

# Screening of extracts of diwal (*Pholas orientalis*) for antimicrobial activities

## Abstract

Diwal or Angel wing (*Pholas orientalis*) is one of the commercially important bivalve in the Philippines. There are areas in Region 6, Philippines that are declared “diwal sanctuaries” to protect the resource from continuous exploitation. Diwal is considered a delicacy and to add to its saleability, extracts of its different parts (siphon, mantle, body meat and gills) were bioactivity screened for antimicrobial properties. Cold methanol and PBS (phosphate buffer saline) extracts were assayed against gram-positive (*Staphylococcus aureus* and *Micrococcus luteus*) and gram-negative (*Pseudomonas aeruginosa* and *Vibrio harveyi*) bacteria for antibacterial activity and against three fungal strains: *Aspergillus niger*, *Trichoderma sp.*, and *Candida albicans* for antifungal activity. Three doses (100 500, and 1,000 mg/mL) of the extracts were used in the bioassay with Chloramphenicol (10 mg/mL) for antibacterial and Ketoconazole (10 mg/mL) for antifungal serving as positive controls. All extracts of Diwal in both cold methanol and PBS extracts did not show any antibacterial activities. It did have antifungal activities but only in a 1000 mg/mL dose of cold methanol and PBS extracts of body meat and cold methanol extract of the mantle. Of the three fungal strains tested, *Trichoderma sp.*, a fungus that caused skin dermatitis in humans, exhibited a zone of inhibition. So, there is potential for Diwal extract as an antifungal agent. Preparations of Diwal body meat and mantle extracts should be tested for its affectivity especially against dermatitis. Extraction of Diwal parts should also be done using nonpolar solvents to further assess its potential as a promising source for identifying novel drug lead compounds that might ultimately benefit the ongoing global search for clinically useful antimicrobial agents.

**Keywords:** Diwal, *Pholas orientalis*, Antimicrobial activity, Antifungal agent

Volume 1 Issue 1 - 2014

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**Received:** October 10, 2014 | **Published:** November 07, 2014

**Abbreviations:** MDR: Multi-Drug Resistant, PBS: Phosphate Buffer Saline, CRM: Coastal Resource Management, NIMBB: National Institute of Molecular Biology and Biotechnology, PNCM: Philippine National Collection of Microorganisms, NA: Nutrient Agar, MHA: Mueller-Hinton Agar, PDA: Potato Dextrose agar, PYGB: Peptone-Yeast Extract-Glucose, UPV: University of the Philippines Visayas

## Introduction

According to the World Health Organization new diseases are emerging at the historically unprecedented rate of one per year. There will be another disease like AIDS, another Ebola or another SARS, sooner or later. Experts say there are now hundreds of new “superbugs”, lethal organisms totally immune to antibiotic treatments. The recent appearance of a growing number of microorganisms resistant to conventional antibiotics has become a serious medical problem. To overcome this resistance, the development of antibiotics with novel mechanisms of action is a pressing issue.<sup>1</sup> In an attempt to keep ahead of bacterial evolution, new antibiotics based on antimicrobial compounds produced by all multicellular organisms are being developed.<sup>2-4</sup> Antimicrobial compounds are widespread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates.<sup>5</sup> A number of molluscs were ranked very high in the priority list of species exhibiting antimicrobial activity.<sup>6</sup> Antimicrobial activities have been reported from bivalves like oyster *Crassostrea madrasensis* and mussel *Perna viridis*,<sup>7</sup> winged oyster *Pteria chinensis*<sup>8</sup> and also on horse mussel *Modiolus*.<sup>9</sup> Bivalve molluscs have compounds of high biomedical properties. They are not only a cheap source of protein for human consumption but also found to possess some complex bioactive

compounds which have tremendous potential in medical science. Brown mussel hydrolysate for example is available for human use in Russian market with trade names Viramid and Midel as antiviral drugs.<sup>10</sup> Luminescent (light-emitting) compounds like Pholasin from *Pholas dactylus* have been extracted and used in inflammation, free radical and oxidative stress research as indicator.<sup>11-14</sup>

The mode of action, the broad activity, the molecular diversity, and the noncytotoxicity of all these circulating antimicrobial compounds make them very attractive as therapeutic agents for pharmaceutical or agricultural applications. Researchers consider them as “natural antibiotics” and as such a new and innovative alternative to chemical antibiotics with a promising future as biotechnological tools. These features make these compounds a powerful arsenal of molecules that could be the antimicrobial drugs of the new century as an innovative response to the increasing problem of multi-drug resistant (MDR) organisms.<sup>15</sup> Considering phylogenetic relationship, as a bivalve species, it is possible that Diwal also contain antimicrobial compounds. To determine the potential of Diwal as a source of antimicrobial compounds, its cold methanol and PBS (phosphate buffer saline) extracts were screened for antimicrobial activities. Therefore, if Diwal is found to have antimicrobial properties, this will surely add to its saleability as a commodity. Not only that the Diwal resource is resuscitated but there would be a ready source when they are used therapeutically, or industrially in agriculture and in aquaculture.

## Materials and methods

### Collection and Extraction

Live specimens of bivalves, *Pholas orientalis* were collected fresh from the fish landing of Barangay Punta Cogan, Roxas City, Capiz,

Philippines, which is the site of the Diwal Rehabilitation Project of Roxas City Coastal Resource Management (CRM) Office. Live Diwal were transported directly to the laboratory of the NIMBB (National Institute of Molecular Biology and Biotechnology), University of the Philippines Visayas (UPV), Miagao, Philippines. Each specimen was weighed individually. The length and width were also measured using a caliper. The specimens used were of 12-13 cm long and 3-4 cm in its widest part (Figure 1). Prior to dissection the specimens were washed to remove the dirt and mud present inside and outside of the shell. Different parts of Diwal were dissected out with a fine scissor, namely: siphon, mantle, body meat, and gills, (Figure 2). The parts were washed thoroughly with distilled water.

Two different methods of extraction were done, that is, extraction in cold methanol and in PBS (phosphate buffer saline) considering that antimicrobial compounds may include proteins or peptides. With PBS extraction, protease inhibitor was added before homogenization to prevent degradation of proteins present in the extracts. In every 100 g (wet weight) sample, 1.5 µl protease inhibitor was added.

Different parts of Diwal were soaked separately in cold methanol and in PBS (phosphate buffer saline) for 48 hrs and homogenized using an osteorizer in chilled condition to avoid degradation of proteins or protein-like compounds. Homogenized samples were stored in the refrigerator and allowed to cool for 24 hrs and then filtered twice using Whatman filter paper no. 42 placed in ice to keep it under low temperature to avoid degradation. The filtrates were evaporated to dryness in rotary evaporator under reduced pressure of 60 mmHg at temperature of 30°C. The collected concentrated extracts were further acetone-dried by centrifugation at a speed of 1650 rpm for 10 mins. The dried extracts were collected and stored in refrigerator to be used all throughout the experiment.

### Antibacterial Assay

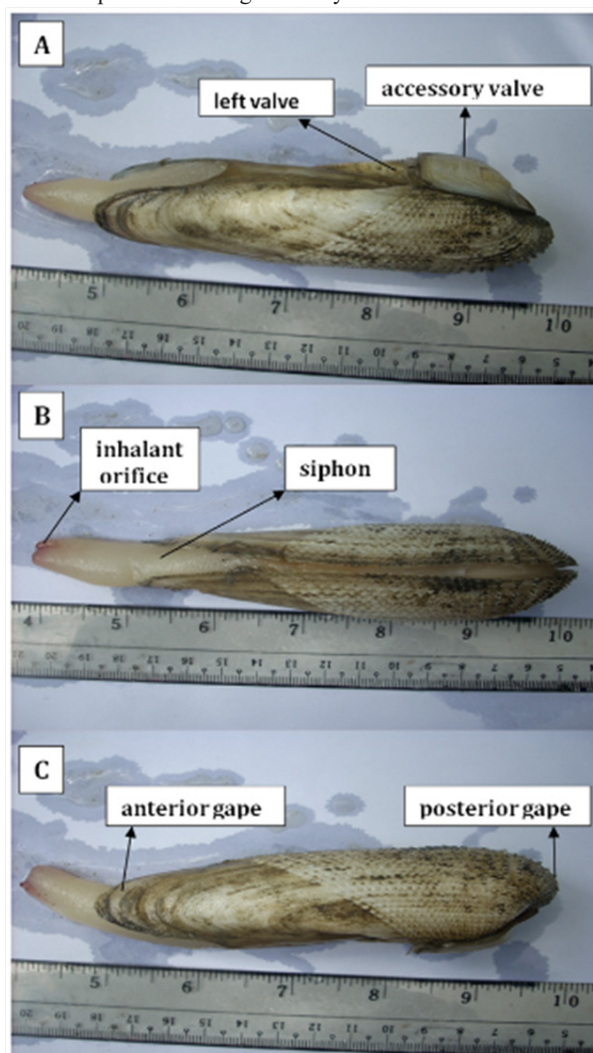
Antibacterial activity of Diwal (*Pholas orientalis*) was determined against four bacterial strains: *Staphylococcus aureus* (ATCC No. 29737) and *Micrococcus luteus* (ATCC No. 4698) for gram-positive bacteria and *Pseudomonas aeruginosa* (ATCC No. 29336), and *Vibrio harveyi* (ATCC No. 1987) for gram-negative bacteria. These strains were obtained from the Division of Biological Sciences Microbiological Collection, UPV, Miag-ao, Iloilo and Philippine National Collection of Microorganisms (PNCM), UP Los Baños, Laguna, Philippines. The antibacterial property of Diwal extracts were assayed using the standard paper disc diffusion method. The preparation of materials and culture media were done according to the procedure compiled by Quinto and Santos.<sup>16</sup>

Antibacterial activity was evaluated by measuring the diameter (mm) of the clear zone of inhibition and corresponding antibacterial index for each inhibition was calculated using the following formula:<sup>17</sup> Antibacterial index = zone of inhibition –

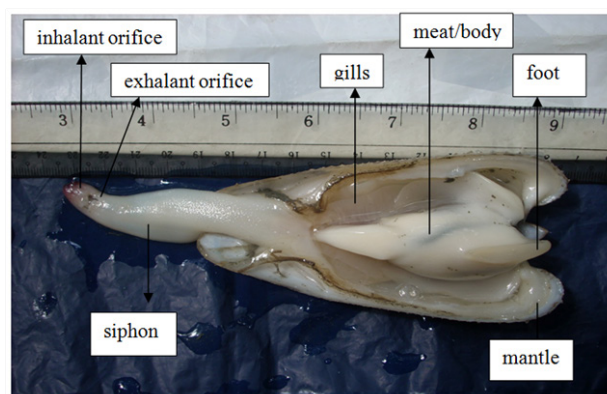
The bacterial isolates were prepared following Ruangpan and Tendencia<sup>18</sup> methods. A loopful of bacteria, gram (+) and gram (-) were inoculated separately in a flask with 50 ml nutrient agar (NA) broth and incubated for 18-24 hrs at 35° C. Five (5) ml of the inoculated bacteria were transferred to a sterile test tube and compared with the prepared 0.5 McFarland standard resulting to 1.5x10<sup>8</sup> CFU/mL of the test organism to be used in the experiment.

Whatman no. 1 filter paper discs with 6 mm in diameter were used for antibacterial tests. Paper discs were wrapped in aluminum foil and sterilized in an autoclave for 15 minutes at 15 psi. A sterile forcep was used to impregnate paper discs with the different extracts of Diwal

(methanolic and PBS) ranging from 100 500, and 1,000 mg/mL A positive control containing commercial antibiotic (Chloramphenicol), solvent control of methanol or PBS solution, and water as negative control were provided during the assay.



**Figure 1** Features of *Pholas orientalis* (Diwal) A. Dorsal view, B. Ventral view and C. Side view.



**Figure 2** *Pholas orientalis* opened up to show the different parts used, extracted and assayed for antimicrobial activities.

The assay was done on the plate containing Mueller-Hinton agar (MHA). The test bacteria were swabbed onto the plate. The

experiment was done in three replicates for each test organism. The test plates were incubated at 37°C for 24 hours.

### Antifungal Assay

Antifungal activity was determined against three (3) fungal strains namely: *Aspergillus niger*, *Trichoderma sp.*, and *Candida albicans*. These strains were obtained from the Division of Biological Sciences Microbiological Collection, UPV, Miag-ao, Iloilo, Philippines. The preparation of materials and culture media were done according to the procedure compiled by Quinto and Santos.<sup>16</sup>

The fungal isolates were prepared using Sabouraud agar or Potato Dextrose agar (PDA) following Ruangpan and Tendencia [18] methods. A loopful of test fungal strains were inoculated separately in a flask with 50 ml Peptone-Yeast extract-Glucose (PYGB) broth and incubated for 18-24 hrs at 30°C. Five (5) mL of the inoculated fungus were transferred to a sterile test tube to be compared with the prepared 0.5 McFarland standard resulting to 1.5x10<sup>8</sup> CFU/ml of the test organism to be used in the experiment.

A positive control containing commercial antifungal (Ketoconazole), solvent control containing methanol or PBS, and water as negative control were provided in dishes containing Potato Dextrose agar. The fungal test organisms were swabbed onto the test plates in three replicates each.

### Results and discussion

In the present study cold methanol and PBS (phosphate buffer saline) extracts of bivalve, Diwal (*Pholas orientalis*) were screened for antimicrobial activities. The extracts were obtained from whole body meat, siphon, mantle, and gills of the bivalve and tested against gram-positive (*Staphylococcus aureus* and *Micrococcus luteus*) and gram-negative (*Pseudomonas aeruginosa* and *Vibrio harveyi*) bacteria for antibacterial activity and against three fungal strains: *Aspergillus niger*, *Trichoderma sp.*, and *Candida albicans* for antifungal activity.

All the assays for antibacterial activity conducted using different extracts from methanol and phosphate buffer saline (PBS) at all concentrations (100, 500 and 1,000 mg/mL) did not show any activity. No extract from any body part of Diwal had antibacterial activity. The absence of antibacterial activity of the extracts may be due to low concentration of the bioactive compounds extracted from the different body parts of Diwal which was insufficient to hamper the growth of the test bacteria. It is also possible that the type of bioactive compounds present in the Diwal extracts may not have intrinsic antibacterial property that, in spite of increasing the test extract concentration to 1,000 mg/ mL, still no antibacterial activity was observed.

It did show antifungal activity. The body meat (cold methanol and PBS extracts) and mantle (cold methanol extract) parts of Diwal at a dose of 1000 mg/mL showed antifungal activity against *Trichoderma sp.*, a fungus that caused skin dermatitis in humans (Table 1). The antifungal activity, however, was not as high as the positive control, Ketoconazole. The antifungal index was only 0.17 for body meat and mantle methanol extracts and 0.33 for body meat PBS extract as compared to 1.33 antifungal index of the positive control, Ketoconazole which was given at the dose of 10mg/mL (Table 1).

Just like in the antibacterial assay, the gills and siphon parts of Diwal in both cold methanol and PBS extracts did not show antifungal activities. It is possible that antimicrobial compounds in these body parts of Diwal are not soluble in polar solvents, methanol and PBS used in the present study. The nature of the antimicrobial constituents

suspected may be non-polar (or partly non-polar). Nonpolar solvents are able to dissolve bioactive compounds for acetone and chloroform crude extracts of whole body meat of winged oyster exhibited broad antibacterial activity.<sup>8,19</sup> *Candida albicans* exhibited high zone of inhibition against n-butanol extracts of marine mollusc *Thais tissoti*.<sup>6</sup> Although, a protease inhibitor was also added before homogenization in PBS, but the amount used may not be enough to prevent degradation, that when the PBS extract was assayed, poor bioactivity was observed. Based on the review by Tincu and Taylor,<sup>1</sup> antimicrobial peptides are major components of the innate immune defense system in marine invertebrates which show antimicrobial properties.

**Table 1** Antifungal activity of Diwal (*Pholas orientalis*) extracts against *Trichoderma sp.* at 1000 mg/L. Ketoconazole as positive control is given at 10 mg/L

Extracts	Ave. zone of inhibition (mm)	Antimicrobial index
Methanol extract Mantle	7	0.17
Body meat	7	0.17
PBS extract Body meat	8	0.33
Ketoconazole (positive control)	14	1.33
Methanol (solvent control)	-	-
PBS (solvent control)	-	-
Water (negative control)	-	-

The mantle and body meat of Diwal, however, showed potential antifungal property. Preparations of extracts of these Diwal parts should be further tested for its therapeutic use against human dermatitis. It is also recommended to do antimicrobial activity screening again with extracts of the different parts of Diwal using nonpolar solvents. Protein assay and an SDS-Page electrophoresis should also be done. The crude protein or peptide content of Diwal extracts should be screened for antimicrobial activities for its potential as sources of antibacterial and antifungal substances is still not fully explored. Our preliminary results reveal that Diwal can produce bioactive compounds which could be of pharmaceutical interest and is sufficient to affirm further research.

### Acknowledgements

We thank the University of the Philippines Visayas, Miagao, Iloilo, Philippines for the research fund.

### Conflicts of interest

None.

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