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THE ANTIFUNGAL ACTIVITY OF THE HOLOTHURIN OF PHILIPPINE LITTORAL HOLOTHURIANS

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ABSTRACT

Ethanolic extracts of the body wall, gut, and Cuvierian tubules of littoral holothurians were tested for their activity against *Aspergillus fumigatus*, *Candida albicans*, and *Saccharomyces cerevisiae* by the surface diffusion method of assay. At dosage of 7 mg/ml, the sensitivity of the three fungi to holothurin did not vary. There were no significant differences among the families of sea cucumbers but, among genera, *Actinopyga* was found to be more effective than either *Bohadschia* or *Holothuria*. Body wall holothurin was effective with its activity varying according to the source of the specimens while gut holothurin showed some variation according to the time of collection.

Introduction

The body fluids of *Holothuria atra* are used to stupefy tide-pool fishes in Guam and Majuro Islands in the Indo-Pacific region (8). The toxicity of the sea cucumbers, however, seemed to be known earlier (cited in 5). Cooper in 1880 recorded the toxicity of a sea cucumber in his book *Coral Lands* which was noted by Halstead (9). Saville-Kent reported in 1892 fatal poisonings by ingestion of *Stichopus variegatus* while Castellani and Chalmers claimed in 1919 that the visceral fluid from *Holothuria argus* could cause dermatitis and blindness upon contact with the skin or the eye (5). With regard to the first reports, Cleland was likely right to argue copper poisoning instead of sea cucumber poisoning (5) inasmuch as since this report there had been no case of human death due to the consumption of the sea cucumber although Russel, as cited by Bakus (3), mentioned, too, of acute conjunctivitis in persons swimming in water containing tissue extracts of some sea cucumbers.

Thus, the toxicity of the sea cucumber particularly to fishes has been interpreted as the defense mechanism of the animals against predation (4) with the con-

centration of the toxin in some parts in each of the species as specific evolutionary development (2). The adaptive function of the sea cucumber toxic material has been correlated with the highest saponin contents of the ovaries and the integuments (12).

By virtue of its toxicity, therefore, the sea cucumber was hinted to possess a pharmacodynamic substance (7, 21). Nigrelli then experimented with Cuvierian tubule extracts on Swiss-Webster mice implanted with sarcoma tumorous cells (13). He reported the first finding of a possible medicinal value of holothurin - *holothurin* was the name given by him to the sea cucumber saponin. Many studies, subsequently, revealed the potential of holothurin as one of the "drugs from the sea" (17).

The study being presented herein investigated the potential of holothurin as an antifungal drug. Previous studies had suggested the efficacy of holothurin for possible topical application on fungal infections (1, 11, 18, and 19), wherefore, the relative abundance of even one or two species of the animals in Philippine littoral waters offered for the sustained interest in the research.

Materials and Methods

Some crude holothurins or ethanolic extracts randomly selected from samples used in the hemolytic assays (14 and 15) were tested *in vitro* for activity against three species of fungi: *Aspergillus fumigatus, Candida albicans,* and *Saccharomyces cerevisiae.* The measurement of antifungal activity was by the surface diffusion or paper disc method (10). *A. fumigatus* grown in potato-dextrose-agar medium and C. *albicans* and *S. cerevisiae* in glucose-yeast-peptone-agar medium were obtained from the U.P. - NSRI Culture Collection. Antifungal assays were performed in February to March, 1989 on stored refrigerated samples - 69 samples of body wall holothurin, 17 gut holothurin, and 7 of Cuvierian tubule holothurin.

Petri dishes, each containing 1 ml of the medium for the fungi, were inoculated with 1 ml suspension of fungal spores in 0.1% peptone. The fungal suspension was spread evenly on top of the agar by means of a cotton swab. Filter disc 6 mm in diameter and impregnated with 7 mg/ml holothurin solution was placed on top of the solid medium that had been previously seeded with the test organisms. The plates were incubated at room temperature, those plates containing *C. albicans* for 18 hours while those with *A. fumigatus,* within three days.

After incubation, the extent of inhibited growth or the diameter of the clear zone surrounding the filter disc was measured and the index of antifungal activity calculated as follows:

$$\text{Antifugal Activity Index} = \frac{\text{Diameter of cleared growth - Diameter of filter disc}}{\text{Diameter of filter paper disc}}$$

The average of three determinations was taken for the inhibitory potency of the different crude holothurins. The activity of holothurin was tested against 7 mg/ml Nystatin (Mycostatin, Squibb) and distilled water.

Analysis of variance and the Duncan's test (20) were the basis for the interpretation of the data.

Results and Discussion

Results of the antifungal assays are shown in Tables 1-3 and summarized in Figure 1.

At the dosage of holothurin used in the assay, it was found that there were no significant differences in the antifungal activity among the families of sea cucumbers but, among genera, *Actinopyga* was found to be more effective than either *Bohadschia* or *Holothuria*. The three test organisms did not differ in their sensitivity. Analysis for the influence of time and locality of collection revealed significantly higher activity of body wall holothurin of collections from San Fernando, La Union and significantly higher activity of gut holothurin of collections in 1983. Duncan's test showed antifungal activities ranked as follows according to time and place of collection: July 1983 > December 1983 > January-March 1984 > April-May 1984 > December 1984 > February-March 1985, collection in January 1982 not included; Lingsat San Fernando La Union > Poro San Fernando La Union > Padre Burgos Lucena Quezon > Kauswagan Lanao del Norte > Balongbato sandbar Calatagan Batangas > Balongbato Burot Point Calatagan Batangas > Tiwi Albay > Nueva Valencia Guimaras Iloilo > Balongbato Alvarez Farms Calatagan Batangas > Silaqui Island Bolinao Pangasinan > Mercedes Camarines Norte.

As in the previous studies (14 and 15), the variation in the composition of the extracts may be inferred from the absence of correspondence in the amounts of crude holothurin and the physiological effects. Actually, this has been revealed later by the thin layer chromatography of the extracts (16). Suggested, too, is that the fungistatic mechanism could be apart from the hemolytic mechanism.

An effective fungistatic action must deal not only with the sterols, principally ergosterol, either in free or combined forms in the cytoplasmic membrane (22) but also with the polymers of hexoses and hexosamines providing main structural wall elements of the fungal cell (6). The intricacy of the antifungal mechanism may then explain variations from the results of the hemolytic assay, such as in potencies of activity and the influence of time and locality of collection.

Comparison of present results with those obtained by the Russians (1, 11) also showed some discrepancies. The Russians had reported most promising antifungal from members of the family Stichopodidae and the genus *Bohadschia*, the groups characteristically yielding unsulfated holothurins. Moreover, of the fungi which they had used namely *Candida albicans, C. tropicalis, C. utilis, C. kruset,* and *Saccharomyces carlbergensis,* the latter was found to be most sensitive.

Table 1. The activity against three fungal species of body wall holothurin of different Philippine sea cucumbers

Species	No. of Samples	Average index of antifungal activity against A. fumigatus	C. albicans	S. cerevisiae
Family Holothuriidae				
Actinopyga echinites	9	3.3	2.1	2.6
Actinopyga mauritiana	1	3.4	2.1	3.7
Actinopyga miliaris	2	2.2	5.1	3.1
Actinopyga sp.	1	3.6	0.3	1.8
Bohadschia argus	1	1.1	0.6	0.8
Bohadschia graeffei	1	1.5	1.3	3.0
Bohadschia marmorata	3	1.2	2.8	3.0
Bohadschia vitiensis	3	0.7	1.2	0.7
Holothuria atra	10	2.3	1.4	2.2
Holothuria coluber	1	1.3	0.0	0.6
Holothuria fuscocinerea	3	1.9	0.9	1.4
Holothuria hilla	1	0.0	0.8	0.8
Holothuria impatiens	1	1.5	1.1	1.1
Holothuria klunzingeri	1	0.0	0.3	0.0
Holothuria nobilis	1	1.4	0.8	1.2
Holothuria pervicax	1	2.6	1.7	4.4
Holothuria pulla	6	2.2	2.4	2.1
Holothuria rigida	1	1.1	2.9	0.6
Holthuria sanguinglenta	1	2.0	0.8	7.9
Holothuria scabra	5	1.9	1.7	1.4
Holothuria tigris	1	1.8	0.0	0.3
Family Stichopodidae				
Stichopus chloronotus	1	1.1	0.0	1.2
Stichopus naso	1	0.2	0.8	1.9
Stichopus sp.	1	0.0	0.0	0.9
Stichopus variegatus	6	2.0	1.7	1.7
Stichopus variegatus var. *hermanii*	2	2.4	2.2	2.6
Family Synaptidae				
Opheodesoma grisea	1	1.0	0.4	1.5
Pendekaplectana nigra	1	0.4	1.3	1.0
Synapta maculata	1	1.4	0.3	0.5
Family Chiridotidae				
Polycheira rufescens	1	3.4	2.4	1.8
CONTROLS				
Nystatin (Positive control)		7.3	6.0	5.7
Distilled Water (Negative Control)		0.0	0.0	0.0

Table 2. The activity against three fungal species of gut holthurin of different sea cucumbers

Species	*No. of Samples*	*Average index of antifungal activity against*		
		A. fumigatus	*C. albicans*	*S. cerevisiae*
Family Holothuriidae				
Actinopyga echinites	1	4.0	5.6	1.3
Bohadschia graeffei	2	3.3	3.3	1.2
Bohadschia marmorata	4	1.4	2.0	4.0
Bohadschia vitiensis	3	2.0	1.7	4.2
Holothuria atra	1	1.7	2.8	0.5
Holothuria pervicax	1	1.6	1.0	1.5
Holothuria pulla	3	1.7	0.9	3.0
Holothuria scabra	1	2.2	2.2	0.6
Family Stichopodidae				
Stichopus variegatus	1	1.8	0.0	3.1
CONTROLS				
Nystatin (Positive control)		7.3	6.0	5.7
Distilled Water (Negative Control)		0.0	0.0	0.0

Table 3. The activity against three fungal species of Cuvierian tubule holothurin of different sea cucumbers

Species	*No. of Samples*	*Average index of antifungal activity against*		
		A. fumigatus	*C. albicans*	*S. cerevisiae*
Family Holothuriidae				
Bohadschia marmorata	2	3.4	6.0	4.8
Bohadschia vitiensis	3	2.2	1.8	2.7
Holothuria fuscocinerea	1	1.9	2.9	3.7
Holothuria pulla	1	2.1	2.8	3.3
CONTROLS				
Nystatin (Positive control)		7.3	6.0	5.7
Distilled Water (Negative Control)		0.0	0.0	0.0

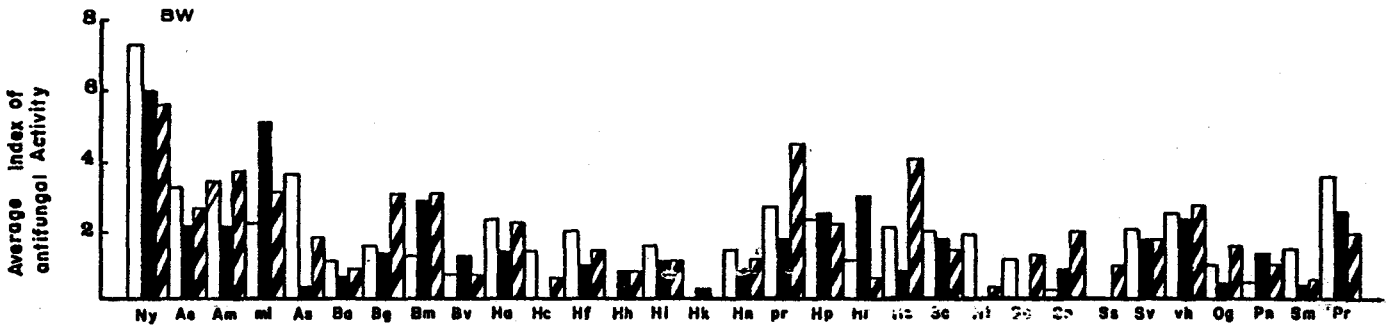

Figure 1. The activity against three fungal species of body wall holothurin of different Philippine sea cucumbers. □ A. fumigatus, ▮ C. albicans, ▨ S. cerevisiae, Ny. Nystatin (Positive Control). Names of different sea cucumbers abbreviated.

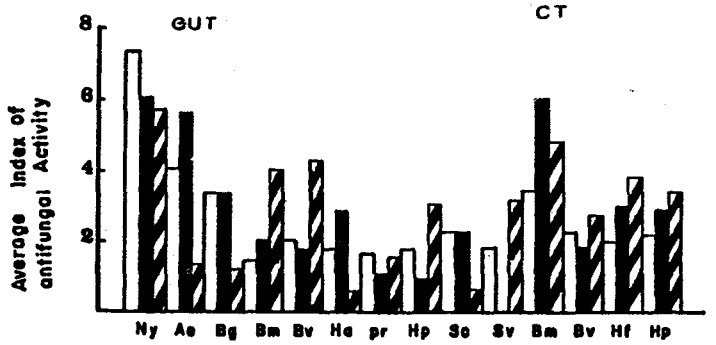

Figure 2. The activity against three fungal species of gut and Cuvierian tubule holothurin of some Philippine sea cucumbers. □ A. fumigatus, ▮ C. albicans, ▨ S. cerevisiae, Ny. Nystatin (Positive Control). Names of different sea cucumbers abbreviated.

The most probable best explanation for the above discrepancies could be the quantitative and qualitative differences in the holothurin content of the different species which may be according to the geographical location (cited in 5). Besides, the Russians had tested with already partially purified holothurins which had not been obtained in the present work. Their results of the significance of the lack of sulfate moeity for antifungal action agreed with the results of tests conducted in the Osborn Laboratories in New York whereby a desulfated extract of one species was demonstrated to possess ten fold greater antifungal activity(18).

In the areas of the Philippine waters which were explored, only very few samples of *Stichopus* were obtained. Members of the genus *Bohadschia* are also becoming depleted due to the unregulated harvests for the "trepang" industry. Between the two relatively more plentiful species, *Actinopyga echinites* and *Holothuria pulla,* the latter being a more unpopular source of "trepang" may be tapped for medicinal purposes. The future study on this will have to compare the activities among the crude, partially purified and isolated holothurins as well as their desulfated forms. The antifungal activity may also be verified by other assay techniques.

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References

1. Anisimov, M. M., V. V. Shcheglov, V. A. Stonik, A. L. Kulga, Z. V. Levina, V. S. Levin, and G. B. Elyakov. 1972. Comparative study of the antifungal activity of triterpene glycosides of Pacific holothurians. *Doklady Akademic Nauk SSSR 207*: 711-713 translated in *Doklady (Proceedings) of the Akademy of Sciences of the USSR (Biology) 205:* 666-668.
2. Bakus, G. J. 1968. Defensive mechanisms and ecology of some tropical holothurians. *Marine Biology (Berlin)* 2: 23-32.
3. _____. 1973. The biology and ecology of tropical holothurians. *In* Jones, O. A. and R. Endean (eds.). Biology and geology of coral reefs. Vol. II. Biology I. Academic Press, New York.
4. Bakus, G. J. and G. Green. 1974. Toxicity in sponges and holothurians: a geographical pattern. *Science* 185: 951-953.
5. Burnell, D. J. and J. W. Apsimon 1983. Echinoderm Saponins. In Scheuer, P. J. (ed.) Marine Natural Products, Chemical and Biological Perspectives. Vol. V. Academic Press, New York.
6. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and B. Wood Jr. 1973. Microbiology, 2nd ed. Harper and Row Publishers, Maryland.
7. Emerson, G. A. and C. H. Taft. 1945. Pharmacologically Active Agents from the Sea. *Texas Reports in Biology and Medicine* 3: 302-338.

8. Frey, D. G. 1951. The use of sea cucumbers in poisoning fishes. *Copeia* 1951: 175-176. *Biological Abstracts* 25: 2900.
9. Halstead, B. W. 1965. Poisonous and Venomous Marine Animals of the World. Vol. I. U.S. Government Printing Office, Washington, D.C.
10. Jawetz, E., J. L. Melnick, and E. A. Adelberg. 1978. Review of Medical Microbiology, 13th ed. Maruzen Asian Edition. Maruzen Co. Ltd., Japan.
11. Kuznetsova, T. A., M. M. Anisimov, A. M. Popov, S. I. Baranova, Sh. Sh. Afiyatullov, I. I. Kapustina, A. S. Antonov, and G. B. Elyakov. 1982. A comparative study *in vitro* of physiological activity of triterpene glycosides of marine invertebrates of the echinoderm type. *Comparative Biochemistry and Physiology C. Comparative Pharmacology* 73: 41-43.
12. Matsuno, T. and T. Ishida. 1969. Distribution and seasonal variation of toxic principles of sea cucumber (*Holothuria leucospilota* Brandt). *Experientia* 25: 1261.
13. Nigrelli, R. F. 1952. The effect of holothurin on fish and mice with Sarcoma-180. *Zoologica* 37: 89-90.
14. Pocsidio, G. N. 1988. Relative Hemolytic Potencies of Holothurin of Thirty Philippine Holothurians. *Transactions of the National Academy of Science and Technology (Philippines)* 10: 267-284.
15. _____. 1990. The Variability in the Holothurin of Philippine Holothurians. *Transactions of the National Academy of Science and Technology (Philippines)* 12: 203-218.
16. _____. The Thin Layer Chromatography of the Holothurin of Philippine Holothurians. *Proceedings of the First National Symposium in Marine Science* (in press).
17. Ruggieri, G. D. 1976. Drugs from the Sea. *Science* 194: 491-497.
18. Ruggieri, G. D. and R. F. Nigrelli. 1974. Physiologically Active Substances from Echinoderms. *In* Humm, H. S. and C. F. Lane (eds.). Bioactive Compounds from the Sea. Vol. 1. Marcel Dekker, Inc., New York.
19. Shimada, S. 1960. Antifungal Steroid Glycoside from Sea Cucumber. *Science* 163: 1462.
20. Steel, R. and J. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., Inc., New York.
21. Taft, C. H. 1945. Poisonous Marine Animals. *Texas Reports on Biology and Medicine* 3: 302-338.
22. Weete, J. D. 1981. Lipids in fungal growth and reproduction. *In* Turran, G. and H. R. Hohl (eds.). The Fungal Spore: Morphogenetic Control. Academic Press, New York.