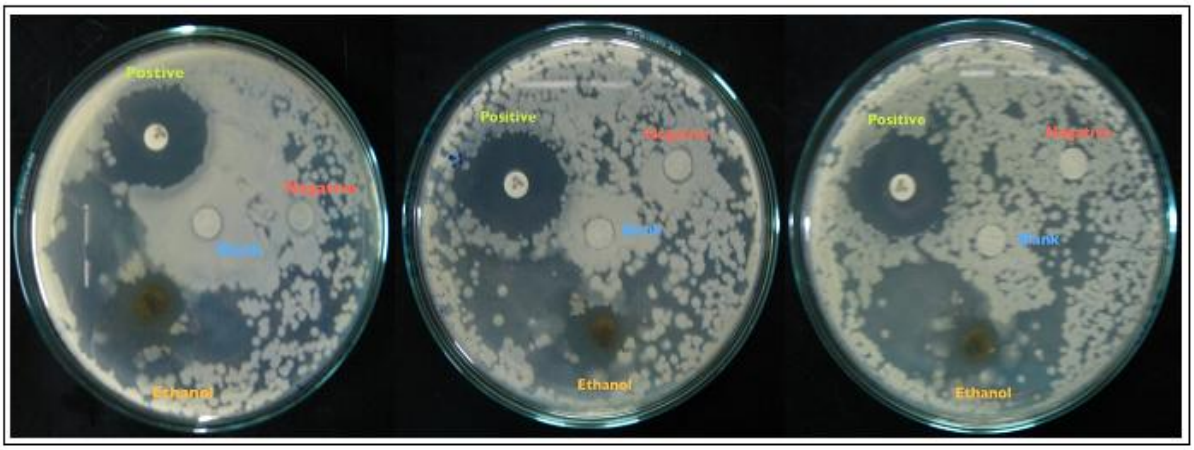

Fig. 1. Antibacterial activity of *S. siliquosum* ethanol extract against *P. vulgaris* using Disc Diffusion Assay.

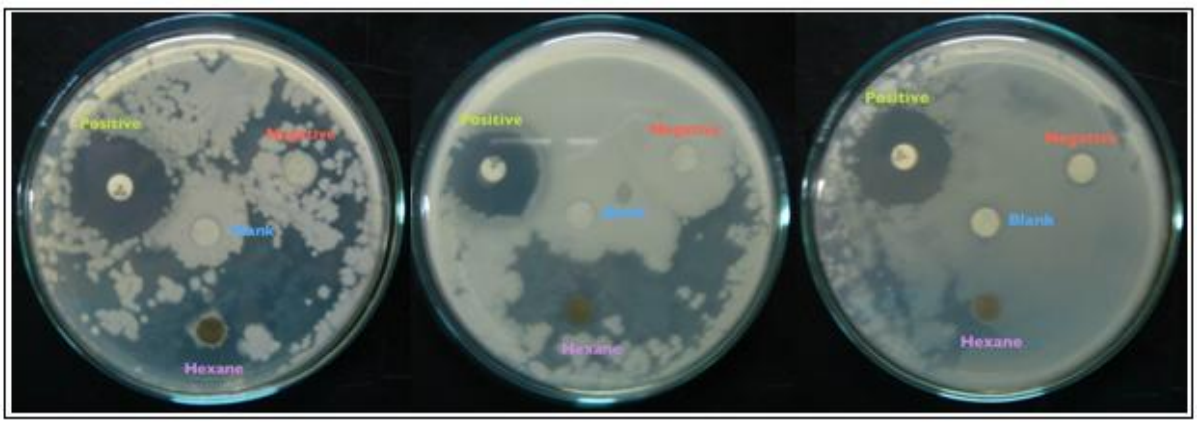

Fig. 2. Antibacterial activity of *S. siliquosum* hexane extract against *P. vulgaris* using Disc Diffusion Assay

In a recent study, it was found out that the dichloromethane extract of *S. siliquosum* contains an abundant amount of alkaloids, flavonoids, saponins, tannins and phenolic compounds. Using thin layer chromatography, they also found out that it encompassed quercetin. Quercetin is a flavonoid with antioxidant, anticancer and antimicrobial activity (Metwally *et al.*, 2010; Corpuz *et al.*, 2013). This might be one of the phytochemicals responsible for the observed antimicrobial activity of *S. siliquosum* in this study.

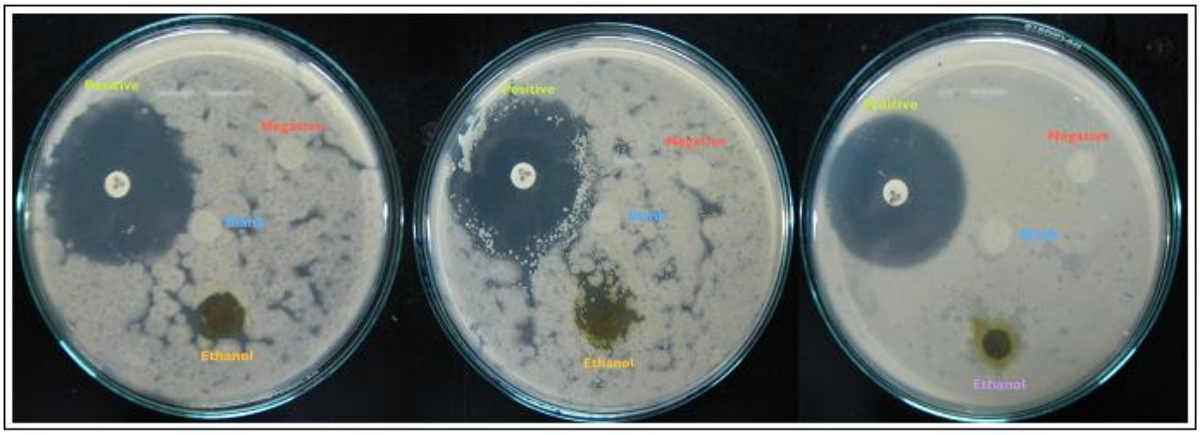

Fig. 3. Antibacterial activity of *S. siliquosum* ethanol extract against *P. aeruginosa* using Disc Diffusion Assay.

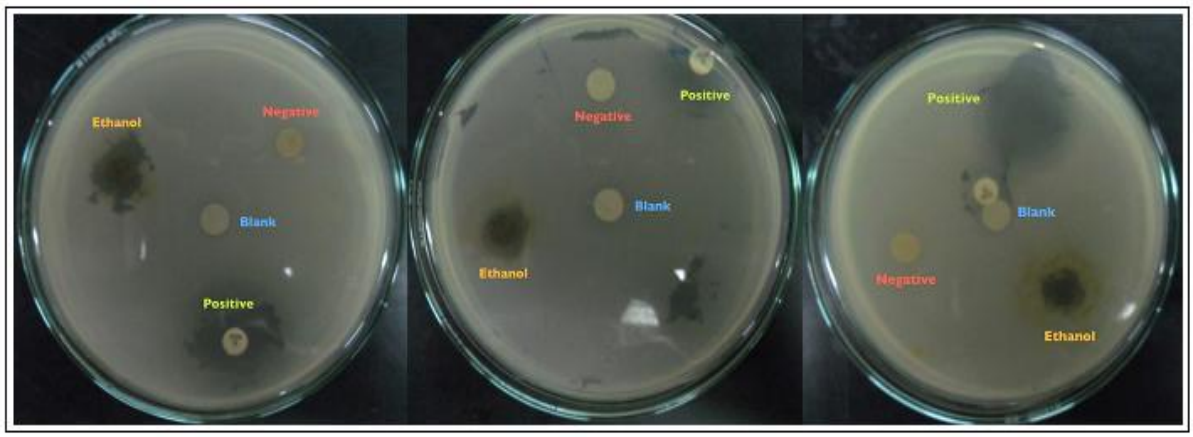

Fig. 4. Antibacterial activity of *S. siliquosum* ethanol extract against *A. hydrophila* using Disc Diffusion Assay.

S. siliquosum extracts tested did not show antimicrobial activity against *E. aerogenes* and *V. alginolyticus*. This might be due to masking of antibacterial activity due to the presence of some inhibitory compounds in the crude extract (Sastry and Rao, 1994). Further purification of the crude extract might improve the antibacterial activity of *S. siliquosum* against these fish pathogens.

Conclusion

This work is likely the first to assess the antimicrobial activity of *S. siliquosum* extracts against fish pathogens. The results show that seaweeds are potential source of bioactive compounds that can be mined for combatting bacterial fish diseases. The search for new antibiotic substances from natural products is very important especially during this time of emerging antibiotic resistant pathogenic bacteria.

Based on the resultsobtained, *S. siliquosum* can be considered for further studies as a potential source of antimicrobial agents against aquaculture pathogens. Phytochemical analysis is recommended for identification and characterization of the composition of the extract. Antibiofilm activity is also highly recommended.

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