Investigation of Antibacterial Activity of Crude Extracts from Marine Snails and Bivalves in the Southern Coast of Vietnam

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Abstract: The primary antibacterial activity of methanol and chloroform crude extracts from marine snails and bivalves was assessed by using the agar diffusion technique against four bacterial strains. Active methanol extracts were then characterized using TLC, SDS-PAGE and FTIR. Methanol extracts from 5 snail species and 8 extracts from 12 bivalve species possessed the ability to inhibit Bacillus subtilis. Methanol extracts from 3 snail species Tectus conus, Maninella alounia and Trochus maculatus inhibited Escheria coli and those from 4 snail species Cerithium chinatum, Maninella alonmia, Tectus pyramidis, Trochus maculatus and the bivalve species Pinna bicolor exhibited activity against Serratia marcescens. Chloroform extracts from 7 snail species and those from 7 bivalve species showed inhibition on Bacillus subtilis. Only chloroform extract from the bivalve Chama cf. dunkeri was active on Salmonella typhimur and that from the snail Trochus maculatus and bivalve Lopha cristalgaali inhibited Escheria coli. TLC and FTIR analysis of active methanol extracts showed the presence of amino acids, peptides and proteins. SDS-PAGE of those extracts also revealed proteins with a molecular weight range between 10 and 28 kDa. The obtained results indicate the potential antimicrobial compounds that could be explored in snail and bivalve in Vietnam.

Keywords: Snail, Bivalve, Crude Extracts, Antibacterial Activity, TLC, FTIR, SDS-PAGE

1. Introduction

Among marine invertebrates, mollusk is the second largest animal phylum, and account for 23% of the total species of marine organisms. They serve not only as a food source but also as a potential drug cabinet due to the richness in proteins and bioactive compounds. Many studies have reported the bioactivity of compounds from mollusk such as antioxidation and cytotoxicity, activities against inflammatory, microbes. For example, mussel Mytilus galloprovincialis plasma contained cytotoxic activity against both vertebrate (erythrocytes and mouse tumors) and protozoan cells [11] and myticin compounds possessing antimicrobial activity [17]. Sulfated beta-galactans from the clam Meretrix petechialis [1] and compounds from the clams Viloraria cyprinoidis, Meretrix casta and green mussel Perna viridis [7], magus cone Conus magus [23] exhibited inhibition on HIV-1. Antibiaterial or antivirus compounds were also isolated from numerous molluse such as the oysters Ostrea eduk, Crassostrea gigas [11], and Crassostrea virginica, the mussels Mytilus edulis, Geukensia demissa [2], and Perna canaliculus [24] and the white rock shell Dicathais orbita [5], the cone snails Conus betulinus and Conus inscriptus, the spiral Babylon Babylonia spirata [18, 19], the cuttlefish Sepia parshadi [21], squids Sepia sp., Loligo sp., snail Tibia insulaechorabcura [10, 16] the sea hare Dolabella auricularia [26], sea slug Armina babai [22], the abalones Haliotis laevigata, H. rubra, and H. rufescens, snails
Littorina littorea, Buccinum corneum, Tegula gallina, Rapana venosa and Buccinum undatum), and hard clams Mercenaria mercenaria, Mya arenaria, Raditapes philippinarum, cockle Cerastoderma edule, mussels Mytilus galloprovincialis and Crenomytilus grayanus), oysters Crassostrea virginica, C. gigas and Ostrea edulis (reviewed by Dang et al. 2015 [9]). The extracts from the green mussel Perna viridis and the Indian volute Melo melo also inhibited the replication of influenza virus, virus causing skin, mucosa, respiratory problems [7] and showed activity against some strains of bacteria and fungi [6, 13, 14].

Vietnam has a long coastline and large territorial waters with the majority of mollusc species distributed along the country. The rich diversity of this animal assumes a great opportunity for exploring bioactive products. However, studies on bioactive compounds in mollusk are rare. In this investigation, we screened the antibacterial activity of organic extracts from snail and bivalve in the Southern waters of Vietnam to identify compounds of biomedical importance.

2. Materials and Methods

2.1. Sampling

Marine snails and bivalves were collected from different coastal habitats in the Southern coast of Vietnam in May 2017 by SCUBA diving during the joint investigation between Vietnam and Russia using the Akademik Oparin vessel. Sample photos were taken and identified by zoologists at the Institute of Oceanography, Vietnam. The marine snails and bivalves were cleaned with freshwater and deshelled, then the whole body tissues were collected and stored in liquid nitrogen. 7 snails and 12 bivalve species were used in this research.

2.2. Extraction

All homogenised tissues were extracted using methanol and chloroform solutions at a ratio of 1:4 (g:ml) and then stored at 4°C for 24 h. The homogenate supernatants of each solvent were collected and filtered through Whatman No1. The tissues were extracted twice with the same solvent. The solvents from combined filtrates were evaporated completely. Dried crude extracts were used for the next experiments.

2.3. Antibacterial Activity Test

The antibacterial activity of the crude extracts was assessed by using the agar well diffusion technique on Mueller Hinton Agar (MHA-Himedia, India) according to Bauer et al (1996) [4]. Each of 100 mg crude extract was dissolved into 1 ml methanol (Merck). Antibacterial activity tests were performed on standard strains, including Bacillus subtilis (ATCC6633), Escherichia coli O157, Salmonella typhimurium (ATCC 6994) and Serratia marcescens PDL100 (ATCC BAA-632).

Bacteria were cultured in Marine agar medium, then inoculated into Marine Broth medium to grow up large amounts of bacteria. Standard strain broths were stained with a solution containing Coomassie blue 0.25%, methanol 40% and acid acetic 10%. The used markers were Precision Plus ProteinTM Standards with molecular weight (MW) range of 10 kDa to 180 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3. Results

3.1. Content (%) of Crude Extracts

The content of methanol extracts varied from 2.45 to 3.81% in snail and 1.68-3.52% in bivalve, and those of
chloroform extracts from 0.18 to 0.72% in snail and 0.17-0.37% in bivalve (Table 1).

Table 1. Content (%) of crude extracts from snails and bivalves.

| Mollusc species | Content of methanol extract (%) | Content of chloroform extract (%) |
|----------------|---------------------------------|-----------------------------------|
| Snail          |                                 |                                   |
| Cerithium chinatum | 3.26                          | 0.72                             |
| Tectus comus    | 2.58                            | 0.41                             |
| Maninella alounia | 3.81                          | 0.41                             |
| Tectus pyramis  | 2.98                            | 0.25                             |
| Trochus histrio | 2.67                            | 0.18                             |
| Trochus maculatus | 2.68                         | 0.52                             |
| Turbo chrysostomus | 2.45                         | 0.31                             |
| Bivalve         |                                 |                                   |
| Lopha cristagali | 2.37                          | 0.18                             |
| Chama lazarus   | 2.52                            | 0.24                             |
| Atrina sp       | 2.96                            | 0.27                             |
| Chama cf dunkeri | 1.68                          | 0.28                             |
| Pinna bicolor   | 2.39                            | 0.17                             |
| Tridacna crocea | 3.05                            | 0.29                             |
| Spondylus squamosus | 1.89                        | 0.19                             |
| Hyotissa spp    | 2.59                            | 0.32                             |
| Barbatia f oliata | 2.32                         | 0.31                             |
| Atrina vexillum | 2.73                            | 0.30                             |
| Hyotissa cf hyotis | 3.52                       | 0.37                             |
| Spondylus sp    | 2.77                            | 0.31                             |

3.2. Antibacterial Activity

Table 2. Diameter of inhibition zone (mean ± SD) (mm) of the antibacterial activity of methanol extracts from snails and bivalves.

| Mollusc species | Inhibition zone (mm) | Bacillus Subtilis | Salmonella typhimurium | Escheria coli | Serratia marcescens |
|----------------|---------------------|-------------------|------------------------|--------------|---------------------|
| Snail          |                     |                   |                        |              |                     |
| Cerithium chinatum | 2.50 ± 0.86     | 0                 | 0                      | 1.26 ± 0.44  |                     |
| Tectus comus    | 3.50 ± 0.50        | 0                 | 2.26 ± 0.44            | 0            |                     |
| Maninella alounia | 4.50 ± 0.50      | 0                 | 2.50 ± 0.86            | 2.50 ± 0.86  |                     |
| Tectus pyramis  | 5.00 ± 1.00        | 0                 | 0                      | 2.00 ± 0.0   |                     |
| Trochus histrio | 0                  | 0                 | 0                      | 0            |                     |
| Trochus maculatus | 1.00 ± 0.0       | 0                 | 1.26 ± 0.44            | 2.00 ± 0.0   |                     |
| Turbo chrysostomus | 0               | 0                 | 0                      | 0            |                     |
| Bivalve         |                     |                   |                        |              |                     |
| Lopha cristagali | 0                  | 0                 | 0                      | 0            |                     |
| Chama lazarus   | 4.26 ± 1.08        | 0                 | 0                      | 0            |                     |
| Atrina sp       | 3.50 ± 0.86        | 0                 | 0                      | 0            |                     |
| Chama cf dunkeri | 5.00 ± 0.70      | 0                 | 0                      | 0            |                     |
| Pinna bicolor   | 4.00 ± 0.70        | 0                 | 0                      | 1.50 ± 0.50  |                     |
| Tridacna crocea | 2.50 ± 0.86        | 0                 | 0                      | 0            |                     |
| Spondylus squamosus | 3.50 ± 0.86     | 0                 | 0                      | 0            |                     |
| Hyotissa spp    | 0                  | 0                 | 0                      | 0            |                     |
| Barbatia f oliata | 0                | 0                 | 0                      | 0            |                     |
| Atrina vexillum | 0                  | 0                 | 0                      | 0            |                     |
| Hyotissa cf hyotis | 6.00 ± 0.0     | 0                 | 0                      | 0            |                     |
| Spondylus sp    | 1.76 ± 0.44        | 0                 | 0                      | 0            |                     |

For methanol extracts, 5 among 7 of samples from snail (except 2 species Trochus histrio and Turbo chrysostomus) and 8 among 12 extracts from bivalve possessed the ability to inhibit Bacillus subtilis with inhibition zone from 1.0-5.0 mm and 2.50-6 mm, respectively. The extracts from 3 species of snail Tectus comus, Maninella alounia and Trochus maculatus inhibited Escheria coli with inhibition zones 2.26 mm, 2.50 mm and 1.26 mm, respectively. Extracts from Cerithium chinatum, Maninella alounia, Tectus pyramis, Trochus maculatus, and the bivalve Pinna bicolor exhibited activity against S. marcescens with inhibition zones recorded at 1.26, 2.50, 2, 2 mm, and 1.50 mm. No extracts from snail and bivalve were active on S. typhimurium (Table 2).
Table 3. Diameter of inhibition zone (mean ± SD) (mm) of the antibacterial activity of chloroform extracts from snails and bivalves.

| Mollusc species | Inhibition zone (mm) | Bacillus subtilis | Salmonella typhimurium | Escheria coli | Serratia marcescens |
|-----------------|----------------------|------------------|------------------------|--------------|-------------------|
| Snail           |                      |                  |                        |              |                   |
| Cerithium chinatum | 2.00 ± 0.00       | 0                | 0                      | 0            | 0                 |
| Tectus conus    | 5.26 ± 0.44         | 0                | 0                      | 0            | 0                 |
| Maninella alounia | 2.50 ± 0.86        | 0                | 0                      | 0            | 0                 |
| Tectus pyramis  | 6.00 ± 0.00         | 0                | 0                      | 0            | 0                 |
| Trochus histrio | 6.50 ± 0.86         | 0                | 0                      | 0            | 0                 |
| Trochus maculatus | 1.50 ± 0.50       | 0                | 1.76 ± 1.08            | 0            | 0                 |
| Turbo chrysostomus | 4.00 ± 0.00       | 0                | 0                      | 0            | 0                 |
| Bivalve         |                      |                  |                        |              |                   |
| Lopha cristagali | 2.50 ± 0.86         | 0                | 2.26 ± 0.44            | 0            | 0                 |
| Chama lazarus   | 3.26 ± 0.82         | 0                | 0                      | 0            | 0                 |
| Atrina sp       | 3.26 ± 1.08         | 0                | 0                      | 0            | 0                 |
| Chama cf dunkeri | 0                   | 1.00 ± 0.00      | 0                      | 0            | 0                 |
| Pinna bicolor   |                      |                  |                        |              |                   |
| Tridacna crocea | 2.50 ± 0.50         | 0                | 2.26 ± 0.44            | 0            | 0                 |
| Spondylus squamosus | 0          |                  |                        |              |                   |
| Hyotissa spp    | 2.00 ± 0.00         | 0                | 0                      | 0            | 0                 |
| Barbatia fowia  | 3.26 ± 0.82         | 0                | 0                      | 0            | 0                 |
| Atrina vexillum |                      |                  |                        |              |                   |
| Hyotissa cf hyotis | 0           |                  |                        |              |                   |
| Spondylus sp    | 2.50 ± 0.50         | 0                | 0                      | 0            | 0                 |

In chloroform extracts, the obtained results revealed that 7 extracts from 7 snail species and 7 among 12 extracts from bivalve (except Chama cf dunkeri, Pinna bicolor, Spondylus squamosus, Atrina vexillum, Hyotissa cf hyotis) showed inhibition on B. subtilis with inhibition zones 2.0- 6.50 mm and 2.0-3.26 mm, respectively. Only extract from the bivalve Chama cf dunkeri inhibited S. typhimurium with inhibition zone 1.0 mm. Extracts from snail Trochus maculatus and bivalve Lopha cristagali exhibited activity against E. coli with inhibition zones 1.76 and 2.26 mm. All chloroform extracts from snail and bivalve were insensitive against S. marcescens (Table 3).

3.3. Protein Pattern

The sheet showing purple to pink spots by TLC indicated the presence of amino acids and peptides in the active samples with Rf of 0.82 and 0.91 (Figure 1). FTIR: The IR spectra of the methanol extracts revealed the characteristic peaks of proteins (data not shown) and Figure 2 shows the IR spectrum of the methanol extract from the representative species Trochus maculatus. The free NH stretching vibration appeared at 3413 cm⁻¹ and the amide I vibration, absorbing at 1629 cm⁻¹, arises mainly from the C=O stretching vibration with minor contributions from the out-of-phase CN stretching vibration, the CCN deformation and the NH in-plane bend. N-deuteration converted the amide II mode to largely a C-N stretching vibration at 1490–1460 cm⁻¹ (named amide II mode). The amide III region (1200-1350 cm⁻¹) is N-H in-plane bending coupled with C-N stretching and also includes C-H and N-H deformation vibrations. The absorbance at 527–601 cm⁻¹ was the result of the vibration of out-of-plane CO bending [3].

Figure 1. TLC profile of methanol extracts: 1. Cerithium chinatum; 2. Tectus conus; 3. Tectus pyramis; 4. Trochus maculatus; 5. Pinna bicolor.

Figure 2. FTIR spectrum of the methanol extract of the representative species Trochus maculatus.
SDS-PAGE analysis of methanol samples revealed the presence of proteins with MW range between 10-28 kDa (Figure 3).

Figure 3. Protein pattern of methanol extracts determined by SDS-PAGE: 1. Maninella alonuia; 2. Tectus conus; 3. Cerithium chinatum; 4. Tectus pyramis; 5. Trochus maculatus; 6. Pinna bicolor; M: Protein standard.

4. Discussion

In this research, the content of crude extracts varied depending on the type of extraction solvents. Indeed, from the same species, the methanol extraction produces more yield than that of chloroform. Methanol and chloroform extracts exhibited antibacterial activity against tested bacteria with varying degrees. Most methanol and chloroform extracts from the snail and bivalve species possessed the ability against Gram-positive Bacillus subtilis. This result is similar to Jayaseeli et al. (2001) [12], who reported that the extracts of four bivalve species showed significant activity against Bacillus subtilis and inhibition of few pathogenic bacteria. The number of methanol extracts possessing the ability against Gram-negative pathogenic bacteria is higher than those of chloroform extracts, the reason could be that methanol is much more polar and extract more hydrophilic compounds than chloroform. In the green mussel Perna viridis, the methanolic extract was more active and showed antibacterial activity at low concentrations, the study showed that the substance involved in producing the antibacterial effect could be a high polar compound [14]. Similar results were observed in Octopus dolfusii with inhibition of methanol extracts against Escherichia coli and Streptococcus pneumonia [21] and in the sea slug Armina babai with the crude methanol extracts displaying good activity on the selected pathogenic bacteria [22]. In our research, the antibacterial activity was observed against some pathogenic bacteria from crude methanol extracts, indicating that this solvent is suitable for the extraction of bioactive compounds in a mollusc.

On the other hand, in our investigation, the TLC and IR results demonstrated the presence of protein compounds. TLC indicated the presence of amino acids and peptides with Rf of 0.82 and 0.91 and this result had the same Rf compared to the amino acids and peptides in the sea slug Armina babai [22]. In addition, the IR spectrum of crude methanol extract from the representative species confirms the existence of protein compounds with amide I, II, III vibrations.

On protein properties, SDS-PAGE analysis of methanol extracts active on bacteria revealed the proteins in the range of very low MW from 10 to 28 kDa. The result of Ramya et al. (2014) [22] showed the existence of peptide or amino acid with purple or violet by TLC and MW of proteins from 13-72 kDa by SDS-PAGE from bioactive antibacterial extracts in the sea slug Armina babai. Periyasamy et al. (2012b) [19] found MW of proteins in the active extract on bacteria from the spiral Babylonia spirata was distributed from 2-110 kDa. MW of antibacterial protein extracted from the gill of the green mussel Perna viridis was 9.7 kDa [6], and that of methanol extract from the tissue of this species was 20-63 kDa [16]. Analysis of the crude proteins from the clams Meretrix meretrix and Meretrix casta showed protein bands ranging from 45 to 261 kDa [25]. More or less similar MW protein was also isolated from 14 kDa and 29 kDa in the clam Meretrix casta and the green mussel Perna viridis. Usually, snail and bivalve contain a variety of molecules which exhibit promising level of antibacterial activity. Small size proteins have been the proven to possess strong antioxidant, antimicrobial activity, therefore they could be potential drug source with great promise for the discovery of novel bioactive compounds instead of a plant. This study indicates the presence of potent antimicrobial compounds in marine snails and bivalves on the Southern coast of Vietnam. Several compounds from some bivalves in Cat Ba, northern Vietnam also showed the antibacterial effect against 1-2 pathogenic bacteria strain and 1 compound inhibited MCF7 and HepG2 [20]. Other researches reported that marine mollusc is considered as a good source of bioactive metabolites possessing inflammatory, microbial, oxidant and cytotoxic activity [8].

5. Conclusions

The preliminary results obtained in the present study indicate that the whole body of some marine snail and bivalve species would be a potential source of antibacterial agents that could be explored and potentially developed in Vietnam. Isolation and structural elucidation of bioactive compounds from these marine molluscan extracts are needed to be carried out in further studies.

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