Various In Vitro Bioactivities of Secondary Metabolites Isolated from the Sponge Hyrtios aff. Erectus from the Red Sea Coast of Egypt

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ABSTRACT

Objectives: The present study revealed the presence of bioactive constituents in Hyrtios aff. erectus sponge (HES) extract collected from the Red Sea using skin and scuba diving.

Materials and Methods: Cytotoxicity was tested against hepatocellular carcinoma cell lines as a prescreening test.

Results: The HES extract had high contents of total phenolic compounds (0.061 mg/g), flavonoids (0.2839 mg/g), and carotenoids (1.976 mg/g). Moreover, the HES extract showed high antioxidant capacity with 93.0% and 99% at 1 mg using 2,2’-Diphenyl-α-pircylhydrazyl and 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), respectively. Cytotoxic activity against cancerous cell lines showed that the HES extract could inhibit cell growth effectively with IC50 = 47.5 µg/mL. Furthermore, anticancer activity using protein tyrosine kinase and sphingosine kinase 1 inhibitor screening assays resulted in 71.66% and 85.21% inhibition activity, respectively. The anti-inflammatory assays showed that the inhibition activity against cyclooxygenase (COX1), COX2, interleukin-6, and tumor necrosis factor-α was 71.82%, 81.13%, 80.89%, and 59.74%, respectively. At the same time, the anti-Alzheimer results using acetylcholine inhibition assay showed high activity at 1 mg with 83.51%. Additionally, the antiviral activity using the reverse transcriptase inhibition assay was 91.70%.

Conclusion: This marine sponge isolated from the Red Sea showed tremendous activity against many diseases and it is considered an excellent source for bioactive pharmaceutical compounds.

Key words: Red Sea, cytotoxic, antioxidant, anti-Alzheimer, anticancer, anti-inflammatory, antiviral

ÖZ

Amaç: Bu çalışma normal ve tüplü dalışlar ile Kızıl Deniz’den toplanan Hyrtios aff. erectus süngerindeki (HES) biyoaktif bileşiklerin varlığını göstermeyi amaçlamıştır.

Gereç ve Yöntemler: Sitotoksitesine hepatosellüler karsinoma hücre hatlarına ön izleme testleriyle belirlenmiştir.

Bulgular: HES ekstresi yüksek derecede fenolik bileşikler, (0.061 mg/g), flavonoidler (0.2839 mg/g) ve karotenoidleri (1.976 mg/g) içermektedir. Ayrıca, 1 mg HES ekstresi 2’-difenil-α-pircylhidrazil ve 2,2’-azino-bis (3-etilbenzotiazolin-6-sulfonik asit) ile sırasıyla %93,0 ve %99 ile yüksek antioksidan kapasite göstermiştir. HES ekstresi kanseröz hücre hatlarına karşı sitotoksik bulunan ve hücre büyümesini inhibe edebilmişdir (IC50 = 47,5 µg/mL). Dahası, protein tirozin kinaz ve sfingosin kinaz 1 inhibitör izleme testleri kullanarak belirlenen antikanser aktivitesi, sırasıyla %71,66 ve %85,21 inhibitör aktiviteyle sonuçlanmıştır. Antifenlamatuar testler siklooksijenaz 1 (COX1), siklooksijenaz 2 (COX2), interleükin-6 ve tümör nekroz faktör-α inhibitör aktivitesinin sırasıyla %71,82, %81,13, %80,89 ve %59,74 olduğunu göstermiştir. Aynı zamanda, asetil kolin esteraz inhibisyonu teti kullanılarak elde edilen anti-Alzheimer sonuçlar 1 mg dozda yüksek aktiviteli (%83,51) belirlenmiştir. Ek olarak, geri transkriptaz inhibisyon yöntemi kullanılarak bakılan antiviral aktivitesi %91,70'dir.
INTRODUCTION

Marine habitats contain a broad range of different organisms having a variety of biochemical and physiological characteristics and ability to adapt to their environment. Marine organisms such as sponges, tunicates, fishes, soft corals, nudibranchs, sea hares, mollusks, echinoderms, bryozoans, prawns, shells, sea slugs, and marine microorganisms are sources of bioactive compounds. Marine sponges belonging to the phylum Porifera (Metazoa), evolutionarily the oldest animals, are the single best source of marine natural products. Very recently, marine sponges of the Red Sea have been recognized as a rich source of bioactive secondary metabolites. A great number of biologically active compounds with potential antitumor, anticancer, antimicrotubule, antiproliferative, cytotoxic, and photoprotective, as well as antibiotic and antifouling properties have been isolated.

The main objective of the present study was to investigate the tremendous activities of sponge secondary metabolites collected from the Red Sea as antioxidant, cytotoxic, anti-Alzheimer, anticancer, anti-inflammatory, and antiviral agents.

MATERIALS AND METHODS

Area of study

The Red Sea (Figure 1a) comprises a wide range of tropical marine habitats, many of which are internationally recognized for their conservation, scientific, economic, or recreational value. It attracts many human activities, which in turn impact its environment and disturb the Red Sea’s natural ecosystems.

Sampling, identification, and prescreening bioassays of the sponge Hyrtios aff. erectus

Hyrtios aff. erectus samples were collected from Hurghada on the Egyptian Red Sea coastline during spring 2014 (Figure 1b). The samples were collected using skin and scuba diving, processed, washed with freshwater, and transferred directly to the laboratory in sterile polyethylene bags under reduced temperature (0°C). Identification of the sponge species was kindly performed by Dr. Nicole Voogd, at the Naturalis Biodiversity Center, Department of Marine Zoology, RA Leiden, the Netherlands. The voucher specimen is incorporated in the collections of the Zoological Museum of the University of Amsterdam under registration number RMNH POR.8633.

Chemicals and solvents

Potassium ferricyanide, ferric chloride, NaOH, chloroform, glacial acetic acid, ferric chloride solution, H₂SO₄, Folin-Ciocalteu reagent, vanillin, methanol, HCl, n-hexane, H₂O₂, HNO₃, Se standard, Mn standard, β-carotene, catechin, (+)-quercetin, sodium nitrite, aluminum chloride, and gallic acid were purchased from Sigma Aldrich.

Instruments

Atomic absorption spectrophotometry [AAS and Graphite Furnace Atomizer (GFA) Shimadzu] and GC-MS (Thermo, USA) were applied.

Preliminary bioactive screening of Hyrtios aff. erectus sponge extract (HES)

The ethyl acetate extract of Hyrtios aff. erectus was subjected to different chemical tests for the detection of different phytoconstituents, i.e. tannins, phlobatannins, saponins, alkaloids, flavonoids, quinines, coumarin, terpenoids, and cardiac glycosides.

Quantitative chemotaxonomy profiling

Determination of total phenolic content in HES extract

Total phenolic compounds in Hyrtios aff. erectus extract were determined as described by Taga et al.

Determination of total flavonoid content in HES extract

Total flavonoid content was determined by a colorimetric method reported by Zhishen et al.

Determination of total tannins in HES extract

Tannins (proanthocyanidins) were determined according to the method described by Sun et al.

Determination of total carotenoids in HES extracts

Total carotenoid content was measured according to Thaipong et al.

Figure 1. Location of sampling stations at the Red Sea (a) and sponge sample Hyrtios aff. erectus RMNH POR.8633 sample (b) and (c) represent the all steps of the present study (c) The schematic diagram of sponge extract constituents and multimedicinal effect.
Preparation and extraction for mineral and metal assessment (Fe, Zn, Co, Mn, Cu, and Se) of HES extract

A 0.5 g dried sample of HES marine extract was digested using 5 mL of concentrated HNO$_3$, the mixture was heated using a hot plate for 1 h and when semidried 5 mL of concentrated HNO$_3$ and 2 mL of H$_2$O$_2$ were added and it was kept on the hot plate for 1 h. The semidried cooled residue was filtered with the help of Whatman filter paper and the residue volume was made up to 25 mL with 2N HNO$_3$. Analysis was carried out using an AAS (GFA Shimadzu atomic absorption spectrophotometer AA-6800) according to the Official Methods of Analysis$^{31}$ for the determination of Fe, Zn, Co, Mn, Cu, and Se.

Elemental analysis of HES extract

The total carbon and hydrogen contents of marine HES extract were determined using a CHNO Elemental Analyzer.

Prescreening bioassays using in vitro cytotoxicity with cell lines

Different concentrations of HES extract (µg/mL) from all samples were tested for each cell line. Samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted with cell culture medium. The final DMSO concentration used was 1% of total volume of the medium in all treatments, including the control group. Cells with no treatment were examined as negative and positive controls.$^{32}$

Primary screening assay

2.2'-Diphenyl-α-picrylhydrazyl radical scavenging effect of HES extract

A 2.2'-Diphenyl-α-picrylhydrazyl (DPPH) radical scavenging assay of the total $Hyrtios$ aff. erectus extract was performed using a modified previously established methodology by Blois$^{33}$ and Amarowicz et al.$^{34}$ The scavenging ratio of the DPPH assay was calculated as follows:

$\%$ scavenging=$\frac{[A_\sigma-A_\chi]}{A_\sigma}\times100$

2.2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity assay of HES extract

The 2.2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^+$) free radical decolorization assay was developed according to Chkraborty et al.$^{35}$ The percentage scavenging of ABTS$^+$ was calculated by the following formula:

Scavenging activity ($\%$)=$\frac{[A_\sigma-A_\chi]}{A_\chi}\times100$

$A_\chi$ and $A_\sigma$ were the absorbance at 734 nm of samples with and without extract, respectively.

Specialized screening assays

Acetylcholinesterase inhibition (AChEI) assay of HES extract

Inhibition of acetylcholinesterase inhibition (AChE) by $Hyrtios$ aff. erectus extract was evaluated as described by Moyo et al.$^{36}$ Percentage inhibition by extracts was calculated using the following equation:

$\%$ inhibition=\frac{[Initial activity–inhibitor]}{Initial activity}\times100$

Determination of protein tyrosine kinase inhibitory activity of HES extract

Sample preparation

The dimethylsulfoxide (DMSO) sample solution of the appropriate extract was diluted with H$_2$O (1:1 v/v) to yield corresponding sample solutions (1 mg/mL). Tyrosine kinase (TK) inhibitory activity was determined using a commercial test kit (TK assay kit, nonradioactively, Takara Cat. #MK410). Protein tyrosine kinase (PTK) activity of the samples was calculated based on the prepared standard curve. The color intensity is stable for 1 h after addition of stop solution at room temperature in a light room.

$\%$ inhibition=\frac{[Initial activity–inhibitor]}{Initial activity}$

Determination of cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) inhibitor screening assay of HES extract

The COX inhibitory activity of the crude extract was determined by using the colorimetric sphingosine kinase 1 (SK1) inhibitor screening assay kit from Cayman. The plate was covered and the fluorescence was measured using an excitation wavelength between 530 and 540 nm and an emission wavelength between 580 and 590 nm.

$\%$ inhibition=\frac{[Initial activity–inhibitor]}{Initial activity}$

Determination of tumor necrosis factor alpha (TNF-α) assay of HES extract

The TNF-α inhibitory activity of the crude extract was determined using a KOMA BIOTECH colorimetric kit. The absorbance was measured at 450 nm.

Determination of interleukin 6 (IL-6) assays of HES extract

The IL-6 inhibitory activity of the crude extract was also determined using a KOMA BIOTECH colorimetric kit. The absorbance was also measured at 450 nm.

Determination of reverse transcriptase (RT) enzyme inhibitor screening assay of HES extract

The RT inhibitory activity of the crude extract against a purified recombinant, human immunodeficiency virus (HIV-1)-RT, was determined using a Roche colorimetric kit. The assay was performed according to Fonteh et al.$^{37}$ with HIV-1 protease enzyme and the substrate, which is a synthetic peptide that
contains a cleavage site Tyr-Pro for HIV protease, as well as two covalently modified amino acids for the detection of cleavage. Acetyl pepstatin was used as a positive control for HIV-1 PR inhibition. The blank treatment consists of an assay buffer with only the substrate; untreated control of enzyme and substrate was also included. The absorbance was measured at 450 nm.

Statistical analysis
All results were analyzed by ANOVA using Prism.

RESULTS AND DISCUSSION
The secondary metabolites isolated from HES extract showed high contents of sulfur compounds (Figure 2). The mineral results showed high iron and zinc contents (Figure 3), in addition to polyphenol contents, which reflected high tannins and flavonoids. The crude extract of the sponge showed also high carotenoids contents (Figure 4). The bioactive profiling and diversity of natural compounds produced by sponge showed the presence of certain chemical classes of steroids, chromones, quinones, alkaloids, fatty acids; diketopiperazine, steroid, lactone, quinolone, anthaquinone, trisindole, phenol, and dihydroxyridine benzoic acid derivatives; terpenoids; macrolactam; ethers; carboxylic acid; and terpenes, which are responsible for antioxidant, anti-inflammatory, antimicrobial, anti-HIV, anticancer, or antitumor activity. The quinolone derivatives are responsible for anti-HIV activity, fatty acid esters and fatty acids are responsible for anti-inflammatory activity, and pentaketides and alkaloids are responsible for neuroprotective activity.38 The present study revealed that this sponge has cytotoxicity against hepatocellular carcinoma (Table 1) (Figure 5). This finding agrees well with other research papers.4,8,39 Cytotoxic activity is considered the first parameter in screening for anticancer agents,4,8 while the cytotoxicity assay needs to be followed by other experiments to confirm their potential activity as an anticancer agent and to determine the mechanism. It has been reported that cell death can be induced through three mechanisms: apoptosis, autophagy, and oncrosis.40 In the present study the anticancer activity was determined through two different experimental models using TK and SK1 as anticancer targets.41 Sphingolipid-metabolizing enzymes have an important role in controlling the balance of the cellular levels of some important bioactive lipids, for example proliferative compound as well as apoptotic and ceramide compounds in addition to sphingosine 1-phosphate.42

Figure 2. The elemental analysis result of *Hyrtios aff. erectus*
Std: Standard

Figure 3. The mineral profiling for *Hyrtios aff. erectus*
sponge extract
Std: Standard

Table 1. Inhibitory activities of *Hyrtios aff. erectus* sponge extract against hepatocellular carcinoma cells

| Sample concentration (µg) | Viability % |
|--------------------------|------------|
| 50.00                    | 47.83      |
| 25.00                    | 69.17      |
| 12.50                    | 80.24      |
| 6.25                     | 93.62      |
| 3.125                    | 97.89      |
| 1.56                     | 100        |
| 0.00                     | 100.00     |

Figure 4. The polyphenol and carotenoid profiling for *Hyrtios aff. erectus* sponge extract
Std: Standard

Figure 5. The anti-human immunodeficiency virus and anticancer profiling of *Hyrtios aff. erectus* sponge extract
Std: Standard, PTK: Protein tyrosine kinase, SKH: Sphingosine kinase
The discovery of new chemotherapeutic resistance is an urgent and important challenge in oncology. Increased level of SK1 is considered a poor prognosis, and overexpression of SK1 means resistance to chemotherapeutics. Sphingosine kinase is involved in the development of different cancers and in chemotherapeutic resistance to drugs. Thus, SK1 represents an important target for anticancer drug therapy. Receptor tyrosine kinases are cell surface transmembrane proteins responsible for intracellular signal transduction. They are expressed in several cell types and, after activation by growth factor binding, trigger a series of intracellular pathways, leading to a wide variety of cell responses such as differentiation, proliferation, migration, invasion, angiogenesis, and survival. The overexpression of protein kinase members is associated with cancers and tumor cells. Therefore, tyrosine kinases are pivotal targets in drug therapy for cancer. The flavonoids, which are remarkably nontoxic and could inhibit PTK and SKH activity, appear to have promising bioactivity as anticancer agents and are worthy of further investigation. Phenolic compounds, especially flavonoids, exhibit anti-inflammatory and anticancer effects by inhibiting PTKs through several mechanisms. The first one is as an antioxidant and as being competitive inhibitors for the ATP binding sites on a variety of kinase enzymes.

Agullo et al. reported that the effectiveness of flavonoids depends mainly on the position, number, and substitution of the hydroxyl group of the β-ring. The saturation of the C2–C3 bond is also an important factor that affects flavonoids inhibition of phosphatidylinositol 3-kinase. This can be easily found in marine natural products as more rings and chiral centers are present in a variety of natural products, and are the most abundant antioxidants in the human diet. Phenolic compounds, especially flavonoids, exhibit antioxidant activities. In the present study, the total polyphenolic assay, i.e. total phenolic and flavonoids, showed a high polyphenolic content (Figure 4). Flavonoids are naturally occurring polyphenolic compounds that are present in a variety of natural products, and are the most abundant antioxidants in the human diet. While there has been a major focus on their antioxidant properties, there is an emerging view that flavonoids and their in vivo metabolites do not act only as conventional hydrogen-donating antioxidants, but also to modulate cell function through actions at protein kinase and lipid kinase signaling pathways (PTK and SHK). These findings are in agreement with many other previous studies. In fact, flavonoids, and their metabolites, have been reported to act at PI 3-kinase, Akt/protein kinase B, tyrosine kinases, protein kinase C, and mitogen-activated protein kinase signaling cascades. Inhibitory or stimulatory actions at these pathways are likely to affect cellular function profoundly by altering the phosphorylation state of target molecules and by modulating gene expression.

An understanding of the mechanism of action of flavonoids, either as antioxidants or as modulators of cell signaling, is key to evaluating the potency of biomolecules as inhibitors of oxidative stress in general and in neurodegeneration. The flavonoid compounds are characterized by their inhibitory effect on tyrosine kinase. Accordingly, the H. aff. erectus extract revealed the highest inhibition activity in PTK and SHK assays. Saponara et al. reported that the activity of pp60src gene product, which is a PTK, has been shown to be inhibited by flavonoids. Two major types of HIV have been identified so far, HIV-1 and HIV-2. HIV-1 is the cause of the worldwide epidemic and is most commonly referred to as HIV. The basic biological processes in the HIV-1 life cycle are now well established, and natural compounds targeting specific steps in this life cycle can be found. HIV RT inhibitors include nucleotide RT inhibitors and non-nucleotide RT inhibitors. Most clinical anti-HIV drugs are HIV RT. In the last decade (2002-2011), 132 anti-HIV natural products were obtained from marine organisms. Of the anti-HIV bioactive marine natural products, before or after 2002, more than half were derived from marine sponges. The present study indicated that the highest activities were from H. aff. erectus by 91.7%, in agreement with previous studies. Moreover, Simmons et al. concluded that sessile marine organisms (sponge and seaweeds) contain substances capable of potent biological activity, which has also been demonstrated against different types of cancer and HIV/Acquired Immune Deficiency syndrome. Restoring acetylcholine levels by inhibiting AChE has become the primary treatment for the cognitive deficits of Alzheimer disease (AD). The inhibition of AChE is beneficial not only to the enhancement of cholinergic transmission in the brain, but also to reduce the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD. In recent decades, researchers have attempted to develop new AChE inhibitors, especially the so-called “multifunctional AChE inhibitors” with additional efficacy in vascular dementia treatment. There have been plenty of phytochemicals found to be effective in inhibiting AChE, which mainly consist of alkaloids, cannabinoids, curcumins, stilbenes, and flavonoids. Among them, flavonoids have attracted more and more interest for their high inhibitory activity and low toxicity. Moreover, their diverse activities such as antioxidation, inhibition of advanced glycation products, and cardio-cerebrovascular protection give them extra advantages as potential multifunctional therapeutic agents for aging-related diseases. The anti-Alzheimer results of the present study (Figure 6) using different extracts showed the highest inhibition ratio by H. aff. erectus, which produces secondary metabolites for defense against other microorganisms and these secondary metabolites serve as a source of bioactive compounds for use in human therapies as they thrive in harsh oceanic climates. Many studies confirm the high activity of secondary metabolites isolated from marine H. aff. erectus including alkaloids, esters, fatty acids, glycosides, ketones, lipids, macrolides, alcohols, peptides, peroxides, polyketides, quinones, steroids, sterols, terpenes, and terpenoids. Chronic inflammation is thought to play crucial roles in the pathogenesis of various diseases, including neurodegenerative conditions such as Alzheimer disease. Therefore, the anti-inflammatory effects of flavonoids may be a potential beneficial effect for treating Alzheimer disease.
diseases. Several types of drugs are used to treat inflammatory disorders, but they cause adverse side effects. Natural products offer a great hope for the discovery of bioactive lead compounds. These compounds can be developed into drugs for treatment of inflammatory disorders. The biological and chemical diversity of marine habitats constitutes a sizeable reservoir of novel compounds. Some of them, like sesquiterpenoids, diterpenes, steroids, polysaccharides, alkaloids, fatty acids, proteins, and other chemical compounds, isolated from marine organisms are found to exhibit anti-inflammatory activity in agreement with our results as shown in Figure 7. Recently different compounds from sponge have been shown to have potent anti-inflammatory activity. The natural products from marine sponge with different structures such as diterpenes, alkaloids, sulfated polysaccharides, and polyphenols inhibit different types of pro-inflammatory biomarkers, IL-6, TNF, NF-κB, IL-1β, COX1, and COX2, through different pathways:

1. The antioxidant effect by inhibition of the production of ROS compounds, which stimulate the pro-inflammatory biomarkers.

2. The direct effect by inhibition of prostaglandin and in sequence inhibition of the NF-κB cascaded stimuli also the TNF and IL-6.

When the human body faces a lot of stress, ROS are produced as a result. Deficiency of antioxidant agents leads to different degenerative diseases, for example cardiovascular, Alzheimer, and various inflammatory diseases. Consumption of antioxidants from natural sources reduces oxidative stress. Many studies showed that flavonoids and phenolic constituents have attributed to the antioxidant activities of natural compounds. Furthermore, many studies cited that minerals, for example Cu, Zn, Mg, Mn, and Se, played a significant role as antioxidants. Additionally, dietary antioxidants including tocopherols, carotenoids, and ascorbic acid have been investigated. Although many synthetic antioxidants have been shown to remediate oxidative stress, their lack of availability, high cost, and side effects remain the main challenge in dealing with oxidative stress, making the need to discover new antioxidant agents urgent. The sponge extract exhibits a potent antioxidant effect as shown in Figures 8 and 9, and the marine extract contains a variety of bioactive compounds known by their effect as antioxidants such as polyphenol (tannins, phenolic compounds, and flavonoids), carotenoids, and different minerals (Cu, Fe, Se, Zn, and Mn).

CONCLUSION

The secondary metabolites isolated from the sponge *Hyrtios aff. erectus* collected from the Red Sea in Egypt have been confirmed to have multimedicinal effects as anticancer, antiviral, anti-inflammation, and anti-Alzheimer agents. Further investigations should be performed to purify the pure compounds.

Conflict of interest: The authors declare that there is no conflict of interest.

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