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Preliminary assessment of bioactive ingredients and antioxidant activity of some Red Sea invertebrates' extracts.

Reham Elshaarawy[1], Esraa Aboali[1], Abdallah Alian[2], Hany Ibrahim[1], Sobhy Hassab El-Nabi[1], Khaled Mohammed-Geba[1], Asmaa Galal-Khallaf[1*].

[1]Zoology Department, Faculty of Science, Menoufia University, Menoufia, Egypt.
[2]Zoology Department, Faculty of Science, Al-Azhar University, Assiut Branch, Assiut, Egypt.

[*]**Corresponding Author: as_kh_22@yahoo.com**

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ABSTRACT

Red Sea organisms represent key and genuine resources for biologically active metabolites. To provide preliminary clues about the biological activities of some Red Sea invertebrates, the soft coral *Sarcophyton convolutum* and the crab *Charybdis natator* were obtained. The whole amount of the coral and the shell part of the crab were subjected to extraction to obtain bioactive metabolites. These metabolites were determined in the extracts using Gas chromatography–mass spectrometry (GC-MS). Next, zebrafish protected with these extracts were challenged with copper sulfate ($CuSO_4$) to identify the status of total antioxidant activity in fish livers. The liver was chosen as the main organ where detoxifying and antioxidant enzymes are expressed, as reported in zebrafish and many other fish species before. The results showed that both invertebrate species possess a diverse array of biologically active ingredients, mainly fatty acids, steroid derivatives, flavonoids, sesquiterpenoids, and thiols. The main compounds found in the crab extract were 1-Heptatriacotanol, Dotriacontane, Ethyl iso-allocholate, tert-Hexadecanethiol, 11-Octadecenoic acid, Hexadecanoic acid, and Tetradecane. The analyzed fraction of soft coral extract contained mainly palmitic acid, Isochiapin B, Isooleic acid, and 7,9-Di-tert-butyl-1-oxaspiro(4,5)dec a-6,9-diene-2,8-dione. Zebrafish protected using these extracts showed differential responses. Whereas coral extract could restore the levels of Hydrogen Peroxide (H_2O_2) scavenging activity to levels that were similar to the negative control group, the crab shell extract seemed to have lesser effects on the same parameter. More work is recommended regarding the in-depth characterization of bioactive ingredients of these organisms using bigger fractions and also to assess further biological activities of them.

INTRODUCTION

Aquatic ecosystems, with their vast biodiversity, represent an important source of novel compounds with various biological activities. These compounds showed biological powers, for example, antitumor, antibacterial, antiviral, antioxidant, and anti-

inflammatory activities (**Hou *et al.*, 2019; Cunha and *Pintado*, 2021**). The Red Sea is one of the main hotspots for biological diversity in the world, being extremely rich with micro- and macro-fauna that exhibit an extremely diverse array of bioactive compounds. Moreover, crude extracts of the Red Sea sponges, seaweeds, algae, and crustaceans proved promising antitumor, anti-inflammatory, and antibacterial agents (**Nasr 2011; El-Damhougy *et al.*, 2017; Madkour *et al.*, 2019**). This is because of their extraordinary capabilities to produce or harbor some microbiomes that produce various bioactive metabolites. For example, algae are rich sources of phenolic compounds; including Flavonoids, phenolic acids, lignans, tannins, and tocopherols; carotenoid pigments; phycobilin pigments, as well as vitamins and minerals (**Gomez-Zavaglia *et al.*, 2019**). Also, sponges exhibit diverse variety of bioactive ingredients, including isoxazolines, oxepinisoxazolines, phenolics, quinolines, isopernoids, sesterterpenoids and macrolides (**El-Demerdash *et al.*, 2019**). Soft corals are usually rich in steroids, sesquiterpenes, diterpenes, and cembranoids (**Abdelhafez *et al.*, 2019; Nurrachma *et al.*, 2021**). Crabs also provide a rich source for fatty acids, pheonlics, terpenoids, and other essential bioactive ingredients (**Galal-Khallaf *et al.*, 2022**).

Crabs ranked the third species, after shrimp and lobster, that offer major health benefits due to the presence of vitamins, proteins, and unsaturated fatty acids as well as their peptides that may help treat different diseases (**Narayanasamy *et al.*, 2020**). Crabs' exoskeletons contain a variety of bioactive compounds that play serious roles as anti-inflammation and antioxidant agents (**Devi *et al.*, 2015; Zhou *et al.*, 2021**). Among the Red Sea crabs is the portunid crab *Charybdis natator* (**Herbst, 1789**), which is widely distributed throughout the Indo-West Pacific from the Red Sea to China and Australia. Recently, soft tissue extract, specifically leg muscle extract of *C. natator* has been assessed and found to have anti-inflammatory properties (**Narayanasamy *et al.*, 2020**).

Also, cnidarians provide some of the most significant resources of marine natural products, particularly for the great diversity of bioactive metabolites they produce in their environments owing to the interactions they have with many organisms living on them (**König *et al.*, 2006**). More specifically, many of the natural compounds derived from soft corals were proven to have a wide range of biological actions, including anti-tumor, antiviral, antifouling, and anti-inflammatory properties (**Nurrachma *et al.*, 2021**). The soft coral *Sarcophyton convolutum* is one of these corals that recently showed promising biological activities (for example, **El-Gendy *et al.*, 2022**).

Yet, both *C. natator* and *S. convolutum* bioactive ingredients still need to be described sufficiently. Identification of these ingredients, that are usually produced by those marine organisms as a way for adaptation to their harsh environments, can precede harnessing them to combat severe health hazards, including inflammatory diseases, cancer, oxidative stress, and immune diseases (**Hamed *et al.*, 2015; Galal-Khallaf *et al.*, 2022**). Furthermore, application of methanol as a solvent has previously been proven to be an efficient method for extracting bioactive ingredients. It resulted usually in lower

yield of inactive compounds and higher yield of phenolic, flavonoid, alkaloid, and terpenoid compounds than other solvents extract, such as EtOH, $CHCl_3$, CH_2Cl_2, and Me_2CO (**Rameli *et al.*, 2018; Truong *et al.*, 2019**). Hence, this study aimed to provide preliminary analysis for some bioactive ingredients in these two Red Sea invertebrate species and to identify whether they have antioxidant properties. Methanolic extracts for *S.convolutum* whole mount and *C. natator* exoskeleton were explored in the current study. As an experimental model, adult zebrafish *Danio rerio* was used. Zebrafish are being extensively used to discover new drugs and study developmental biology, especially owing to their low maintenance costs, quick embryogenesis, transparency, and high transcriptomic similarity to humans, especially in metabolic and immunological pathways (**Khan and Alhewairini, 2018; Cassar *et al.*, 2019).**

MATERIALS AND METHODS

1. *Sampling of target invertebrates*

Samples from the soft coral *S. convolutum*, were collected from Marsa Alam city, on the Red Sea, Egypt. The species was morphologically confirmed according to **Fabricious and Aldersdale (2001)** (Fig. 1). Also, fresh crab *Charybdis natator* (Crustacea: Decapoda) (Fig. 2) was collected from the Abu Zenima area, Sinai, in the Northeast of Egypt (Coordinates: 29°02'36.8"N 33°07'21.1"E). The crabs were directly euthanized by placing them in ice until complete immobilization, then 500 gm of the carapace were removed from each one, thoroughly rinsed with distilled water to remove any residues of soft tissues or field contaminants, dried at room temperature, and thoroughly ground.

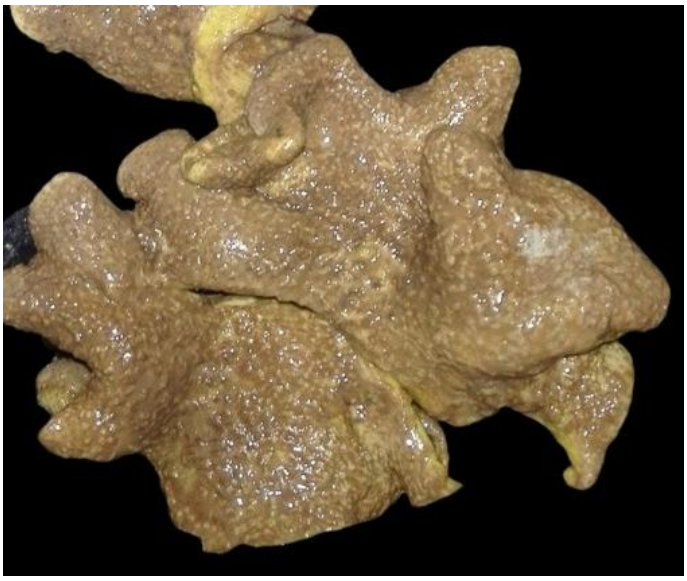


Fig. 1. *Sarcophyton convolutum* collected for extraction in the current study.

Fig 2. *Charybdis natator* collected for extraction in the current study.

2. Extraction and isolation of bioactive metabolites

Srcophyton convolutum samples were chopped into small pieces and extracted by maceration in methanol (750 ml/three times). A rotary evaporator distilled off the solvent at a low temperature (50 °C) to obtain a dry residue, which weighed 2.5 g of the mentioned coral. Meanwhile, the ground *Charybdis natator* shells (about 300 gm) were extracted by placing them in 500 ml methanol and continuing the same procedures as in Galal-Khallaf *et al.* (2022). For each crab and coral extract, the precipitates were reconstituted in 10 ml of absolute Ethyl alcohol until further processing for Gas chromatography–mass spectrometry (GC-MS) analysis.

3. Identification of bioactive ingredients using GC-MS

Fatty acids present in each extract were determined by GC-MS after being H_2SO_4-derivatized into their methyl esters (FAMEs) (**Hewavitharana *et al.*, 2020**). Gas chromatography–mass spectrometry data were acquired using Trace GC-ISQ mass spectrometer (Thermo Scientific, USA) equipped with A3000 autosampler and TG-5MS Capillary column of 30 m length, 0.25 mm i.d., and 0.25 μm film thickness. All conditions were the same as in **Galal-Khallaf *et al.* (2022)**. GC-MS procedures were carried out according to **Abd El-Kareem *et al.* (2016)**. The chemical ingredients contained in the extract were tentatively identified by comparing their relative retention times and mass spectra to the WILEY database (Wiley Registry of Mass Spectral Data, 9th Edition, Version 1.02) and NIST 05 library (NIST/EPA/NIH mass spectral library version 2.0d).

4. *Experimental procedures*

Mixed-sex adult zebrafish were obtained from a local pet shop. Thirty fish were acclimated to normal, dechlorinated freshwater at a temperature of 20 °C and a normal 12h:12h light regime. The lab-acclimation period was for two weeks. Fish were fed a daily ration of 2 % of their total body weight with tropical aquaria standard fishmeal twice a day. Later, twenty fish were transferred into 4 separate, 5 L-capacity aquaria filled with dechlorinated, well-aerated freshwaters (Fish mass: 2±0.2 g).

A brief protection experiment was carried out. First, both positive and negative control groups received an intraperitoneal injection with 20 µl of 1 % DMSO (n=5 fishes/each group). A *coral* group received a single intraperitoneal injection with a coral extract at a dose of 25 µg/µl in 1 % DMSO (n=5 fishes). A *crab* group received a single intraperitoneal injection with the crab extract at a dose of 2.5 µg/µl in 1 % DMSO (n=5 fishes). Then, all groups, except the negative control one, were transferred into new, 5-L aquaria, where they were immersed in 30 µg/L of CuSO4 for 48 hours. Then, all zebrafish were collected by netting and euthanized in ice-cold water. No mortalities were recorded in any of the experimental groups. The fish were dissected, and the livers were excised to be used for immediate homogenization and measurement of total antioxidant activity.

5. Measurement of total antioxidant capacity

Total H_2O_2 scavenging activity was measured according to **Khalil *et al.* (2018)**. Fish livers were individually homogenized in 10 % (weight/volume) ice-cold, 0.1 M phosphate buffer ($Na_2HPO_4.7H_2O$-NaH_2PO_4, pH 7.2). Then, the homogenates were individually centrifuged at 4 °C, as 10,000g for 30 min. The homogenates were transferred into new, sterile, 1.5 ml tubes. The clear homogenates were used for measurement of total antioxidant activity using a commercial kit (cat. no. TA2513, Biodiagnostic Co., Egypt). 10 µl from each sample were added, in triplicates, to the well of a U-shaped bottom, 96-well plate suitable for spectrophotometric measurements. After adding the diluted kits´s first reagent, R1, according to the manufacturer's instructions, the plate was incubated at 37 ºC for 20 min. Then, the working reagents (R2 & R3) were added in equal volumes; the plate was incubated at 37 º C again for 5 min. The absorbance was read immediately using the spectrophotometric plate reader (TECAN, infinite F50, Switzerland) at 500 nm. Finally, total antioxidants concentration was measured in mM / L through subtracting Absorbance of the sample from absorbance of the blank and multiplying the result by 3.33, according to the manufacturer's instructions.

6. Statistical analysis

Total GC/MS data were identified by **Abd El-Kareem *et al.* (2016)**. Total antioxidant activity results were analyzed using (ANOVA) One-Way analysis of variance in the software STATGRPHICS Centurion XVI. Tukey-HSD test was used as test *a posteriori*. Differences were considered significant at p-value <0.01.

RESULTS

GC-MS analysis showed that extracts from both Red Sea invertebrates had a diverse array of bioactive chemicals. However, the types and quantities of bioactive ingredients varied completely between the two extracts, to the extent that almost no common ingredients were found among the most abundant categories of natural products identified. For the crab *C. natator*, the major components detected using GC-MS analysis were 11-Octadecenoic acid (vaccenic acid, 20 %), Hexadecanoic acid (17 %), tert-Hexadecanethiol (14 %), -Heptatriacotanol (12 %), Dotriacontane (13 %), Ethyl iso-allocholate (13 %), and Tetradecane (11 %) (Fig. 3, Table 1). Most of these derivatives were fatty acids. However, Ethyl iso-allocholate is a steroid-derivative, and tert-Hexadecanethiol is a thiol.

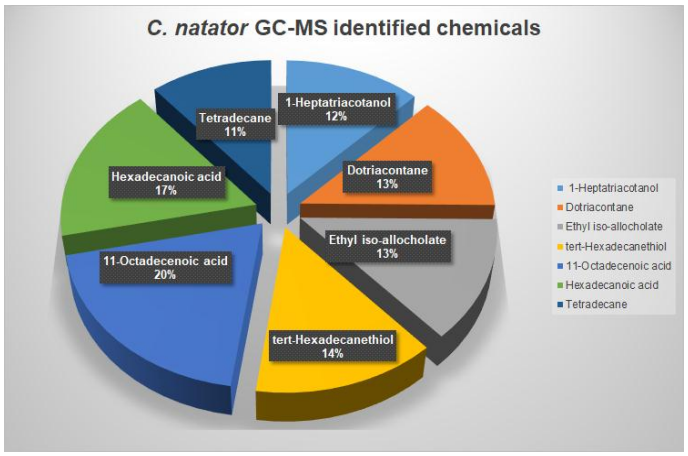
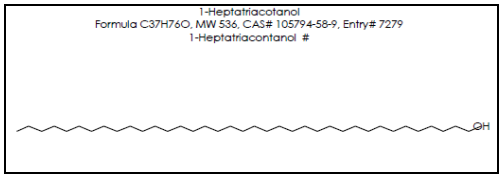
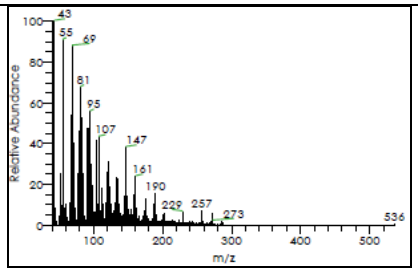
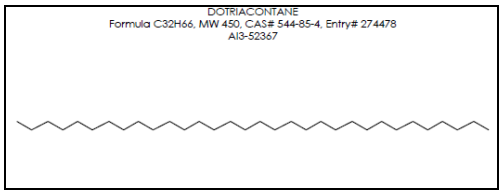
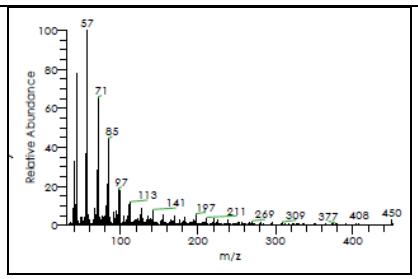
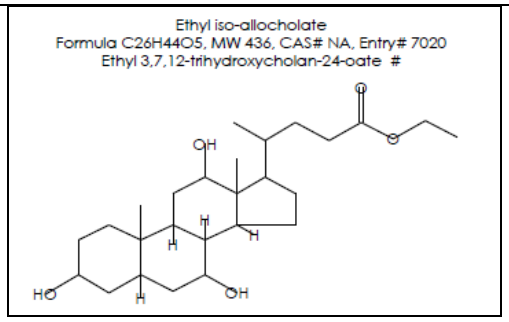
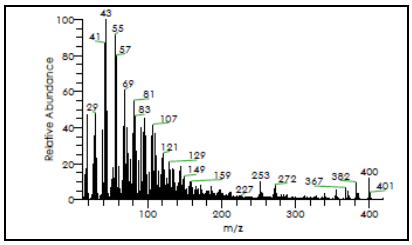
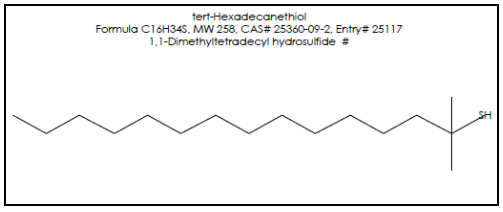
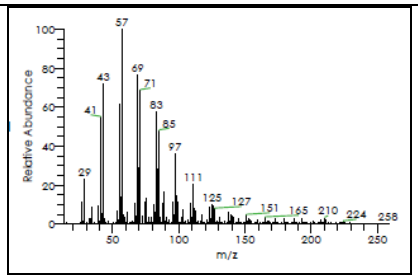
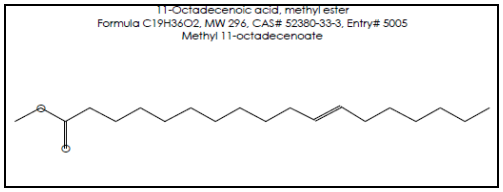
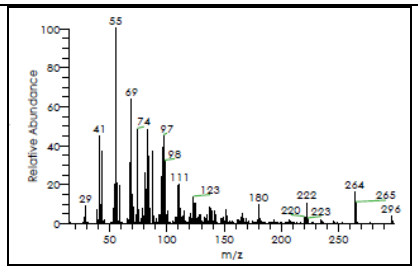
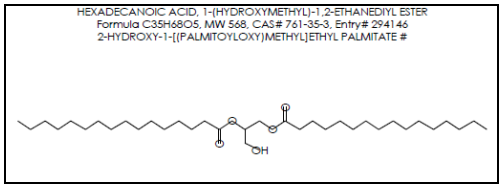
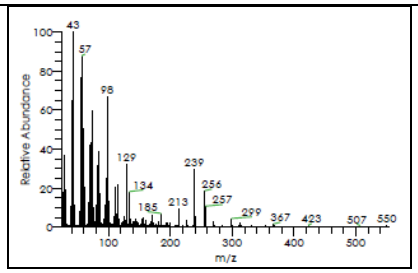
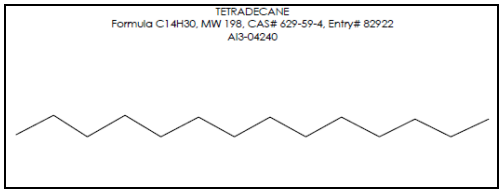
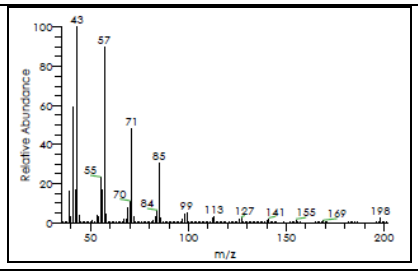


Fig. 3. Main components of *C. natator* shell methanolic extract identified in the current study.

Table 1. Main bioactive ingredients found in the crab *C. natator* shell extract using GC-MS analysis

Chemical compound	Structure	Mass Spectra
1-Heptatriacotanol		

Dotriacontane		
Ethyl iso-allocholate		
tert-Hexadecanethiol		
11-Octadecenoic acid		
Hexadecanoic acid		
Tetradecane		

The soft coral *S. convolutum*, however, showed lesser amount of bioactive ingredients that could be detected by GC-MS. The main identified components were Palmitic acid and its derivatives (fatty acid, 54 %), Isooleic acid (fatty acid, 21 %), Isochiapin B (Sesquiterpen, 16 %), and 7,9-Di-tert-butyl-1-oxaspiro(4,5)dec a-6,9-diene-2,8-dione (flavonoid, 9 %) (Fig. 4, Table 2).

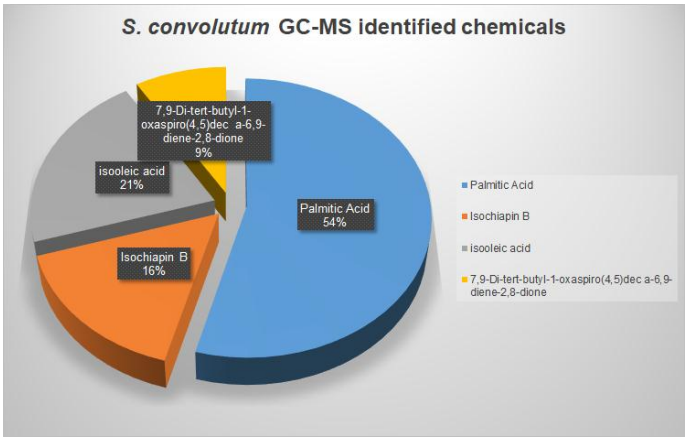
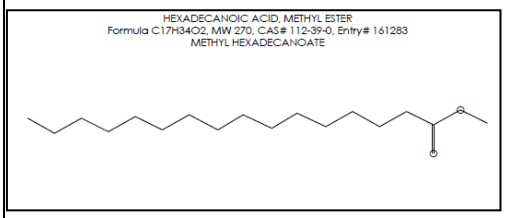
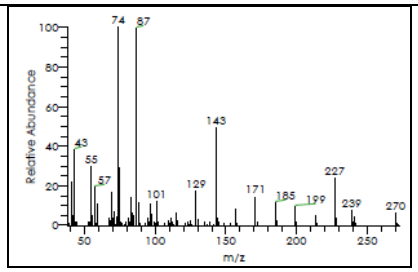
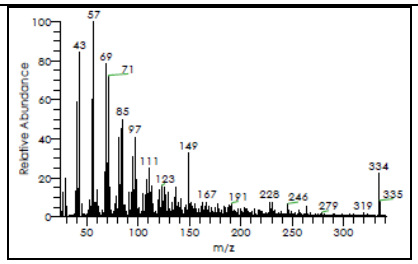
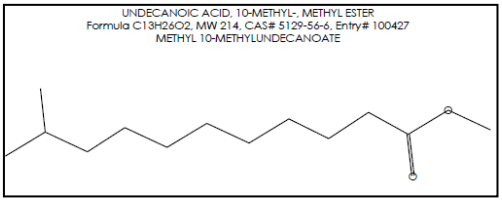
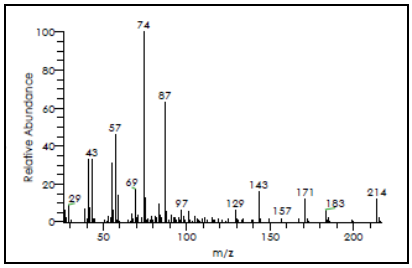
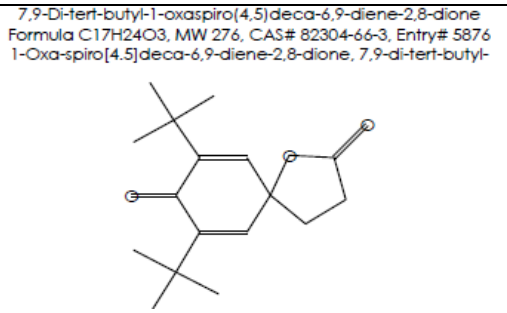
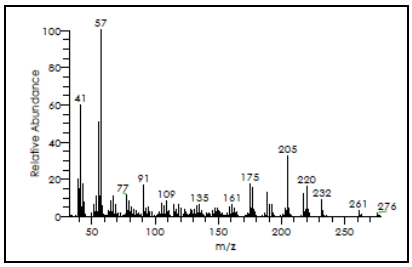


Fig. 4. Main components of *S. convolutum* methanolic extract identified in the current study.

***Table* 2**. Main bioactive ingredients found in the soft coral *S. convolutum* using GC-MS analysis

Chemical compound	Structure	Mass Spectra
Palmitic Acid		
Isochiapin B		

Isooleic acid (Undecanoic acid, 10-methyl-)		
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione		

No mortalities were recorded in all fish aquaria applied in the current short experiment. Also, the fish showed normal feeding and swimming behaviors. Furthermore, no apparent changes in water temperature or other assessed conditions for water quality during the whole lab conditions-acclimation and experimental times. However, zebrafish showed apparently variable responses to the treatments with soft coral and crab extracts and the subsequent challenge by $CuSO_4$. The H_2O_2 scavenging capacity of liver tissues decreased significantly in the positive control (i.e., DMSO-injected, $CuSO_4$-challenged) group in comparison to the negative control group (Fig. 5). It appeared that the applied dose of the coral extract (25 µg/µl) could partially restore the level of the total antioxidants to a similar level as the negative control. Yet, this change was not significant (Fig. 5). Meanwhile, the applied dose of crab extract (2.5 µg/µl) failed to alter the total antioxidant activity back to the negative control level (Fig. 5).

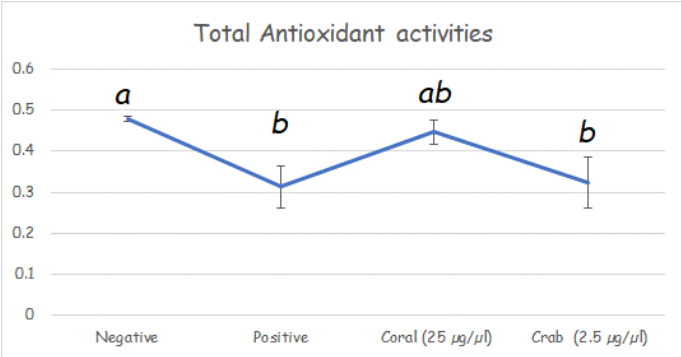


Fig. 5. Total H_2O_2 scavenging activity (in mM/L) in zebrafish protected with either coral, i.e.,*S. convolutum,* extract (25 µg/µl) or crab, i.e., *C. natator*, shell extract (2.5 µg/µl) and challenged with 30 µg/L of $CuSO_4$ for 48 hours. Neagtive: "DMSO-injected only" goup, positive: "DMSO-injected+$CuSO_4$-challenged" group. Data are represented as mean ± standard error of the mean (SEM; n = 5). Different letters (a,b) indicate significant differences among experimental groups (one way ANOVA, and Tukey-HSD as post-hoc test, $P< 0.01$.

DISCUSSION

The types and amounts of bioactive ingredients in marine organisms affect, to a deep extent, their extraordinary capabilities to combat different stresses and diseases, such as oxidative stress, inflammations, and parasitic infections, among many others. In this preliminary analysis for two invertebrate species from the Red Sea, i.e., the crab *C. natator* and the soft coral *S. convolutum*, differential contents of bioactive ingredients were found. Also, it seems that their capabilities to reduce oxidative stress were also differential. Soft coral had palmitic acid as the main unsaturated fatty acid in its extract. A similar abundance of this acid was found in extracts of some other marine soft corals that showed potent antioxidant activities, such as the soft corals belonging to *Dendronephythya* sp. **(Shahbudin *et al.*, 2011)** and *Lobophytum* sp. **(Mu'nisa *et al.*, 2023)**. The sesquiterpenoid compound isochiapin-B was also present in several extracts that showed antioxidant activities (**Değirmenci *et al.*, 2020; Espinoza *et al.*, 2020**). However, it has been relatively recently elucidated from Red Sea sponge species, i.e. *Ircinia* sp. and *Iotrochota purpurea* marine soft corals (**Hassan *et al.*, 2022a**). Likewise, the Undecanoic acid was recently characterized in Red Sea invertebrates, such as the soft coral *Nepthea* sp. (**Hassan *et al.*, 2022b**), where it showed the potential to inhibit microbial growth. However, to the best of the authors' knowledge, no clear data is available regarding its antioxidant activity. Finally, the flavonoid 7,9-Di-tert-butyl-1-oxaspiro(4,5)dec a-6,9-diene-2,8-dione has been characterized as a part of extracts that provide strong antioxidant capabilities (**Khaled *et al.*, 2021**; **El-Sayed *et al.*, 2022**). The long-chain thiol, tert-Hexadecanethiol, exhibited both antioxidant and antibacterial activities (**Begum *et al.*, 2016**). Vaccenic (11-Octadecenoic) acid had also a wide spectrum of biological activities as a fatty acid, including hypolipidemic (Wang *et al.*, 2008), anti-inflammatory (**Jacome-Sosa *et al.*, 2016**), and antioxidant activities (**Qadir *et al.*, 2020**).

On the other hand, crab shell extract also demonstrated a plethora of bioactive components. 1-Heptatriacotanol, a fatty acid, was present in extracts that exhibited antioxidant and anticancer activities (**Hadi et a. 2016**; **Junwei *et al.*, 2018**). The steroid derivative Ethyl iso-allocate was found to have antimicrobial (**Malathi and Ramaiah, 2017**), anticancer (Thakur and Ahirwar 2019), and antiviral activity (**El-Naggar *et al.*, 2022**). Hence, the contradictory presence of this richness with pharmacologically active components and the reduction in antioxidant activity in zebrafish protected with *C. natator* extract necessitate more in-depth work with different amounts of the crude extract and with more extensive experimental protocol, including further doses, for example, of the extract, and further analyses for bioactivities, rather than antioxidant activity only.

Moreover, some of the identified bioactive ingredients were found to have some ecological significance, especially in the interactions between crabs and soft corals and their environments. For crabs, the presence of hexa-and Octadecenoic acids were found

in sediments where high densities of spider crabs *Encephaloides* sp. are present, and they were believed to be biomarkers for quality of organic matter in sediments where these crabs were found **(Smallwood *et al.*, 1999)**. Furthermore, some derivatives from these acids were proven to have a probiotic activity **(Arruda *et al.*, 2022)**. For soft corals, palmitic acid was found to be the most abundant fatty acid in scleractinian corals, which indicated omnivorous or carnivorous feeding modes **(Samorì *et al.*, 2017)**. However, he ecological roles of many of the obtained bioactive substances in the current studies are ahead to be elucidated.

Finally, the differential capability of both extracts in relation to antioxidant activities can be either related to the contents of the extracts themselves, or to the experimental protocol applied in the current preliminary study. *Sarcophyton* extracts proven potent antioxidant activities in several studies before, especially owing to their high contents of terpenoids, carotenoids, cembranoids, and other natural product classes (**Kusmita *et al.*, 2017; Tanod *et al.*, 2019; Bawakid *et al.*, 2023**). *Sarcophyton convolutum* extract tested in the current study for antioxidant activity was not extensively assayed before for this activity, to the best of authors knowledge. Furthermore, the classes of natural products identified in the coral in the current study were mainly flavonoids and fatty acids, which were proven previously to have this antioxidant activity. However, not finding the other bioactive ingredients that are usually found in soft corals methanolic extracts makes the authors estimate that increasing the amount of the extract and/or using other solvents may exhibit a wider pattern of natural products identified in the current work. Furthermore, absence of antioxidant-inducing capability from the crab shell extract was not in agreement with the known for this group of animals. For example, antioxidant activities were proven for extracts from the crabs *C. lucifera. Callinectes sapidus,* and *Scylla olivacea* (**Soundarapandian *et al.*, 2014; Kaya *et al.*, 2016; Wan Yusof *et al.*, 2019**). Although the major components, as found by GC-MS analysis, were like the known for many other crab species, this contradictory pattern necessitates more works also, especially in the quantity of the biological material used form the crab for extraction, and for the experimental exposure protocol of zebrafish to this extract. Based on the current study, more future works have been planned to include more abundant biological material for extracting bioactive ingredients, as well further doses of each extract. These will aim to assess the presence of other bioactive substances, and to test whether there are dose-dependent effects for these extracts.

CONCLUSION

In conclusion, both Red Sea invertebrates showed promising diversity of bioactive ingredients that are worth more future works for their further elucidation and analyses. These bioactive substances could provide the observed antioxidant protection for the tested experimental model, especially in case of the coral extract. Further works are expected to investigate more activities for both crab and coral extract.

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