ORIGINAL ARTICLE

Zoochemical Analyses and *In vitro* Antimicrobial Activity of Crude Methanolic Extract of *Perna viridis*

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ABSTRACT

Introduction. The rise of antibiotic resistance and superbugs drives the search for new antibiotics today. Meanwhile, the green mussel *Perna viridis* is a cultivated and marketed staple bivalve in the Philippines due to its fast reproduction, high protein content, and tolerance to environmental variables. Although some studies have analyzed the antimicrobial activity of *P. viridis*, zoochemical analyses and further evaluation of its antimicrobial activity, such as determining the minimum inhibitory concentration (MIC), remains unexplored.

Objectives. The study evaluated the zoochemicals present in crude methanolic extract of *P. viridis* by qualitative screening and thin-layer chromatography analysis. It further evaluated the crude extract for its antimicrobial activity against common pathogenic bacteria and plant pathogenic fungi.

Materials and Methods. The zoochemicals in crude methanolic extract of *P. viridis* were screened using qualitative spotting methods and thin-layer chromatography (TLC). The antimicrobial activity of the extract was evaluated against the bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*, and the fungi *Colletotrichum capsici*, *Lasiodiplodia theobromae*, and *Rhizopus* sp. using disk diffusion assay and two-fold microdilution.

Results. Qualitative screening and thin-layer chromatography analysis of the crude extract revealed detectable amounts of alkaloids, saponins, terpenoids, sterols, and polyphenols. All of the tested bacteria were susceptible to the extract with *P. aeruginosa* (19.00±0.82 mm) and *S. aureus* (19.33±0.47 mm) as the most inhibited with MICs of 2.60±0.63 and 3.65±1.69 mg/mL, respectively. However, for the three fungi tested, only the growth of the fungus *L. theobromae* (7.33±0.94 mm) was inhibited with a MIC of 33.33±11.79 mg/mL.

Conclusion. It can be inferred that the zoochemicals detected in the crude extract of *P. viridis* contributed to its antimicrobial activity.

Key Words: Antibacterial agent, Antifungal agent, Green mussel, Plant pathogenic fungi, Secondary metabolites

INTRODUCTION

The ocean is home to organisms that produce bioactive metabolites. The biodiversity that the ocean houses may lead to the ecosystem becoming a renewable repository of solutions for global issues such as the threat of antibiotic resistance. With the current trend on the evolution of resistance of pathogens to existing antibiotics, marine faunae are sought for their biomolecules to counter the demand for novel compounds.[1] Currently, there is an increased interest in the screening of antimicrobial compounds isolated from molluscan species.[2] The bivalve *Perna viridis* gained attention in the research field in the Philippines due to its varying economic and trade dynamics despite the industrial biofouling issue.[3] Some studies have investigated the anti-

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inflammatory, antioxidant, antibacterial, and antifungal bioactivities of *P. viridis*.[4-6] The antimicrobial compounds present in the methanol and ethanol extracts of *P. viridis* was identified in another study using high-performance thin-layer chromatography (HPLC). Based on the results, the fractionated components were protein by nature instead of possessing properties that were related to secondary metabolites.[7] From the mentioned bioactivities, the majority of the investigations inferred proteins to be responsible for their bioactive potential. However, only a few studies have suggested secondary metabolites as the ones responsible for its bioactivity.

Secondary metabolite is a broad classification of biomolecules under the umbrella term zoochemicals found in fauna.[8] With the current interest in extracting valuable metabolites, such as terpenoids and sterols, in marine organisms, *P. viridis* may serve as a prolific source of secondary metabolites mainly active for defense.[9] Hence, a baseline study to further identify bioactive compounds, not only proteins in *P. viridis,* is needed.

Furthermore, studies conducted on the antimicrobial bioactivity of *P. viridis* extracts poorly studied the Minimum Inhibitory Concentration (MIC) against the tested organisms even if the zones of inhibition observed were inferred to as susceptible compared to the standards. Bridging its activity on both fungal and bacterial test organisms, this study serves as baseline on a wide range of spectrum for the methanolic extract of *P. viridis* exhibiting antimicrobial capacity using secondary metabolites. It also determined the MIC on susceptible test organisms. This also pioneers the testing of a zoochemical-based extract on plant pathogens. To our knowledge, there are no known studies on the antifungal activity of animal extracts against the plant pathogen fungi *Colletotrichum capsici* and *Lasiodiplodia theobromae.*

MATERIALS AND METHODS

Mussel Methanolic Extraction

Perna viridis samples were bought from Jiabong, Samar. These were sent to the Bureau of Fisheries and Aquatic Resources for identification and certification. The samples were freed of adhering debris using tap water and followed with distilled water. One hundred fifty-two (152) grams *Perna viridis* whole mussel tissue was separated from the shells. These were homogenized with a blender and macerated following a 1:2 and 1:1 homogenate weight to methanol volume ratios. Initially, the homogenates were macerated (1:2) with 304 mL Methanol (MeOH) and incubated for 48 hours at 4°C. The mixture was centrifuged at 6000 rpm for 20 minutes and the supernatant was collected and vacuum-filtered (Nalgene filtration bottle) using Whatman paper no.1. The resulting residue was extracted using 152 mL MeOH (1:1) following the same 1:2 weight-to-MeOH volume ratio. The supernatant were pooled and concentrated using a rotary evaporator at 35-55°C under reduced pressure.[5]

Zoochemical Screening

Test for Alkaloids

Ten (10) mg of the sample was obtained and 2 mL of 2% HCl was added. The sample was boiled in a hot water bath and the mixture was filtered. Two drops of 1% picric acid solution was added to 1 mL of the filtrate. The formation of a yellow precipitate indicated the presence of alkaloid.[10]

Test for Flavonoids

Ten (10) mg of the crude sample was mixed with 5 mL ethyl acetate. The mixture was heated for 3 minutes and then filtered. The filtrate (4 mL) was mixed with 1 mL diluted ammonia solution. The formation of an intense yellow coloration indicated the presence of flavonoids.[11]

Test for Polyphenols

Two (2) drops of ferric chloride (5%) was added to the 10 mg sample extract dissolved in 2 mL distilled water. The appearance of brown precipitate confirmed the presence of polyphenols.[12]

Test for Saponins

Ten (10) mg of the crude extract was obtained and added to 5 mL distilled water. The mixture was shaken and boiled. The appearance of frothing which was stable for 15- 30 min or longer confirmed the presence of saponins.[13]

Test for Sterols – Liebermann-Burchard Test

One (1) mL glacial acetic acid and 1 mL chloroform solution was prepared and cooled to 0°C. A drop of concentrated sulfuric acid was added. Five (5) mL of the aqueous suspension sample was then added. The blue, green, red, or orange colors that changed with time confirmed the presence of sterols.[13]

Test for Terpenoids – Salkowski Reaction

One to two (1-2) mg of the sample was dissolved in 1 mL chloroform and 1 mL concentrated sulfuric acid was added. The formation of two phases with a red or yellow coloration confirmed the presence of terpenoids and sterols.[14]

Test for Cardiac glycosides – Keller-Kiliani Test

Ten (10) mg sample was mixed with 1 mL of glacial acetic acid. Then, two drops of 5% Ferric chloride followed by two drops of concentrated sulfuric acid was added. A green-blue coloration indicated the presence of cardiac glycosides.[15]

Thin-Layer Chromatography Profiling

Thin-layer chromatography was used to further evaluate the methanolic extract of *P. viridis* whole mussel tissues. TLC plates precoated with silica gel were cut into 4x10 cm plates using a common household cutter. The plates were marked using a pencil and were loaded with 2000 ppm of the extract 1 cm from the bottom. The TLC plates were

developed in a 400 mL beaker with 10 mL of the solvent systems, Chloroform: Methanol: Water (6.5:2.5:1)[16], Chloroform: Methanol: Glacial Acetic Acid (83:17:10)[17], Methanol: Dichloromethane: chloroform (30: 35: 35)[4], Ethyl acetate: Methanol: Water (100: 13.5: 10)[18], and Toluene: Chloroform: Acetone (5:3.1:4.3)[19]. The plates were developed using one-dimensional ascending method then viewed under UV light at 366 nm.

The solvent system which developed the best chromatogram in terms of resolution and number of spots was further used for the derivatization of spots using the detection reagents Dragendorff's and Liebermann-Burchard. Separate chromatograms were developed for each derivatization reagent. In the case of Liebermann-Burchard Reagent, the chromatogram was heated at 100°C after spraying to develop the spots.[20] The Rf values were measured using a ruler and the plates were photographed and analyzed using ImageJ 1.51j8 in order to estimate the intensity and to measure the Rf value of each spot.

Pathogen Procurement

The bacterial cultures of *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 23857), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923) were obtained from the Department of Science and Technology VIII, Leyte, Philippines. All bacterial cultures were stored in Nutrient agar (NA) slants at 4°C.

Plant pathogenic fungi *Lasiodiplodia theobromae*, *Colletotrichum capsici*, and *Rhizopus* sp. were obtained from the Plant Disease Diagnostic Laboratory, Visayas State University, Philippines. The fungal cultures were recently isolated from infected plants and crops, as noted by the Research Specialist. All fungal cultures were maintained in Potato Dextrose Agar (PDA) slants at 4°C. The microbial pathogens were stored and handled in a research laboratory with Biosafety level 2 (BSL-2). Upon execution of the microbiological aspect of the study, the antimicrobial testing was performed in a culture room with accessible UV-radiation for room decontamination before the execution of experiments in the biosafety cabinet.

Antimicrobial Activity Assay

The Disk diffusion method was used to perform the antibacterial assay and the antifungal assay.[21,22] The 100 mg/mL crude methanolic extract was prepared by dissolving 200 mg of the extract in 2 mL sterile distilled water. The extract was stored in a sterile vial at 4°C until the antimicrobial assays were performed. A 0.5 McFarland standard solution was also prepared before culture inoculations as turbidity standard for the culture suspensions. Briefly, 0.05 mL of 1.175% Barium chloride dihydrate ($BaCl \cdot 2H_20$) was mixed with 9.95 mL of 1% sulfuric acid (H_2SO_4). For standardization, the prepared solution was adjusted to an absorbance reading range of 0.08 to 0.1 at 625nm using a spectrophotometer.[23,24]

Before bacterial and fungal broth suspension preparation, all bacteria were subcultured in NA slants and incubated at 35°C for 18-24 hours. Meanwhile, all fungi were maintained and subcultured in Potato Dextrose Agar (PDA) slants and incubated at 35°C for five days.

The bacteria were inoculated in a Luria Bertani Broth (pH 6-7.5) and incubated at 35°C for at least 18-24 hours to prepare a bacterial suspension. The bacterial suspension was compared to a 0.5 McFarland solution and was adjusted accordingly. If the opacity does not coincide with the 0.5 McFarland standard, the opacity was reduced by adding sterile broth or increased by adding more bacterial inoculum. The swab method was then used to inoculate the bacteria to a Mueller-Hinton Agar. The inoculum was dried at room temperature for 10-15 min to ensure that excess moisture was absorbed and a confluent lawn of bacteria was produced.

In the fungal test organisms, the fungal suspensions were prepared by flooding the previously incubated culture with a 4 mL 0.9% Sterile Saline solution. The cultures were gently swabbed to dislodge the spores or conidia from the mycelial mat. The resulting suspension was allowed to settle for 5 minutes at room temperature and then transferred to sterile screw-capped test tubes. The suspensions were then standardized to $1x10^6$ spores/mL using a hemocytometer. The swab method was employed to inoculate the 200µL fungal suspension to the Potato Dextrose Agar.[23]

Three types of disks were separately prepared by impregnating 6 mm sterile Whatman no. 1 filter paper disks with sterile water as negative control, streptomycin as positive control for bacteria and fluconazole for fungi, and the crude MeOH extract.

For the crude extract, a 100 mg/mL concentration was prepared from storage and 50 µL was added to the disks. Sterile water (50 µL) was added as negative control and 50 µL streptomycin or fluconazole (10 mg/mL) was eluted as positive control. After an hour of drying, the disks were placed on the surface of the agar and incubated at 35°C for 24 h (bacteria) or 3-5 days (fungi) in an inverted position. The three disks were placed equidistantly. The set-ups were performed in triplicates. Zone of inhibition (ZOI) was measured using a ruler.[23,24]

Minimum Inhibitory Concentration Determination

The Minimum Inhibitory Concentration (MIC) was determined for the crude extract which showed the highest antibacterial activity against the microorganisms tested as suggested by the National Committee for Clinical Laboratory Standards.[24] The two-fold microdilution method was used to determine the MIC of the crude extract. The stock solutions of the crude extract were prepared by dissolving in distilled water (100 mg/mL). The crude extract was then tested at a concentration ranging from 100 mg/mL to 0.78 mg/mL.[24] All bacterial suspensions were prepared, and the turbidity was compared to the 0.5 McFarland standard to signify 10^5 CFU/mL.

On the other hand, the prepared standardized fungal suspensions at $1x10^6$ spores/mL were used. A 50 µL bacterial/fungal suspension was inoculated to the medium and extract dilution and was incubated at 35˚C for 18-20 h (bacteria) or 3-5 days (fungi). The lowest concentration which displayed inhibition of bacterial growth was recorded as the MIC.

Biological samples disposal

After the execution of the microbiological assays, glasswares containing bacterial and fungal cultures were all autoclaved at 121˚ C and 15 psi (1.02 atm). Similarly, the same decontamination and sterilization procedure was followed for all of the glasswares used. Waste and used materials were disposed of properly in waste bags intended for biological samples provided by the General Laboratory of the University of the Philippines Visayas Tacloban College, Philippines.

Data Analysis

All tests were carried out in three replications and the data obtained were presented as mean ± standard deviation.

RESULTS

Zoochemical classes of *Perna viridis*

The methanolic extract of the bivalve contained detectable levels of zoochemicals such as saponins, sterols, alkaloids, polyphenols, and terpenoids (Table 1).

Thin-Layer Chromatography Profile

Semi-quantification by TLC analysis yielded Rf values of zoochemicals present in the extract. Retention factor (Rf) value refers to the ratio of the distance moved by the compound and the distance moved by the solvent. The crude extract has various zoochemicals (Table 2). Development of the crude extract in all of the solvent systems except Chloroform: Methanol: Water (6.5:2.5:1) gave only one spot as viewed under UV light at 366 nm. The development of the chromatogram at the solvent system Chloroform: Methanol: Water (6.5:2.5:1) gave four spots with Rf values of 0.87, 0.82, 0.77, and 0.63. Among the spots, the spot with an Rf value of 0.87 was the most intense with a percentage of 33.90%. On the other hand, the spot with an Rf value of 0.63 gave off the least intense color under UV light with an intensity percentage of 19.30%. Nevertheless, out of all the solvent systems, the latter gave the highest number of spots; hence, it was used to develop a chromatogram to be sprayed further by derivatization reagents.

The chromatograms developed at the solvent system Chloroform: Methanol: Water (6.5:2.5:1) were sprayed with Dragendorff's reagent and Liebermann-Burchard reagent separately to identify the classes of zoochemicals present in the sample extract. Dragendorff's reagent and Liebermann-Burchard reagent gave colored spots that can be viewed under UV light at 366 nm (Table 3). Two red spots were generated using Dragendorff's reagent with Rf values of 0.92 (Alkaloid 1) and 0.85 (Alkaloid 2). The red coloration indicated that the compounds present after spraying were alkaloids. The Rf values of the spots were also relatively high which signified that the separated compounds have medium polarity or were slightly polar. Results also showed that the spots generated after spraying with Dragendorff's reagent did not match any of the Rf values generated in the different solvent systems. This implied that the spots generated were separate or distinct from the spots generated from the different solvent systems.

Two spots were generated after derivatization with Liebermann-Burchard Reagent (Table 3). The spots were differently colored where the spot with an Rf value of 0.93 was colored gray and the spot with an Rf value of 0.40 was blue. The gray coloration indicated that the spot was

Table 1. Qualitative zoochemical screening of the crude methanolic extract of *P. viridis*

Zoochemical	Observation	Result
Saponins	Two centimeters honeycomb-like froth	+
Polyphenols	Brown precipitate	+
Flavonoids	Pale Yellow Coloration	-
Alkaloids	Yellow Precipitate	+
Sterols	Green Coloration	+
Terpenoids	Yellow layers with reddish interface	+
Cardiac glycosides	Pale green coloration	-

Note: "+" indicates positive reaction; "-" may mean negative or undetectable amount

Table 2. Rf values and intensity percentages of the spots generated in different solvent systems from the Thin-Layer Chromatography (Silica gel) analysis

Solvent System	No. of Spots	Rf Values	Intensity (%)
Chloroform: Methanol: Water (6.5:2.5:1)	4	0.87	33.90
		0.82	21.77
		0.77	25.03
		0.63	19.30
Chloroform: Methanol: Glacial Acetic acid (83:17:10)	1	0.94	-
Methanol: Dichloromethane: Chloroform (30:35:35)	1	0.96	-
Ethyl acetate: Methanol: Water (100:13.5:10)	1	0.96	-
Toluene: Chloroform: Acetone (5:3.1:4.3)	1	0.97	-

Table 3. Colors, Rf Values, and Intensity Percentages of the spots generated in Chloroform: Methanol: Water (6.5:2.5:1) after derivatization with spraying reagents

Derivatization Reagent	No. of spots	Color of spots	Rf Values	Intensity (%)	Compound Class
Dragendorff's Reagent	2	Red	0.92	3.36	Alkaloid 1
			0.85	42.59	Alkaloid 2
Liebermann-Burchard Reagent	2	Gray	0.93	44.54	Terpenoid 1
		Blue	0.40	9.51	Saponin 1

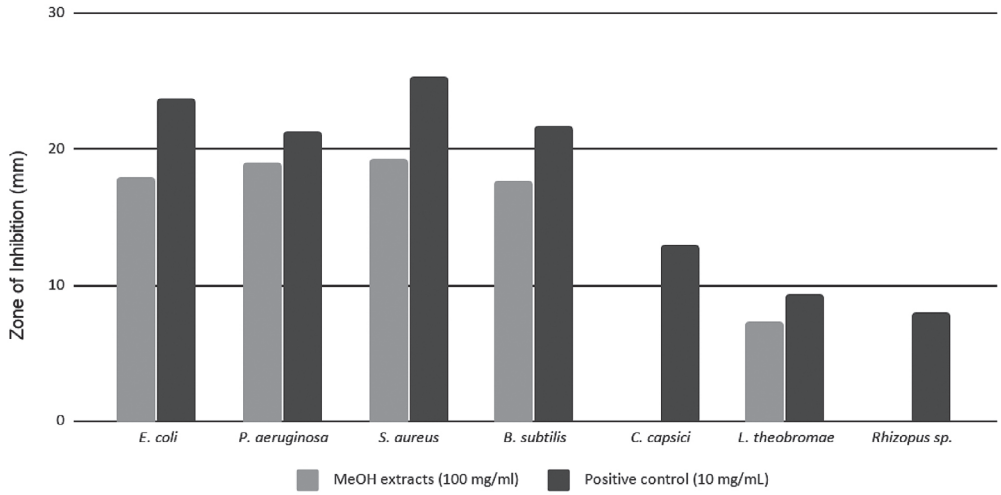

Figure 1. Antimicrobial activity of the crude methanolic extract of *P. viridis* (disk diameter is 6 mm).

classified as terpenoid while the blue coloration indicated the compound as saponin (Wagner and Bladt 2001). The Rf values did not match the Rf values in the different solvent systems. Even more, a spot with an Rf value of 0.40 was generated which implied that it was the most polar among the other compounds present in the extract. Among the spots, Alkaloid 2 and Terpenoid 1 gave the most intense spots compared to the other two spots with an intensity percentage of 42.59% and 44.54%, respectively. Hence, Alkaloid 2 and Terpenoid 1 comprised the majority of compounds separated by the solvent system.

Antimicrobial Activity

In this study, the antimicrobial activity of the crude methanolic extract at 100 mg/mL of *P. viridis* was screened using the disk diffusion assay. The study reported that all of the test bacteria were susceptible to the crude methanolic extract as compared to the standards published by the Clinical and Laboratory Standards Institute on antimicrobial susceptibility testing[25] (Figure 1). Moreover, the results also revealed that the growth of *P. aeruginosa* and *S. aureus* were highly inhibited with mean zones of inhibition at 19.00±0.82 mm and 19.33±0.47 mm, respectively. Meanwhile, the bacteria *E. coli* and *B. subtilis* were the least inhibited with a mean zone of inhibition of 18.00±1.25 mm and 17.67±0.94 mm, respectively. In the case of fungi, only *L. theobromae* was inhibited by the crude extract with 7.33±0.94 mm of the zone of inhibition but interpreted as resistant based on the standards published by CLSI on non-dermatophyte fungi.[26] The extract had no antifungal activity against the rest of the test fungi, *Rhizopus* sp. and *C. capsici.*

Minimum Inhibitory Concentration

The MIC assay using broth microdilution was performed on the bacteria and fungi that were inhibited by the extract, which included *E. coli, P. aeruginosa, S. aureus, B. subtilis,* and *L. theobromae* (Table 4). However, standard MICs by CLSI suggested that the observed values in this study were still classified as resistant.[25] Nevertheless, the extract was able to inhibit bacterial growth in various concentrations. The least MIC recorded was on *S. aureus* with a concentration of 2.60±0.63 mg/mL.

Table 4. Minimum Inhibitory Concentration (MIC) of the crude methanolic extract of *P. viridis* against susceptible bacteria and fungus

Test Organism	MIC (mg/mL)
E. coli	8.33±2.55
S. aureus	2.60±0.63
P. aeruginosa	3.65±1.69
B. subtilis	10.42±2.55
L. theobromae	33.33±11.79

Meanwhile, among the bacteria, the most excellent MIC recorded was against *B. subtilis* with a MIC of 10.42±2.55 mg/mL. For the sole fungi, the recorded MIC was 33.33±11.79 mg/mL. Due to the unavailability of data for *L. theobromae*, no standard reference range could be compared in terms of MIC. However, CLSI suggests that any MIC value less than 64µg/mL in comparison to fluconazole as the positive control is inferred to as resistant.[26]

DISCUSSION

The results of this study agreed with Sreejamole and Radhakrishnan that alkaloids, saponins, sterols, and polyphenols are present at detectable amounts in the crude methanolic extract of *P. viridis* whole mussel tissue.[4] This study, however, revealed that terpenoids were also present in the methanolic extract of *P. viridis*.

Terpenoids have also been isolated in bivalve clams. Terpenoids, such as isopimarane norditerpenoids, act as precursors for the synthesis of sterols and steroids in bivalves. Since bivalves are filter and detritus feeders,

algae and plankton are also sources of a wide variety of zoochemicals found in their system which include terpenoids and alkaloids.[27,28]

Sterols, such as cholesterol, are dominant in the class Bivalvia partly because of their antibacterial and protective role in spawns during their reproductive cycles.[29] Polyphenols and saponins are present at detectable amounts in the crude methanolic extract of *P. viridis*. Novel polyphenols and saponins, however, are seldom isolated from bivalves.[30] The occurrence of polyphenols and saponins in the extract of *P. viridis* using methanol as solvent have been studied and published.[4,31] Gallic acid is a possible identifier of the polyphenol compound because they are commonly isolated from mytilids as part of their endogenous defense system.[32] Meanwhile, further identification of the saponin compound present in this study is needed as there are no published reports of the occurrence of novel saponins from *P. viridis*.[33,34] Possibly, the saponins may have originated from the plankton diet of *P. viridis* such as the diatoms *Coscinodiscus* spp. and *Phaeodactylum* spp.[35]

In this study, the solvent system chloroform-methanol-water gave the best results, which could mean that the compounds separated well in slightly polar solvents. This result was expected because the solvent used in the study was 95% Methanol, a polar solvent. Alkaloid 2 and Terpenoid 1 gave the most intense spots compared to the other two spots providing an insight that the dominant compounds present in the extract originated from the diet of *P. viridis,* particularly planktonic microorganisms.[28] Marine microalgae such as the genera *Chlorella*, *Skeletonema, Dunaliella, Tetraselmis,* and *Isochrysis* produce a considerable amount of phytochemicals such as alkaloids and terpenoids which could have been pooled in the tissues of bivalves because of their diet.[36-38]

In previous studies, *P. viridis* has been screened for its antibacterial activity against *E. coli, Klebsiella pneumonia, Vibrio parahaemolyticus, Vibrio cholerae* and many other bacteria.[5] The literature suggests that the antibacterial activity of *P. viridis* was attributed to zoochemicals namely proteins, peptides, free amino acids, and lipids present in the crude methanolic extract. Literature also suggested that the screened molecules are polar by nature because methanol was used as the solvent.[5,39] Nevertheless, the results agreed with the study conducted by Shanmugam et al. that the crude methanolic extract of *P. viridis* has higher growth inhibition against *Pseudomonas* sp. (13.2 mm) and *S. aureus* (13.23 mm) as compared to *E. coli* (5.26 mm) and *B. subtilis* (5.06 mm) at a dose of 2 mg/mL.[5] Also, the results agreed with the study conducted by Madhu et al. that the extract has a growth inhibitory effect against *E. coli* (10 mm) at a dose of 100 µg/mL.[39] In these studies, the authors implied that the antimicrobial activity of the methanolic crude extract may have been derived from the zoochemicals present such as antimicrobial peptides and narrowly, secondary metabolites such as alkaloidal compounds.[40,41]

The fungal species in this study have efflux pumps in their cell membrane which can decrease the concentration of the antifungal agent which led to the antifungal resistance.[42] Both *Rhizopus* sp. and *C. capsici* have been reported to be characterized by ATP-driven efflux pumps.[43,44] This provided an insight into the resistance of these fungi against the crude methanolic extract. Because *L. theobromae* was inhibited in terms of growth, a possible mechanism of antifungal activity of the candidate terpenoid and alkaloid in the extract would be the inhibition of germ tube formation and the inhibition of membrane-localized enzyme modifications, respectively.[45-47] The antibacterial activity of the alkaloids and terpenoids present would possibly have mechanisms involving inhibition of the synthesis of ergosterol, the formation of aqueous pores, and inhibition of macromolecules.[48,49] Particularly, candidate terpenoids may exhibit antibacterial activity by cytoplasmic degradation and cell membrane damage by promoting K^+ ion leakage.[48] Consequently, candidate alkaloids can exhibit antimicrobial activities by inhibiting biofilm formation and enzyme modification such as membrane transporters.[49] To date, most reports published on the antifungal activity of the extract against *L. theobromae* and *C. capsici* are of plant origin. No reports have been published on the screening of the antifungal activity of animal extracts against *L. theobromae* and *C. capsici*. Publications for zoochemicals screened against plant pathogens are limited, hence, little is known on their mechanism against the plant pathogenic fungi.

Meanwhile, the MICs reported in this study were comparable to the MICs reported in other literature, with the extract in this study inhibiting the growth of *S. aureus* at a lower concentration (2.60 mg/mL) compared to the study of Borquaye et al. which was at 5 mg/mL.[50] Likewise, it was hypothesized that the compounds responsible for the inhibition of growth of the test bacteria were zoochemicals which were present in the tissues of the mollusks *Littorina littorea* and *Galatea paradoxa*.[50]

For the fungus inhibited in this study, the MIC against *L. theobromae* (33.33 mg/mL) was not as remarkable compared to other studies made on the inhibition of *L. theobromae* using plant extracts.[51,52] The study hypothesized that the result was attributed to the natural defense metabolites of plants which were not present in *P. viridis* because of the bivalves' low exposure to pathogenic fungi.

CONCLUSION

Both qualitative zoochemical screening and TLC analysis revealed that the crude methanolic extract of *P. viridis* comprised of the following zoochemicals: alkaloids, saponins, sterols, terpenoids, and polyphenols. All the test bacteria were susceptible to the extract, with *S. aureus* and *P. aeruginosa* being the most inhibited in terms of growth. Among the three fungi tested, *L. theobromae* was the only fungus inhibited by the extract – with MIC of 33.33±11.79

mg/mL. The extract exhibited a promising MIC against *S. aureus* and *P. aeruginosa* with values of 2.60±0.63 and 3.65±1.69 mg/mL, respectively.

Recommendation

The proponents recommend evaluating zoochemicals in different locations and evaluating it further to establish a trend on the effects of the environment on their antimicrobial bioactivities. It is also suggested that the MIC per test organism should be determined and tested against the test organisms using the disk diffusion assay to further narrow the gap of the disparity of ZOIs in literature. Furthermore, better bioactivities might be achieved if zoochemicals were purified and isolated.

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Statement of Authorship

All authors participated in data collection and analysis and approved the final version submitted.

Author Disclosure

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