Secondary Metabolites Screening of Ink from Sea hare, *Dolabellaauricularia* and Its Anti-bacterial Activity

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Abstract*: Secondary metabolites present in the purple ink of sea hare (Dolabellaauricularia) resource from Davao Oriental, Philippines were determined. Different qualitative test namely Froth test (for saponins),Libermann-Buchard(sterols), Bate-Smith and Metacalf (flavonoids),Dragendorff and Maeyer(alkaloids), Keller-Kiliani(glycosides), ferric chloride (tannins) andBortnträger(anthraquinones) were used. Its anti-bacterial activity was also determined against Escherichia coli and Staphylococcus aureus using agar well diffusion method. Results revealed that ink contains saponins, steroids and flavonoids. The test for alkaloids, glycosides, tannins and anthraquinonesshowed negative results. Further, the ink extract showed antibacterial factor.*

Keywords: antibacterial activity, *Dolabellaauricularia*, *Escherichia coli*, *Staphylococcus aureus,* secondary metabolites.

1. Introduction

Sea hare (*Dolabella sp*.) is a soft bodied marine organism belonging to Phylum Mollusca. It is often called “sea bunnies” because of their two long ear-like projections or extensions of their bodies [1].Their scientific name (Anaspidea) is derived from the Greek word “without shield”. This marine organism is a rich source ofsecondary metabolites which are being obtained from their algal diet and plays a vital role in its chemical defense via the purple ink [2].

Sea hare, *Dolabella*species have been reported to contain some other biologically active substances including antibacterial factor, haemagglutins, cytotoxins, and chemical defense substances. The studies on the purple fluid of *D. auricularia*implicate the potentials of biologically active compounds for medicinal purpose. It exhibited a broad antibacterial spectrum against some of the tested pathogens. It shows highest activity against *P. aeroginosa* and minimum activity was observed against *V. cholera*[3]. Further, Dolabellanin and dolastatin compounds have been isolated from *D. auricularia* (Indianocean sea hare) which have extreme potential for chemical use as anticancer agent. In fact, Dolastatin 10 is now already in its phase II clinical trials [4].

The province of Davao Oriental has an abundant supply of sea hare. It produces egg strings which is a source of food and livelihood among the local fisher folks. The potential of this resource is not only for sustenance but as a source of pharmacological compounds as well. There is not much scientific information available about the composition of the ink of the sea hare coming from Davao Oriental. Hence, this study aimed to determine the presence of secondary metabolites of ink from sea hare and its antibacterial activity. Hopefully, sea hare from Davao Oriental could be a good source of another bioactive andnovel compounds.

2. Material and Methods

The sea hares were collected along the coast of Guang-guang, Pujada Bay, Mati, Davao Oriental (Figure1).The area includes a fishing ground and a stretch of mangrove reforestation. The sea bed is sandy-muddy with sea grasses making it more preferable by the burrowing mollusk. The absence of an extreme low tide in the area makes it a good breeding ground and hence a rich marine site.

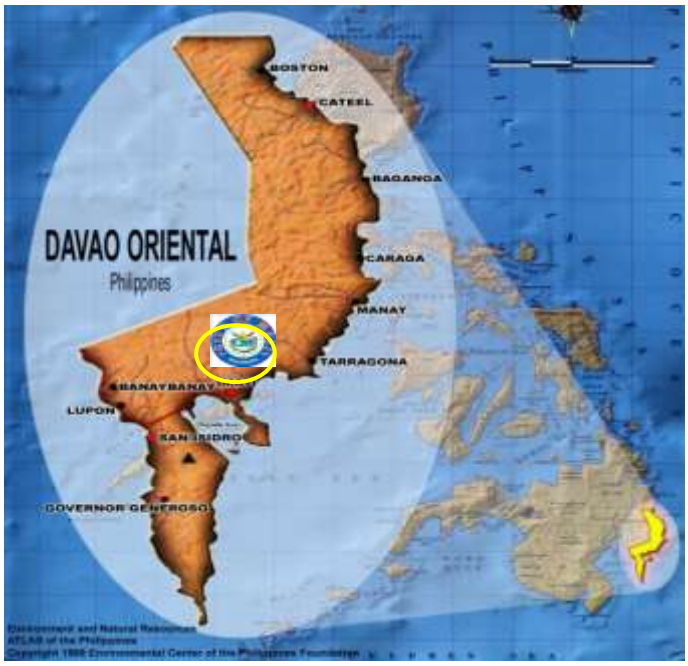

Figure 2. Map of Davao Oriental showing the study site, PujadaBay,City of Mati (Courtesy of Environmental Center of the Philippines

2.1 Sample Collection and Preparation

Ink samples was extracted from sea hare by exerting pressure into the organism for it to release ink. Collected ink samples about 100-200 mL was transferred into a reagent bottle. About 100 mL of 95% ethanol was added to the sample and allowed to stand for 24 -48 hours. The mixture was then filtered and concentrated *in vacou* at temperature below 50^{o}C using rotary evaporator. The concentrate was used for the screening of secondary metabolites and its antibacterial activity.Standard procedures were followed for secondary metabolites screening and test for antibacterial activity[6].

2.2 Secondary Metabolite Screening

2.2.1Test for Saponin (Froth Test)

A volume of the extract was placed in a test tube added with one mL of gogo extract. After standing, 10 mL distilled water was added. The test tube was then stoppered and shaken vigorously for 30 seconds. The formation of honeycomb froth after 10 minutes is a positive indication of saponins.

2.2.2 Test for Steroids (Liebermann-Buchard)

One portion of the prepared mixture from the Keller-Kilianimethod was stirred. Allowed to stand and added with 6mL dichloromethane followed by the addition of 3 drops of acetic anhydride. Color was then observed. A change in color of dichloromethane means a positive result.

2.2.3 Test for alkaloids (Laboratory Test Tube Method)

The ink extract was evaporated to a syrupy consistency. It was added with 5 mL 2M HCl, heated for 5 min and cooled. A 0.5 g NaCl was added and the mixture was stirred and filtered. One mL of the filtrate was added with 3 drops of Mayer's Reagent and another 1 mL with Dragendorff's reagent. Positive results will give white and orange precipitate for Mayer's and Dragendorff's reagent respectively.

2.2.4 Test for Glycosides (Keller-Kiliani)

The ink extract was evaporated to dryness and defatted with petroleum ether. Gentle mixing was done followed by removal of the excess petroleum ether. It was then filtered and divided into 4 tubes with one portion as the control. One portion was added with 3 mL ferric chloride reagent followed by the addition of 1 mL concentrated sulfuric acid. The mixture was allowed to stand and color observation was noted.

2.2.5 Test for Flavonoids (Bate-Smith &Metacalf)

The ink extract was evaporated to dryness and defatted with petroleum ether. The ether was discarded after thorough mixing. Dilution with 80% ethanol was done followed by filtration and dividing the solution into 5 equal parts. One part was treated with 0.5 mL concentrated HCl. Strong red or violet color is an implication for the presence of flavonoids.

2.2.6 Test for Tannins (Ferric Chloride Test)

An incipient dried sample was extracted with hot distilled water and added with 5 drops of 10% NaCl solution. Filtered and divided into equal portions. One portion was treated with 3 drops of 1% Ferric chloride reagent. A blue-black or brownish green color is an indication of the presence of tannins.

2.2.7 Test for Anthraquinone(Bortnträger'sTest)

An incipient dried sample was added with 10 mL distilled water, filtered and the residue was then discarded. The aqueous filtrate was twice extracted with benzene. The combined extract was divided in to two fractions. One portion was treated with 5 mL ammonia solution and was shaken. Development of red color in the lower ammoniacal layer indicated the presence of anthraquinones.

2.3 Antibacterial activity

2.3.1 Preparation of Test Organism

A loopful of bacteria was incubated in Muller-Hinton Broth for 24 hours at 35^{o}C. The turbidity of the culture browth is an indication of positive growth. A portion of the culture broth was transferred in a sterile screw capped tube, agitated on a vortex mixer.

2.1.2.2 Antibacterial Activity

The ink extracts were subjected to antimicrobial screening against pathogenic gram positive and gram negative microorganisms such as *Staphylococcus aureus* and *Eschericia coli* by agar well diffusion method. Ampicillin and penicillin were used as standard drugs respectively for studying the activities of the extract.

The melted agar was then poured into dry sterile petri dishes and solidified. A sterile cotton swab moistened with the test suspension was used to steak over the entire surface of the agar and allowing it to stand for 5 minutes. Acork borer was sterilized by immersing it in an erlenmeyer flask containing ethanol and heated over an alcohol flame. The agar was stabbed using the cooled cork borer all the way to the bottom of the dish to create a well. The well was filled with the extract. The incubation period was 24 hours at 35^{o}C. The activity of the extract was determined by measuring the diameter of zone of inhibition and compared with the values produced from the standard drugs.

3. Result and Discussions

Secondary Metabolite Screening revealed that sea hare's ink contained saponins, steroids and flavonoids. The honey comb formation indicated the presence of saponin using Froth test whileblue color formation resulted with Libermann-Buchard test for steroids. Furthermore, development of red color solution using Bate-Smith and Metacalfindicated the presence of flavonoids. The test for alkaloids, glycosides, tannins and anthraquinonesshowed negative results using laboratory test tube method (Dragendorff'sand Mayer's reagent), Keller-Kiliani, ferric chloride and Bortnträgerrespectively (Table 1).

There were only seven secondary metabolites tested qualitatively which limits the results of this study. Maybe, as we increase the number of secondary metabolites to be tested, more will be determined. Specific compounds that belong to other groups like terpenes, polyketides, peptides and porphyrins may also be isolated from the test organism, *D. auricularia* from Davao Oriental, Philippines.Accordingly, sea hares are a rich source of novel secondary metabolites. Different sea hare species accumulates different secondary metabolites depending on their algal diet. For instance , secondary metabolites terpenepalisadin A from red algae, *Laurenciaobtusa*and halogenated furanone 3 from *Deliseapulchra*were quantified from two species of sea hare, *Aplysiaparvula*and*Aplysiadactylomela*on varying amount [7]. On the otherhand, the *D. auricularia* contains compound aplysioviolin, prepacifinol epoxide and its derivative johnstonol and(-)-7-dehydrocholesterol [9].

The ink extract showed 6.0 mm zone of inhibition against *E. coli* and *S. aureus* whereas standard ampicillin and penicillin exhibited zone of inhibition at 18 and 26 mm in diameter respectively as shown

in Table 2. The antimicrobial activity of the ink extract was comparatively less than the standard drugs. Accordingly, sea hares have different microbial component. *A californica* contains escapin, an L-amino acid oxidase while *A. datylomela* contains dactylmeil-P. Both compounds exhibit antimicrobial activity at varying concentrations. The concentration of escapin is as high as 2mg/mL and dactylomelin is 30 micromol/L which are both above the antimicrobial threshold [9]. Hence, the low antimicrobial activity *D.auricularia*of Davao Oriental, Philippines maybe attributed to the type of antimicrobial compound present and its concentration. Moreover, the presence of saponins, steroids and flavonoids could be responsible or had contributed to its antimicrobial activity since these compounds generally have antibacterial factor [10,11].

This study showed that sea hare ink extract contains saponins, steroids and flavonoids which may be responsible for the seemingly weak antibacterial factor. The quantitative analyses of flavonoids and other secondary metabolites and its anti-oxidative property are currently being done. Moreover, collaborative efforts are also explored for the isolation, structure elucidation and identification of specific bioactive compounds from the three general secondary metabolites which will hopefully increase the medicinal value of sea hare, *D. auricularia*from Davao Oriental, Philippines.

Table 1. Secondary metabolites screening of Ink extract from sea hare.

Secondary Metabolites	***Test***	***Results***	***Conclusion***
Saponin	*Froth Test*	*Honey comb formation*	*+*
Steroids	*Liebermann-Buchard*	*Blue color formation*	*+*
alkaloids	*Laboratory Test Tube Method*	*No color change*	*-*
Glycosides	*Keller-Kiliani*	*No color change*	*-*
Flavonoids	*Bate-Smith &Metacalf*	*Red color formation*	*+*
Tannins	*Ferric Chloride Test*	*No color change*	*-*
Anthraquinones	*Bortnträger's Test*	*No color change*	*-*

Table 2. Antimicrobial activity of ink extract from sea hare.

Trial	***Gram positive*** ***Staphylococcus aureus***	***Gram negative*** ***Eschericia coli***
1	*+*	*+*
2	*+*	*+*
3	*+*	*+*
Ampicillin	*+++*	*Not tested*
Penicillin	*Not tested*	*++++*

Note: (-) indicate no activity, (+) weak activity with inhibition 1-6 mm, (++) slight activity with inhibition 7-12 mm, (+++) moderate activity with inhibition 13-18 mm and (++++) high activity with inhibition >19 mm.

4. Conclusion

Secondary metabolites screening showed the presence of saponins, steroids and flavonoids. The absence of alkaloid, glycosides, flavonoids, tannins and anthraquinones were confirmed from its negative results. The ink extract of sea hare exhibited antibacterial activity against *E. coli* and *S. aureus* relatively lower compared to the standard ampicillin and penicillin used. Preliminary results of this study showed that ink of sea hare from Davao Oriental is a good source of secondary metabolites and it has antimicrobial factor. These findings can be a basis for further chemical studies that will hopefully led to the discovery of some pharmaceutical substances.

Acknowledgement

The authors are grateful to Davao Oriental State College of Science and Technology for funding this study.

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