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Preliminary bioactivity screening of dichloromethane and methanol crude extracts from the fire coral *Millepora dichotoma*

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ABSTRACT

Marine organisms are a very good source of unique bioactive secondary metabolites, although they have been comparatively underexplored relative to their terrestrial counterparts. Preliminary investigation of crude extracts from the fire coral Millepora dichotoma revealed that the hemolytic potential of the MeOH extract, at a concentration of 5 mg/mL, was significantly lower at less than 5% hemolysis while DCM extract at the same concentration resulted to almost 100% hemolysis. Furthermore, antimicrobial assay revealed that the MeOH extract (5 mg/mL) mildly inhibited the fungi T. mentagrophytes and resulted in the thinning of growth of S. aureus, while DCM extracts showed no detectable activity against these organisms. These results demonstrated that MeOH extracts of M. dichotoma, without showing hemolytic side effects, could be further analysed for potential use against T. mentagrophytes and S. aureus infections.

Keywords: Millepora, fire corals, hemolytic, antimicrobial

INTRODUCTION

Marine organisms continue to be a rich source, albeit underexplored compared with their terrestrial counterparts, of novel bioactive secondary metabolites with unique structures and interesting biological activities. The number of new compounds reported from marine sources have steadily increased over the years and the most recent review of the 2014 literature puts it at over 1,300 novel molecules [1].

Millepora spp., which are hydrozoan corals, are common inhabitants of the world's reef systems, contributing to approximately 10% of the total surface cover, and even higher in some regions [2,3]. Being a cnidarian, a phylum that has traditionally been a good source of new compounds [1], millepores are potential and promising candidates for biological activity screening research. These animals are well documented to cause a painful, burning sensation and other pathological reactions in humans upon contact, brought about by a mix of toxins injected into the skin [4,5]. These resulting symptoms have led to them being more colloquially known as "fire corals." Early investigations on fire coral envenomation has revealed the proteinaceous nature of the causative toxins, the purified samples of which showed similar biological manifestations to recorded human envenomation cases [6-8]. More recently, various research groups have successfully characterized new protein toxins from fire corals that exhibit diversebioactivites such as phospholipase A2, cytotoxic, vasoconstrictor, hemolytic, vasopermeable, dermonecrotic, and calcium-dependent smooth muscle excitatory effects [9-13].In addition to these studies, almost all of which dealt with protein toxins, the chloroform: methanol crude extract of two species of fire corals, *M. platyphylla* and *M.*

dichotoma, collected from the Red Sea has been demonstrated to exhibit slight to moderate fungicidal and bactericidal activities [14].

In this report, a preliminary screening of the antimicrobial and hemolytic properties of the dichloromethane and methanol extracts of *M. dichotoma* was investigated and compared. To the best of the author's knowledge, this is the first report of a comparison of these two activities in extracts obtained from fire corals.

MATERIALS AND METHODS

Sample Collection

Samples of *M. dichotoma* were collected by Prof. Wilfredo Licuanan of the Biology Department of De La Salle University by scuba, in Puerto Galera, Oriental Mindoro, Philippines on March 2015. The specimens were immediately put in ice and transported to the laboratory and stored in a freezer prior to use. Identification of the samples were carried out by Prof. Abdulmohsin Abdullah M. Al-Sofyani of the Marine Biology Department, King Abdulaziz University, Saudi Arabia.

General Extraction Procedure

M. dichotoma samples were lyophilized for 24 hours, after which it was grounded into smaller pieces and soaked either in DCM or MeOH for 72 hours. Afterwards, the samples were filtered and the solvent was removed using a rotary evaporator to complete dryness to obtain the crude DCM and MeOH extracts. The extracts were re-dissolved in a mixture of dimethylsulfoxide: methanol (1:1 v/v) for bioactivity testing.

Hemolysis Assay

Two mL of fresh human blood were suspended in 18 mL of PBS buffer (0.1 M phosphate, containing 2.7 mMKCl and 137 mMNaCl, pH 7.4), mixed and centrifuged at 2000 rpm for 5 minutes, repeated twice. The residue was collected and re-suspended in 20 mL of the same PBS buffer, after which 2 mL of the resulting suspension was further obtained and diluted to 20 mL PBS buffer to give a 1% hematocrit suspension.

For the hemolysis assay, 190 µL of the 1% suspension was transferred to a 0.5 mL eppendorf tube, followed by addition of 10 µL of the test samples and incubation at 37 °C for 1 hour. Afterwards, the samples were centrifuged at 2000 rpm for 5 minutes and 50 µL of the supernatant was subsequently aspirated to a 96-well microplate. The amount of released hemoglobin was analysed by measuring the absorbance of the supernatants at 450 nm.

For both the DCM and MeOH extracts, hemolytic activity was tested at 5 concentrations; 0.01, 0.1, 0.5, 1, and 5 mg/mL. Vehicle and 100% hemolysis control used was a solution of dimethylsulfoxide:methanol (1:1 v/v) and 1 mg/mL detergent (sodium dodecyl sulfate) in water respectively. All samples were performed in triplicates. Percent hemolysis was calculated using the following formula:

$$\%\, hemolysis = \frac{As}{A100}\, x\, 100$$

Where As and A100 refers to sample absorbance and absorbance at the condition of 100% hemolysis, respectively.

Antimicrobial Assay

The DCM and MeOH extracts were tested for their activity against various bacteria, fungi, and yeast, namely *Bacillus subtilis* (UPCC 1295), *Escherichia coli* (UPCC 1195), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (UPCC 1143), *Candida albicans* (UPCC 2168), *Aspergillusniger* (UPCC 3767), and *Trichophytonmentagrophytes* (UPCC 3767). The bacterial/yeast and fungal suspensions were prepared from 24-hour old and 5-day old cultures of the microbes, respectively.

Corresponding microbial suspensions were inoculated in 3-mm thick pre-poured plates of nutrient agar (NA; for bacteria), glucose yeast peptone agar (GYP; for yeast) and potato dextrose agar (PDA; for fungi) by swabbing the agar surface. The cotton swab on the applicator stick was streaked over the entire agar surface, and repeated twice by rotating the plate 60° each time to ensure full and even distribution of the inoculum. Then, three (3) equidistant wells were made on the plates using a 10-mm cork borer, followed by the addition of 200 µL of the extracts, both at a concentration of 5 mg/mL. A vehicle control, dimethylsulfoxide:methanol (1:1 v/v), was also used.

The NA and GYP plates were incubated at 35 °C and observed after 24 hours, while the PDA plates were incubated at room temperature and observed after 72 hours. The clearing zone, in millimetres, was determined and the antimicrobial index (AI) was calculated using the formula:

$$AI = \frac{diameter\ of\ clearing\ zone - diameter\ of\ well}{diameter\ of\ well}$$

Statistical Analysis

Data from the hemolysis assay were compared and analyzed using an unpaired *t*-test ($p< 0.05$) with Graphpad Prism ver. 6.07.

RESULTS AND DISCUSSION

Fire corals belonging to the genus *Millepora* are known to cause painful stings upon contact with humans. These effects, including some other pathological reactions, have been blamed on proteinaceous toxins injected by the animals [4-13]. Not surprisingly, most of the reports on fire corals in the literature have focused on these molecules, and very few have investigated the bioactive potential of its crude solvent extracts. Hence, in this report, the hemolytic and antimicrobial potential of the DCM and MeOH extracts from *M. dichotoma* were explored.

One of the biggest challenges in today's healthcare system is the rapid emergence of bacterial and fungal strains that are resistant to common antimicrobials, even to those drugs of last resort [15,16], brought about by widespread and inappropriate use of these medications. The dangers posed by these previously susceptible microbes in terms of human and financial aspects has resulted in the increase of the number of research dedicated to finding new and potent antimicrobials to combat this growing threat.

Hemolysis, the destruction of red blood cells, is an unwanted side effect that could limit the potential use of extracts or drugs in the treatment of various diseases. Figure 1 shows the percent hemolysis caused by both the DCM and MeOH extracts against human erythrocytes. Both extracts were found to be comparable and not hemolytic up to a concentration of 1 mg/mL, but the DCM extract was significantly more hemolytic at 0.5 mg/mL. Unexpectedly, a very clear difference emerged at a concentration of 5 mg/mL; it showed an almost 100% hemolysis caused by the DCM extract, while on the other hand, the MeOH extract exhibited a negligible activity of less than 5% hemolysis. To ensure that hemolysis was due to the extract and not the solvent, a control experiment usingdimethylsulfoxide:methanol (1:1 v/v) was also carried out, which showed no hemolysis after solvent addition (data not shown).

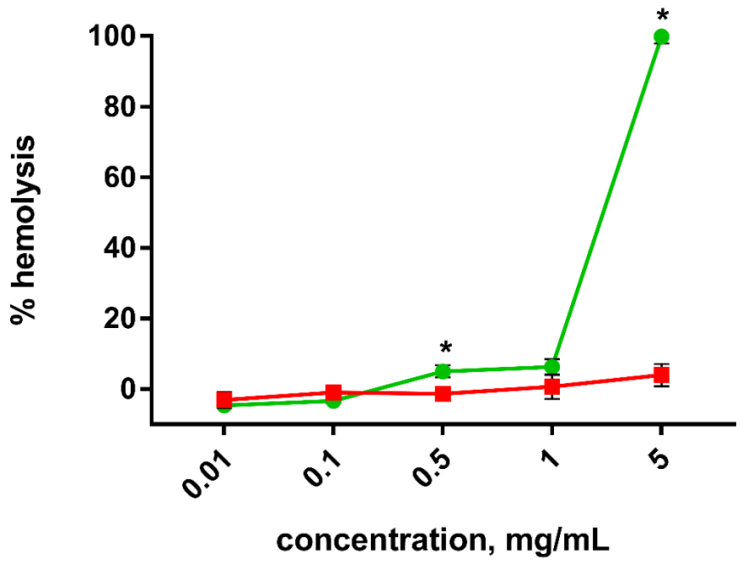

Figure 1. Hemolytic activities of the DCM (green) and MeOH (red) extracts from *M. dichotoma*. Results show the average of three trials. Error bars indicate standard deviation. * indicate significant difference between the two extracts ($p< 0.05$)

From the hemolysis results, both extracts were then tested for their antimicrobial activity at a concentration of 5 mg/mLagainst four bacteria and three fungal species. Results showed that both extracts were inactive at the concentration tested against *B. subtilis*, *E. coli*, *P. aeruginosa*, *C. albicans*, and *A. niger*. However, the MeOH extract showed a slight inhibition against the fungi *T. mentagrophytes*, approximately 30% of the standard drug's activity (a clearing zone of 13 mm against 45 nm). This result was comparable to the activity of a chloroform:methanol crude extract of *M. dichotoma*, collected from the Red Sea, against a related fungi, *T. schoenleinii* [14]. In addition, although MeOH extracts from this work did not inhibit *S. aureus* growth, a noticeable thinning of bacterial growth was observed within a 16-mm diameter from the sample well. This result indicates that the growth of the bacteria was retarded by the extract, and this thinning could be due to a higher tolerance of the bacteria to the sample. Ultimately, the extracts could be inhibitory at higher concentrations. A similar thinning of microbial growth have been reported before for a strain of yeast that have been implicated in protecting certain fruits against postharvest pathogens [17]. On the other hand, DCM extracts still did not show any activity against these organisms and results are summarized in Table 1. The finding that the MeOH extract of *M. dichotoma* exhibited some antimicrobial activity, while devoid of any significant hemolytic effect, suggests a potential for further investigating this extract to identify its active compound.

Table 1. Summary of the antimicrobial activities of the DCM and MeOH extracts of *M. dichotoma*

Test Organism	Sample	Clearing Zone (mm)			AI
		1	2	3	
B. subtilis	MeOH extract	-[a]	-	-	0
	DCM extract	-	-	-	0
	Chloramphenicol disk[c]	20			2.3
E. coli	MeOH extract	-	-	-	0
	DCM extract	-	-	-	0
	Chloramphenicol disk	27			3.5
P. aeruginosa	MeOH extract	-	-	-	0
	DCM extract	-	-	-	0
	Chloramphenicol disk	15			1.5
S. aureus	MeOH extract	(16)[b]	(16)	-	0
	DCM extract	-	-	-	0
	Chloramphenicol disk	33			4.5
C. albicans	MeOH extract	-	-	-	0
	DCM extract	-	-	-	0
	Canestensolution[d], 100 µL	32			2.2
A. niger	MeOH extract	-	-	-	0
	DCM extract	-	-	-	0
	Canesten solution, 100 µL	23			1.3
T. mentagrophytes	MeOH extract	13	13	13	0.3
	DCM extract	-	-	-	0
	Canesten solution, 100 µL	45			3.5

[a] *No clearing zone*
[b] *No inhibition of growth of the test organism but thinning of growth was observed within diameters in parenthesis*
[c] *Contains 30 µg chloramphenicol, 6 mm*
[d] *Contains 1% clotrimazole*

CONCLUSION

In this study, the hemolytic and antimicrobial activities of the DCM and MeOH crude extracts of the fire *M. dichotoma* were evaluated. The data revealed that MeOH extracts only exhibited a negligible hemolytic action, even at the highest concentration tested at 5 mg/mL, while showing activity against *T. mentagrophytes* and *S. aureus*. On the other hand, the DCM extracts was not active against any of the microbes tested, and it was also very hemolytic at 5 mg/mL. These results indicate the potential of the MeOH extract as an antimicrobial, without the unwanted side effects, in the treatment of infections from *T. mentagrophytes* and *S. aureus*. However, further investigations will have to be carried out to identify the active component of the MeOH extract of this fir coral.

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