Original Article

Antimicrobial Activities of the Soft-Tissue Ethanolic Crude Extracts of Corbiculid Clam, *Corbicula fluminea* and Cyrenid Clam, *Geloina expansa*

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Abstract

Objective: The emergence of new infectious diseases and the increased in bacterial resistance to diseases have led to the search for potential sources of antimicrobials. Bivalves have been found to possess bioactive compounds that have tremendous potential in medical science. *Corbicula fluminea* and *Geloina expansa* are bivalves that can survive in extreme environmental conditions and are surrounded by various microbes; these may have facilitated the potential production of different bioactive compounds with unique features to protect against pathogenic microorganisms. **Materials and Methods:** Antimicrobial activity of various concentrations of the ethanolic crude extracts (ECEs) of *C. fluminea* and *G. expansa* were tested against bacteria and fungi using the standard disk diffusion technique. There were three bacteria used in the antibacterial assay, namely *Escherichia coli* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive), and for antifungal assay, the fungi, *Candida albicans,* and *Aspergillus niger* were used. **Results:** The ECEs of *C. fluminea* showed 12.67-h activity on *E. coli* with an inhibition zone (IZ) range of 10–12.8 mm and for *G. expansa* showed *an* IZ range of 9.6–14.4 mm. A similar IZ range for *C. fluminea* (10.6–12.4 mm) and *G. expansa* (9.8–13.6 mm) was observed in *P. aeruginosa* with an efficacy time of 14 h and 17.3, respectively. *S. aureus* test cultures also showed activity of *C. fluminea* ECEs with an IZ range of 0.00–17 mm and efficacy time of 15 h, while *G. expansa* showed a mean of 15.6 mm. The two fungal strains tested showed activity of *C. fluminea,* and *G. expansa* ECEs, an IZ range of 8.3–12.2 mm was observed in *A. niger* while *C. albicans* showed activity with an IZ range of 9.6–12.8 mm. The ECE's concentrations of 50 and 100 mg/ml of *C. fluminea* and *G. expansa* showed a significantly higher result than positive control against *P. aerigunosa*. **Conclusions:** The study showed that *C. fluminea* and *G. expansa* are potential sources of antimicrobial compounds. Identification, extraction, and purification of such compounds are recommended for future studies.

Keywords: Antimicrobial activities, *Corbicula fluminea,* efficacy time, *Geloina expansa*, inhibition zone

Introduction

The emergence of new infectious diseases and the increased in bacterial resistance of diseases transmitted between animals and humans have created studies to develop new antimicrobial drugs.[1,2] The World Health Organization also reported that new diseases are emerging at a historically unprecedented rate causing at least 10 million deaths per year[3] That leads to the search for more antimicrobial metabolites from natural sources, including the aquatic environment.[4,5] Over 7000 bioactive marine compounds have been identified, analyzed, and isolated from different species of aquatic organisms.[6] Several aquatic organism groups have been frontrunners in research that contain bioactive metabolites, and mollusks are becoming a promising source of new compounds. The priority list of species exhibiting antimicrobial activity includes several mollusks.[7] Several species of bivalves have been reported with antimicrobial activities, like oyster *Crassostrea madranensis*, mussel *Perna veridis*, and clam *Polymesoda expansa*.[8] These only suggest that mollusks are a cheap source of protein for human consumption and are also found to possess some

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complex bioactive compounds that have tremendous potential in medical science.

The species *Geloina expansa* and *Corbicula fluminea* are clams that belong to the family of Cyrenidae and Corbiculidae, respectively. *G. expansa* have been found on the landward side of the high intertidal area of mangrove forests and estuarine rivers,[9-12] where it thrives in extreme conditions. It has also been known that mangrove areas harbor various pathogens. Yet, *G. expansa* thrives in such conditions, suggesting that this clam might have developed a natural immunity to pathogenic microbes. In addition, *G. expansa* has also been found to reduce the infection of influenza virus type-A and B. While *C. fluminea* is usually found in lakes and streams with silt, mud, sand, and gravel substrate.[13] *C. fluminea* has a high reproductive output, rapid growth, and great powers of dispersal[14] and has its ability to thrive in both standing and flowing, oligotrophic and eutrophic waters and tolerates salinities approximately 5–14 ppt.[15] *C. fluminea* has also been considered to have a wide range of therapeutic benefits in traditional Chinese medicine, including better appetite and vision, liver illness, dieresis and measles therapy, anti-alcoholism, cough relief, decreased sputum, and fever relief.

Thus, the distinct characteristics of these species, exemplify a significant potential to inhibit microbial growth.

Materials and Methods

Location of the study

The clams were collected on the streams of Casiguran, Aurora, for *C. fluminea* and in mangrove areas of Binmaley, Pangasinan, for *G. expansa*.

The laboratory experiment was conducted at the Microbiology laboratory (Biosafety Level II) of the Bureau of Fisheries and Aquatic Resources National Integrated Fishery Technology Demonstration Center in Bonuan, Binloc, Pangasinan.

Experimental treatments

Range-finding tests using logarithmic concentrations (1, 10, 100, and 1000 mg/ml) were conducted to determine the concentrations of the ethanolic crude extracts (ECEs) to be used in the definitive tests. The result showed that the concentration that has shown the zone of inhibition is the concentration of 100 mg/ml.

Based on the range-finding test result, the test concentrations of 10, 50, and 100 mg/mL were set for definitive tests. Amoxicillin (bactericide) and clotrimazole (fungicide) were used as positive controls and 95% ethanol as the negative control [Table 1]. All the negative control assays showed no inhibition zone (IZ) in all the test organisms. The negative control results were not indicated on the graph of the results.

Test microorganisms

Human pathogenic microbes were used as the test microorganisms for the antibacterial and antifungal assays of ECEs of *C. fluminea* and *G. expansa*.

There were three bacteria used in the antibacterial assay, namely *Escherichia coli* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative bacteria), and *Staphylococcus aureus* (Gram-positive), which were cultivated and maintained in the Mueller–Hinton Agar. These bacteria cultured in blood agar are requested from the Region 1 Medical Center Dagupan City, Pangasinan.

In the antifungal assay, the fungi, *Candida albicans*, and *Aspergillus niger* were cultivated in a Potato Dextrose agar medium. These fungi were purchased from the Philippine Center for Postharvest Development and Mechanization (PHILMECH) that were cultured in blood agar. The pathogenic microbes were cultivated and maintained at 37°C. After 24 h of incubation, the cultures were used in the study.

Data gathering procedure and instruments

Collection and preparation of test microorganisms

The pathogens from the blood agar were revived and streaked in a nutrient agar plate. After 24 h of incubation, when microorganisms had fully grown into the agar, it would now be ready to be the bacterial and fungal solution source.

Collection of Asian clam and mangrove clam and preparation of the soft tissue

Clams attached to the substrate were gathered with bare hands. They were then washed externally to wash away mud and other foreign objects adhering to them. After, they were allowed to remain in clean, fresh water for 24 h to depurate. They were then brought to the laboratory. Each sample was shucked using a knife on its posterior edge to prevent the meat from dismantling and being ready for ECE preparation.

Preparation of ethanolic crude extract

ECEs of *C. fluminea* and *G. expansa* were prepared using 300 g soft-tissue samples soaked and macerated in 300 mL of laboratory-grade ethanol. The mixture of the macerated soft tissue and ethanol was kept in a glass jar at room temperature (30°C) for 24 h at NIFTDC, Bureau of Fisheries and Natural Resources Bonuan Dagupan, City. It was then centrifuged at 1500 rpm for 15 min, and the supernatant was filtered through Whatman grade GF/C glass microfiber filter. Finally, the solvent was concentrated into a gummy dark-brown residue in a rotary evaporator. The ECEs were stored in a covered beaker at 4°C until usage for antimicrobial activity.

Preparation of discs

The 6–mm discs were prepared from a Whatman Grade Filter Paper 1, 125 mm. After the raw preparation, discs were placed on a Petri plate and covered. The autoclave was then set to 121°C and 15 psi for 15 min for the discs to be sterilized. After the sterilization process, the discs were ready to be treated with different concentrations.

Antimicrobial activity

The antimicrobial activity of the ECEs was determined using the standard disc diffusion method.[16] Microbial inoculums

were then prepared by having 3–5 distinct colonies from the bacterial and fungal cultures. They were then homogenized in a 5–ml sterile saline solution (0.85%). The turbidity of the bacteria and fungus saline solution was compared and visually adjusted to that produced by a 0.5 McFarland standard.[17,18]

The test plates were prepared using 20 ml of the Mueller Hinton Agar medium for bacteria and Potato Dextrose Agar medium for fungus and mold. Inoculations of the pathogenic microbial strains were carried out using the spread plate method, in which three drops of the solutions were placed on each plate, and were speeded several times on the surfaces of the Petri plates. Three concentrations of the ECEs, negative (ethanol) and positive controls (100 mg/mL clotrimazole for fungi and 25 mg/mL amoxicillin for the bacteria) were then applied to 6-mm sterile discs, which were impregnated on the top of the test plates using a sterilized forceps, these test plates were then seeded with pathogenic microbial strains.[19-21]

Measurement of diameters of inhibition zones

The diameter of the IZ was then measured in millimeters. The test plates were observed hourly, and the IZ diameter was measured on the area where the pathogens had not proliferated.

Determination of efficacy time

The efficacy time was then determined by observing the test plates hourly for 24 h. From the time, the pathogens are seeded with the ECEs concentrations, positive and negative controls mark the time, in which the test plates are observed.

Data analysis

A one-way model analysis of variance with three replications was used to determine the effects of various ECEs concentrations on the susceptibility of the pathogenic microbes. It has been set at the 5% significance level. Duncan's multiple range test was used as a *post hoc* test.

Results

Inhibition zone and efficacy time of *Corbicula fluminea* ethanolic crude extracts against Pathogenic Microbes *Escherichia coli, Pseudomonas Aeruginosa,* and *Staphylococcus aureus*

All of the concentrations of *C. fluminea* ECE exhibited mean inhibitions zones ranging from 10 to 12.8 mm when tested against *E. coli.* Pure ethanol discs (negative control) did not show activity [Figure 1].[22-24] Amoxicillin showed a 24.6 mm mean IZ diameter. In the case of the *E. coli* growth, IZ showed a significant difference ($P < 0.05$) among the five concentration means). Furthermore, a comparison among means showed that amoxicillin discs produced significantly higher IZ than the other treatments. After 12.67 h, the IZ was still distinct but already masked by *E. coli.*

Regarding *P. aeruginosa* zone of inhibition, all concentration means (10.6–12.4 mm) were numerically higher than the amoxicillin, with IZ mean of 10.4 mm [Figure 1]. Analysis of variance showed a significant difference ($P < 0.05$) among five concentration means. Likewise, a comparison among means showed that concentrations of 50 mg/ml and 100 mg/ml produced significantly higher IZ than the other concentration. While concentrations 100 mg/ml, 10 mg/ml and amoxicillin discs mean growth inhibition shows no significant difference.[25,26] The IZs of all ECE discs start to be masked with *P. aeruginosa* after 14 h. On the other hand, the highest mean IZ of 15.4 mm was recorded at 50 mg/ml against the *S. aureus* test culture [Figure 1]. All the ECE concentrations showed a significant difference ($P > 0.05$) and a comparison among means showed that the amoxicillin is higher than the three ECE concentrations.[27]

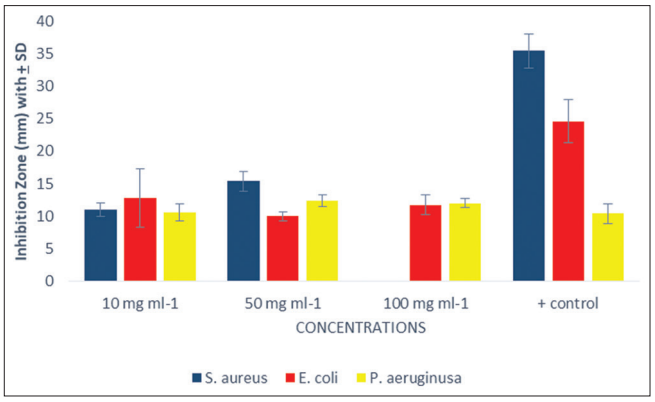

Figure 1: Inhibition Zone of *Corbicula fluminea* (ECEs) against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. ECE: Ethanolic crude extract

Inhibition zone and efficacy time of *Corbicula fluminea* ethanolic crude extract against Pathogenic Microbes *Aspergillus niger* and *Candida albicans*

The three concentrations of ECE of the soft tissues of Asian Clam have exhibited mean inhibitions zones ranging from 8.3 to 10.5 mm when tested against *A niger* [Figure 2]. Analysis of variance in the growth IZ showed a significant difference ($P < 0.05$) among the five concentration means, and comparison among means showed that clotrimazole discs produced significantly higher IZ than the ECE concentrations. The zone of inhibition of all ECE discs started to be masked with *A. niger* after 21.9 h.[28-30]

The ECE of *C. fluminea* showed antifungal activity against the *C. albicans,* with mean IZ ranging from 9.6 to 12.8 mm [Figure 2]. Furthermore, the growth IZ on *C. albicans* showed a significant difference ($P < 0.05$) and showed that clotrimazole is higher than the three ECE concentrations. The IZs of all ECEs discs start to be masked with *C. albicans* after 21.62 h.

Inhibition Zone and Efficacy Time of *Geloina expansa* ethanolic crude extract against Pathogenic Microbes *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*

The ECE of the soft tissues of *G. expansa* has exhibited mean inhibitions zones ranging from 9.6 to 14.4 mm when tested

against *E. coli* [Figure 3]. In the case of *E. coli* growth IZ, analysis of variance showed a significant difference ($P < 0.05$) among five concentration means, and comparison among means showed that amoxicillin discs produced significantly higher IZ than the ECE concentrations. These were followed by 100 mg/ml and 50 mg/ml, which are not significantly different. After 12 h, the IZs were still distinct but already masked by *E. coli.*

About *P. aeruginosa* zone of inhibition, it appeared that most of the concentration means (9.8–13.6 mm) were higher than the amoxicillin (positive control) except for 10 mg/ml with IZ mean of 10.2 mm. After 17.3 h, the IZs were still distinct but already masked by *P. aeruginosa* [Figure 3].[31]

Numerically, the highest mean IZ was recorded in concentration three against the *S. aureus* test culture with 15.6 mm [Figure 3]. All of the ECE concentration mean yielded a significant difference from each other ($P > 0.05$), and comparison among means showed that IZ mean of amoxicillin (37.4 mm) is higher than the three ECEs. This positive control was followed by 100 mg/ml and 50 mg/ml, which were not significantly different. After 15.6 h, the IZs were still distinct but already masked by *S. aureus.*[32]

Inhibition zone and efficacy time of *Geloina expansa* ethanolic crude extract against Pathogenic Microbes *Aspergillus niger* and *Candida albicans*

The three *G. expansa* ECE concentrations have exhibited mean inhibitions zones ranging from 11.6 to 12.2 mm [Figure 4]. Likewise, no activity was observed on pure ethanol discs (negative control). Analysis of variance on the growth IZ on *A niger* showed a significant difference ($P < 0.05$) among five treatment means, and comparison among means showed that clotrimazole discs (37.4 mm) produced significantly higher IZ than the ECE concentrations. Moreover, there are no significant differences observed among the ECE treatments. The IZs of all ECEs discs start to be masked with *A. niger* after 22.67 h.[33]

The *G. expasna* ECE concentrations have exhibited mean inhibitions zones ranging from 11.4 to 12.4 mm [Figure 4] against *C. albicans*. Likewise, no activity was observed on pure ethanol discs (negative control). Based on the analysis of variance, concentrations showed a statistically significant difference ($P < 0.05$).

Moreover, a comparison among means showed that clotrimazole produced higher I. Z. Regarding the ECE concentrations, there were no significant differences observed among the ECE concentration.[34,35]

The IZs of all ECE discs start to mask with *C. albicans* after 25 h.

Discussion

It has been documented that *E. coli* can thrive in warm moist conditions[36] and persist outside of the host in

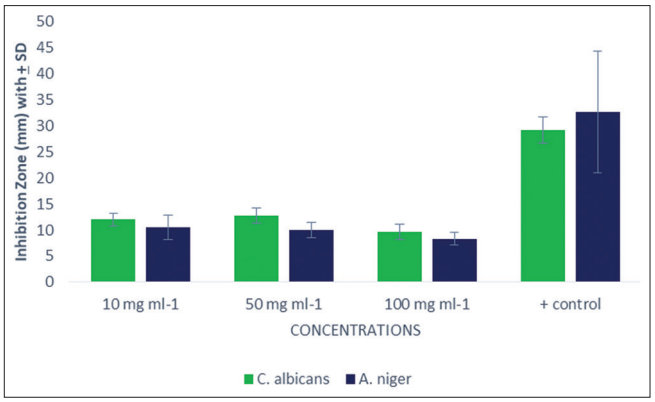

Figure 2: Inhibition Zone of *Corbicula fluminea* (ECE) against *Aspergillus niger* and *Candida albicans*. ECE: Ethanolic crude extract

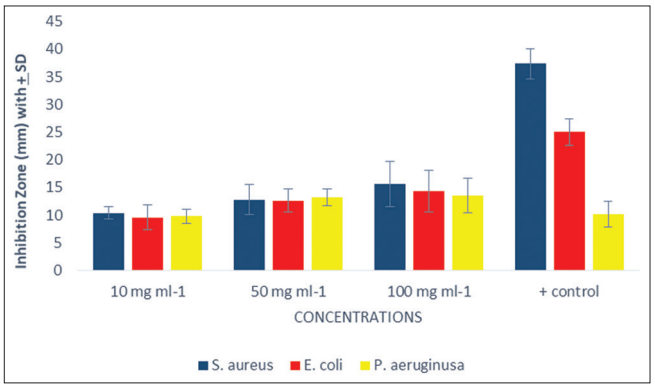

Figure 3: Inhibition Zone of *Geloina expansa* (ECE) against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. ECE: Ethanolic crude extract

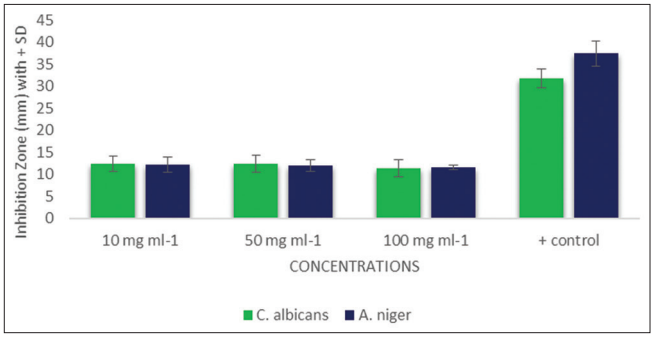

Figure 4: Inhibition Zone of *Geloina expansa* (ECE) against *Aspergillus niger* and *Candida albicans*. ECE: Ethanolic crude extract

varying environments.[37] Bottom sediments have also been recognized as a significant reservoir of *E. coli* in freshwater environments.[38] Many studies indicated that sediments could harbor much higher populations of both fecal coliforms and *E. coli* than the overlying water column.[39] The presence of *E. coli* in recreational marine beach,[40] seawater,[41-43] river water,[44,45] estuary,[46] and in subtropical freshwater have also been reported. Therefore, it appears that *E. coli* is a common bacterium that could be found in aquatic environments. Immunity against such bacteria might have been developed by

Table 1: Experimental treatments

Treatment number	Concentrations
1	10 mg/ml (ECE)
2	50 mg/ml (ECE)
3	100 mg/ml (ECE)
4	Negative (ethanol)
5	Positive (100 mg/mL clotrimazole - fungicide) (25 mg/mL amoxicillin - bactericide)

ECE: Ethanolic crude extract

C. fluminea and *G. expansa*. Similar results were reported in other clams, such as *Polymesoda expansa, Anadara granosa, Mactra chinensis,* and *Tridacna maxima.*[47-49]

The bacterium *P. aeruginosa* is widely distributed in the environment,[50] and it can grow at deficient nutrient levels, such as in tap water.[51] The existence of *P. aeruginosa* has also been found in lake water,[52-54] river water,[55,56] creek water,[57] and freshwater. With the presence of this *P. aerigunosa* in the said environment, it is possible that the *C. fluminea* and *G. expansa* developed resistance that resulted in its antibacterial activity against *P. aerigunosa*. ECE concentrations of *C. fluminea* 50 and 100 mg/ml exhibited significantly higher antibacterial activity than amoxicillin. There is a possibility that *C. fluminea* contains bioactive compounds that is more potent to inhibit the growth of *P. aerigunosa.* Similar results were also reported in other clams such as *Anadara granos*.

S. aureus bacterium is a highly adaptable pathogen.[58-60] Several potential sources of this bacteria contamination in the marine environment have been examined, including shedding from recreational bathers,[61,62] untreated wastewater,[63] urban runoff,[64] marine, and freshwater recreational beaches.[65] Due to the existence of *S. aureus* in the aquatic environment, it is possible that *C. fluminea* and *G. expansa* developed resistance that resulted in its antibacterial activity. Similar results were reported in other clams, such as *Polymesoda expansa* and *Mactra chinensis*.

It is reported that fungi can be found in aquatic habitats with harsh environmental conditions, such as sulfidic springs,[66] acidic peat bogs and lakes,[67] volcanic lakes,[68] and mangrove forests. Arif Ibrahim reported the existence of several species of *Aspergillus*, one of which is *A. niger,* that can be found in freshwater.[69] Moreover, this fungus is classified as aero-aquatic fungi that can grow on plant debris fallen in the water, which serves as substrate for its growth.[70] *C. fluiminea* has also been reported to harbor *A. niger* Rodolfi, *et al*., and it seems to coexist naturally. Thus, it is possible that *C. fluminea* and *G. expansa* developed resistance.

C. albicans is one of the most studied opportunistic pathogenic yeasts frequently associated with human disease Valdes-Collazo *et al.*[71] These fungi can cause superficial infections of the skin, nails, mucosae, gastroenteritis, vaginitis, and urethritis and are frequently the cause of nosocomial infections in compromised hosts.[72-74] Furthermore, Bonde believes that the members of the genus *Candida* should be considered waterborne pathogens.[75] After that, several researchers reported the presence of *C. albicans* in sewage, tropical marine, and freshwater. Due to the presence of *C. albicans* in the environment, *C. fluminea* and *G. expansa* may have developed resistance that resulted in its antifungal activity.

Conclusions

The ECEs of *C. fluminea* and *G. expansa* showed antibacterial against *E. coli*, *P. aeruginosa*, and *S. aureus* and antifungal properties against *C. albicans,* and *A. niger*. Preparations of extracts of these organisms should also be tested for their antioxidants. It is also recommended different solvents should also be used for the extraction procedure of the clams. Identification, extraction, and purification of bioactive compounds are recommended for future studies.

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Conflicts of interest

There are no conflicts of interest.

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