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Antibacterial and Antifungal Activity with Minimum Inhibitory Concentration (MIC) Production from *Pocillopora verrucosa* collected from Al-Hamraween, Red Sea, Egypt

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ABSTRACT
The trouble of antimicrobial drug resistance has presupposed a search for new antimicrobial substances from other exporters including natural sources. Marine micro-organisms are known to produce metabolites to safeguard themselves against pathogens and therefore can be deemed as a potential source of antimicrobial substances. This research intended to evaluate the antimicrobial activity of six hard coral species namely *Acropora hemprichii, Acropora austera Seriatopora hystrix, Seriatopora pistillata, Pocillopora verrucosa* and *Millepora dichotoma* against some pathogenic microbes, and the bioactive compounds were extracted using ethyl acetate. The antimicrobial activity of the extracts was estimated using the disc diffusion method. The organic extract from *Pocillopora verrucosa* was the most effective against all selected microorganisms except *Bacillus subtillus* ATCC6633 and *Aspergillus flavus* while the highest effect was showed against *Fusarium solani* (22mm). Moreover, a partial description of these agents was carried out using the gas-liquid chromatography (GC-Mass). The main ingredient of *Pocillopora verrucosa* crude extract organic acids, aldehydes, esters, carotene, and their derivatives. That is the first research about the extraction of natural bioactive compounds from *Pocillopora verrucosa* which located in Al-Hamraween, Red Sea, Egypt.

INTRODUCTION

The Red Sea has long been known as a region of high biodiversity (Stehli and Wells 1971; Berumen e*t al.* 2013) Physiologically, marine organisms have the ability to develop unparalleled metabolites, to stay live in hard marine environments. These metabolites including a lot of novel secondary products with useful chemical and biological properties which couldn't be produced by terrestrial organisms (Blunt *et al.* 2009). For more than 30 years, marine natural products have attracted the attentiveness of scientists to isolate new biologically active components. Actually, of the 36 phyla of life, 34 are founded in the marine environment. The ocean represents a bushy exchequer for the discovery of potential therapeutic agents. However, despite the onerousness of

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survival, marine organisms have developed mechanisms to synthesize bioactive compounds to overcome these snags (Debitus 1998). In general, the prospect of natural bioactive compounds contents of almost all forms of life in the marine environment has been screened. In the marine life, sponges (37%), coelenterates (21%), and microorganisms (18%) are the large exporter of bioactive compounds followed by algae, echinoderms, tunicates, molluscs, and bryozoans (Kim 2012).Although sponges are considered the major contributing marine organisms of novel bioactive compounds, little are known on the antimicrobial activity of stony corals (Qaralleh *et al.* 2014). Corals are qualified for prohibit unwanted bacteria by various means, like the self-cleaning of mucus from their surface. Furthermore, microbes in the surface mucus layer change the immune system by stimulating specific responses and the immune system in return influences the microbial composition (Rivera-Ortega and Thomé 2018). on antimicrobial and antifungal activity of extracts of marine organisms and the subsequent purified antibiotics isolated from these organisms were tested against human pathogens as potential novel clinically useful drugs (Kelman *et al.* 2006; Afifi *et al.* 2016; AH Ibrahim *et al.* 2020; Sabdaningsih *et al.* 2017). The effectiveness was tested and found fundamentally in marine sponges and gorgonian octocorals. a few is known on the antimicrobial activity of other corals, especially reef-building (hermatypic) stony (scleractinian) corals. This is rather astonishing, considering that these latter organisms are the most predominant and distinguished members of many reefs. The Red Sea is a resource-rich area with a distinctive ecosystem of coral reefs where soft corals are the most abundant species (Ismail *et al.* 2017).

The aim of the current study is to compare the antibacterial and antifungal activity of extracts of six stony coral species from the coral reef of red sea Egypt.

MATERIALS AND METHODS

Study area

Al-Hamraween is located about 60 km southern of Safaga, 20 km northern of Qusier City and about 120 km from the Capital City of the Red Sea governorate (Hurghada) Fig.1. The harbor occupies the southern part of the settlement lying directly on small embayment. The tidal flat off Al-Hamraween area is very narrow and extends smoothly with a very gentle slope towards the sea. The environmental system of the marine area includes dense seagrass beds, algal blooms and mostly bleached coral communities in the lee side of the tidal flat (Dar 2005; Madkour *et al.* 2006).

Sampling and identification

All species of hard corals were collected using SCUBA and snorkeling equipment, using under water digital camera in Al-Hamraween area. The assemble coral reefs were assembled were hard branching reefs from the parallel to the reef edge within the water at 1 m, 1.5 m, 2 m, 3 m to 5m in depth. Each sample was cut into small pieces

and then promptly frozen and maintained at -20°C before extraction (Mohamadizadeh *et al.* 2014). Identification of hard coral species that were collected from the study area was carried out using available references (Veron 2000).

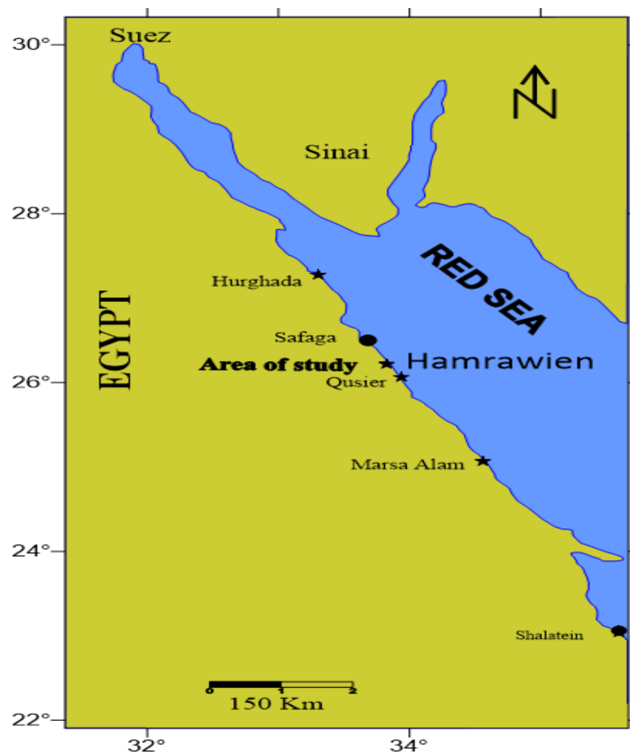

Fig.1. Map of the Al-Hamraween area and the location of sampling site

Hard coral crude extracts

The frozen samples were freeze-dried, prostrate with dry ice by a blender and drenched overnight in sterilized distilled water. The supernatants were then removed, centrifuged at 10,000 rpm, filtered and collected. The collected water extracts were freeze-dried (aqueous extract). The insoluble solid materials were then successively extracted with ethyl acetate successively by percolation (72 hours) at room temperature. The organic extracts were combined and the solvent removed by rotary evaporation at 40ºC to eschew the compounds degradation. The crude extract was kept at -20ºC until further processing (Qaralleh *et al.* 2014).

Test microorganisms

The bacterial strains were *Escherichia coli* ATCC 19404, *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC29212, *Bacillus subtillus* ATCC6633 and *Pseudomonas aeruginosa 9027* while fungal strains were *Aspergillus flavus, Aspergillus terreus* and *Fusarium solani*. All strains were obtained from microbiological lab (National Institute of Oceanography and Fishers, Egypt). The Pathogenic bacterial strains in the current study were maintained on nutrient agar slants and stored at 2°C. For long preservation, the slants were folded with 25% glycerol.

Antibacterial activity

The biological culture was performed using an agar well diffusion method. Antimicrobial activities of hard corals extract using organic solvent were performed against pathogenic selected bacteria. The antibacterial activity was done by Kir bye Bauer disk diffusion method. The pure cultures of pathogens were sub cultured in nutrient broth at 35°C on a rotary shaker at 120 rpm. For bacterial growth, 100 ml fresh culture having 10^6 colony forming units (CFU)/ml of each test pathogen were grown on nutrient agar plates using a sterile L-rod spreader. After solidification, 8 mm wells were punched into the nutrient agar plates for testing extract. Using a micropipette, 100 µl of the test sample was poured onto each well on all plates with a concentration of 100 mg/ml. After overnight incubation at 35°C, the radius of each inhibition zone was linearly measured in mm (Suresh *et al.* 2016).

Antifungal activity

The fungal isolates were growing in YEPD media (peptone 20g; Yeast extract; Dextrose 20g and distilled water 1000 ml) at 25 °C for 7 days then, spores were harvested from slants, to prepare inoculums containing 10^5 spores/cells ml^{-1} of fungi. For solidification media were add 1.5% (w/v) agar was added to the broth medium. Into sterilized plates 100 µl of the indicator strain suspension was poured and overlaid with a soft agar medium cooled to 45°C and mixed. A culture supernatant (100 µl) was added into each well with a concentration of 100 mg/ml. After incubation, the antifungal activities were determined by measuring diameter of each inhibition zones (mm) (Cizeikiene *et al.* 2013).

Minimum inhibitory concentration (MIC)

Different concentrations of the tested coral sample from *Pocillopora verrucosa* (100, 75, 50, 25, 12.5, 6.25 and 3.12 mg/ml) were introduced on wells onto agar plates inoculated with the various pathogenic cultures. Minimum inhibitory concentration (MIC) values were taken as the lowest concentration of extract that inhibited the growth of the pathogen after 24 h of incubation at 37C. Microbial growth was determined by measuring the diameter of the inhibition zone area (Velmurugan *et al.* 2012).

Statistical analysis

Data was presented as the mean of three replicates ± standard error (SE). XLSTAT program (version 5.03) was used for statistical analyses (Pagès *et al.* 2014).

Characterization of crude extract

The produced extracts from *Pocillopora verrucosa* were willing for GC-Mass analysis. Analysis of GC / MS was performed using a GC instrument (Agilent 7890A) equipped with an HP-5MS column (30 m/250 µm/0.25 µm film thickness) and a MS detector (Agilent 5975C).The initial temperature of the oven was set for holding at 90 ° C for 1 min, then rising at 8 ° C / min to 300 ° C for 30 min. Helium was used at a flow rate of 1.5 ml / min as a carrier gas. The injection volume of the sample was 1 µl in the splitless mode where the injector temperature was 290°C. Mass spectrum was operated at 70ev and mass range from 60-600 amu. Extracts prepared using ethyl acetate solvent and

concentrated until complete dryness and finally resuspended in convenient volume of ethyl acetate (Ibrahim *et al.* 2012).

RESULTS

Identification of coral species

The collected hard corals from Al-Hamraween area as well as encountered hard corals were identified according to (Veron 2000). A total of 6 species belonging to 3 families and 4 genera were recorded in (Table.1 and Fig.2).

Table1: Red Sea hard corals identification at the study area

PHYLUM	CNIDARIA
FAMILY	*Acroporidae*
GENUS	*Acropora*
SPECIES	***A. hemprichii*** ***A. austera***
FAMILY	Pocilloporidae
GENUS	*Seriatopora*
SPECIES	***S. hystrix*** ***S. pistillata***
GENUS	*Pocillopora*
SPECIES	***P. verrucosa***
CLASS	*Hydrozoa*
FAMILY	*Milleporidae*
GENUS	*Millepora*
SPECIES	***M. dichotoma***

Antibacterial and antifungal activity of corals

In this study, crude extracts of six identified hard corals collected from Red Sea, Egypt were screened for their antibacterial and antifungal activity against selected bacterial and fungal indicators (*Escherichia coli* ATCC 19404, *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC29212, *Bacillus subtillus* ATCC6633, *Pseudomonas aeruginosa 9027*, *Aspergillus flavus, Aspergillus terreus* and *Fusarium solani).* Using ethyl acetate solvent which were selected according to different polarities. Extracts were prepared and then screened against the aforementioned bacterial andfungal pathogens using well cut diffusion method. In general, the crude extracts showed positive records against most of selected pathogens. The crude extraction from aqueous extracts tested against the pathogen and showed in (Table 2 Figure 3). The zone of inhibition (mm) ranged from (22 to 10) by *Pocillopora verrucosa, Acropora austere, Seriatopora pistillata Acropora hemprichii, Millioporadichomata* and *Seriatopora hystrix***.** The highest value (22.0 mm) was recorded against fungal pathogens *F. solani* by the crude

extract produced from *P.verrucosa.* while lowest activity (10 mm) showed against *F. solani* by the crude extract produced from ***Seriatopora pistillata.*** The antimicrobial study revealed that: The extract of *Pocillopora verrucosa* has marked effect against most tested bacterial strains either gram positive or gram-negative bacteria as well as most tested fungus strains. The MIC values of *Pocillopora verrucosa* curd extract against pathogenic bacteria were determined and tabulated (Table 3). Statistical analysis showed that, data do not pass the normality test, even after various treatment processes (e.g. log (x+1) transformation). So, a non-parametric Friedman test of differences among coral species was conducted and rendered Chi-square ($\chi 2$) values of 11.08 which were significant ($p < 0.05$) for ethyl acetate extractions (Table 4).

Fig.2. Red Sea hard corals at the study area and used for preparing crudes extracts.

Table 2. Antibacterial and antifungal activity of ethyl acetate extraction of hard corals species using well cut diffusion method

Pathogens	**Antibacterial and Antifungal activity (mm) of Coral species a concentration of 100 mg ml^{-1}**					
	Acropora hemprichii	***Seriatopora hystrix***	***Milliopora dichomata***	***Acropora austera***	***Pocillopora verrucosa***	***Seriatopora pistillata***
E. coli	0.0	12.0	16.0	0.0	**12.0**	0.0
S. aureus	18.0	16.0	18.0	12.0	**20.0**	20.0
E. faecalis	16.0	16.0	0.0	12.0	**16.0**	0.0
B. subtillus	0.0	0.0	0.0	0.0	**0.0**	0.0
P. aeruginosa	0.0	12.0	0.0	0.0	**16.0**	0.0
A. flavus	0.0	0.0	0.0	0.0	**0.0**	0.0
A. terreus	0.0	14.0	14.0	0.0	**16.0**	0.0
F. solani	0.0	0.0	16.0	16.0	**22.0**	10.0

(A) **(B)**

Fig.3. Antimicrobial and antifungal activity production from *Pocillopora verrucosa* crude extracts against (A) *Staphylococcus aureus* ATCC6538 and (B) *Aspergillus terreus*

Table3: Minimum inhibitory concentration of crud extracts from *Pocillopora verrucosa* against bacterial and antifungal pathogens

Pathogens	MIC of *P.verrucosa* (g/ml)
E. coli	50±1
S. aureus	12.5±1
E. faecalis	25±1
P. aeruginosa	25±1
A. terreus	25±1
F. solani	12.5±1

Table 4: Friedman's test of inhibition zone differences among coral species for different extractions.

	Ethyl acetate
Q (observed value)	11.08
Q (Critical value)	11.07
DF	5.00
p-value (Two-tailed)	0.0498
alpha	0.05

GC-MS chromatogram analysis

The ethyl acetate extract of *P. verrucosa* was exposed to GC-MS analysis to identify its components (Fig. 4). These compounds were recognized through mass spectrum attached with GC. The active principles with their retention time [RT], molecular formula (MF), Molecular weight (MW) and concentration (%) are accessible in Table 5. Table 2 shows the presence of bioactive compounds in *P. verrucosa*and the major compounds were: Piperidine, 1-methyl-,1-butanamine, 2-methyl-n-(2-methylbutylidene),3-methylbutyl-(3-methylbutylidene) amine, Dimethyl trisulfide, Ethanethioic acid, S-[2-(dimethylamino)ethyl] ester, Cyclooctasiloxane, hexadecamethyl-, N-Phenethyl-2-methylbutylidenimine, 1h-purin-6-amine,[(2-fluorophenyl)methyl], 1-Hexadecanol, hexadecanoic acid, Phthalic acid butyl dodecyl ester, 9-Octadecenoic acid, 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-, 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-, 2-([(2-ethylhexyl)oxy]carbonyl)benzoic acid, Tetradecanoic acid, hexadecyl ester, cis-9-Hexadecenoic acid, heptyl ester, 9-Octadecenoic acid (Z)-, tetradecyl ester.

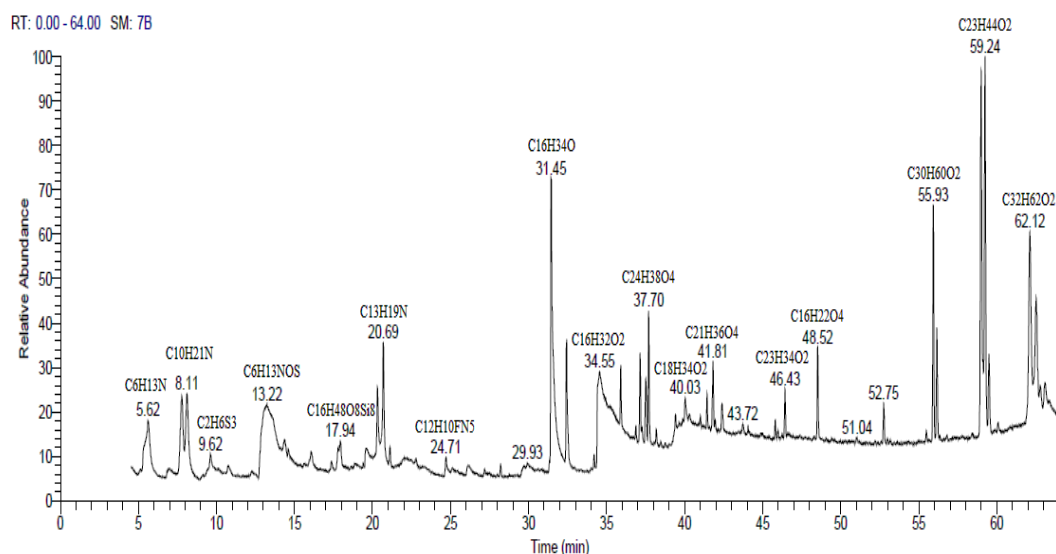

Fig.4: GC-MS chromatogram and structure formulas of compounds of the bioactive compounds extracted from *Pocilloporaverrucosa*.

Table 5: Components detected in *Pocilloporaverrucosa*crude extraction using ethyl acetate.

No	R T Value (In Min.)	Compound	Mol. Formula	Molecular Weight	Peak area (%)
1	5.62	Piperidine, 1-methyl-	C6H13N	99	3.63
2	7.78	1-butanamine, 2-methyl-n-(2-methylbutylidene)	C10H21N	155	2.76
3	8.12	3-methylbutyl-(3-methylbutylidene) amine	C10H21N	155	2.45
4	9.62	Dimethyl trisulfide	C2H6S3	126	0.46
5	13.03	Ethanethioic acid, S-[2 (dimethylamino)ethyl] ester	C6H13NOS	149	4.78
6	17.94	Cyclooctasiloxane, hexadecamethyl-	C16H48O8Si8	592	0.94
7	20.69	N-Phenethyl-2-methylbutylidenimine	C13H19N	189	2.83
8	24.17	1h-purin-6-amine,[(2-fluorophenyl)methyl]	C12H10FN5	243	0.68
9	31.45	1-Hexadecanol	C16H34O	242	8.16
10	34.43	Hexadecanoic acid	C16H32O2	256	4.94
11	37.69	Phthalic acid butyl dodecyl ester	C24H38O4	390	2.76
12	40.3	9-Octadecenoic acid	C18H34O2	282	0.68
13	41.81	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	C21H36O4	352	1.72
14	46.43	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	C23H34O2	342	1.05
15	48.52	2-([(2-ethylhexyl) oxy] carbonyl)benzoic acid	C16H22O4	278	1.97
16	55.93	Tetradecanoic acid, hexadecyl ester	C30H60O2	452	5.25
17	59.24	cis-9-Hexadecenoic acid, heptyl ester	C23H44O2	352	8.83
18	62.11	9-Octadecenoic acid (Z)-, tetradecyl ester	C32H62O2	478	6.44

DISCUSSION

The aggregate objective of our study was to parallel the ability of extracts of Red Sea hard corals collected from the same habitat, to inhibit the growth of some pathogens. The biotechnological possibility of marine invertebrates such as corals has attracted the attention of researchers because of their ability to produce powerful bioactive compounds (Blunt *et al.* 2017). Marine corals are known to contain a rich variety of marine secondary metabolites and are considered a very diverse group of marine organisms. The bioactive compounds from marine corals have not only great significance in chemical ecology but also rapid various biological activities such as antitumor, antibacterial, antiviral and antifungal (Afifi *et al.* 2016). Our results have obviously shown in (Tables 2 and 4) and the result of antimicrobial activity in laboratory assays do not necessarily signalize a deficiency of antimicrobial chemical defense. Chemicals produced by higher organisms against cooccurring microorganisms may not simply kill or inhibit the growth of the target microorganism, but can act selectively against particular characteristics that are expressed by the bacteria. Both water and organic solvents were used to extract the bioactive compounds and all extracts give positive results. In similar study organic solvent was used to make extraction the hydrophilic and hydrophobic compounds from some hard corals species (Qaralleh *et al.* 2014; Mohammadizadeh *et al.* 2013). However, the extract using ethyl acetate of *P.verrucosa*was the most effective in the inhibition of most tested microorganisms. On antithesis that, the extracts of *A. Hemprichii*, *A. austera* and *S. pistillata* had the lowest effective one against all pathogens. Extracts from Scleractinia corals have been recorded to contain antimicrobial compounds (Geffen and Rosenberg 2005). The extracts of stony corals had no antimicrobial activity compared to extracts of soft corals that present good antimicrobial activity (Kelman *et al.* 2006). The coral *Pocillopora damicornis* prompt production antibacterial agents following a mechanical stress. Only antimicrobial compounds have also been notified in coral-associated bacteria (Geffen and Rosenberg 2005). Thus, antimicrobial activity is possible to engendered from both microbes and host sources (Mohamadizadeh *et al.* 2014).The alteration in the environment or the health conditions of the coral allows for changes to the microbial community. This also may suggest that production and extractions of bioactive compounds by mucus-associated bacteria is part of the scleractinian coral's defense strategy against pathogens (Ritchie 2006). In sundry studies the antibacterial activity of hard corals has been, resolved. Koh and his team reported, the alcohol extract of a great percentage of coral samples had antibacterial activity against a number of pathogenic bacteria (Koh 1997), while results acquired by (Kim 1994) explained antibacterial activity for both polar and non-polar (coral-derived) fractions with best activity associated with non-polar fractions. Antimicrobial activity was found particularly, among extracts obtained with non-polar rather than hydrophilic solvents (Gantar *et al.* 2011). Antibacterial activity in extracts of this hard species suggests a possible ecological function for their secondary metabolites. The antibacterial property of

the hard corals extracts detect that they are potent enough to be less affected by bacterial organisms than the fungal (Mohamadizadeh *et al.* 2014). The ethyl acetate extract of *P.verrucosa* was subjected to Gas chromatography/mass spectrometry studies. Our data revealed that the main constituents of *P.verrucosa*ethyl acetate crude extract were; organic acids, aldehydes, esters, carotene and their derivatives. These major compounds have all shown to have antimicrobial, antioxidant and cancer preventive in many reports (Al-Rubaye *et al.* 2017; Dhinakaran and Lipton 2014).Most of these compounds have been extracted from sea cucumber (*Holothuriaatra*) which collected from the Indian Ocean and the extraction had various compounds such as the flavonoids, phenolic components, terpenoids, saponins, alkaloids which were seen on their ability as anti-bacterial and anticancer activity (Dhinakaran and Lipton 2014). They are constituent of simple basic oils such as aromatic phenolic compounds, and diterpenoids, 1-Dodecene, Piperidine, Hexadecenoic acid, 9-Octadecenoic acid (Z)-, tetradecyl ester which has been notify to have numerous medicinal properties, they are used as antibacterial, antineoplastic, anti-carcinogenic, antimalarial, anti-ulcer and other pharmaceutical functions (Anadakumar *et al.* 2018; Thenmozhi and Rajan 2015; Vijisaral Elezabeth and Arumugam 2014; Tanod *et al.* 2018; Idan *et al.* 2015).

CONCLUSION

Some of the identified compounds in hard corals of Red Sea, Egypt, are rare compounds which are not yet announced in hard corals in the literatures. For future research, it is important to focus on finding the mechanisms used by corals to protect themselves against invasion, infection mechanisms and the type of chemical compound in coral extracts that inhibit bacterial and fungal growth. Therefore, extraction and purification that compounds can be useful for identification of their anti-microbial properties. On the other hand, although the amounts of identified compounds were not high to warrant economic value of studied hard corals, but identification of the bioactive compounds can be a good steering for showing chemical synthesis methods of identified compounds.

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