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TERPENOIDAL SAPONINS FROM *Holothuria atra* J. EXHIBIT ANTIBACTERIAL ACTIVITY AGAINST *Escherichia coli*

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ABSTRACT

Antibacterial activity of both crude and fraction extract of sea cucumber *Holothuria atra* J. were detected against bacterial pathogen *Escherichia coli* ATCC 2592; AN 1964. Antibacterial activity was evaluated using disc – diffusion method. Considerable antibacterial activity was observed in chloroform extract of *H. atra* which had a comparable activity (32.27mm) as the amoxicillin (34.82mm). Antibacterial activity of extract fractions of *H. atra* has inhibitory effect against bacterial pathogen *E. coli*. Fractions 1, 2, and 3 yielded zone of inhibition with 24.79 mm, 5.70 mm and 8.98 mm respectively. Subsequently, another trial was done and in the case that isolated fraction was combined. Results showed that combined fraction 1, 2 and 3 inhibit the growth of *E. coli* with 31.17 mm which would indicate synergistic effect of compounds.

Keywords: *Holothuria atra*, antibacterial activity, thin layer chromatography

INTRODUCTION

Globally, the advancement of technology and the science of natural products are focused on sea floor diversity. Researchers want to increase their knowledge on the potential pharmacological functions and identify new bioactive metabolites in marine species with commercial values [1].

Some marine species produce substances for their defense. Aside from the nutritional value they posses, they can also be functional food. Example of bioactive molecule isolated from marine organism is a triterpene glycoside which exhibits anticarcinogenic, antifungal, and antibacterial activity [2]. Saponins were also identified in Holothuroids and Echinoderms with pharmacological value [3]. These bioactive metabolites were also reported present in sponges and starfishes which serve as their defense mechanism from the highly competitive environment [4].

Holothuroids, or sea cucumbers, are abundant and diverse group of marine invertebrates. Sea cucumbers are marine animals, which are important as human food source in some parts of Asia [5]. They are usually soft-bodied echinoderms comprising a diverse group of flexible, elongated, worm-like organisms, with a leathery skin and gelatinous body, looking like a cucumber. Normally, they live on the sea floor in deep seas [6].

Holothuroids are able to produce toxins and various metabolites from lanosterol with pharmacological activity [7]. A number of biological and pharmacological activities by the Holothuroids include: anti-angiogenic [8], anticancer [9], anticoagulant [10], anti-hypertension [11], anti-inflammatory [12], antimicrobial [13], antioxidant [14], antithrombotic [15], antitumor [16], and wound healing [17]. The medicinal and health functions of sea cucumbers can be attributed to the presence of the bioactive molecules namely: triterpene glycosides (saponins) [18], chondroitin sulfates [19], glycosaminoglycan [20], sulfated polysaccharides [21], sterols (glycosides and sulfates) [22], phenolics [23], peptides [24], cerberosides [25] and lectins [26].

Along the coast of Barangay Diguisit, Baler, Aurora, not much is known about these organisms, but different species have been described. Due to the high demand, this species is about to become extinct which may cause an imbalance in natural populations. Only a limited study had been conducted on Holothuroids in the Philippines. Due to this gap, this study was conceptualized. This

research study determined the potential of sea cucumber as source of drugs. In order to determine the importance of this organism, the study aimed to evaluate the antibacterial and determined the presence of terpenes and steroids in *H. atra*.

MATERIALS AND METHODS

Collection and Sample Preparation

Holothuria atra were collected along the intertidal zone of the coastal ecosystem of Barangay Diguisit, Baler Aurora. Collected samples were placed in an icebox and was immediately brought to the laboratory. Samples were washed thoroughly with running water and distilled water. The viscera were separated from the body wall. The body wall was placed on a steel screen away from light and heat.

Extraction of Holothuroids

The extraction procedure was adopted from Campagnuolo *et al*. [27], Van Dyck *et al*., [28] Garneau *et al*., [29] and Grassia *et al.,* [30] with some modifications. One hundred fifty-six grams of dried sample were added with liquid nitrogen and fully crushed with porcelain mortar and pestle and extracted with chloroform. The crushed body wall was transferred into a flask and added with 300 milliliters of chloroform for 72 – hours. Then it was filtered using Whatman filter paper No.1. The filtrate was then concentrated at 45°C in a rotary evaporator. The extract was stored in sterile vial and refrigerated prior to use.

Evaluation of Antibacterial Property

Test Organisms

Escherichia coli ATCC 2592; AN 1964 were used as bacterial pathogens for the antibacterial potential of *Holothuria atra* species and 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 [31] 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 10 ml Mueller - Hinton broth and incubated at 37°C for 6 to 8 hours to obtain the turbidity for the 0.5 McFarland standard with the density of $1.5x10^8$ cells /ml.

The turbidity of the growing culture was adjusted with sterile broth to obtain turbidity comparable to that of 0.5 McFarland standards. 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 was dissolved and melted in one – liter distilled water and sterilized in an autoclave at 121°C at 15psi. 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 Discs

Paper discs (Whattman No.1) measuring approximately 6 millimeters in diameter were prepared using a paper puncher. The paper discs were placed in a petri plate and sterilized in an autoclave.

Antibacterial Assay

Disk diffusion [32, 33] was used to study the antibacterial property of Holothuroids with minor modifications. The extract of Holothuroids was subjected to preliminary assessment. The plates were inoculated 100µL of bacterial culture and aseptically swab using a sterile bent rod and allowed to dry for a few minutes. Five to ten microliter was spotted on paper disc and allowed to dry the extract for 30 minutes. The plates were incubated at 37°C and zone of inhibitions measured and recorded in millimeters (mm) using a caliper after 8, 16and 24 – hours.

Separation and Identification of Terpenes and Steroids

The methods of Bahrami *et al.,* [34] and Dhinakaran and Lipton [35] were adopted with some modifications such as the use of different solvents and ratios as developing systems.

Thin Layer Chromatography

The chloroform extract of *H. atra* was loaded onto 7x1.5cm silica gel 60 F_{254} sheets and developed with toluene: chloroform (4.5:5.5), toluene: acetone: chloroform (4:2.5:3.5), n – butanol: acetic acid: water (4:1:5), chloroform: acetic acid: water (5:4.5:5), chloroform: methanol (5:5), ethyl acetate: hexane (7:3), hexane: ethyl acetate (7:3), hexane: chloroform (3:7; 1:1; and 7:3), pure toluene, acetone and chloroform solvent system. The profile of separated compounds on the TLC plate was visualized under UV light and sprayed with vanillin- sulfuric acid for terpenes and acetic anhydride for the presence of steroids. The sprayed TLC plates were allowed to dry at 100°C for 10 minutes.

The retention factor (Rf) was calculated as:

$$\mathbf{Rf = \frac{\text{distance of the spot on the TLC plate}}{\text{distance of the solvent front}}}$$

RESULTS

Table 1 presents the zone of inhibition of *H. atra* on Mueller – Hinton agar after 24- hours of incubation. The highest zone of inhibition (Figure 1) was recorded in amoxicillin (34.82±1.71) followed by *H. atra* with a mean of 32.37±0.5 after 24 – hour of incubation. Significant differences were noted between negative control (chloroform and distilled water), amoxicillin and *H. atra* extract had similar zone of inhibition against *E. coli*.

Thin Layer Chromatography Analysis

The presence of terpenes in *H. atra* was detected in TLC and calculated using the standard formula as Rf = distance moved by spot/distance moved by solvent front.

Out of thirteen solvent system, three of these elicited separation of spots namely toluene: acetone: chloroform, toluene: chloroform and chloroform.

More spots were visible in toluene: acetone: chloroform (Figure 2 and 3). Thin layer chromatography resulted in separation of spots with Rf values of 0.08, 0.51, and 0.74. Visible spots in the crude extract of *H. atra* had terpenoid and steroidal group of metabolites. Red – violet spot (Rf 0.08) indicated the presence of terpenoids. Rf value of 0.51 with strong blue – violet color revealed the presence of steroids while the Rf value of 0.74 had the combination of terpenoids and steroids which was blue to red violet color.

Antibacterial activity of the extract fraction, *Holothuria atra*

Antibacterial activity of extract fractions of *H. atra* was assessed by disc – diffusion method on Mueller – Hinton agar (MHA). Wide range of secondary metabolites had been isolated from sea cucumbers which have large and diverse group of organisms. Some of these compounds possess biological activity such as toxicity, antibacterial, antifungal, antiviral, antitumor and other specific activities [36], [37], [38], [39]. In this study, the antibacterial effect of extract fractions of *H. atra* was assessed and the results are presented in Table 2.

The highest zone of inhibition (Figure 4) was observed in positive control (amoxicillin) with 30.77±2.63 mm zone of inhibition which is not significantly different to fraction 1 ((0.08; terpenes) with a mean zone of inhibition of 24.79±4.70 mm but statistically different to fraction 2 (0.51; steroids) and fraction 3 (0.74; terpenes and steroids) with zone of inhibition of 5.70±8.05 mm and 8.98±12.69 mm respectively. No zone of inhibition was observed at negative control (0.00±0.00).

Table 3 presents the data on the zone of inhibition of combined extract fractions using thin layer chromatographic method. Highest zone of inhibition was observed at positive control (amoxicillin) 34.28±1.27 mm after 24-hours of incubation. Zone of inhibition of combined fraction 1,2, and 3 was 31.17±0.97 mm . In these results, the data gathered were significantly different from each other because the p – value is 0.00 which was less than the level of significance of 0.05 indicated that the antibacterial activity of combined fraction of *H. atra* had different means and not all equal.

The first trial of antibacterial potential of extract fractions of *H. atra* (Table 2) showed that fraction 1 (terpenes) was comparable to positive control but not to fraction 2 (steroids) and fraction 3 (terpenes and steroids). Subsequently, another trial was done and the separated extract fractions were combined. Results showed that zone of inhibition of combined extract fractions 1, 2 and 3 (combination of terpenes and steroids) were significantly different from the positive control (Figure 5). Despite the statistical analysis that data were significantly different, interesting results showed that combination of extract fractions is a proof of concept that the isolates delivered synergistic combination of bioactive metabolites such as terpenes and steroids. Inasmuch that the synergetic action combination of bioactive metabolites could not be supported and justified; it seems more probable that the combination of three isolates had inhibited the bacteria than a state of isolated one fraction.

Table 1: Zone of inhibition (mm) of different treatments by disc – diffusion method

TREATMENTS	DIAMETER ZONE OF INHIBITION AFTER		
	8 – hour	16 – hour	24 – hour
Chloroform	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}
Distilled water	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}
Amoxicillin	30.28±0.62^{a}	30.53±0.62^{a}	34.82±1.71^{a}
Holothuria atra	22.33±7.95^{a}	28.10±0.96^{b}	32.37±0.59^{a}

Values presented are means and standard deviation

Treatments means in each source with the same letter of superscript in each column are not significantly different from each other at 5% level of significance using DMRT.

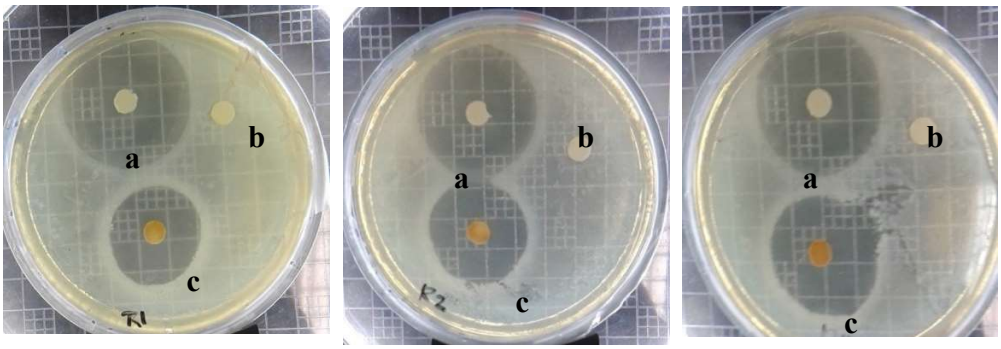

Figure 1: Antibacterial test for *E. coli* after 24 – hours of incubation. (a) Amoxiciilin(b) distilled water (c) *H. atra*

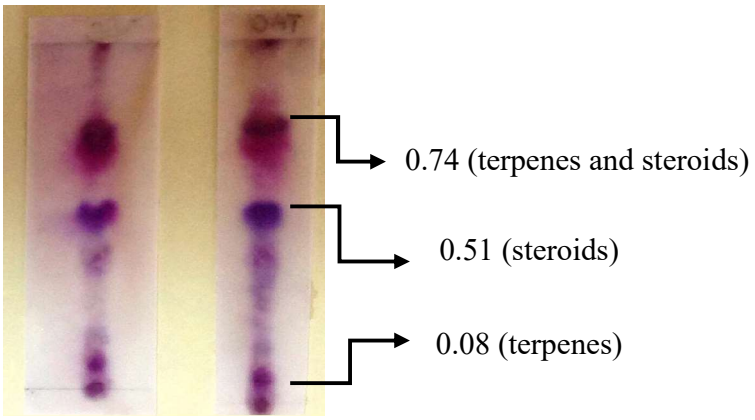

Figure 2: Thin layer chromatography of chloroform extract, *H. atra* using toluene:acetone: chloroform solvent system and vanillin – sulfuric spray reagent

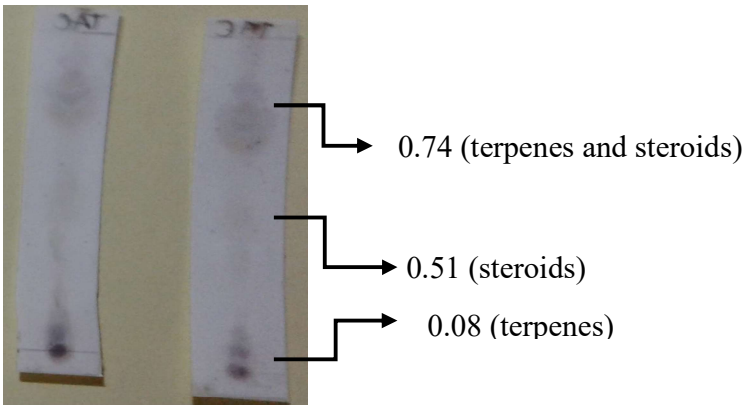

Figure 3: Thin layer chromatography of chloroform extract, *H. atra* using toluene: acetone: chloroform solvent system and acetic anhydride spray reagent

Table 2: Zone of inhibition (mm) of different treatments of active fractions by disc diffusion method

TREATMENTS	DIAMETER ZONE OF INHIBITION AFTER		
	8 – hour	16 – hour	24 – hour
Chloroform	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Distilled water	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Amoxicillin	27.13±1.81^{a}	29.44±3.50^{a}	30.77±2.63^{a}
Fraction 1 (0.08; terpenes)	21.66±4.52^{ab}	22.54±3.93^{ab}	24.79±4.70^{ab}
Fraction 2 (0.51; steroids)	6.42±9.07^{bc}	6.11±8.64^{bc}	5.70±8.05^{c}
Fraction 3 (0.74; terpenes and steroids)	8.23±11.63^{bc}	8.74±12.36^{bc}	8.98±12.69^{bc}

Values presented are means and standard deviation.
Treatments means in each source with the same letter of superscript in each column are not significantly different from each other at 5% level of significance using DMRT.

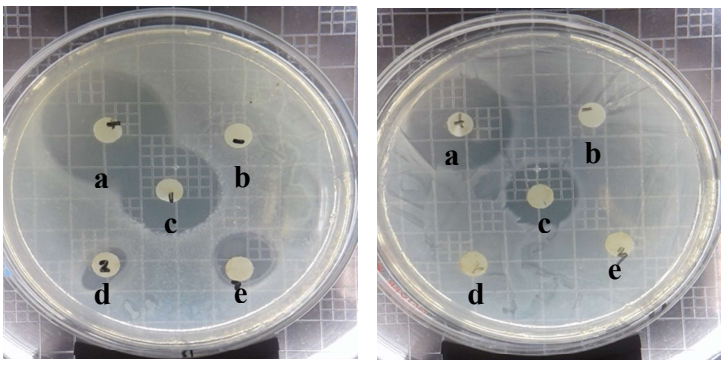

Figure 4: Antibacterial test for *E. coli* after 24 – hours of incubation. (a) Amoxiciilin(b) distilled water (c) terpenes (d) steroids (e) terpenes and steroids

Table 3: Zone of inhibition (mm) of different treatments of active fractions by disc –diffusion method

TREATMENTS	DIAMETER ZONE OF INHIBITION AFTER		
	8 – hour	16 – hour	24 – hour
Chloroform	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Distilled water	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Amoxicillin	30.83±3.71^{a}	31.98±2.21^{a}	34.2±1.27^{a}
Fraction 1,2 and 3 (combination of terpenes and steroids)	25.48±1.16^{b}	26.74±0.95^{b}	31.17±0.97^{b}

Values presented are means and standard deviation
Treatments means in each source with the same letter of superscript in each column are not significantly different from each other at 5% level of significance using DMRT.

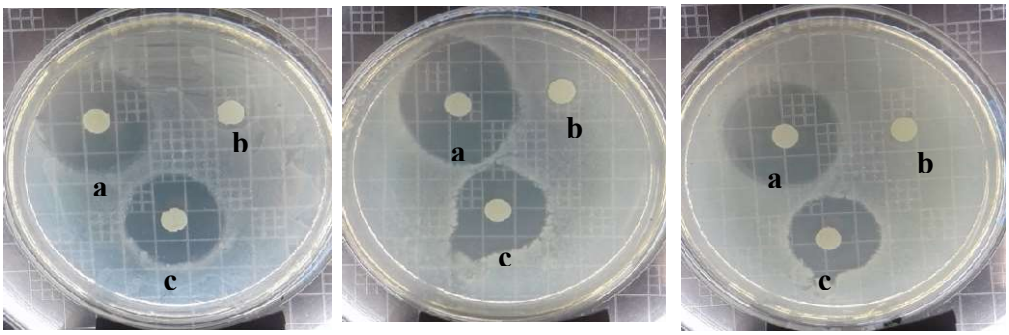

Figure 5: Antibacterial test for *E. coli* after 24 – hours of incubation. (a) Amoxiciilin(b) distilled water (c) combined extract fractions of *H. atra*

DISCUSSION

The crude extracts of both coelomic fluid and flesh of *H. scabra, H. leucospilota* and *H. atra* produced antagonistic effect against *S. aureus ATCC 6538, P. aeruginosa ATCC 8739, V. damsela, S. faecalis* and *E. coli* [40]. Similar to results of Shakouri *et al.,* [41] aqueous methanol extract had an inhibitory effect on *E. coli* and *A. niger*. Moreover, *H. scabra* and *A. miliaris* extracts showed higher degree of antibacterial activity followed by *H. atra* [42].

Furthermore, the findings from the TLC analysis were further supported by Abraham *et al.,* [42] concluding that the highest ethanolic soluble fraction was in *A. miliaris* followed by *H. atra, A. echinites* resulted in the isolation of spots with Rf values of 0.73, 0.47, 0.67 and 0.65 and 0.77 respectively. The visible spots of *H. atra* indicated a positive result for some compound of triterpenes with a blue – violet to violet coloration. Terpenoids had been isolated from a variety of marine organisms such as echinoderms, sponges and corals [43].Another study concluded that triterpene glycosides were the predominant secondary metabolites of sea cucumber [44]. Although this study focused on the triterpenes, it can be occurring as free triterpenes and several components of steroid as triterpene glycosides [45]. Several reports that marine steroidal and triterpene glycosides had mainly been reported from echinoderms, specifically from the Asteroidea (sea stars), which possessed steroidal glycosides, and the Holothuroidea (sea cucumbers), which possessed triterpene glycosides [46]. Although it had been suggested that these compounds play a defensive role in echinoderms, experimental evidence is inconclusive and mostly related to seasonal fluctuations in metabolite levels and varying concentrations in different echinoderm tissues [46, 47]. Also, Seigler [48] reported that triterpenes had been found in many plant species and in some marine animals [49].

The presence of bioactive compounds and some various biological activities in methanol extract of *H. atra* to *E. coli* showed less effect at 50% and 60% of fractions (100 µl concentration) which showed the zone diameter range of 2 and 3mm [50]. In another study, Ridzwan *et al.* [51] evaluated the antibacterial activity of the extracts from sea cucumbers harvested from coastal areas of Sabah (Malaysia) using *in vitro* tests. According to their results, the extracts, the lipid fraction and methanol fraction, derived from sea cucumber species, *Holothuria scabra*, *Holothuria atra* and *Bohadshia argus* did not show considerable antibacterial

action. Findings of Stonik *et al.,* [52] suggested that sea cucumbers produce water – soluble glycosides and other secondary metabolites such as the flavonoids, phenolic components, terpenoids, saponins and alkaloids that elicited their antimicrobial properties. Moreover, one of the important factors that could cause the antibacterial activity of the sea cucumber extracts is secondary metabolites such as triterpene glycosides [53].

REFERENCES

[1] Sottorff, I., A., V. Aballay, L.X. Hernández, L.R. Munoz, M. Silva, J. Becerra, and A. Astuya. 2013. Characterization of bioactive molecules isolated from sea cucumber *Athyonidium chilensis*. Revisita de Biologia Marina y Oceanografia, 48(1): 23 – 25.

[2] Aminin, D. L., E.S. Menchinskaya, E.A. Pisliagin, A.S. Silchenko, S.A. Avilov, and V.I. Kalinin. 2015. Anticancer activity of sea cucumber triterpene glycosides. Marine Drugs, 13(3): 1202–1223.

[3] Li, R., Y. Zhou, Z. WU, and L. Ding. 2008. ESI-QqTOF- MS/MS and APCI-IT-MS/MS analysis of steroid saponins from the rhizomes of *Dioscorea panthaica*. Journal of Mass Spectrometry, 41(1): 1–22.

[4] Levina, E.V., A.I. Kalinovsky, P.S. Dmitrenok, E.A. Martyyas, and V.A. Stonik. 2010. Two new steroidal saponins, hylodoside A and novaeguinoside Y, from the starfish *Leptasterias hylodes reticulata* and *Culcita novaeguineae* (juvenile). Natural Product Communications, 5(11): 1737-1742.

[5] Taiyeb-Ali, T.B., S.L.A. Zainuddin, D. Swaminathan, and H. Yaacob. 2003. Efficacy of "Gamadent" toothpaste on the healing of gingival tissues: A *preliminary report*. *Journal* of *Oral Science,* 45(3): 153-159.

[6] Conand, C. 1990. The fishery resources of Pacific island countries Part 2: Holothurians. FAO Fisheries Technical Paper 2722. Rome: Italy. Food and Agriculture Organization of the United Nations.

[7] Makarieva, T.N., V.A. Stonik, I.I. Kapustina, V.M. Boguslavsky, A.S. Dmitrenoik, V.I. Kalinin, M.L. Cordeiro, and C. Djerassi. 1993. Biosynthetic studies of marine lipids. 42. Biosynthesis of steroid and triterpenoid metabolites in the sea cucumber *Eupentacta fraudatrix*. Steroids, 58: 508-517.

[8] Tian, F., X. Y. Zhang, Y. Tong, S. Yi, L. Zhang, P. Li, P. Sun, L. Lin, and J.P.E. Ding. 2005. A new sulfated saponin from sea cucumber, exhibits anti-angiogenic and anti-tumor activities *in vitro* and *in vivo*. Cancer Biology Therapy, 4:874–882.

[9] Roginsky, A., B. Singh, X.Z. Ding, P. Collin, C. Woodward, M.S. Talamonti, R.H. Bell and T.E. Adrian. 2004. Frondanol (R)- A5p from the sea cucumber, *Cucumaria frondosa* induces cell cycle arrest and apoptosis in pancreatic cancer cells. Pancreas, 39(5): 646 – 652.

[10] Chen, S, C. Xue, L. Yin, Q. Tang, G. YU, and W. Chai. 2011. Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers. Carbohydrate polymers, 83: 688 – 696.

[11] Hamaguchi, P., M. Geirsdottir, A. VRAC, H.G. Kristinsson, H. Sveinsdottir, O.H. Fridjonsson, and G.O. Hreggvidsson. 2010. *In vitro* antioxidant and antihypertensive properties of Icelandic sea cucumber (*Cucumaria frondosa*). Presented at Institute of Food Technologies Annual Meeting and Food Expo, Chicago, IL: USA, presentation no. 282-04.

[12] Collin, P.D. 2004. Peptides having anti-cancer and anti-inflammatory activity. United State Patent 6,767,890.

[13] Hing, H.L., M.A. Kaswandi, R. Azraul-Mumtazah, S.A. Hamidah, A.Z Sahalan, S. Normalawati, M.W. Samsudin and B.H. RIDZWAN. 2007. Effect of methanol extracts from sea cucumbers *Holothuria edulis* and *Stichopus chloronotus* on *Candida albicans*. Microscopy and Microanalysis Journal, 13: 270–275.

[14] Althunibat, O.Y., B.H. Ridzwan, M. Taher, M.D. Jamaludin, M.A. Ikeda and B.I. Zali. 2009. In vitro antioxidant and antiproliferative activities of three Malaysian sea cucumber species. European Journal of Scientific Research 37: 376–387.

[15] Mourao, P.A.S., B. Guimaraes, B. Mulloy, S. Thomas, and E. Gray. 1998. Antithrombotic activity of a fucosylated chondroitin sulphate from echinoderm: Sulphated fucose branches on the polysaccharide account for its antithrombotic action. British Journal of

Haematology, 101(4): 647-652.

[16] Zou, Z., Y. Yi, H. Wu, J. Wu, C. Liaw, and K. Lee. 2003. Intercedensides A–C, three new cytotoxic triterpene glycosides from the sea cucumber *Mensamaria intercedens* Lampert. Journal of Natural Products, 66: 1055–1060.

[17] San Miguel-Ruiz, J.E. and J.E. García-Arrarás. 2007. Common cellular events occur during wound healing and organ regeneration in the sea cucumber *Holothuria glaberrima*. BMC Developmental Biology, 7: 115.

[18] Kerr R, and Z. Chen. 1995. *In vivo* and *in vitro* biosynthesis of saponins in sea cucumbers (Holothuroidea). Journal of Natural Products, 58: 172–176.

[19] Vieira, R.P., B. Mulloy, and P.A. Mourao. 1991. Structure of a fucose-branched chondroitin sulphate from sea cucumber. Evidence for the presence of 3-*O*-sulfo-β-D-glucuronosyl residues. Journal of biological chemistry, 266 (21): 13530-13536.

[20] Pacheco, R.G., C.P. Vicente, P. Zancan, and P.A.S.Mourão. 2000. Different antithrombotic mechanisms among glycosaminoglycans revealed with a new fucosylated chondroitin sulfate from an echinoderm. Blood Coagulation and Fibrinolysis, 11(6): 563-573.

[21] Mourao, P.A.S. and M.S. Pereira. 1999. Searching for alternatives to heparin: sulfated fucans from marine invertebrates. Trends Cardiovascular Medicine, 9: 225-232.

[22] Goad, L.J., Garneau, F.X., Simard, J.L., Apsimon, J.W. and Girard, M. 1985. Isolation of Δ9 (11)-sterols from the sea cucumber. Implications for holothurin biosynthesis. Tetrahedron Letters, 26: 3513–3516.

[23] Mamelona, J., E. Pelletier, K. Girard-Lalancette, J. Legault, and S. Karboune. 2007. Quantification of phenolic contents and antioxidant capacity of Atlantic sea cucumber *Cucumaria frondosa*. Food Chemistry, 104: 1040-1047.

[24] Rafiuddin, A.M., U. Venkateshwarlu, and R. Jayakumar. 2004. Multilayered peptide incorporated collagen tubules for peripheral nerve repair. Biomaterials, 25: 85–94.

[25] Sugawara, T., N. Zaima, A. Yamamoto, S. Sakai, R. Noguchi, and T. Hirata. 2006. Isolation of sphingoid bases of sea cucumber cerberosides and their cytotoxicity against human colon cancer cells. Bioscience Biotechnology Biochemistry, 70: 2906–2912.

[26] Mojica, E.R.E. and F.E. Merca. 2005. Isolation and partial characterization of a lectin from the internal organs of the sea cucumber (*Holothuria scabra* Jaeger). International Journal of Zoological Research, 1: 59-65.

[27] Campagnuolo, C., E. Fattorusso, and O. Taglialatela-Scafati. 2001. Feroxosides A–B, two norlanostane tetraglycosides from the Caribbean sponge *Ectyoplasia ferox*. Tetrahedron, 57: 4049 – 4055.

[28] Van Dyck, S., P. Gerbaux, and P. Flammang. 2009. Elucidation of molecular diversity and body distribution of saponins in the sea cucumber *Holothuria forskali* (Echinodermata) by mass spectrometry. Comparative Biochemistry and Physiology, 152(2009): 124–134.

[29] Garneau, F.X., J. Simard, O. Harvey, J. Apsimon, and M. Girard. 1983. The structure of Psoluthurin A: the major triterpene glycoside of the sea cucumber *Psolusfabricii*. Canadian Journal Chemistry, 61: 1465–1471.

[30] Grassia, A., I. Bruno, C. Debitus, S. Marzocco, A. Pinto, L. Gomez-Paloma, and R. Riccio. 2001. Spongidepsin, a new cytotoxic macrolide from *Spongia* sp. Tetrahedron, 57: 6257-6260.

[31] Lalitha, M. K. 2004. Methods of antimicrobial susceptibility testing. In Coyle, M. B. (Ed.), Manual on antimicrobial susceptibility testing. Washington, DC: American Society for Microbiology.

[32] Mokhlesi, A., S. Saeidnia, A.R. Gohari, A.R. Shahverdi, A. Nasrolahi, F. Farahani, and R. Khoshnood. 2012**.** Biological activity of sea cucumber *Holothuria leucospilota*. Asian Journal of Animal and Veterinary Advances, 7(3): 243-249.

[33] Mariana, N.S., K.A. Norfarrah, N.I. Nik, N.F. Yusoff, and A. Arsdad. 2009. Evaluating the antibacterial activity and *in vivo* assay of methanolic extract of *Stichopus*

badionotus. International Journal of Pharmacology, 5: 228-231.

[34] Bahrami, Y., W. Zhang and C. Franco. 2014. Discovery of novel saponins from the viscera of the sea cucumber *Holothurianlessoni*. Marine Drugs, 12(5): 2633–2667.

[35] Dhinakaran, I. and A. Lipton. 2014. Bioactive compounds from *Holothuria atra* of Indian Ocean. Springer Plus, 3: 673.

[36] Bryan, P., Mcclintock, J., Marion, K., Watts, S. and Hopkins, T. 1992. Feeding deterrence and chemical defense in echinoderm body wall tissues from the Northern Gulf of Mexico. American Zoologist, 32: 100.

[37] Villasin, J. and Pomory, C. 2000. Antibacterial activity of extracts from the body wall of *Parastichopus parvimensis* (Echinodermata: Holothuroidea). Fish Shellfish Immunology, 10: 465-467.

[38] Haug, T., Kjuul, A., Styrvold, O., Sandsdalen, E., Olsen, M. and Stensva G, K. 2002. Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). Journal of Invertebrate Pathology, 81: 94-102.

[39] Han, H., Yi, Y., Li, L., Liu, B., La, M., and Zhang, H., 2009. Antifungal active triterpeneglycosides from sea cucumber *Holothuria scabra*. Acta Pharmacologica Sinica, 44(6): 620 – 624.

[40] Ibrahim, H.A.H. 2012. Antibacterial carotenoids of three *Holothuria* species in Hurghada, Egypt. The Egyptian Journal of Aquatic Research, 38(3): 185–194.

[41] Shakouri, A., M. R. Shoushizadeh and F. Nematpour. 2016. Antimicrobial Activity of Sea Cucumber (*Stichopus variegatus*) Body Wall Extract in Chabahar Bay, Oman Sea. Jundishapur Journal of Natural Pharmaceutical Products, In Press (In Press):e32422.

[42] Abraham, T. J., J. Nagarajan and S.A. Shanmugam. 2002. Antimicrobial substances of potential biomedical importance from holothurian species. Indian Journal of Marine Sciences, 31(2): 161-164.

[43] Edrada, R.A., V. Wray, D.H. Handayani, P. Schupp, M. Balbin – Oliveros and P. Proksch. 2000. In

Atta – ur- Rahman (Ed.), Studies in Natural Product Chemistry. Karachi, Pakistan: Elsevier Science, pp. 251 – 286.

[44] Chludil, H.D., C.C Muniain, A.M. Seldes and M.S. Maier. 2002. Cytotoxic and antifungal triterpene glycosides from the Patagonian sea cucumber *Hemoiedema spectabilis.* Journal of Natural Products, 65(6): 860-865.

[45] Boar, R. B. and J. Allen. 1973. β-Amyrin triterpenoids. Phytochemistry, 12: 2571 – 2578.

[46] Stonik, V.A. and G.B. Elyakov. 1988. Secondary metabolites from echinoderms as chemotaxonomic markers. In Scheuer PJ (Ed.) Bioorganic marine chemistry, Vol 2. Springer-Verlag, Berlin, pp. 59–86.

[47] Urnell D.J. and J.W. Apsimon. 1983. Echinoderms. In Scheuer PJ (Ed.) Marine natural products chemistry and biological perspectives. Academic press, New York, 5: 287 – 389.

[48] Siegler, D.S. 1998. Triterpenes and steroids in plant seconday metabolism. Kluwer Academic Publishers, Boston/Dordrecht/London, pp. 427-455.

[49] Anisimov, M.M. 1987. Triterpene glycosides and the structural – functional properties of membranes. Nauchnye Doklady Vysshei Shkoly. Biologicheskie Nauki, 10: 49-63.

[50] Dhinakaran, I. and A. Lipton. 2014. Bioactive compounds from *Holothuria atra* of Indian Ocean. Springer Plus, 3: 673.

[51] Ridzwan, B.H., M.A. Kaswandi, Y. Azma, and M. Fuad. 1995. Screening for antibacterial agents in three species of sea cucumbers from coastal areas of Sabah. General Pharmacology, 26(7): 1539–1543.

[52] Stonik, V.A., Chumak, A. D., Isakov, V. V., Belogortseva, N. I., Chirva, V. YA and Elyakov, B. 1979. Glycosides of marine invertebrates VII. Structure of holothurin-B from *Holothuria atra*. Chemistry of Natural Compounds, 15: 453 – 457.

[53] Mulyndin, V.A. and V.V. Kovalev. 2001. Effects of the extraction of internal organs of the holothurian *Cucumaria japonica* on the indices of nonspecific resistance. Russian Journal of Marine Biology, 27: 406-408.