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Evaluation of the Phytochemical Components and Antimicrobial Property of *Gracilaria edulis* Extracts Against Selected Aquaculture Pathogenic Bacteria

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ABTRACT

With pathogenic bacteria in aquaculture becoming increasingly resistant to antibiotics, there is a compelling need to look into bioactive chemicals present in seaweed as novel treatment options for fish infections. The purpose of this study was to evaluate the phytochemical characteristics of G. edulis extracts and its antimicrobial activities against selected aquaculture pathogenic bacteria. Phytochemical screening of G. edulis was carried out following qualitative test tube screening methods. The antimicrobial activities of the methanolic and ethanolic extracts of G. edulis were tested using the paper disc agar diffusion method against Aeromonas hydrophila, Escherichia coli, and Staphylococcus aureus. Amoxicillin and distilled water were used as a positive and negative control respectively. Based on the phytochemical analysis, the methanolic crude extract of G. edulis contained tannins and flavonoids while the ethanolic crude extract included only tannins. Further, the results of the antimicrobial assay test showed that both methanolic and ethanolic extracts of G. edulis inhibited the bacteria with inhibitory activity comparable to that of the positive control (Amoxicillin). The study therefore suggests that G. edulis can further be investigated for the possible formulation of therapeutics and drugs in light of its potential as an antibacterial agent.

Keywords: *aquaculture pathogenic bacteria, ethanolic crude extract, methanolic crude extract, paper disc diffusion method, zone of inhibition*

INTRODUCTION

Several incidences of bacterial pathogens on cultured aquatic species have been reported worldwide. The occurrence of S. aureus in farmed shrimps required the development of a vaccine to prevent the disease caused by it (Arfatahery et al., 2015). Escherichia coli was also confirmed in Nile tilapia (Oreochromis niloticus) from a fish farm in Sylhet, Bangladesh (Reza et al., 2021). The bacteria E. coli is usually non-pathogenic inside the gut of fish, but when it spreads outside the intestine, it can cause disease, resulting in entero-toxigenic. There has also been an ample information documented on the impact of A. hydrophila on aquaculture, with significant mortality in recorded on silver carp (Hypophthalmichthys molitrix) (Rashid et al., 2013).

To treat bacterial diseases in fish farming, various chemotherapeutics, vaccines, immunostimulants, and probiotics have been used, but the emergence of mutants and drug-resistant microorganisms has become a major issue. Antibiotic-resistant bacteria are causing an increase in the number of infections worldwide (Levy and Marshall, 2004). Furthermore, decreased efficiency and pathogen resistance to antibiotics have necessitated the development or discovery of new mutations (Bolanos et al., 2017).

Studies revealed that seaweeds contain amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones and alkanes, and cyclic polysulphides (Anjum et al., 2014). They are a rich source of bioactive compounds, capable of producing secondary metabolites that can be used as antimicrobial agents, and have the potential to be used as new pharmaceutical materials (Maftuch et al., 2016). Such bioactive compounds are also referred to as phytochemicals which have potential biological activities (Ghannadi et al., 2016); and are primarily responsible for the protection of plants against insect infestations and microbial infections (Escobido and Orbita, 2016). Unlike pharmaceutical chemicals, such phytochemicals have no adverse effects; hence, they are considered as “friendly medicines” that play a vital role against numerous diseases (Banu and Cathrine, 2015). Some of these phytochemicals include, alkaloids, anthraquinones, tannins and polyphenols, saponins, flavonoids, steroids, terpenoids, cyanogenic glycosides, etc.

Antimicrobial activity, on the other hand, is thought to be a characteristic of seaweeds that allows them to synthesize bioactive secondary metabolites or organic compounds that are not directly involved in the normal growth, development, and reproduction of an organism. Different organic solvents such as aqueous, chloroform, methanol, acetone, petroleum ether, diethyl ether, ethanol, n-hexane, dichloromethane, ethyl acetate, and toluene have been used to test the bioactivity of various types of algae against a wide range of microorganisms such as gram positive and negative bacteria, fungus, and even viruses (Qari and Khan, 2019). According to Pérez et al. (2016), different solvent extracts of red seaweeds demonstrated potent antimicrobial activity.

Gracilaria edulis is widely distributed in coastal areas throughout the Philippines, particularly the coastal of Cagayan like Buguey, Sta. Ana, and Claveria, Cagayan. However, the research of G. edulis as antimicrobial substance against aquaculture pathogens and potential source of bioactive compounds is poorly documented yet which gives the impetus of this study. Therefore, in this study we focus on exploring the bioactive compounds contained within G. edulis found and cultured in Claveria, Cagayan, with the aim of using them as antimicrobial agents and natural immunostimulants for aquaculture.

Objectives of the Study

This study generally aimed to evaluate the phytochemical characteristics and antimicrobial properties of G. edulis extracts against selected aquaculture pathogenic bacteria. Specifically, it sought to evaluate the bioactive compounds present in the thallus of G. edulis and its antimicrobial activity against S. aureus, E. coli, and A. hydrophila using methanolic and ethanolic extraction.

MATERIALS AND METHODS

Research Design

The seaweed samples used in the experiment were collected from the Bureau of Fisheries and Aquatic Resources – Claveria Brackishwater Technology Outreach Station (BFAR-CBTOS) located in Pata East, Claveria, Cagayan. For the bacteria, two of the microorganisms were requested from the Department of Agriculture – Bureau of Fisheries and Aquatic Resources Regional Office 2 (DA- BFAR R02) Regional Fish Health Laboratory (E. coli and S. aureus) while the A. hydrophila was obtained from the Central Luzon State University – College of Fisheries (CLSU-COF), Science City of Munoz, Nueva Ecija. The antimicrobial screening was also conducted at the BFAR-RO2 Regional Fish health Laboratory while the phytochemical analysis has been carried out in the Department of Science and Technology Regional Office 02 - Regional Standards and Testing Laboratory (DOST R02-RSTL). The flow process is shown in Figure 1. From field collection or gathering to air drying of G. edulis samples for a week, which have been pulverized for the maceration process. The solution was filtered and concentrated thru the vacuum-pressured rotary evaporator to obtain the crude extracts. Such crude extracts derived were eventually used for the phytochemical and antimicrobial analysis.

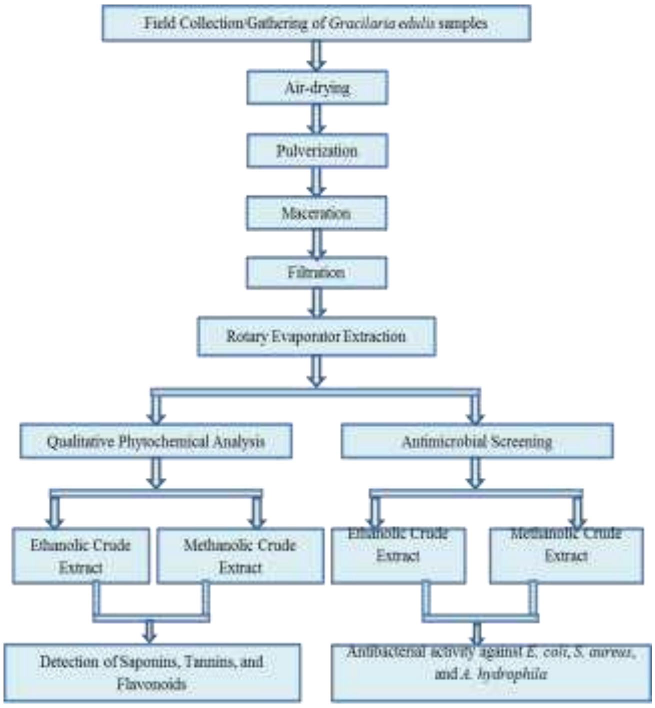

Figure 1. Research Methodological Flow

Phytochemical analysis

About fifty milliliters (50 mL) of each extract of the G. edulis were brought to the DOST R02-RSTL for the phytochemical screening following the qualitative screening procedure of Guevarra et al. (2005): Ferric Chloride test for the detection of tannins; the Bate-Smith and Metcalf method for the test of Flavonoids; and the Froth test for the detection of saponins.

Antimicrobial Screening

Two test microorganisms have originated from the DA-BFAR RO2 Regional Fish Health Laboratory, where the experiment was conducted and another one has been obtained from the CLSU-COF. These test microorganisms are as follows: E. coli, S. aureus, and *A. hydrophila* cultured at the said laboratories.

The paper disc agar diffusion method was used to determine the antimicrobial properties of the sample. About 5 mL of ethanolic and methanolic crude extracts (assay solution), distilled water (negative control) and amoxicillin (positive control) were dispensed into the sterile paper discs and was performed in five trials or replicates. The impregnated discs were aseptically applied and pressed into the seeded nutrient agar in an equidistant manner. The assay plates were then labelled for antimicrobial potential. Sets of assay plates were arranged inside the laminar airflow or biosafety cabinet. The Petri dishes were incubated at 35 degrees Celsius for 18-24 hours.

After 18-24 hours of incubation, the diameter of zones of inhibition were measured in millimeters using the Vernier caliper with the aid of a black paper background. Clear and well-defined zones of inhibition (ZOI) around the discs were observed if the sample tested possessed antimicrobial potential while failure of the disc to exhibit zones of inhibition indicates the absence of antimicrobial effects.

Analysis of the Data/ Statistical treatment

Mean values ± SD were used to present the antimicrobial efficacy of the various extracts of G. edulis against the selected aquaculture pathogens based on the average measurement of the "zones of inhibition," which were expressed in millimeters using a Vernier caliper. The data were initially tested for normality and homogeneity of variance using Shapiro-Wilk's and Kolmogorov-Smirnov tests, respectively. Whenever necessary, data are subjected to arcsine or square-root transformation to meet the parametric assumptions. Non-parametric data were analyzed using Kruskal-Wallis test followed by pairwise Mann-Whitney U test as post hoc comparison procedure. Parametric data were also analyzed using one-way ANOVA (Analysis of Variance) followed by Tukey's HSD Test.

RESULTS AND DISCUSSION

Table 1. Result of the Qualitative Phytochemical Analysis

Sample Description	Parameter	Result
Ethanolic Crude Extract	*Flavonoids* *Tannins* *Saponins*	- + -
Methanolic Crude Extract	*Flavonoids* *Tannins* *Saponins*	+ + -

Table 1 reveals the result of the phytochemical analysis of *G. edulis* extracts (i.e., ethanolic and methanolic crude extracts) showing the various test parameters such as flavonoids, tannins, and saponins. Based on the results of the phytochemical analysis, the ethanolic crude extract of the thallus of G. edulis contains tannins and does not contain flavonoids and saponins, whereas the methanolic crude extract has flavonoids and tannins and is deficient in saponins, that is, observed however in both extracts.

Table 2 indicates the mean and standard deviation of zone of inhibition of G. edulis extracts against selected aquaculture pathogenic bacteria namely: A. hydrophila, E. coli, and S. aureus. Of the three selected aquaculture pathogenic bacteria, the A. hydrophila was found to be the most susceptible to the ethanolic crude extract as compared to the other bacteria, obtaining mean ± SD zone of inhibition (ZOI) of 8 ± 1.32476, followed by S. aureus and E. coli with means and standard deviations of 6.44 ± 0.69857 and 6.425 ± 0.61305 respectively. The trend in the responses of these bacteria was also demonstrated to be similar to that of the methanolic crude extract of G. edulis, that is, sensitive with E. coli as the least susceptible (6.7 ± 0.65828), followed by S. aureus (8.1 ± 0.68191) and A. hydrophila (8.26 ± 1.60717) as the most susceptible bacterium.

Table 2. Mean and Standard Deviation (Mean ± SD) of Zone of Inhibition of G. edulis Extracts against Selected Aquaculture Pathogenic Bacteria

Test Microorganism	Sample Code	Sample Description	Zone of Inhibition (mm)
A. Hydrophila	MCE	Methanolic Crude Extract	8.26 ± 1.60717
	ECE	Ethanolic Crude Extract	8 ± 1.32476
	+	Positive Control	7.1 ± 1.27083
	-	Negative Control	---
E. coli	MCE	Methanolic Crude Extract	6.7 ± 0.65828
	ECE	Ethanolic Crude Extract	6.425 ± 0.61305
	+	Positive Control	33.25 ± 0.07071
	-	Negative Control	---
S. aureus	MCE	Methanolic Crude Extract	8.1 ± 0.68191
	ECE	Ethanolic Crude Extract	6.44 ± 0.69857
	+	Positive Control	6.42 ± 0.57619
	-	Negative Control	---

Values are means of five replicates (n=5) ± standard deviation; No Inhibition Activity (---)

It is revealed, however, that at a significant level of 5 % (ANOVA), the sensitivity (ZOI) of each of the selected aquaculture pathogenic bacteria to the methanolic and ethanolic extracts of G. edulis, including the amoxicillin (positive control), has no significant differences (p>0.05); otherwise, it has no specific sensitivity and resistance to such antimicrobial agents. Nonetheless, E. coli was the most sensitive to the positive control or Amoxicillin relative to the other aquaculture bacteria which exhibit resistance to the commercially available antibiotic; not to mention that S. aureus was the most resistant to the commercially available antibiotic, i.e., likewise observed in A. hydrophila having however a lesser resistance.

In the present study, the two crude extracts (methanolic and ethanolic) of G. edulis were screened for the occurrence of three phytochemicals named saponin, tannins, and flavonoid. Based on the result, the methanolic crude extract has contained more phytochemicals or showed a higher number of bioactive compounds present than the ethanolic crude extract, which means that the methanol solvent used is more efficient in terms of extracting bioactive compounds from the sample compared to the other solvent used, which is ethanol. The ethanolic crude extract of the thallus of G. edulis contains tannins and no flavonoids or saponins, whereas the methanolic crude extract contains flavonoids and tannins but lacks saponins, as observed in both extracts. According to Rayapu et al. (2017), such flavonoids detected in the methanolic crude extract of G. edulis are compounds with various medicinal properties and health benefits. It can operate as an antibacterial because it can damage the bacterial cell wall, followed by the discharged intracellular compounds, which disrupts the permeability of cell membranes and the synthesis process for protein and DNA (Firdausy et al., n.d.). On the other hand, tannins are considered to be healing agents in inflammation and burns with antidote, anti-ulcer, and antioxidant properties (Escobido et al., 2016). Such saponins, however, which are absent in both extracts, are known to possess numerous biological properties like antimicrobial, anti-inflammatory, and antifeedant, as cited in the study of Qari and Khan (2019).

The current observation is also underpinned by the findings of Sobuj et al. (2021), who reported that methanolic extract contained a significant amount of phenolics and maximum quantity of total flavonoid content (TFC) when compared to ethanol and water extracts, which contained fewer amounts. It is important to note, however, that the degree of polarity affects the components of the extracted phytochemicals; otherwise, phytochemicals can be extracted with an appropriate solvent, not to mention phytochemical flavonoids, alkaloids, and saponins are capable of dissolving in polar solvents such as ethanol and methanol (Dayuti, 2018). Similarly, the fractionation and separation of different compounds such as pigments, alkaloids, and different groups of phenolic compounds, among others, is possible using different extraction solvents based on their polarity (Afonso et al., 2021).

Both extracts of the G. edulis exhibited inhibitory activity against the selected aquaculture pathogenic bacteria as opposed to the findings of Assaw et al. (2018), where the methanolic extract of Gracilaria sp. has possessed a moderate inhibitory activity against Bacillus subtilis, Staphylococcus aureus, S. epidermidis, Escherichia coli, Vibrio cholera, and Enterobacter cloacea, i.e., in agreement as well with the report of Qari and Khan (2019), where the ethanolic and methanolic extracts of selected Gracilaria spp. have exhibited good and greater zone of inhibition or antibacterial activity, respectively, against E. coli and Salmonella typhi (enteric pathogens).

However, it is interesting to note in the current investigation that the methanolic and ethanolic extracts resulted in higher ZOI for A. hydrophila and S. aureus when compared to the positive control. This can be due to the phytochemicals present in such extracts which have the potential to possess antibacterial activity, as reported in the study of Firdausy et al. (n.d.). The difference of inhibition zone, particularly in both extracts, where the ethanolic has shown lesser inhibitory activity when compared with methanolic is possible due to the presence of single bioactive compound (tannins), contrary to the latter with two constituents, namely flavonoids and tannins, exacerbated by the lack of saponins with antimicrobial properties (Dayuti, 2018; Qari and Khan, 2019). Dayuti (2018) further suggests that extracting G. verrucosa using more concentration of methanol can obtain bioactive compounds resulting in higher antibacterial activity and thereby producing more inhibitory potency than that produced by ethanol.

According to Salem et al. (2011), the discrepancies in results could be attributed to the differences in the susceptibilities of the microbial strains utilized. In general, earlier study indicated that seaweed extracts affected Gram-positive bacteria more than Gram-negative bacteria (Cagalj et al., 2022). Gram-negative bacteria's cell walls are more complex, making it more difficult for antibacterial chemicals to penetrate the cell, resulting in a firmer inhibitory zone (Gonelimali et al., 2018). This function of the outer cell wall explains why gram-positive species were more susceptible and less resistant to antibiotics than gram-negative bacteria (Alsenani et al., 2020). In the current investigation, the antibacterial activity of methanolic and ethanolic crude extracts of G. edulis were tested against a Gram-positive bacterium (S. aureus) and two Gram-negative bacteria (E. coli and A. hydrophila). Though G. edulis extracts demonstrated nearly similar inhibition zones against S. aureus (Gram-postive) and A. hydrophila (Gram-negative), it is worth noting that the Gram-negative bacterium E. coli was the least susceptible.
In view of the present study, knowledge of the inhibitory activity or zone of inhibition of G. edulis extracts against selected aquaculture pathogenic bacteria basically indicates or suggests its utilization as a possible antimicrobial agent in the future, in particular for diseases associated with aquaculture caused by certain bacterial pathogens; otherwise, its possible usage can be a potential source of natural therapeutics in the long term, thus beneficial for the pharmaceutical use.

CONCLUSIONS

In conclusion, this study aimed to assess the phytochemical constituents and antimicrobial properties of G. edulis extracts against select aquaculture pathogenic bacteria, namely A. hydrophila, E. coli, and S. aureus, using both ethanolic and methanolic extraction solvents.

The phytochemical analysis of G. edulis revealed the presence of tannins in the ethanolic extract and the presence of tannins and flavonoids in the methanolic extract. However, both extracts were found to be deficient in saponins.
Regarding antimicrobial activity, both ethanolic and methanolic extracts exhibited inhibitory effects against the tested microorganisms. Notably, the methanolic extract displayed a higher inhibition zone activity compared to the ethanolic extract. These findings indicate the potential of G. edulis extracts as antibacterial agents against these specific bacterial species.
Remarkably, the extracts demonstrated inhibitory activity comparable to that of the positive control, Amoxicillin. It is particularly intriguing that these extracts contain two phytochemicals, tannins and saponins, known for their biological activities, including antimicrobial properties. This suggests that G. edulis holds promise for further exploration and development in the field of therapeutic and medicine production, given its potential as a natural antimicrobial agent.
In summary, the study underscores the antimicrobial potential of G. edulis extracts, emphasizing their suitability for further investigation and development in the quest for novel antimicrobial agents in both aquaculture and pharmaceutical industries.

RECOMMENDATIONS

The government, in collaboration with academic societies and research institutions, should initiate and sponsor comprehensive programs and seminar workshops aimed at educating various stakeholders, including students, on the diverse and essential applications of red seaweeds, particularly Gracilaria edulis (G. edulis). These educational efforts should highlight its significance in food production, animal feeds, fertilizer manufacturing, medicinal purposes, and various commercial and industrial applications. Moreover, if such initiatives are not pursued, there is a need for the government to implement more robust policies and broader awareness campaigns to ensure that the knowledge and understanding of G. edulis usage are disseminated effectively.
To enhance our understanding of G. edulis, research efforts should extend to testing its antibacterial properties against a wider range of bacterial pathogens in aquaculture and among various fish pathogenic bacteria species. Moreover, exploration of alternative extraction methods, solvents, and analytical techniques for identifying and quantifying its

phytochemical constituents, including its antibacterial attributes, should be considered.
Additionally, it is advisable to further concentrate the extracted compounds into a dry, powdered form or vary the extract concentrations to assess their efficacy as antibacterial agents. Further research should involve conducting alternative antimicrobial susceptibility tests or confirmatory assays to detect and confirm the antibacterial activity of G. edulis extracts.
Lastly, future studies should delve into exploring other potential biological uses and activities of G. edulis extracts to potentially advance its role in aquaculture technology and interventions.

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