International Journal of Agricultural Technology 2024 Vol. 20(4):1341-1352
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Phytochemical screening and antibacterial activity of seaweed extracts from Bolinao, Pangasinan

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Cachin, E. J. D., Benico, G. A. and Waing, K. G. D. (2024). Phytochemical screening and antibacterial activity of seaweed extracts from Bolinao, Pangasinan. International Journal of Agricultural Technology 20(4):1341-1352.

Abstract Different samples that were collected and identified as *Laurencia flexilis* Setchell (Rhodophyceae), *Hormophysa cuneiformis* (J.F. Gmelin) P.C. Silva (Phaeophyceae), and *Sargassum polycystum* C. Agardh (Phaeophyceae). Tannins were detected in ethanol extracts of *L. flexilis* and *H. cuneiformis,* and in hot water extract of *S. polycystum*. Flavonoids were observed in both of the extracts of *H. cuneiformis,* but only in the hot water extract of *S. polycystum*. Saponins and terpenes were only detected in ethanol extract of *L. flexilis.* Meanwhile, steroids and alkaloids were not observed among all of the extracts. For the antibacterial activity, only the ethanolic extract of *H. cuneiformis* exhibited an activity against *S. auereus* after 12 hours of incubation. Whereas, all of the extracts showed no inhibitory activity against *E. coli.*

Keywords: Antibacterial activity, *H. cuneiformis, L. flexilis,* Phytochemical analysis, *S. polycystum*

Introduction

In recent years, the issue of drug resistance is constantly growing as synthetic antibiotics began to be ineffective against some pathogenic microorganisms (Patra *et al.*, 2009). Thus, the demand for new drug discoveries is highly increasing. Marine organisms are one of the most abundant sources of bioactive compounds and chemical diversity (Kijjoa and Sawangwong, 2004). These organisms are known to produce compounds with biological activities including antibacterial, antifungal, antiviral, etc. and have the potential as new therapeutic agents (Pérez *et al.*, 2016).

In the marine environment, marine algae are considered as one of the largest biomass producers (Bhadury and Wright, 2004). Seaweeds are multicellular marine macroalgae which act as primary producers and forms coastal ecosystems along the seashores.

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The chemical characterization of macroalgae extracts reveals a large range of compounds with interesting biological activities such as antibacterial, antifungal, anti-inflammatory, antivirus, and antioxidant activities (Rosa *et al.*, 2019).

According to Dr. Santiañez (2020) of the University of the Philippines – Marine Science Institute (UP-MSI), the Philippines has a very high diversity of seaweeds. It was also mentioned that the Philippines has the most diverse seaweed flora in the tropical Western Pacific. Locally, the country has more than 1, 000 seaweed taxa. However, this information regarding the seaweed resources is limited to a few areas only. Yet, to further reveal the diversity of seaweeds, more collection and identification efforts must be done.

In Pangasinan, Bolinao is known to have a productive coastal ecosystem that harbors a great diversity of marine species. In the taxonomic report written by Trono and Ohno (1992), there were 98 different species of seaweeds collected from several sites along the coast of Bolinao, Pangasinan. This only shows that there are diverse seaweed species which abound in this area. However, besides the studies regarding species diversity present in the province, the information related to the potential of antimicrobial activity is still lacking. Hence, this study intended to screen phytochemical constituents of seaweeds species present in Bolinao, Pangasinan using hot water and ethanol extracts and to determine their antibaterial potentials against bacterial pathogens – *Escherichia coli* and *Staphylococcus aureus.*

Materials and methods

Collection and identification of seaweed species

The collection of seaweeds having different morphology was done in Brgy. Patar, Bolinao, Pangasinan through handpicking using purposive sampling method. Collected samples were placed in plastic containers containing seawater. The seaweeds were washed with running water to remove extraneous materials.

The morphological evaluations were observed by measuring the holdfast, stipe, blade, and frond size. The characteristics that were taken into consideration include the comparison of structures, pigmentation, size, and shape among the species that were collected. The identification was based on the manual of Field Guide and Atlas of the Seaweed Resources of the Philippines (Trono, 1997).

Processing of identified seaweeds

The seaweeds samples were air-dried for 7 days under shady condition to prevent possible denaturation of the constituents. Then the samples were grinded into powdered form with the use of a blender and were used for further experiments.

Extraction procedure

Hot water and ethanol were used as solvents for the extraction of the seaweeds. 25 g of powdered seaweeds was mixed with 200 ml of solvent (1:8; sample: solvent ratio). In hot water extraction, the mixture was placed in a water bath with 80-90 °C for 2 hours. Consequently, the mixture was filtered using Whatman filter paper No. 1 and the filtrate was poured into a sterile amber bottle. In ethanol extraction, the powdered seaweeds was mixed with 80% ethanol in a sterile flask for 72 hours, and was filtered and subjected into rotary vacuum evaporator set at 45 °C, 60 rpm (Baguistan *et al.*, 2017).

Phytochemical screening

Phytochemical screening was determined based on the chemical test described by Trease and Evans (1983), Guevara and Recio (1985), Sofowora (1988), and Trease and Evans (2009). The phytochemical screening of the different seaweed extracts followed the standard methods as described in the Laboratory Manual for the UNESCO (1986) and noted as present (+) or absent (-). The test was done in triplicates.

Disc diffusion assay

To determine the antibacterial activity of seaweed extracts against *E. coli* and *S. aureus*, disc diffusion method after 12, 24, 36, and 48 hours of incubation was used. The bacterial cultures were grown in Nutrient Agar and transferred in Nutrient broth for 24 hours and was standardized to 1.5 x 10^8 cells/ml using 0.5 McFarland standards which was prepared by mixing 0.05ml of 1.175% Barium Chloride Dihydrate ($BaCl_2 \cdot 2H_2O$) with 9.95ml of 1% Sulfuric Acid (H_2SO_4) (Austria *et al*., 2017).

Statistical analysis

The statistical analysis was evaluated using one-way Analysis of Variance (ANOVA) at 5% level of significance in Statistical Package for the Social

Sciences (SPSS). The individual comparison of means was obtained using Duncan's Multiple Range Test (DMRT).

Results

Morpholigcal identification

The collected samples were identified using morphological characteristics following published taxonomic references of the species. Morphological assessments of the three samples show that they resembled *Laurencia flexilis* Setchell (Figure 1A), *Hormophysa cuneiformis* (J.F. Gmelin) P.C. Silva (Figure 1B), and *Sargassum polycystum* C. Agardh (Figure 1C).

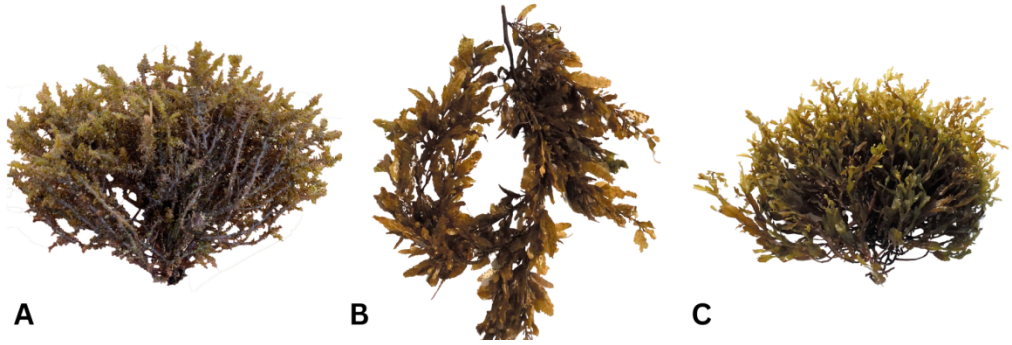

Figure 1. Collected samples in Bolinao, Pangasinan: A) *Laurencia flexilis* Setchell, B) *Hormophysa cuneiformis* (J.F. Gmelin) P.C. Silva, and C) *Sargassum polycystum* C. Agardh

L. flexilis Setchell (Figure 1A) is a species of red algae from the family Rhodomelaceae. It was mentioned in the study of Villanueva *et al*. (2010) that this species is atypical in the genus *Laurencia* and is an intermediate species between *Laurencia* and *Chondrophycus*. The thallus of the collected species appears to be cartilaginous and measures ~ 7 to 10 cm. The discoid holdfast measures about 5 mm which hold several a cluster of stipe. The stipe extends ~ 5 to 8 cm with tiny branchlets which is ~ 2 to 3 mm with decreasing length from the base to the tips. The pigmentation appears to be reddish brown in the stipe and yellowish green in the blades. This species is technically untapped as it has limited information for morphological characteristics.

H. cuneiformis (J.F. Gmelin) P.C. Silva (Figure 1B) or commonly known as "wedgeshaped chain-weed" is a species of brown algae in the family Saragassaceae. This species locally distributed in Bolinao, Pangasinan all year round from January to December. The thallus of the collected species is ~ 15-20 cm tall. The discoid holdfast responsible for attachment measures ~ 5-7 mm

in diameter. The stipe was approximately 3-5 cm long with primary branches which consists leaf-like winged branches with varying measurements that extends from 10-15 cm and is 3-4mm wide. The pigmentation spans from yellowish-brown to dark-brown and the marginal structure of the blades is serrated and possesses cryptostomata.

S. polycystum C. Agardh (Figure 1C) or locally known as "layog-layog" is also a species of brown algae in family Saragassaeceae (White, 2001). It is locally distributed and abundant in Bolinao, Pangasinan during the months of February to April, July to August, and December. The thallus of the collected species was ~ 35-45 cm in length. It has a small holdfast which measures ~ 7-10 mm in diameter. The main stipe is cylindrical which has a diameter of ~ 5 mm and has alternating branches with leafy appendages. The size of the blade varies in terms of their position ranging from 3-5 cm in length and ~ 5 to 10 mm wide. It also has varying size of vesicles ranging from 1-2.5mm long and 3-5 mm wide. The pigmentation of this species is yellowish brown to dark brown. The margin of the blade is serrate-dentate and it also possesses numerous cryptostomata.

Phytochemical screening

Results showed the phytochemical screening of the hot water and ethanol extract of three different seaweed species. Secondary metabolites such as flavonoids were presented on *H. cuneiformis* (HWE & EE) and *S. polycystum* (EE) (Table 1). Tannins were observed in *L. flexilis* (EE), *H. cuneiformis* (EE), and *S. polycystum* (HWE). Meanwhile, saponins and terpenes were only detected in *L. flexilis* (EE). Steroids and alkaloids were not observed in either of the extract of seaweed species.

Table 1. Phytochemical analysis of hot water and ethanol extracts of the different seaweed species

PHYTOCHEMICALS	*L. flexilis*		*H. cuneiformis*		*S. polycystum*	
	HWE	EE	HWE	EE	HWE	EE
Flavonoids	-	-	+	+	-	+
Tannins	-	+	-	+	+	-
Saponins	-	+	-	-	-	-
Terpenes	-	+	-	-	-	-
Steroids	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-

Antibacterial activity against E. coli

The antibacterial activity of hot water and ethanolic extracts of the different seaweeds species were tested against *E. coli*. Table 2 shows the zones of inhibition of the treatments after 12, 24, 36, and 48 hours. However, all of the extracts did not exhibit antibacterial activity against *E. coli*, but was susceptible to streptomycin.

Table 2. Diameter of zones of inhibition (mm) of the different treatments against *E. coli* after 12, 24, 36, and 48 hours of incubation

TREATMENTS	12 HOURS	24 HOURS	36 HOURS	48 HOURS
L. flexilis (EE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
L. flexilis (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
H. cuneiformis (EE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
H. cuneiformis (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
S. polycystum (EE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
S. polycystum (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
Streptomycin	26.80±0.08[b]	25.65±0.23[b]	24.12±0.23[b]	22.87±0.81[b]
Distilled Water	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]

*Values are expressed as mean±SD of the zones of inhibition of the three-replicate test. Values with the same letters of superscript are not significantly different using DMRT.

Antibacterial activity against S. aureus

The antibacterial activity of the hot water and ethanolic extracts of different seaweeds species were also tested against *S. aureus.* Results revealed that zone of inhibition was only observed in ethanolic extract of *H. cuneiformis* (Figure 2, T3) at 12 hours of incubation with 6.63mm±0.44 (Table 3). However, it was significantly lower than the positive control.

Table 3. Diameter of zones of inhibition (mm) of the different treatments against *S. aureus* after 12, 24, 36, and 48 hours of incubation

TREATMENTS	12 HOURS	24 HOURS	36 HOURS	48 HOURS
L. flexilis (EE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
L. flexilis (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
H. cuneiformis (EE)	6.63±0.44[c]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
H. cuneiformis (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
S. polycystum (EE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
S. polycystum (HWE)	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]
Streptomycin	33.47±0.41[b]	29.72±0.50[b]	22.13±0.19[b]	20.77±0.59[b]
Distilled Water	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]	5.00±0.00[a]

*Values are expressed as mean±SD of the zones of inhibition of the three-replicate test. Values with the same letters of superscript are not significantly different using DMRT.

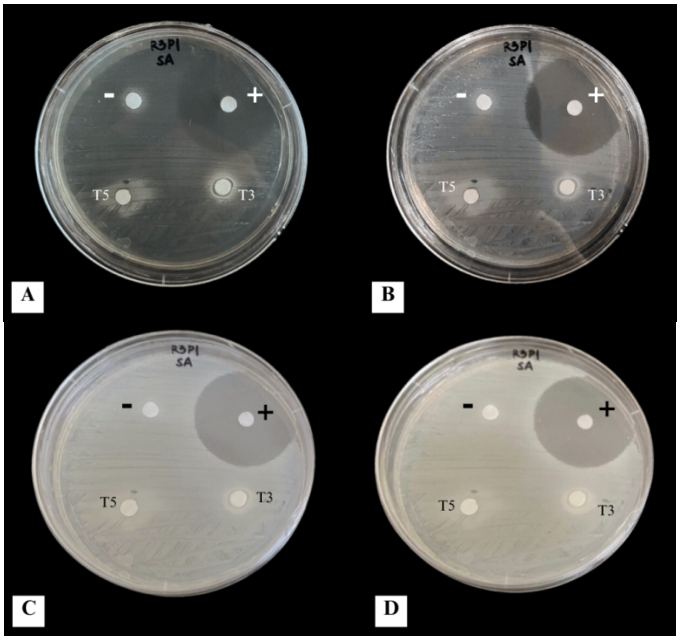

Figure 2. Zones of inhibition of the treatments after A) 12, B) 24, C) 36, and D) 48 hours of incubation against *S. aureus*

Discussion

Studies revealed that marine organisms, such as seaweeds, are considered as one of the most abundant sources of bioactive compound with biological activities such as antibacterial, antifungal, antiviral, etc. having the potential as new therapeutic agents.

With the great diversity of seaweed species in Bolinao, Pangasinan, three different seaweeds were collected and identified as *Laurencia flexilis* Setchell*, Hormophysa cuneiformis* (J.F. Gmelin) P.C. Silva, and *Sargassum polycystum* C. Agardh.

Phytochemical screening based on Laboratory Manual for the UNESCO (1986) revealed that flavonoids, tannins, saponins, and terpenes are present in some of the extracts of the different seaweed species.

The presence of flavonoids was observed in both hot water and ethanolic extract of *H. cuneiformis*. Also, its presence was observed in the ethanolic extract of *S. polycystum,* but neither of the extracts of *L. flexilis.* These results coincide with the findings of Arsianti *et al.* (2020) which reported the presence of flavonoids in the ethanolic extract of *S. polycystum* and El-Nuby *et al.* (2021) which demonstrated positive results for aqueous and ethanolic extract of *H. cuneiformis*.

Tannins was observed in the ethanol extracts of *L. flexilis* and *H. cuneiformis.* Meanwhile, it was also observed in *S. polycystum* but only in the hot water extract. This result is in accordance with the reports of Johnson *et al.* (2015) which revealed the presence of tannins in aqueous extract of *S. polycystum* and El-Nuby *et al.* (2021) which depicted a positive result for tannins in the ethanolic extract of *H. cuneiformis*.

Saponins was only detected in the ethanol extract of *L. flexilis.* There were limited studies with regards to the phytochemical analysis of *L. flexilis*. However, based on other species under *Laurencia* genus, *Laurencia obtusa* specifically exhibited the presence of saponins (Johnson *et al.*, 2015).

Terpenes was only observed in the ethanolic extract of *L. flexilis.* In the study of De Nys *et al.* (1993)*,* they have determined that presence of a class of terpenoid which coincides with the result of this study. In addition, the species used in their investigation was collected locally from Ilocos Norte, Philippines.

Meanwhile, the findings of the study revealed that both steroids and alkaloids were not present among the extracts of the three different seaweed species.

For the antibacterial activity against *E. coli*, the main mechanisms by which microorganisms exhibit resistance to antimicrobials include impermeability, modification, efflux pumps, and inactivation through a phosphate group (Lomartire and Goncalves, 2023). Resistance of gram-

negative bacteria is highly correlated with the presence of additional protection by the outer membrane (Epand *et al*., 2016). In addition, the effectiveness of seaweed extracts in inhibiting the bacterial growth is related to the synergistic effect between the active compounds of the extracts (Wagner and Ulrich-Merzenich, 2009). Based on the conducted phytochemical screening in this study, it is depicted that only few secondary metabolites were present among the extracts. Also, the result was in accordance with the study of Madkour *et al.* (2019) wherein the seaweed extracts displayed no inhibitory action against gram negative bacteria. The mechanism of bioactive compounds against bacterial pathogens is through the alteration of permeability and loss of internal macromolecules (Arguelles, 2018). Thus, gram negative bacteria having multi-layered cell walls make it hard for active compounds to penetrate within bacterial cells.

On the other hand, zone of inhibition was observed in ethanolic extract of *H. cuneiformis* at 12 hours of incubation with 6.63mm±0.44. However, it is significantly lower than the positive control. The resistance of *S. aureus* after 24 hours of incubation can be explained by the capability of the bacteria to adapt overtime and develop genetic changes allowing them to resist antibacterial agents (Biggers, 2022). This may also be the reason behind the decrease of efficiency and zone of inhibition of the positive control.

Organic solvents like ethanol can extract active compounds that exhibit antimicrobial activities. Based on the phytochemical screening, flavonoids and tannins were detected in the ethanolic extract of *H. cuneiformis.* Previous studies have reported that flavonoids can supress cytoplasmic membrane function, nucleic acid synthesis, and energy metabolism (Xie *et al*., 2015; Górniak *et al,* 2018; Donadio *et al.,* 2021). In addition, it was found out that it reduces adhesion and biofilm formation, membrane permeability, and pathogenicity which are vital for bacterial growth (Abreu *et al.,* 2015; Song *et al.,* 2021). On the other hand, tannins also have the ability to penetrate bacterial cell wall up to the internal membrane and interfere with the metabolism of the cell which leads to destruction (Kaczmarek, 2020). Thus, the antibacterial activity exhibited by the ethanolic extract of *H. cuneiformis* is probably due to the presence of the aforementioned secondary metabolites. The non-inhibitory activity of other extracts against *S. aureus* can be attributed with the absence of these metabolites.

Acknowledgements

The author would like to thank Kristine Grace D. Waing, MSc. and Dr. Garry A. Benico, her thesis adviser and thesis critic respectively for sharing their knowledge and expertise throughout the conduct of this study. Also, to the whole faculty of the Department of

Biological Sciences, Biodiversity Laboratory, and Philippine Center for Postharvest Development and Mechamization. Without the help of these people and institution, completing this undertaking would not have been possible.

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(Received: 7 November 2023, Revised: 28 June 2024, Accepted: 30 June 2024)