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Pharmacological activity of the methanolic extract of sea urchins against *Escherichia coli* and *Staphylococcus aureus*

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Abstract

This study elucidated the pharmacological potential of sea urchins using methanol as extracting medium. The antibacterial potential was evaluated using the paper disc method and zone of inhibition against *Escherichia coli* and *Staphylococcus aureus* was measured. Antioxidant properties of sea urchins were evaluated using DPPH radical scavenging assay. Three species of sea urchin randomly collected along the intertidal zone of Diguisit, Baler Aurora were identified using diagnostic keys by the National Museum of the Philippines and they were identified as follows; *Echinothrix diadema, Echinometra mathaei,* and *Echinometra oblonga*. *E. diadema* recorded the highest diameter zone of inhibition against *E. coli* and *S. aureus* after 24 hours of incubation with 11.03 ± 1.75mm and 13.52 ± 1.13mm respectively while *E. mathaei* only inhibited *S. aureus* with zone of inhibition of 9.27 ± 2.06mm in 24 hours of incubation as well. As the zone of inhibition prolongs, the zone of inhibition decreases as observed in 48 hours of incubation. *E. oblonga* did not show inhibitoy effect, however it recorded the highest radical scavenging activity with 64.46% among the three species of sea urchins. This was followed by *E. mathaei* (51.52%) and *E. diadema* (37.38%). All collected species manifested antioxidant potential. Based on the results, the collected species of sea urchins has a pharmacological potential.

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Introduction

Marine invertebrates are excellent sources of bioactive compounds with antibacterial and antioxidant dynamics. Recent discovery on pharmacological dynamics has stimulated the search for natural agents or natural sources that will lead to a desirable antibacterial and secondary metabolites (Abubakar *et al.*, 2012).

The diversity of marine organisms in our ecosystem, secondary metabolites has been identified as one of very important compound available produced in marine organisms. Sea urchins are small, spiny, globular animals belong to the class Echinoidea of the echinoderm phylum (Shankarlal *et al.*, 2011). Additionally, sea urchins have orbicular bodies coated with a strict shell and thoroughly covered with many sharp spines (Amarowicz *et al.*, 2012).

As stated in the study of Bich *et al.*, (2004), echinoderms have pharmacologically active secondary metabolites. The antibacterial activity of sea urchin is generally assayed through various extracts with different solvents. Methanol extract of *Tripneustes gratilla* showed highest antimicrobial activity against *Pseudomonas aeruginosa* (Abubakar *et al.*, 2012). Also, methanol extract of *Diadema setosum* exhibited higher zone of inhibition against *Salmonella typhimurium*, *Staphylococcus epidermidis, Citrobacter freundii,* and *Klebsiella pneumoniae* (Rahman *et al.*, 2015).

An investigation report of Bragadeeswaran *et al.* (2013) on the bioactive compounds of sea urchin *Temnopleurus toreumaticus* showed remarkable hemolytic and cytotoxic activities. The spines of purple sea urchin *Strongylocentrotus nudus* showed excellent activity by using DPPH scavenging activity indicating the presence of PHNQ as potential sources of natural antioxidants (Zhou *et al.*, 2011).

The present work focused on the screening of the antibacterial and antioxidant activity of whole globular body and tissues of *Echinothrix diadema*, *Echinometra mathaei,* and *Echinometra oblonga* collected from the intertidal zone of the coastal ecosystem of Barangay Diguisit, Baler, Aurora, Philippines.

Materials and methods

Collection and Sample Preparation

Live specimens of sea urchins were randomly collected along the intertidal zone of the coastal ecosystem of Barangay Diguisit, Baler Aurora. Collected sea urchins were placed in a styrofoam box containing crushed ice and immediately brought to the laboratory. After dissection, all tissues were frozen in liquid nitrogen for 5 min and stored in unilluminated condition at -70°C to avoid thermo-degradation of secondary metabolites until extraction.

After removal of the internal organs, the shells (with spines) were washed with a stream of cold-water ground and stored at -40°C until used.

Specimen Identification

After taking digital photographs of the live specimen, sea urchins were sent to the National Museum of the Philippines for identification using diagnostic keys.

Methanolic Extraction of Echinoidea

The extraction procedure was adopted from Abubakar *et al.* (2012) with some modifications. Twenty-one grams of *Echinothrix diadema*, 31g of *Echinometra mathaei*, and 17g of *Echinometra oblonga* were homogenized and extracted with 10 volumes/1 gram (v/w) of 70% (v/v) methanol for 48 hours. Sample was filtered using Whatman filter paper No.1. The filtrate was reduced at 55°C in a rotary evaporator. The extract was stored in sterile vial and was stored in 4°C prior to use.

Screening of Antibacterial Activity

Test Organisms

E. coli (ATCC 2592; AN1964) and *S. aureus* (ATCC 6538; AN1823) were used as bacterial pathogens for the pharmacological potential of sea urchin species. Pathogens were obtained from the Philippine National Collection of Microorganisms, University of the Philippines, Los Baños, Laguna.

Preparation of Inoculum

The method in the "Manual on Antimicrobial Susceptibility Testing" by Lalitha (2004) was adopted in the preparation of the inoculum. Three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. After 24 hours of incubation, the growth culture was transferred in prepared nutrient agar slants and incubated for 18 hours. A loopful of bacteria was transferred into 10mL nutrient broth and incubated at 37°C for 6 to 8 hours.

Turbidity of the growing culture was adjusted with sterile broth to obtain turbidity comparable to that of 0.5mc Farland standard. The comparison was done visually against a white background and black lines under adequate light condition.

Preparation of Assay Plates

Thirty-eight grams of Mueller Hinton Agar II was dissolved and melted in one – liter distilled water and sterilized in an autoclave at 121°C, 15psi for 30 minutes. It was cooled down enough to maintain the liquid form and minimize moist inside the petri plates. It was poured on the sterilized petri plates and allowed to solidify.

Preparation of Paper Disc

Discs of Whatman no. 1 filter paper with a diameter of 6mm was cut out using an office punch and was placed in a petri plate and sterilized in an autoclaved at 121°C, 15 psi for 30 min.

Antibacterial Assay

Disc diffusion (Mokhlesi *et al.,* 2011) was used to study the pharmacological property of collected species of sea urchin. Petri plates were inoculated 100µL of bacterial culture and were aseptically swab using a sterile cotton swab and allowed to dry for a few minutes. Each sterile disc was dipped in 100 µl of the various extracts and allowed to dry for 15-30 minutes and carefully placed on the agar plate using sterilized forceps, ensuring that the discs were at least 2cm separate from one another.

There were three - discs inoculated in each petri plate (Streptomycin, methanol, extract). Three replicates were set for each treatment (T_1 - *Echinothrix diadema*, T_2- *Echinometra oblonga*, T_3- *Echinometra mathaei*, T_4- Streptomycin sulfate, and T_5- methanol). After 30 min, the plates were properly labeled, inverted and incubated at 37°C. After the incubation of the plates, zones of inhibition were observed and diameter was measured using a digital vernier caliper. Zones of inhibition were measured after 12, 24, 36 and 48 hours of incubation.

Antioxidant Activity Evaluation

DPPH Radical-Scavenging Assay

TwomL of extracted samples were added in 2mL of 4mm DPPH methanolic solution. An 85% methanol solution was prepared by placing 850mL of absolute methanol into a 1000mL volumetric flask and diluted to mark with distilled water. A 4mM DPPH stock solution was prepared by weighing 0.1577g of 2,2-diphenyl-1-picrylhydrazyl (DPPH)in a dark/dim condition and transferred to a 100-mL volumetric flask covered with aluminum foil.

For DPPH working solution, a 0.1mm was prepared by pipetting out 12.5mL of the DPPH stock solution into 500-mL volumetric flask and diluted to mark with 85% methanol. The DPPH scavenging activity was done by pipetting 0.5mL each of 6-hydrohy-2,5,7,8-tetramethylchroman-2-carboxyli cacid (Trolox) standards (0, 20, 40 ,60, 100, 160, 200, 240, 280, 320µM), checks (Ascorbic acid and BHA) and extracted samples into test tubes. Five milliliter of DPPH working solution was added and mixed using vortex and incubated for 1-hour. The absorbance was read using UV-vis at 517nm. The scavenging activity was calculated as follows: µM trolox /g sample. The antioxidant assays were done at Saint Mary's University, Bayombong, Nueva Vizcaya, Philippines.

Statistical Analysis

The characteristic observed as affected by different treatments and was analyzed using analysis of variance (ANOVA). The comparison of mean was done using Duncan Multiple Range Test (DMRT) at 5% level of significance using SPSS v16.0.

Results and discussion

Sample Identification

Digital photographs of live specimen were sent to the National Museum of the Philippines for identification. Using diagnostic keys, specimens were identified as follows:

Echinothrix diadema

Fig. 1. *Echinothrix diadema.*

Kingdom: Animalia
Phylum: Echinodermata
Class: Echinoidea
Order: Diadematoida
Family: Diadematidae
Genus: *Echinothrix*
Species: *diadema*

The diadema urchin is a species of tropical sea urchin, member of the Diadematidae family. *E. diadema* (Fig. 1) is a long - spine urchin. With its spines, the typical diameter is 10–20cm (3.9–7.9 in). It is generally black or blue-black in colour, and always dark. The spines are closed at the tip; the anal sac is small and dark. *E. diadema* occurs in shallow coral and coral rubble areas at depths of 1 to 40 m. *E. diadema* is herbivore displaying nocturnal feeding behavior. It is known to graze on organic material and adults may also feed on live hard corals (Schoppe, 2001).

Echinometra mathaei

Fig. 2. *Echinometra mathaei.*

Kingdom: Animalia
Phylum: Echinodermata
Class: Echinoidea
Order: Camarodonta
Family: Echinometridae
Genus*: Echinometra*
Species*: mathaei*

E. mathaei (Fig. 2) are roughly spherical in shape and exhibit pentamourous symmetry. The urchin consists of the main body known as the test and spines on the ventral surface of the urchin are smaller in size and are parted in the center where the feeding appendage occurs, and spines are similarly smaller on the aboral surface where they give way to the anus. *E. mathaei* is a dark species digging itself into the basaltic and calcareous rock where it lives (Horton, 2012).

Echinometra oblonga

Fig. 3. *Echinometra oblonga.*

Kingdom: Animalia
Phylum: Echinodermata
Class: Echinoidea
Order: Camarodonta
Family: Echinometridae
Genus: *Echinometra*
Species: *oblonga*

Rock crevices are the natural habitat of *E. oblonga* (Fig. 3). The body of regular sea urchins possesses a pseudo-spherical radially symmetric body with hard prominent spines and prefers hard substratum. Body shape of irregular sea urchin marked bilateral symmetry. Soft spines are present that facilitate life style as sand and mud burrowing animals (Yasmin, 2015).

Antibacterial Property of Echinothrix diadema, Echinometra mathaei, and Echinometra oblonga

This study elucidates the antibacterial activity of methanolic extract of different species of sea urchin namely *E.diadema, E.mathaei* and *E.oblonga* against *E. coli* and *S. aureus*. Diameters of zone of were used as a measure of the degree of the antibacterial activity on each strain and recorded after 12, 24, 36 and 48 hours using a digital vernier caliper.

The result of antibacterial activity of *E. diadema*, *E. obolonga, E. mathaei,* streptomycin sulfate and methanol against *E. coli* was shown in Table 1. Results showed that after 12, 24, 36 and 48 hours of incubation. The highest mean value was observed in streptomycin sulfate which serves as the positive control. It was also observed that *E. diadema* is the only extract of sea urchin inhibited the growth of *E. coli* with the highest diameter of 11.03 ± 1.75mm at 24 hours of incubation.

The zone of inhibition of *E. diadema* extract decreases but still effective at 48 hours of incubation with diameter of zone of inhibition of 7.52 ± 2.15mm (Fig. 4).

Table 1. Mean diameters of zone of inhibition by different treatments on *E. coli* after 12, 24, 36 and 48 hours of incubation.

Treatment	Diameters of Zone of Inhibition (mm)			
	12 hrs	24 hrs	36 hrs	48 hrs
Echinothrix diadema	5.65± 0.80^{b}	11.03 ± 1.75^{b}	9.38 ± 1.99^{b}	7.52 ± 2.15^{b}
Echinometra mathaei	0.00± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Echinometra oblonga	0.00± 0.00 ^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
(+) Control	18.25± 0.70^{a}	25.31± 0.82^{a}	22.37 ± 0.87^{a}	21.45 ± 0.90^{a}
(-) Control	0.00 ± 0·00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

*Values with the same letter are not significantly different at P<0.05 according to DMRT.

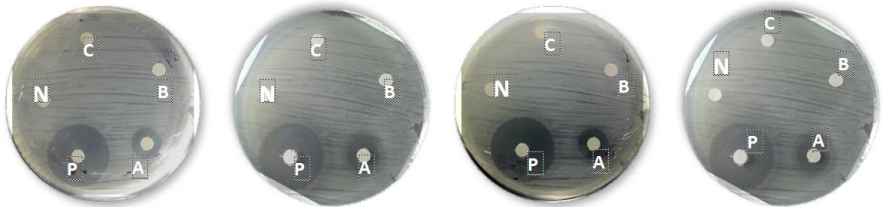

Fig. 4. Assay plates of extracts against *E. coli*, A: *E. diadema* methanol extract; B: *E. oblonga* methanol extract; C: *E. mathaei* methanol extract; P: streptomycin sulfate (positive control); N: negative control (methanol) after 12, 24, 36 and 48 hours of incubation.

Analysis of variance revealed that at 5% level of significance, there is significant difference among the treatments. Statistical analysis showed that positive control was significantly different from the rest of the treatments. The extract of *E. diadema* exhibited mean zone of inhibition which is significantly different among the other sea urchin extract. The *E. mathaei* and *E. oblonga* showed comparable results with the negative control. Correspondingly, Table 2 present the data for the zone of inhibition for *S. aureus*. After 12, 24 36, and 48 hours it is evident that the extract of *E.diadema* inhibited the growth of *S. aureus* with the highest diameter of 13.52 ± 1.13mm at 24 hours of incubation and *E. mathaei* with the highest mean

zone of inhibition of of 9.27 ± 2.06mm after 24 hours of incubation (Fig. 5). It was observed that as the incubation prolongs, zone of inhibition decreases in both extract of *E. diadema* (10.53 ± 1.04mm) and *E. mathaei* (6.48 ± 2.45mm) but is still effective at 48 hours of incubation. Analysis of variance revealed that at 5% level of significance, there is significant difference among the treatments. Statistical analysis showed that positive control was significantly different from the rest of the treatments. The extract of both *E. diadema* and *E. mathaei* exhibited mean zone of inhibition which is significantly different form *E. oblonga*. The *E. oblonga* showed comparable results with the negative control.

E. mathaei only inhibited the growth of *S. aureus* and inactive against the bacterial strain *E. coli*. According to the study of Silhavy *et al.* (2010), gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide which serves as a physical barrier providing the bacteria protection from its surroundings. For that reason, gram-negative bacteria are more resistant to antibiotics.

Table 2. Mean diameters of zone of inhibition by different treatments on *S. aureus* after 12, 24, 36 and 48 hours of incubation.

Treatment	Diameters of Zone of Inhibition (mm)			
	12 hrs	24 hrs	36 hrs	48 hrs
Echinothrix diadema	9.03± 1.45^{b}	13.52 ± 1.13^{b}	11.71 ± 1.35^{b}	10.53 ± 1.04^{b}
Echinometra mathaei	4.85± 0.63^{c}	9.27 ± 2.06^{c}	6.45 ± 3.64^{c}	6.48 ± 2.45^{c}
Echinometra oblonga	0.00± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
(+) Control	26.23± 2.13^{a}	25.31± 0.82^{a}	33.06 ± 1.29^{a}	31.80 ± 1.63^{a}
(-) Control	0.00 ± 0·00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

*Values with the same letter are not significantly different at P<0.05 according to DMRT

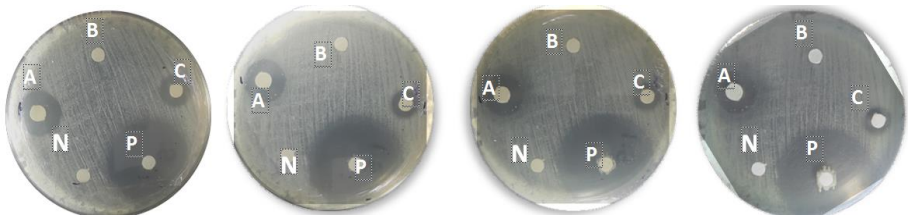

Fig. 5. Assay plates of extracts against *S. aureus*, A: *E.diadema* methanol extract; B: *E. oblonga* methanol extract; C: *E. mathaei* methanol extract; P: streptomycin sulfate (positive control); N: negative control (methanol) after 12, 24, 36 and 48 hours of incubation

The antibacterial property of *E. diadema* can be explained based on the study of Pettit & Ode (1979), that states that *E. diadema* has 2,5-Dihydroxy-3-ethylbenzoquinone ($C_8H_8O_4$) that has been isolated and identified using spectroscopic analysis by Awino *et al.* (2016) as an active antibacterial compound.

On the other hand, *E. oblonga* produced no antibacterial activity. This result is in accordance with the study of Kazemi *et al.* (2016) wherein *E. oblonga* produced no antibacterial activity against tested bacteria. The study of Kazemi *et al.* (2016) also concluded tissues of these sea urchins have just a physical role against environmental stresses including prey and predator and have no chemical function such as antibacterial activity.

Antibacterial activity has also been reported in gonad extracts of echinoid. In the latter study of Abubakar *et al.* (2012) the antibacterial compound was shown to be a lysozyme. These compounds have inhibitory effect on both gram-positive and gram-negative bacteria by disturbing the bacterial metabolism. This may possibly explain its inhibitory effect on the test organisms. The fact that the methanol extract of *E. mathaei* inhibit the growth of *S. aureus* and *E. diadema* inhibit the growth of both gram positive and gram-negative bacteria indicates its broad spectrum

of antibacterial activity. However, since its antibacterial activity is significantly weaker than streptomycin (positive control), this specifies the antibacterial activity is not comparable to the commercially available antibiotics, specifically streptomycin. Its presence of minimal antibacterial activity confirms its use as an anti-infection agent.

Antioxidant Activity of Echinothrix diadema, Echinometra mathaei, and Echinometra mathaei

Scavenging effects of methanolic extracts of species of sea urchin was presented in Table 3. The methanolic extract of *Echinometra oblonga* produced the highest antioxidant activity with the RSA of 64.46%, followed by *Echinometra mathaei* (51.52%). The lowest RSA was observed in *Echinothrix diadema* with 37.38%.

Spinochromes are colored molecules which are involved in both the pigmentation and the antioxidant activity of sea urchins. This suggests and spinochrome pigments are highly concentrated in the shells *E. oblonga*. In accordance to the study of Minh *et al.* (2004) and Shankarlal *et al.* (2011) that compounds isolated which is thespinochrome, specifically 2,7 dihydroxy-3-acetyl-naptazarinexhibits antioxidant activities and this was confirmed by the strong orange to the brown-like color of the extract.

Table 3. Scavenging effects of methanolic extracts of different species of sea urchin and standard cathechin on DPPH.

Treatment	Radical Scavenging Activity (%)
Echinothrix diadema	37.38%
Echinometra mathaei	51.52%
Echinometra oblonga	64.46%
(+) Control (Cathechin)	72.13%

*Values with the same letter are not significantly different at P<0.05 according to DMRz

Their chemical structure suggests other bioactive roles such as antioxidant property. Their antioxidant potential and their capacity to absorb UV light—which protects sea urchins from UV-induced damages have already been reported by Brasseur *et al.* (2017). According to the study of Lebedev *et al.* (2008) that it is well documented that structures having several phenolic OH groups can act as radical scavengers, radical scavenging is the most abundant activity reported for naphthoquinone pigments from sea urchins. Antioxidant properties of pigments have been studied in several different models. Interestingly, pigments in anionic and neutral forms appear to be capable of free-radical scavenging.

This suggests that sea urchin shells and spines, most of which are discarded as waste after removal of gonads, would be a new bio resource for natural antioxidants.

Conclusion

In conclusion, bioactive components which are responsible for antibacterial and antioxidant activity has been successfully extracted using methanol. *E. diadema* and *E. mathaei* which showed positive results can be a promising source of antibacterial activity. The antioxidant activity of both *E. mathaei* and *E. oblonga* indicates that the examined echinoderms could represent a new source of natural antioxidants. These findings suggest that echinoderm species is encouraged as a new tool for biotechnological applications in the pharmaceutical and nutraceutical field.

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