Iranian Journal of Fisheries Sciences 21(5) 1141-1155 2022
DOI: 10.22092/ijfs.2022.127710

Research Article

Extraction and investigation of biological activities of dioctyl phthalate and dibutyl phthalate from marine sponge *Haliclona* (*Soestella*) *caerulea* Larak Island, Persian Gulf

Shafeian E.[1]; Ghavam Mostafavi P.[*1]; Moridi Farimani M.[2]; Mashinchian Moradi A.[3]; Nazemi M.[4]

Received: November 2020 Accepted: December 2021

Abstract

Marines are unique resource that provides a diverse array of natural products, primarily from invertebrates such as sponge. As infectious diseases evolve and develop resistance to existing pharmaceuticals, these ecosystems provide novel leads against microbial, cancer, and viral diseases. The purpose of this study is to investigate antimicrobial and antifungal activities of derivatives of phthalates extracted from *Haliclona* (*Soestella*) *caerulea* on some pathogenic organisms. Sponge samples from Larak Island were collected by divers at a depth of 10 meters. After that, marine sponge extract collection was done by chloroform-methanol extraction as a solvent and through chromatography column and nuclear magnetic resonance (NMR) spectroscopy on the sponge sample, components of dioctyl phthalate (DOTP) and dibutyl phthalate (DBP) were purified and identified. Anti-microbial and antifungal activity of extracted components were investigated by means of bacterial broth dilution method and microdilution broth method and minimum inhibitor concentration (MIC), minimum bactericide concentration (MBC), and minimum fungal concentration (MFC) were determined. The results indicated that DOTP of *H.* (*Soestella*) *caerulea* was active on *Bacillus subtilis*, *Bacillus cereus* bacteria. MBC values of DOTP were determined as 2000 μg/m. DBP of *H.* (*Soestella*) *caerulea* was active on gram positive bacteria. MBC values of DOTP were determined as 1000 μg/m for *B. cereus* and *Staphylococcus aureus* and 500 μg/mL for *B. subtilis*. MFC values of DOTP and DBP were determined as 1000 μg/m and 2000 for *Candida albicans*. Hence it is assumed that the sponge *Haliclona* (*Soestella*) *caerulea* exhibited high antimicrobial activity.

Keywords: Marine sponge, Extraction, natural compounds, Dibutyl phthalate, Dioctyl phthalate, Antibacterial activity, Antifungal activity, Larak Island.

1-Department of Marine Science, Faculty of Natural Resources and Environment, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2-Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.
3-Department of Marine Science, Faculty of Natural Resources and Environment, Science and Research Branch, Islamic Azad University, Tehran, Iran.
4-Persian Gulf and Oman Sea Ecological Research Center, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research. Education and Extension Organization (AREEO), Bandar Abbas, Iran.
[*]Corresponding author's Email: mostafavi_pa@srbiau.ac.ir

Introduction

Natural marine products or secondary metabolites, isolated from marine organisms, attracted attention of biologists and chemists in the last five decades. More than 19,000 natural marine compounds are identified from marine organisms; numerous bioactive compounds are considered in synthesis, biology, biological activity, chemistry, ecology, and other fields. Since many of the natural marine compounds have biologically active properties, scientists found oceans as sources of great medicinal potential (Blunt *et al.*, 2008; Ebada *et al.*, 2008; Mehbub *et al.*, 2016). Most significant by-products and derivatives are related to aquatic invertebrates, such as sponges, mollusks without shells, and soft corals. They are soft-bodied animals that are either sessile or have slow movement and usually lack tools for physical defense, such as protective shell or spines, so presence of chemical defense mechanisms such as ability to synthesize toxins and inhibitory compounds in them seems necessary (Haefner, 2003; Ebada *et al.*, 2008). These chemicals or secondary metabolites are organic compounds that do not participate directly in developmental to reproduction stages of the living organism and play an important role in the immune system. They can be used as drugs that have interesting impacts such as antiviral, antimicrobial, antifungal, anti-cancer and anti-AIDS effects (Anjaneyulu *et al.*, 1999; De Marino *et al.*, 2000).

Sponges are most important sources of natural marine products with rich and new sources of biologically active metabolites, such as terpenes, polysaccharides, peptides, and alkaloids (Taylor *et al.*, 2007). Sponges are widely distributed organisms found in all oceans and are one of the most important communities of animals belonging to phylum Porifera (Barnes, 1995; De Weerdt, 2000). *Haliclona* (*Soestella*) *caerulea* is one of the most widespread species of sponges, it is present in most habitats, from shallow waters to depths of at least 40 meters, from unclean environments to clear water, and from warm waters of bays to colder latitudes. Interesting metabolites are reported in 190 combinations of different chemical types with different functions from *Haliclona* sp., among which a series of phthalate derivatives can be new and controversial candidates as natural compounds with medicinal properties of marine organisms (Santalova *et al.*, 2004; Viegelmann *et al.*, 2014; Zhang *et al.*, 2018). Regarding prominence of sponges as important source of beneficial secondary metabolites and organisms that have been able to preserve their offspring from Cambrian period until now, in many countries extensive research is done to identify and investigate secondary metabolites of sponges, as well as their biological effects. Their potential can be exploited to produce new and more effective drugs in treatment of the new century diseases. Antifungal and antibacterial properties are biological effects of sponges' secondary metabolites. Therefore,

identifying and reporting biological effects of secondary metabolites of Persian Gulf sponges as valuable organisms seems important. To expand identification of bioactive compounds and investigate antibacterial and antifungal effects of *H.* (*Soestella*) *caerulea*, the current study investigated bioactive compounds of this sponge in part of tidal zone of Lark Island as one important island of Persian Gulf, Iran.

Materials and methods

Sampling

Marine species *Haliclona* (*Soestella*) *caerulea*, was collected by divers at depth of 10 meters on the coast of Larak Island; then the samples were transferred to the laboratory of Persian Gulf Fisheries Research Center in the city of Bandar Abbas. Identifying the intended sponge according to identification key of Hooper and Van Soest (2002), by means of spicule brand observations and electronic microscope was done by M. Nazemi. Scientific name of this species of sponge is *Haliclona* (*Soestella*) *caerulea* and samples were preserved at -10°C (Hooper and Van Soest, 2002) (Fig. 1).

Figure 1: Persian Gulf marine sponge, *Haliclona (Soestella) caerulea*.

Extraction and isolation

Sponge samples were freeze-dried and then powdered. Extraction from 315g of sponge was done separately and alternately for three times and each time for a period of 48 hours by using 3 liters of chloroform (Merck) solvent and methanol (Merck) at ambient temperature. Then the sponge was vaporized by means of a rotary evaporator (or Rotavapor) equipped with a vacuum pump at, below 40ºC until a dark (black or gray) solid matter appears. Afterwards, liquid-liquid extraction was done and dry extract was prepared. In order to separate the compounds, the obtained extract was loaded on chromatography column with

a height of 126 cm and a diameter of 3.5cm along with 600g of silica gel (70-230 mesh) (Merck,1.7734.1000). The column was washed using gradient elution technique (i.e., which was eluted with gradient) and through gradual increase in polarity to wash it first with solvents of hexane %100 and then by ethyl acetate and methanol %100. Their obtained fractions were studied at each stage by any percentage of solubility using thin-layer chromatography (TLC) method (the stain of each fraction on TLC under UV ray (254-366 nm) and also by spraying the phosphomolybdate reagent (3g/100cc ethanol) and then heating at 110ºC (Smyrniotopoulos *et al.*, 2015). HRMS was recorded on a micrOTOF-Q time-of-flight spectrometer from Bruker Daltonics. All spectra were acquired at 298 K (25°C). The samples were dissolved in 300 μL of deuterated solvent and tetramethylsilane (TMS) served as internal standard.

H NMR spectrum was acquired with 10 scans. COSY experiment was achieved with 2 scans and 1024 data points in F1-dimension using non-uniform sampling, while NOESY (8 scans) was acquired with 512 in F1 dimension. HSQC spectrum was acquired with 16 scans and 1024 data points in F1-(C)-dimension using non uniform sampling, while HMBC spectrum was run with 24 scans and 400 data points in F1-(C)-dimension.

Separation of phthalate fraction

After preliminary separation, components of the extract of *Haliclona (Soestella) caerulea* were obtained. Regarding TLC analysis of fractions, B11 fraction (mixture of fraction 43-52) indicated some transparent stains on the TLC paper because of being adjacent to the reagent of phosphomolybdic acid at 100-150ºC. This fraction was obtained according to the proportion of dissolution 80-20 of hexane–ethyl acetate from main column by 31 mg. Based on the quantity of the fraction, a column with a diameter of 1.5 cm and the height of 100 cm was selected and filled by approximately 30g silica gel powder; then the fraction was loaded after being prepared on the column.

To wash the column by means of gradient elution technique, the solvents of hexane-dichloromethane in a ratio of 90-10 and two drops of isopropanol were used. Afterwards, 78 lateral fractions were separated among which, just one compound was purified. The pure compound which was obtained by merging the lateral fractions of 41, 42, 43 of D1 was encoded with a weight of 4mg and prepared by means of NMR as a chromatography detector. In addition, the mixture of fractions of 135-155 with a code of B_{46} with 116mg weight was achieved by the solubility ratio of 20-80 and 30-70 hexane and ethyl acetate from main column and was loaded on the column with a diameter of 1.5cm and height of 100 cm along with 30g silica gel. Then, it was washed by a mixed solvent system of dichloromethane and acetone by a ratio of 80-20. After merging similar fractions, a mixed pure, by merging 12-15 fractions, with a weight of 5mg with the code of $B_{46\text{-}S}$, was achieved and prepared in order to be

identified by means of NMR technique. Various spectra of NMR (Bruker Advance III for (600MHZ) proton and (125/77MHZ) carbon) were registered.

Biological measurement of antibacterial and antifungal impact

Investigation of antibacterial activity was done by bacterial broth dilution method on strains of gram-positive and gram-negative bacteria *Escherichia coli* ATCC 15224, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* PTCC 1609, *Bacillus cereus* ATCC 1715, *Staphylococcus aureus* 1764, and *Bacillus subtilis spizizenii* PTCC 1715. Investigation of anti-fungal activity was done by microdilution method on strains of fungi and yeast of *Candida albicans* ATCC 10231 and *Aspergillus fumigatus* PTCC[2] 5009, taken from institute for the industrial fungi and bacteria culture collection of Iran and were prepared in a lyophilized manner. Then, each strain was pre-cultured in specified microbiological culture; from single created colonies, bacteria were injected into broth, and fungi were injected into microdilution broth in test tubes, respectively, for bacteria against half of the quantity of 0.5 McFarland. For fungi, the obtained suspension was measured under the wavelength of 530 nm and by spectrophotometry device with optical transmittance index of %90. Then, out of purified fractions, various concentrations, including 50, 100, 200, 300, 500, 1000, and 2000 µg/mL were added to the tubes and finally the turbidity of test tubes were studied (i.e., turbidity testing was conducted).

Concentration of consumed materials in the turbidity-free test tubes indicates the amount of minimum inhibitory (MIC) factor that means prevention of growth and increased number of bacteria and fungi. Then, the least rate of concentration in which the fractions led to death of bacteria and fungi was determined as MBC (minimum bactericidal concentration) and MFC (minimum fungicidal concentration), respectively (Rosenblatt, 1991; Green *et al.*, 1994).

Results

D_1 compound was obtained by merging fractions 41-43 with a weight of 4mg and it was dissolved in deuterochloroform solvent. Since, spectrum of HRMS from this compound showed *m/z* $[M + H]^+$ 279.2344 (calcd. for $C_{16}H_{22}O_4$ 279.2396) (Fig. 2), D_1 compound was identified as dibutyl phthalate, which was further approved by 1D and 2D NMR, including ^{1}H-NMR as well as COSY, HSQC, and HMBC spectra (Fig. 3). The ^{1}H-NMR revealed present of aromatic ring at δ_H 7.5-79 and ethoxy signal at δ_H 4.3 as well as two triplet methyl resonance at δ_H 0.8 (Fig. 4). Further, HSQC spectrum approved presence of aromatic moiety by correlations between H at δ_H 7.5 and 7.8 with carbon at δ_C 120 and 125 (Fig. 5). HMBC correlation showed correlations between proton of two ethoxy groups (δ_H 4.3) with carbonyls δ_C 150 (Figs. 6, 7 and 8).

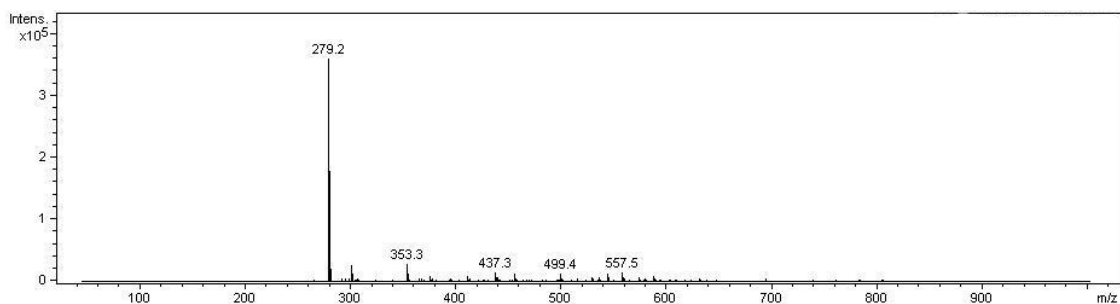

Figure 2: HRMS of D_1, Solvent (MeOH).

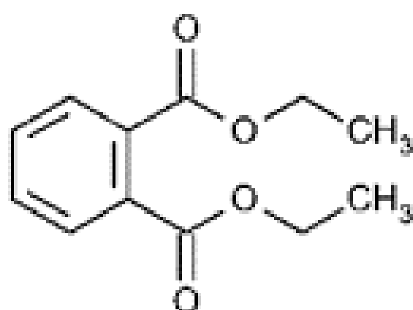

Figure 3: Molecular structure of dibutyl phthalate.

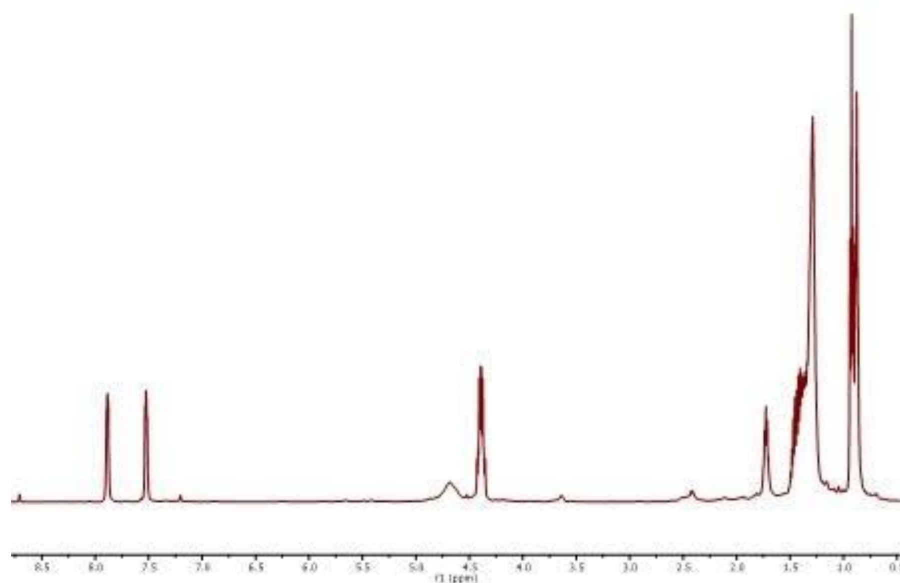

Figure 4: The ^{1}HNMR of D_1 (solvent $CDCl_3$, 600MH).

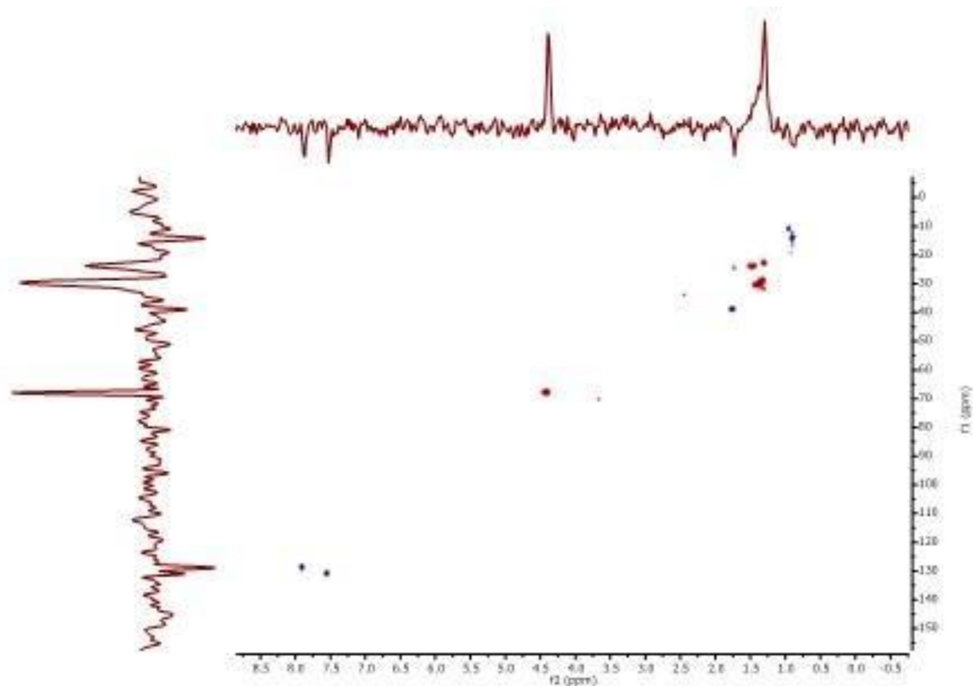

Figure 5: HSQC of D_1 (solvent $CDCl_3$, 600MHZ).

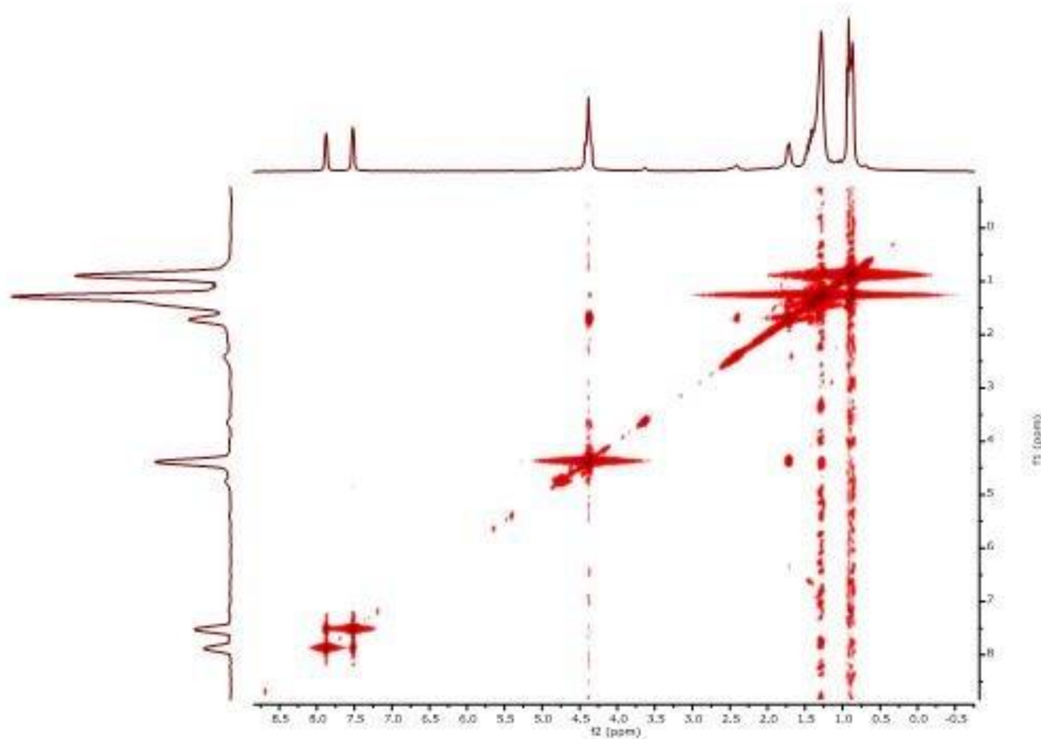

Figure 6: COSY of D_1 (solvent $CDCL_3$, 600MHZ).

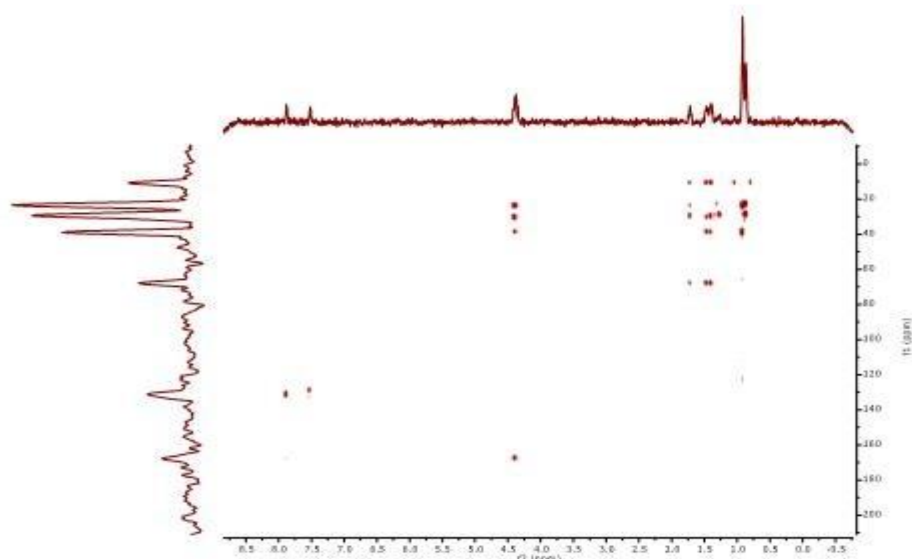

Figure 7: HMBC of D_1 (solvent $CDCl_3$, 600MHZ).

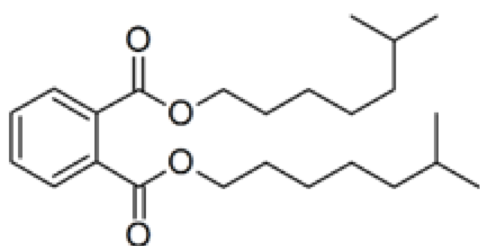

Figure 8: Molecular structure of dioctyl phthalate.

Also, B_{46} compound was purified with a weight of 5 mg showed HRMS at *m/z* $[M + H]^+$ 413.3414 (calcd. for $C_{24}H_{38}O_4$ 413.3411) (Fig. 9). The result of HRMS spectra was compare with the database that was suggested as the known compound (the category of phthalates), namely dioctyl phthalate.

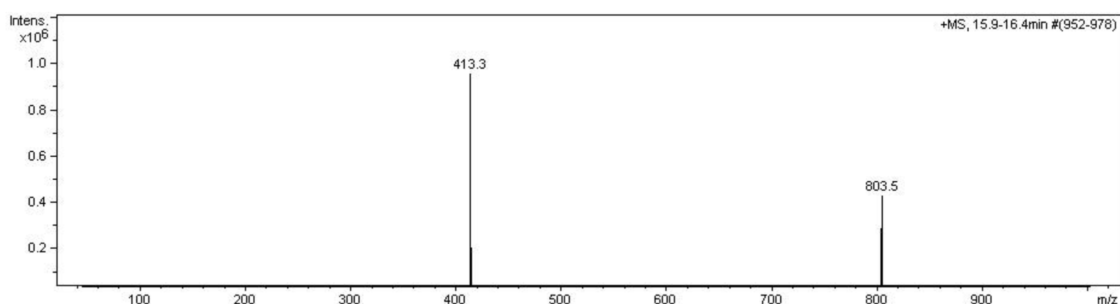

Figure 9: HRMS of B_{46-s} solvent (MeOH).

The least amount of concentration, which prevents growth of bacteria (MIC), and minimum bactericidal concentration (MBC), which underwent an experiment by means of dioctyl phthalate and dibutyl phthalate (DBP) are observed in Table 1.

As shown in Table 1 the least amount of concentration, which prevents growth (MIC) for the purified compounds, dioctyl phthalate and dibutyl phthalate,

obtained from chloroform-methanol based extract of the sponge species *Haliclona (Soestella) caerulea* from Larak Island for two bacterial species of *Bacillus subtilis* and *B. cereus* was 500 µg/mL. MBC of dioctyl phthalate for these two species was 2000 µg/mL and for dibutyl phthalate for *Bacillus subtilis* was 500 µg/mL and for *B. cereus* and *Staphylococcus aureus* were 1000 µg/mL. However, dioctyl phthalate showed no signs of lethality on the bacterium *S. aureus*. In addition, these two compounds showed no signs of lethality on gram-negative bacterial species of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*.

Table 1: Results of determining sensitivity of dioctyl phthalate (DOP) and dibutyl phthalate (DBP) on pathogen bacteria using broth method (R indicates non-impressionability; µg/mL).

Microorganisms	DOP		DBP	
	MIC	MBC	MIC	MBC
Bacillus subtilis	500	2000	500	500
Bacillus cereus	500	2000	500	1000
Staphylococcus aureus	1000	R	500	1000

In Table 2, the least amount of concentration which prevents growth of fungus (MIC) and least fungus lethality concentration (MFC) for which the compounds of dioctyl phthalate and dibutyl phthalate were added are observed.

Table 2: Results of determining sensitivity of dioctyl phthalate (DOP) and dibutyl phthalate (DBP) on fungus according to microdilution Broth method (R indicates non-impressionability µg/mL).

Microorganisms	DOP		DBP	
	MIC	MFC	MIC	MFC
Candida albicans	500	1000	1000	2000
Aspergillus fumigatus	1000	R	2000	R

As shown in Table 2, least amount of dioctyl phthalate concentration, which prevents growth (MIC) for the yeast *Candida albicans,* was 500 µg/mL and for *Aspergillus fumigatus* was 1000 µg/mL. Nevertheless, this compound had just lethality effect with a concentration of 1000 µg/mL for the yeast *C. albicans* and indicated no sign of lethality effect on the fungus *A. fumigatus*. In addition, the compound dibutyl phthalate indicated no sign of lethality effect on the fungus species *A. fumigatus*, however, concentrations 1000 µg/mL and 2000 µg/mL indicated least amount of preventability and lethal concentration on the yeast species *C. albicans*, respectively.

Discussion

Marine sponges are among the best sources of secondary metabolites; thousands of compounds are extracted from marine resources and many of them

are relevant to sponges. This diversity includes specific chemical compounds, which play a significant role in the defense system and can be used as medicine with outstanding antiviral, antimicrobial, antifungal, anticarcinogenic property, as well as preventing HIV transmission (Anjaneyulu *et al.*, 1999; De Marino *et al.*, 2000).

On the other hand, antimicrobial, antifungal, and cytotoxic activities of sponge *Haliclona* sp. in Persian Gulf have been substantiated up to now (Nazemi *et al.*, 2014; Seydi *et al.*, 2015; Karimpoor *et al.*, 2018; Shushizadeh *et al.*, 2018; Nazemi *et al.*, 2020). Species of bacteria that once were found in preliminary antibiotics showed no spectacular response.

Today, it is probable that they are resistant to even the strongest and the most complicated antibiotic compounds as well. In addition, fungus is one of the prevalent pathogenetic factors all over the world due to which the number of patients who are susceptible to be infected due to opportunistic microorganisms such as strains of *Candida* spp. has increased considerably in many countries. Therefore, given the ever-increasing resistance of the strains against medicine among various species of microorganisms, finding anti-microbial and anti-fungal compounds from among natural materials with fewer side effects is necessary.

Nigrelli *et al.* (1959) were presented a preliminary report on the antimicrobial effects of an extract from sea sponges (Newbold *et al.*, 1999). Afterwards, many reports were prepared on the antibacterial activities of sea sponges. Throughout a series of studies in the year 2000, antibacterial and antifungal activities in sea sponge species (*Haliclona* sp.) was investigated by Blackman and Faulkner. Results of this research indicated presence of sulfate, terpenoid and alkaloid compounds with antibacterial and antifungal activities. In addition, in 2014 Nazemi *et al.* reported antibacterial and antifungal activities of sea sponge of species (*Haliclona* sp.) in Persian Gulf.

Their results indicated that species *Haliclona* sp. affects gram-positive bacterial cells and this finding is consistent with the results of this study. Also, they studied antifungal activities on the species *Candida albicans* (MFC:1/5mg/ml) and *A. fumigatus* (MFC:3mg/mL) and mentioned that *Haliclona* sp. can be considered a source of novel antibiotic and antifungal activity. Shushizadeh *et al.* in 2018 reported antibacterial activities of species *Haliclona* sp., which was collected from Khark Island (Persian Gulf) on bacterial cells of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus. subtilis*. Antibacterial activities of methanol extract of sponge species *Haliclona (Soestella) caerulea* on *Bacillus subtilis* (5mg/mL), *Staphylococcus aureus* were also reported by Karimpoor *et al.* (2018).

In the present study, using the marine sponge *Haliclona (Soestella) caerulea* pure dioctyl phthalate and dibutyl phthalate were identified by means of

spectra of NMR and their antimicrobial and antifungal activities were studied. The results of this study indicate that dioctyl phthalate and dibutyl phthalate extracted from the sponge *Haliclona* (*Soestella*) *caerulea*, can be used as bioactive compounds against gram-positive bacteria species of *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and the fungal species of *C. albicans*.

In a similar study in 2006, Roy *et al.* identified dibutyl phthalate compounds by means of GC in sponges and reported them as bioactive compounds against gram-positive and gram-negative bacteria. Nguyen *et al.* (2007) introduced the two compounds, dioctyl phthalate and dibutyl phthalate, under secondary metabolites and bioactive compounds category, which have anti-bacterial effect on species *B. subtilis* and contrary to the current research, they had lethal effect on gram-negative bacteria *Pseudomonas aeruginosa*. The results of the study of Zhang *et al.* (2009) were similar to these findings. In a study by Li *et al.* (2008), phthalate compounds extracted from seahorse species *Hippocampus kuda* were introduced as bioactive compounds.

Moreover, in other studies by Saleem *et al.* (2009) and Qian *et al.* (2012) some phthalate compounds were introduced as treatments for some diseases; they also reported that marine sponge and seahorses are the only animal sources to obtain phthalate. These results are compatible with the results of the study by Blazević *et al.* (2010) in which compound (DBP) prevented growth of gram-positive bacteria species *Staphylococcus aureus* and the yeast *C. albicans*. In an experiment conducted by Islam *et al.* (2013) on the herb *Avicennia officinalis*, diethyl phthalate (DEP) compound was introduced as a secondary metabolite with antibacterial bioactivity on *B. subtilis*. Also, Lee *et al.* (2000) introduced derivatives of diphthalate as anti-tumor compounds. In addition, in a study by Manivasagan *et al.* (2014) some phthalate derivatives were reported as bioactive compounds. Sun *et al.* (2015) extracted di-phthalate, n-butyl phthalate, di-isobutyl phthalate compounds from sponge species *Halichondria* sp. as secondary metabolites from South China Sea. In a similar study, Phuong *et al.* (2018) identified dioctyl phthalate compounds in the sponge species *Streptomyces* sp. using GC and reported them as antifungal compounds of *C. albicans*. In addition, Behroozi *et al.* (2014) proved the presence of diethyl phthalate (DEP) compounds in marine sponge species *Haliclona* sp. located in Persian Gulf through chromatography. Nevertheless, in general, Zhang *et al.* (2018) believed that some derivatives of phthalates are poisonous and are identified as plasticizer and some other derivatives of it are identified as secondary metabolites in animals, plants and microorganisms. Moreover, majority of phthalates come from marine organisms that have bioactive properties. Depending on the environmental conditions, various compounds are produced, among which a series of phthalate compounds are considered as new controversial

medicine candidates extracted from marine creatures. In addition, they reported dioctyl phthalate and dibutyl phthalate compounds as secondary metabolites similar to the results of this study.

The present study indicates that the purified derivatives of phthalates extracted from the marine sponge species *Haliclona (Soestella) caerulea* were considered as secondary metabolite and they were not plasticizer and had antibacterial and antifungal bioactivity; it is hoped that in future investigations, they could be considered as new medicines extracted from sea sponge. In addition, due to the unique ecological conditions of Larak Island and long-term period of ebb and tide there, the creatures can tolerate harsh ecological conditions and show their resistance by producing secondary metabolite. Accordingly, manufacturing chemical compounds is possible. As Wang *et al.* (2008), declared various ecological conditions direct impact on manufacturing secondary metabolite, structural change and biological activity of sea creatures.

Acknowledgements

We hereby thank authorities of Medicinal Plants Research Center affiliated to Shahid Beheshti University and authorities of Persian Gulf and Gulf of Oman Ecology Research Center in Hormozgan Province who assisted us in this project. We thank the contributions of Dr. Zahra Sadeghian for analyzing NMR spectra.

References

Anjaneyulu, A.S.R., Gowri, P.M. and Krishna Murthy, M.V.R., 1999. New sesquiterpenoids from the soft coral *Sinularia intacta* of the Indian Ocean. *Journal of Natural Products*, 62(**12**), 1600-1604. https://doi.org/10.1021/np9901480.

Barnes, D.K.A., 1995. Sublitoral epifaunal communities at Signy Island, Antarctia. II. Below the ice-foot zone resources. *Marine Biology*, 121, 565-572. https://doi.org/10.1007/BF00349467.

Behroozi, S., Shoshizadeh, M.R., Nazemi, M. and Tahan Son, A., 2014. Identification of diethyl phthalate in n-hexane extract of *Haliclona* marine sponge. 4th National Conference on Chemical Applications in New Technologies, in Persian.

Blazević, I., Radonić, A., Mastelić, J., Zekić, M., Skocibusić M. and Maravić, A., 2010. Hedge mustard (*Sisymbrium officinale*), chemical diversity of volatiles and their antimicrobial activity. *Chemistry and Biodiversity*, 7(**8**), 2023-2034. https://doi.org/10.1002/cbdv.200900234.

Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H.G., Northcote, P.T. and Prinsep, M.R., 2008. Marine natural products. *Natural Product Reports*, 25(**1**), 35-94. https://doi.org/10.1039/b701534h.

De Marino, S., Iorizzi, M., Zollo, F., Debitus, C., Menou, J.L., Ospina, L.F., Alcaraz, M.J. and Payá, M., 2000. New pyridinium alkaloids from

a marine sponge of the genus *Spongia* with a human phospholipase A2 inhibitor profile. *Journal of Natural Products*, 63(**3**), 322-326. https://doi.org/10.1021/np990374+.

De Weerdt, W.H., 2000. A monograph of shallow-water Chalinidae (Porifera, Haplosclerida) of the Caribbean. *Beaufortia*, 50(**1**), 1- 67.

Ebada, S.S., Edrada, R.A., Lin, W. and Proksch, P., 2008. Method for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. *Nature Protocols*, 3(**12**), 1820-1831. https://doi.org/10.1038/nprot.2008.182.

Green, L., Petersen, B., Steimel, L., Haeber, P. and Current, W., 1994. Rapid determination of antifungal activity by flow cytometry. *Journal of Clinical Microbiology*, 32(**4**), 1088-1091. https://doi.org/10.1128/jcm.32.4.1088-1091.1994.

Haefner, B., 2003. Drugs from the deep: marine natural products as drug candidates. *Drug Discovery Today*, 8(**12**), 536- 544. https://doi.org/10.1016/s1359-6446(03)02713-2.

Hooper, J.N.A. and Van Soest, R.W.M., 2002. *Systema porifera, a guide to the classification of sponges*, 1st edition. Springer Science and Business Media, Berlin, Germany, 1801P.

Islam, M.T., Yun, H.K., Cho, S.M. and Soon-Young, A., 2013. Isolation of antibacterial compounds from hairy vetch (*Vicia villosa*) against grapevine crown gall pathogen. *Horticulte, Environment and Biotechnology*, 54(**4**), 338-345. https://doi.org/10.1007/s13580-013-0028-8.

Karimpoor, M., Kamrani, E., Yousefzadi, M. and Nazemi, M., 2018. Antibacterial and antioxidant potential of *Haliclona caerulea* extracts from tidal Island Larak, Persian Gulf. *Modares Journal of Biotechnology*, 9(**3**), 347-353.

Lee, K.H., Kim, J.H., Lim, D.S. and Kim, C.H., 2000. Anti-leukaemic and anti-mutagenic effects of di (2-ethylhexyl) phthalate isolated from *Aloe vera* Linne. *Journal of Pharmacy and Pharmacology*, 52(**5**), 593-598. https://doi.org/10.1211/0022357001774246.

Li, Y., Qian, Z.J. and Kim, S.K., 2008. Cathepsin B inhibitory activities of three new phthalate derivatives isolated from seahorse, *Hippocampus kuda* Bleeler. *Bioorganic and Medicinal Chemistry Letters*, 18(**23**), 6130-6134. https://doi.org/10.1016/j.bmcl.2008.10.016.

Manivasagan, P., Venkatesan, J., Sivakumar, K. and Kim, S.K., 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiology Research*, 169(**4**), 262-278. https://doi.org/10.1016/j.micres.2013.07.014.

Mehbub, M.F., Perkins, M.V., Zhang, W. and Franco, C.M.M., 2016. New

marine natural products from sponges (Porifera) of the order Dictyoceratida (2001 to 2012); a promising source for drug discovery, exploration and future prospects. *Biotechnology Advances*, 34(**5**), 473-491. https://doi.org/10.1016/j.biotechadv. 2015.12.008.

Nazemi, M., Alidoust Salimi, M., Alidoust Salimi, P., Motallebi, A., Tamadoni Jahromi, S. and Ahmadzadeh, O., 2014. Antifungal and antibacterial activity of *Haliclona* sp. from the Persian Gulf, Iran. *Journal de Mycologie Médicale*, 24(**3**), 220-224. https://doi.org/10.1016/j.mycmed.20 14.03.005.

Nazemi, M., Ghaffari, H., Moradi, Y., Amiran, M.R. and Aghaei Dargeri, S., 2020. Cytotoxic activity of extracts of demosponges *Haliclona caerulea, Axinella sinoxea* and *Ircinia mutans* from Persian Gulf. *Indian Journal of Experimental Biology*, 58(**4**), 249-253.

Newbold, R.W., Jensen, P.R., Fenical, W. and Pawlik, J.R., 1999. Antimicrobial activity of Caribbean sponge extracts. *Aquatic Microbial Ecology*, 19, 279-284. https://doi.org/10.3354/AME019279.

Nguyen, D.T.M., Nguyen, D.H., Lyun, H.L., Lee, H.B., Shin, J.H. and Kim, E.K., 2007. Inhibition of melanogenesis by dioctyl phthalate isolated from *Nigella glandulifera* Freyn. *Journal of Microbiology and Biotechnology*, 17(**10**), 1585-1590.

Nigrelli, R.F., Jakowska, S. and Calventi, I., 1959. Ectyonin an antimicrobial agent from the sponge *Microciona prolifera* verrill. Zoologica(NY)44, 173-175. **https:**//doi.org/10.5962/p.2033.72

Phuong, T.V., Lam, P.V.H. and Diep, C.N., 2018. Bioactive compounds from Marine *Streptomyces* sp. by gas chromatography-mass spectrometry. *The Pharmaceutical and Chemical Journal*, 5(**1**), 196-203.

Qian, Z.J., Kang, K.H. and Kim, S.K., 2012. Isolation and antioxidant activity evaluation of two new phthalate derivatives from seahorse, *Hippocampus kuda* Bleeler. *Biotechnology and Bioprocess Engineering*, 17(**5**), 1031-1040. https://doi.org/10.1007/s12257-012-0115-1.

Rosenblatt, J.E., 1991. Laboratory tests used to guide antimicrobial therapy. *Mayo Clinic Proceedings*, 66(**9**), 942-948. https://doi.org/10.1016/s0025-6196(12)61583-3.

Roy, R.N., Laskar, S. and Sen, S.K., 2006. Dibutyl phthalate, the bioactive compound produced by S*treptomyces albidoflavus* 321.2. *Microbial Research*, 161(**2**), 121-126. https://doi.org/10.1016/j.micres.2005 .06.007.

Saleem, M., Nazir, M., Akhtar, N., Onocha, P.A., Riaz, N., Jabbar, A., Ali, M.S. and Sultana N., 2009. New phthalates from *Phyllanthus muellerianus* (Euphorbiaceae). *Journal of Asian Natural Products Research*, 11(**11**), 974-977. https://doi.org/10.1080/1028602090 3341388.

Santalova, E.A, Makarieva, T.N., Gorshkova, I.A., Dmitrenok, A.S., Krasokhin, V.B. and Stonik, V.A., 2004. Sterols from six marine sponges. *Biochemical Systematics and Ecology*, 32, 153-167. https://doi.org/10.1016/S0305-1978(03)00143-1.

Seydi, E., Motallebi, A., Dastbaz, M., Dehghan, S., Salimi, A., Nazemi, M. and Pourahmad, J., 2015. Selective toxicity of Persian Gulf sea cucumber (*Holothuria parva*) and sponge (*Haliclona oculata*) methanolic extracts on liver mitochondria isolated from an animal model of Hepatocellular carcinoma. *Hepatitis Monthly*, 15(**12**), e33073. https://doi.org/10.5812/hepatmon.33073.

Shushizadeh, M.R., Behroozi, S., Behfar, A.A. and Nazemi, M., 2018. Antibacterial activity and GC-Mass analysis of organic extract from Persian Gulf *Haliclona* sp. *Pharmacophore*, 9(**2**), 19-24.

Smyrniotopoulos, V., Rae, M., Soldatou, S., Ding, Y., Wolff, C.W., McCormack, G., Coleman, C.M., Ferreira, D. and Tasdemir, D., 2015. Sulfated steroid-amino acid conjugates from the Irish marine sponge *Polymastia boletiformis. Marine Drugs*, 13(**4**), 1632-1646. https://doi.org/10.3390/md13041632

Sun, J.F., Wu, Y., Yang, B. and Liu, Y.G., 2015. Chemical constituents of marine sponge *Halichondria* sp. from south China Sea. *Chemistry of Natural Compounds*, 51(**5**), 975-977. https://doi.org/10.1007/s10600-015-1467-5.

Taylor, M.W., Radax, R., Steger, D. and Wagner, M., 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, 71(**2**), 295-347. https://doi.org/10.1128/MMBR.00040-06.

Viegelmann, C., Parker, J., Ooi, T., Clements, C., Abbott, G., Young, L., Kennedy, J., Dobson, A.D.W. and Edrada-Ebel, R.A., 2014. Isolation and Identification of antitrypanosomal and antimycobacterial active steroids from the sponge *Haliclona simulans*. *Marine Drugs*, 12(**5**), 2937-2952. https://doi.org/10.3390/md12052937

Wang, C., Liu, H., Shao, C., Wang, Y., Li, L. and Guan, H., 2008. Chemical defensive substances of soft corals and gorgonians. *Acta Ecologica Sinica*, 28(**5**), 2320-2328. https://doi.org/10.1016/S1872-2032(08)60048-7.

Zhang, Y., Mu, J., Gu, X., Zhao, C., Wang, X. and Xie, Z., 2009. A marine sulfate-reducing bacterium producing multiple antibiotics: biological and chemical investigation. *Marine Drugs*, 7(**3**), 341-354. https://doi.org/10.3390/md7030341.

Zhang, H., Hua, Y., Chen, J., Li, X., Bai, X. and Wang, H., 2018. Organism-derived phthalate derivatives as bioactive natural products. *Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews*, 36(**3**), 125-144. https://doi.org/10.1080/10590501.2018.1490512