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# Bioactive Compounds from Omani Sea Cucumbers

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**المركبات البيولوجية النشطة لدي خيار البحر العماني**

**سيرجي دوبريتسوف وإيمان محمد المعمري وبسام سوسي**

الخلاصة: هدفت هذه الدراسة إلى إختبار قدرة بعض المواد الأيضية الذائبة، (مستخلصات مياه البحر) وغير الذائبة (مستخلص الميثانول:كلوروفورم بنسبة ١:١) والمستخلصة من حيوان خيار البحر (*Holothuria atra Holothuria edulis*,) من منطقة بندر الخيران في عمان، على منع نشاط الميكروبات والدياتوم واليرقات. وقد بينت الدراسة عدم وجود أي تأثير من المستخلصات على نمو البكتيريا (٣ من بكتريا مياه البحر و *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus puogenes, Staphylococcus epidermidis*) أوالدياتوم من نوع *Chaetoceros* sp.، بينما أدت كل المستخلصات إلى وفاة يرقات *Artemia salina*. وعليه فإن هذه الدراسة تشير إلى إمكانية إحتواء بعضا من مركبات حيوان خيار البحر العماني على سموم مضادة لليرقات.

كلمات مفتاحية: خيار البحر، الترسبات الحيوية، مضادات الترسبات، مواد أيضية ثانوية، بحر عمان

ABSTRACT: Antimicrobial, anti-diatom and anti-larval activities of both water soluble (water extracts) and non-water soluble metabolites (methanol: chlorophorm, 1:1 extracts) of the sea cucumbers *Holothuria atra* and *Holothuria edulis* from Bander AL-Khiran region, Oman were tested in this study. There was no significant effect of the extracts from sea cucumbers on bacterial (3 reference bacteria from seawater and pathogens *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus epidermidis*) and the diatom *Chaetoceros* sp. growth. Both water extracts and methanol: chlorophorm extracts caused significant mortality of *Artemia salina* nauplia. This study suggests that Omani sea cucumbers might be a good source of toxic anti-larval compounds.

Keywords: Sea cucumber, biofouling, antifouling, secondary metabolites, Sea of Oman.

## Introduction

Any natural and man-made substrates in the marine environment are quickly colonized by micro- (bacteria, diatoms and protozoa) and macroorganisms (algae and invertebrates) in a process known as "biofouling" (Railkin, 2004). The adverse effects of biofouling on ships and boats are high frictional resistance, speed reduction, increase of corrosion and high fuel consumption (Yebra *et al*., 2004). Biofouling can also clog water intake lines in power plants and membranes in desalination plants (Flemming and Ridgway, 2009). So far, the most effective methods of biofouling control are based on the application of highly toxic substances like tributyl tin (TBT), copper or organic compounds (e.g. Sea-Nine, Isothiazolone) (Thomas, 2001; Yebra *et al*., 2004). All these antifouling compounds kill marine organisms and pollute the marine environment (see reviews of Evans, 1999; Yebra *et al*., 2004). Therefore, novel antifouling compounds are urgently needed (Dobretsov *et al*., 2006).

Class *Holothuroidea* includes approximately 1200 known species of sea cucumbers, which are found throughout the world's oceans and in great ranges of latitudes and depths, but their greatest abundance occurs in the Indo-Pacific region (Conand, 2004). While sea cucumbers have a massive exoskeleton, they are less susceptible to predation and biofouling compare to other marine organisms. This suggests that sea cucumbers might have a chemical defense mechanism (Fusetani, 2004). Therefore, chemical compounds from sea cucumbers could be used for antifouling defense and biomedical applications.

Sea cucumbers contain numerous polar and non-polar secondary metabolites that can be used for drug discovery (Paul *et al*., 2008). These compounds are commonly utilized for treating weakness, impotence, constipation and frequent urination (Hamel and Mercier, 2004). Recently, antitumour, antiviral, anticoagulant and antimicrobial compounds have been isolated from sea cucumbers (Kelly, 2005). These

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organisms are also remarkably rich in vitamins, trace elements, and polysaccharides (condroitin sulfate), which reduce arthritis pain and inhibit viral activities (Hamel and Mercier, 2004). Saponins, such as holothurins, are one of the major natural products isolated from sea cucumbers (Bhakuni and Rawat, 2005). These water soluble glycosides showed haemolytic and cytotoxic activity *in vivo* and *in vitro* (Kelly, 2005) and can be used for treatment of cancer and fungal infections. Antifouling compounds have not been isolated from sea cucumbers so far (Fusetani, 2004).

The main aim of this study was to investigate the anti-microbial, anti-diatom and anti-larval potential of water soluble and non-soluble extracts from the sea cucumbers *Holothuria atra* and *H. edulis* in laboratory experiments *in vitro.*

## Material and Methods

### *Preparation of the Extracts*

Several specimens of the sea cucumbers *Holothuria atra* and *H. edulis* were collected 24.09.07 from Bandar Al-Khiran area (23°31'13.9"N 58°43'58.68"E) at the depth 4 m. These sea cucumbers were kept on ice and frozen at -20°C in the laboratory. After 2 weeks, the sea cucumbers *Holothuria atra* and *H. edulis* were defrosted and cut into small pieces and separated into 2 approximately equal portions. The first portion of the sea cucumbers was extracted with distilled sterile water. For this, 500ml of water was added to 649g (wet weight) of the black sea cucumber *H. atra* or 663g (wet weight) of the red sea cucumber *H. edulis* and incubated at 4°C in the fridge for 2 days. The second portion the sea cucumbers was extracted with 1:1 methanol:chloroform solution. For this, 500 ml of 1:1 methanol:chloroform solution was added to 660g (wet weight) of the sea cucumbers *H. atra* and *H. edulis*. These samples were extracted for 2 weeks at room temperature (23°C). After that, the samples containing water soluble metabolites were filtered and preserved in the freezer for the bioassays (see below), while the methanol:chloroform extracts were filtered and concentrated under vacuum using a rotary evaporator. The water bath temperature was +40-50°C. After evaporation the remaining sample was mixed with new 1:1 methanol:chloroform solution and transferred into small labelled containers. Finally, all extracts were dried at room temperature.

### *Bioassays*

### *Preparation of the bacterial media*

Bacteria were growing either in liquid marine broth (Difco) or on the surface of nutrient agar (Oxoid). Broth were prepared on distilled water according to the manufacturer's protocols. Flasks with media were autoclaved at 121°C in order to sterilize them.

### *Anti-bacterial assay*

Three strains of unidentified marine bacteria (Reference1, Reference2, and Reference5) isolated from Bandar Al-Khiran water column (23°31'13.9"N 58°43'58.68"E) and bacterial pathogens *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus epidermidis* obtained from SQU Hospital were used in this bioassay.

Before the assay, all strains were sub-cultured in the marine broth for 24h at + 30°C. Two hundred µl of the bacterial culture was spread on each Petri dish. Water extracts of sea cucumbers were pipetted and loaded on paper disks (1 $mm^2$ in diameter). There were 3 disks containing 50 µl (WB50) and 20 ml (WB20) of the water soluble extracts of *Holothuria atra*, as well as with 20 µl (WR20) and 50 µl of *H. edulis* (WR50). For the controls, 3 disks containing 20 and 50 µl of sterile water were used. Twenty µl of chloroform: methanol extracts of *H. atra* (B20) and *H. edulis* (R20) were added to 3 disks. All disks were dried at room temperature (23°C) and placed into Petri dishes containing bacterial strains under investigation. The experiment was run with 3 replicates. Petri dishes were kept at room temperature (23°C) for 2 days. The observed zones of growth inhibition between the disc and the bacterial film were measured to the nearest 0.2 mm.

### *Anti-diatom assay*

Prior to the experiment, the diatom *Chaetoceros* sp. (provided by Prof. U. Riebesell, IFM-GEOMAR) was cultivated in the F2 media for 2 weeks at room temperature (23°C). When a visible film developed in the culture flask, diatom suspensions were prepared by brushing the culture flask with a sterile paint brush. This algal suspension was then used in the following experiments.

Anti-diatom activity of the sponge extracts was tested according to a protocol developed by Dobretsov and Qian (2002). Five hundred µl of the water extracts of *H. edulis* or *H. atra* were added to Petri dishes in 3 replicates containing 5 ml of diatom suspension (about 12 x 104 cells $ml^{-1}$). Methanol: chloroform extracts of *H. atra* and *H. edulis* were evaporated and re-dissolved in dimethylsulfoxide (DMSO). Then, 100 µl extracts were added to Petri dishes in 3 replicates containing 5 ml of diatom suspension. Five hundred µl of sea water or 100 µl of DMSO were used as the controls. The Petri dishes were incubated for 4 days with continuous light at 23°C. After that, the unattached diatoms and water were poured out. Then, 15ml of 90% acetone was added to each Petri dish to extract chlorophyll *a*. The amount of chlorophyll *a* in each sample was measured using a spectrophotometer following Lorenzen (1967).

### *Anti-larval assay*

Prior to the experiment, the brain shrimp *Artemia salina* nauplia were cultivated. For this, we added 2g of the *A. salina* eggs into 1L of sterile seawater. Eggs with seawater were kept in a covered container (volume 1L) with aeration at room temperature (23°C). After two days, the larvae hatched. Photopositive nauplia were collected and used in the bioassay. Fifty mµl of *A. salina c*ulture

containing 10-20 larvae was added to each cell of multi-well dishes containing 500 mµl of sterile sea water. Then, 500 µl or 50 µl of the water extracts from *H. edulis* and *H. atra* were added to the cells. Additionally, 50 µl of methanol: chloroform extracts of these sea cucumbers evaporated and re-dissolved in DMSO was applied to other cells. Five hundred µl of seawater or 100 µl of DMSO were used as the controls. Each treatment was repeated 4 times. After 24h, the amounts of swimming and dead larvae were counted using a microscope.

***Statistical Analysis***

Chlorophyll *a* concentrations were log transformed to ensure normality and homogeneity of variance (Zar, 1996). The percent values of larval survival were arcsine-transformed. To improve the arcsine-transformation, those replicates with zero survival were given the value of 1/(4n) (n = number of larvae in a single replicate) (Zar, 1996). In all cases, the normality assumption was verified by the Shapiro-Wilk test (Shapiro and Wilk, 1965). The differences between the experimental and control treatments were determined by one-way ANOVA followed by an LSD post-hoc test (Zar, 1996). In all cases, the threshold for significance was 5%.

**Results**

***Anti-bacterial Assay***

None of the tested extracts (water extracts and chloroform: methanol extracts) of *H. atra* and *H. edulis* inhibited growth of reference bacteria and pathogens. At the same time, we observed halos (reduction of bacterial growth) in the case of reference bacteria 1 in the presence of water extracts from *H. edulis* (WR20) and from *H. atra* (WB20 and WB50).

***Anti-diatom Assay***

The results of the diatom test showed that there were no significant changes (P>0.05, ANOVA) in diatom growth after 4d of experiment (Fig.1). None of the extracts caused reduction of chlorophyll *a* concentration.

***Anti-larval Assay***

All tested extracts changed mortality of *Artemia salina* larvae (Fig. 2). In all cases, water and methanol: chloroform extracts of *H. edulis* and *H. atra* caused significantly high (ANOVA, P<0.05) mortality of larvae compared to the controls. All larvae died in the presence of methanol: chloroform extracts of *H. atra* and *H. edulis.* Water extracts of both species at 50 µl and 500 µl caused moderate mortality of *A. salina* larvae.

**Discussion and Conclusions**

In this study, we have investigated anti-microbial, anti-diatom and anti-larval activities of both water-soluble and non-water soluble metabolites from two species of sea cucumber specimens from the Sea of Oman. Our findings suggest that polar and non-polar metabolites of the sea cucumbers *H. atra* and *H. edulis* can kill larvae of invertebrates and cannot be used as non-toxic antifouling compounds.

Our results show that neither chloroform: methanol (non-polar) nor water (polar) extracts of the sea cucumber

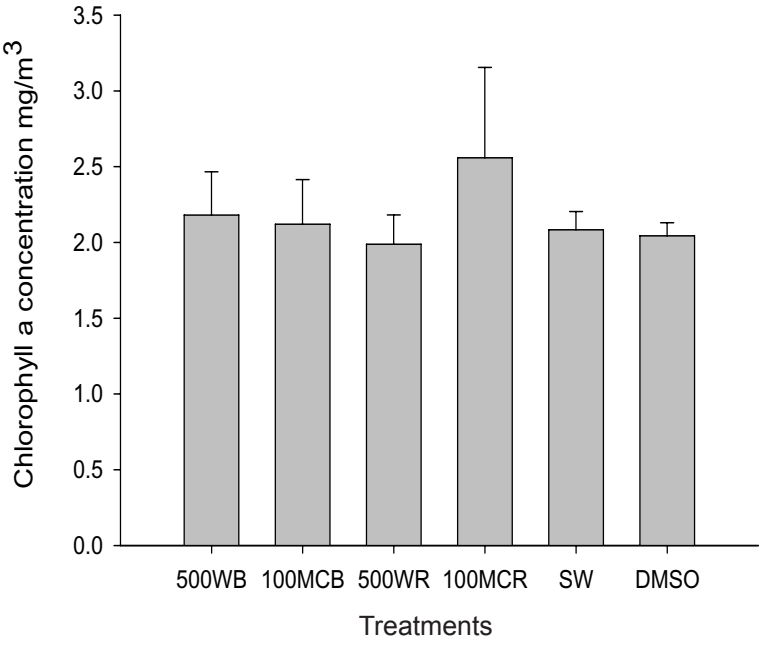


**Figure 1**. The effect of water and chloroform: methanol extracts (1:1) of the sea cucumbers *H. atra* and *H. edulis* on growth of the diatom *Chaetoceros* sp. The data are the mean chlorophyll *a* concentration (mg $m^{-3}$) ± SE (standard error) measured after 4d experiments with 500 µl of the water extracts o*f H. edulis* (500WR) an*d H.atra* (500WB), as well as 100 µl methanol:chloroform extracts of *H. edulis* (100MCR) and *H.atra* (100MCB) re-dissolved in dimethylsulfoxide (DMSO). Five hundred µl of seawater (SW) and 100 µl of DMSO (DMSO) were used as the controls.

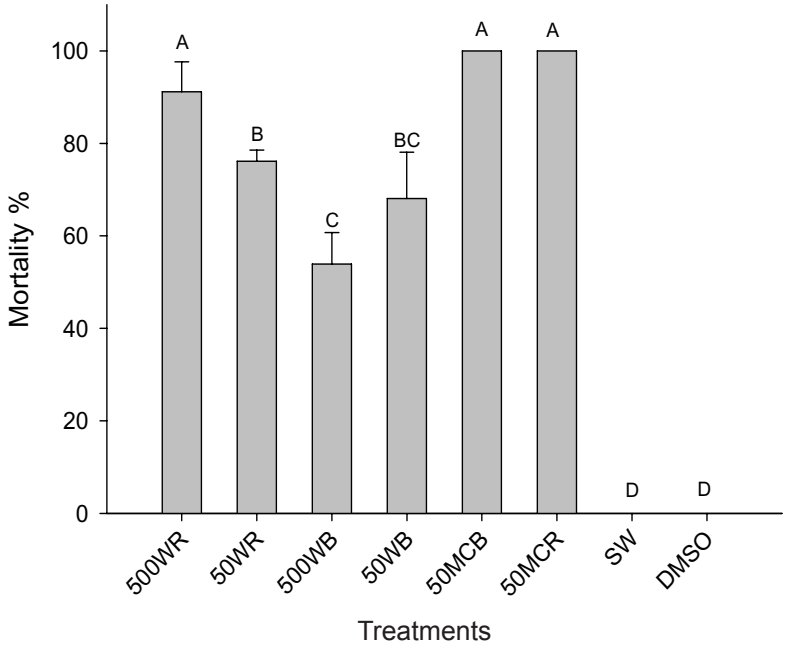


**Figure 2.** The effect of water and chloroform: methanol extracts (1:1) of the sea cucumbers *H. atra* and *H. edulis* on mortality of *Artemia salina* nauplia. The data are the mean percentage of mortality ± SE (standard error) in experiments with 500 µl (500) and 50 µl (50) of the water extracts o*f H. edulis* (WR) and *H. atra* (WB), as well as 50 µl (50) of methanol: chloroform extracts (MCB and MCR) of sea cucumbers re-dissolved in dimethylsulfoxide (DMSO). Five hundred µl of seawater (SW) or 100 µl of DMSO (DMSO) were used as the controls. Different letters above the bars indicate data that are significantly different in the LSD test ($P < 0.05$, one-way ANOVA).

*H. edulis* and *H. atra* affected the growth of tested pathogens. This can be explained by the fact that the investigated sea cucumbers most likely have never been exposed to the human pathogens tested in this study and, therefore, may lack such a defense. Furthermore, the tested extracts did not demonstrate antimicrobial activity against naturally occurring bacterial strains. This suggests that *H. edulis* and *H. atra* lack antimicrobial defense in the present experiment set up. This result contradicts findings of Stonik *et al.* (1979) suggesting that sea cucumbers produce different antimicrobial water-soluble glycosides. Additionally, tested reference bacteria might not be harmful to these sea cucumbers and, therefore, the sea cucumbers have not evolved any antimicrobial defense against them.

None of the tested compounds affected the growth of the diatom *Chaetoceros* sp. This result suggests that the sea cucumbers *H. atra* and *H. edulis* have no or very low anti-diatom activity. To our knowledge, anti-diatom activity of sea cucumbers has not been reported previously.

We found that the methanol: chloroform and water extracts from *H. atra* and *H. edulis* caused high mortality and low survival of Artemia salina larvae compared to the controls. These results suggest that some toxic secondary metabolites are present in the extracts of sea cucumbers. These metabolites potentially can be used as cytotoxic compounds for cancer treatments. Previously, holothurins and asterosaponins that are toxic to the larvae of marine invertebrates have been isolated from sea cucumbers (Fusetani, 2004). Similarly, several triterpene glycosides isolated from *Psolus patagonicus* (Dendrochirotida: Psolidae) showed a high level of mortality against the brine shrimp *Artemia salina* and revealed antifungal activity against the fungi *Cladosporium fulvum, Fusarium oxysporum* and *Monilia* sp. (Muniain *et al*., 2008). Thus, in line with these findings, our data suggest that secondary metabolites from sea cucumbers have promising potential for biomedicine applications.

Overall, very little information is available about the biology, ecology and biotechnological potential of Holothuroids along the Arabian Sea coast of Oman (Rashdi *et al*., 2007). The present study is the first investigation of anti-microbial, anti-diatom and anti-larval activity of both water-soluble and non-water soluble metabolites from two species of sea cucumbers from the Sea of Oman coast. Further studies of sea cucumber metabolites are needed in order to elucidate the structure of possible novel secondary metabolites useful in biomedical applications.

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**References**

Bhakuni, D.S. and D.S. Rawat. 2005. Bioactive Marine Natural Products. Springer Anamaya, India.

Conand, C. 2004. Present status of world sea cucumber resources and utilization: An international overview. In: *Advances in Sea Cucumber Aquaculture and Management.* A. Lovatelli, C. Conand, S. Purcell, S. Uthicke, J.F. Hamel and A. Mercier (Editors). 13-23. Geneva: FAO Fisheries Technical Paper.

Dobretsov, S. and P.Y. Qian. 2002. Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. *Biofouling* 8:217-228.

Dobretsov, S., H.U. Dahms and P.Y. Qian. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling* 22:43-54.

Evans, S.M. 1999. TBT or not TBT?: that is the question. *Biofouling* 14:117-129.

Flemming H.C. and H. Ridgway. 2009. Biofilm Control: Conventional and Alternative Approaches. In: *Marine and Industrial Biofouling.* R. Venkatesan, P.S. Murthy, K. Cooksey and H.C. Flemming (Editors), 4:103-119.

Fusetani, N. 2004. Biofouling and antifouling. *Natural Product Reports* 21:94-104.

Hamel, J.F. and A. Mercier. 2004. Synchronous gamete maturation and reliable spawning induction method in holothurians. pp 359–372. In: *Advances in Sea Cucumber Aquaculture and Management.* A. Lovatelli, C. Conand, S. Purcell, S. Uthicke, J.F.Hamel and A. Mercier (Editors), 425 pp. FAO Fisheries Technical Reports No. 463, FAO, Rome.

Kelly, M.S. 2005. Echinoderms: their culture and bioactive compounds. *Progress in Molecular and Subcellular Biology* 39:139-165.

Lorenzen, C. 1967. Determination of chlorophyll and phaeo-pigments: spectrophotometric equations. *Limnology and Oceanography* 12:343-346.

Muniain, C., R. Centurioìn, V.P. Careaga and M.S. Maier. 2008. Chemical ecology and bioactivity of triterpene glycosides from the sea cucumber *Psolus patagonicus* (Dendrochirotida: Psolidae). *Journal of Marine Biology Association,* UK, 88:817-823.

Paul, V.J. and R. Ritson-Williams. 2008. Marine chemical ecology. *Natural Product Reports* 25:662-695.

Railkin, A.I. 2004. Marine Biofouling: Colonization Processes and Defenses. Boca Raton, Fl, USA CRC Press.

Rashdi, K.M., M.R. Claereboudt and S.S.Al-Busaidi. 2007. Density and size distribution of the sea cucumber, *Holothuria scabra* (Jaeger, 1935), at six exploited sites in Mahout Bay, Sultanate of Oman. *Journal of Agricultural and Marine Sciences* 12:43-51.

Shapiro, S.S. and M.B. Wilk. 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52: 591-611.

Stonik, V.A., A.D. Chumak, V.V. Isakov, N.I. Belogortseva, V.Ya, Chirva and B. Elyakov. 1979. Glycosides of the sea invertebrates. 7. Structure of *holothurin-B* from *Holothuria atra* Khim. *Prir Soedin* 15:522-52.

Thomas, K.V. 2001. The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling* 17:73-86.

Yebra, D.M., S. Kiil and K. Dam-Johansen. 2004. Antifouling technology - past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings* 50: 75-104.

Zar, J.H. 1996. Biostatistical Analysis. 3rd Edition. Prentice Hall International, Inc., Upper Saddle River.

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