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METABOLITES PROFILING AND *IN VITRO* ANTICANCER AND ANTIOXIDANT ACTIVITIES OF THREE DIFFERENT MARINE SPONGES METHANOLIC EXTRACTS FROM THE RED SEA EGYPT.

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ABSTRACT

In the current study, three different marine sponges methanolic extracts *Clathria arbuscula* (*SR), Crella cyathophora* (S), and *Xenia macrspiculata* (CS) were collected from the Red Sea in Hurghada and examined for their metabolite profiles, antioxidant, and anticancer properties. Flavonoid, total phenolic , (2,2-diphenyl-1-picrylhydrazyl)DPPH, and (Ferric Reducing Antioxidant Power)FRAP tests for antioxidants were used to identify the different types of marine sponges methanolic extracts. The anticancer action of the cancer cells MDA-MB 231, HUH-7, and NB4-APL was dose- and extract-dependent. Total phenolic content (TPC) of *Xenia macrspiculata* methanolic extract has been found to be higher than that of *Clathria arbuscula* methanolic extract and *Crella cyathophora* methanolic extract . While the total flavonoid content (TFC) of *Xenia macrspiculata* methanolic extract has been shown to be higher than that of *Clathria arbuscula* methanolic extract and *Crella cyathophora* methanolic extract. Different levels of FRAP and DPPH activity have been found in all methanolic extracts of marine sponges. It has been worked the UPLC-TOF-MS analysis for the three different extracts. A total of 1,054 metabolites were found in the metabolome analysis. It was divided the primary groups of chemicals were and peptides, amino acids ,fatty acids and conjugates, organic dicarboxylic acids, purines, benzoic acids, indoles, pyrimidines, benzenes, phenylacetic acids, and pyridines compounds. The multivariate statistical analysis identified 32 metabolites, including 2-Chloro-N, N-dimethyl- 3- oxobutanamide, 4-Methyl-2-oxo-pentanoic acid, ,6-Dihydrouracil, 3-Hydroxy-3-methylglutaric acid, (E)-3-hydroxy-2-(2-hydroxypentadecanoylamino) dec-4-ene-1-sulfonic acid, Cysteine sulfinic acid,10-Hydroxydecanoic acid, 1-Aminocyclopropane-1-

carboxylate, 4-Hydroxyphenyllactic acid, [3-carboxy-2-[(Z)-octadec-11-enoyl]oxypropyl]-trimethylazanium, 1,4 Androstadiene-3,17-dione, 5-Aminolevulinic acid, 1H-Isoindole-1,3(2H)-dione, Olivetol, 3-Methylhistamine, 3-Ureidopropionic acid, Piperine, 3-Hydroxyanthranilic acid, 2-Oxobutyric acid, 2,4-Heptadienal, 2-Aminophenol, Nylidrin, 3-Methyloxyindole, Terbutaline, Cholecalciferol, Carnosine, 2-Hydroxybenzothiazole, Proscillaridin A, 3-(Trifluoromethyl)benzoic acid, Kojic acid, Indole-3-acetamide, as well as Acitretin, which have more beneficial associations with anticancer and antioxidant properties. This extensive library of metabolites from marine sponges may be used to target natural anticancer potentials in conjunction with anticancer actions. Both the pharmaceutical and nutraceutical sectors would benefit greatly from this research.

Keywords: *Antioxidant, Anticancer,* Biomarker compounds, *Clathria arbuscula*; *Crella cyathophora*; *Xenia macrspiculata*; Untargeted metabolomics.

INTRODUCTION

A vast variety of marine life forms exist, such as corals, sponges, gorgonians, ascidians, sea pens, fungus, algae, and marine-associated microorganisms **[1].** These creatures are thought to be significant sources for the identification of subsequent metabolites that are both structurally varied and bioactive. **[2].** Marine sponges have soft bodies, are sessile, and filter-feed on minute food particles that rise through the seawater, The size of a sponge may vary from a few millimeters to over 1-2 meters. Numerous bioactive substances, including sterols, peptides, alkaloids, macrolides, and terpenes, have been shown to be produced by sponges **[3].** These substances often have antiviral, anticancer, antitumor, anti-inflammatory, and antibacterial qualities. **[4,5].** Marine sponges A highly reliable supply of medicinally substances effective compounds from marine species, functioning enzyme clusters derived from sponges and the accompanying microorganisms are responsible for producing the beneficial secondary metabolites extracted from sponges**.[6,7].** The taxonomic classification of the order *Dictyoceratida* is as follows: it belongs to the phylum *Porifera* and the class *Demospongiae*. Which has provided more than 20% of novel secondary metabolic products obtained from all sponges hitherto, the compounds were shown to have antiparasitic , cytotoxic, antimicrobial, antiviral ,anti-H. pylori, , anti-inflammatory, antiallergic, antioxidant, , atherosclerosis inhibition, and other biol*ogic*al effects, It has been classified Phylum *Porifera* into the, *Calcarea* , *Demospongiae, Homoscleromorpha* and *Hexactinellida*, main classes**[8].** The Demospongiae class has been considered some of the most fascinating invertebrates. **[9].** *Clathria (Microciona) aceratoobtusa*, an invasive or locally spreading red sponge, causing destructive outbreaks on coral reefs in the Gulf of Manner. The *Crellidae* family comprises of five taxa distinguished by a tangential crust of acanthostyles or acanthoxeas and associated pore fields composed of parallel spicules. *Crella* is the genus with

the most species, with 63 species and four subgenera *(Crella, Grayella,* Pytheas, and Yvesia) **[10].** Corals are phylum *Cnidaria* and class *Anthozoa* sessile marine invertebrates that are in heavy colonies of numerous identical individual polyps. Corals are divided into two types: stony corals and soft corals. Stony corals are primarily reef builders. Coral Scleractinia **[11].** The soft corals belonging to the genus *Xenia and Heteroxenia* are rich in sesquiterpenoids, diterpenes, and sterols. Coral may be separated into the subclasses of *Octocorallia and Hexactinellid* coral based on the number of digestive cavity branches **[12].** In the present study, three types of marine sponge and coral and, identifying novel antioxidants, comparing specific sponge species and using methanolic extract to shown the most plentiful source of bioactive elements such flavonoids, phenol, and antioxidant activity. The current study sought to investigate the metabolic profiles, in addition to possessing antioxidant and anticancer characteristics of phytochemicals collected in several marine sponges, which may be classified as biomarker substances with antioxidant or anticancer potential. The three sea sponges were studied using an untargeted metabolome profiling technique to uncover phytochemical substances underpinning antioxidant and anticancer effects. Additionally, a multivariate analysis was conducted performed to demonstrate biomarker-key chemicals with antioxidant and anticancer activities, which might be useful in nutraceutical applications.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), acetic acid, crystal violet, quercetin, Dulbecco's Modified Eagle Medium (DMEM), TPTZ, Trolox, HPLC grade methanol, $FeCl_3$, acetate buffer, and 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Marine Biological Station (MBS)

Location Situates at 5 kilometers north to Hurghada city. The coral reefs directly about to the mainland and other three main reefs independently are enclosed by water. The specimens collected at depths ranges between 1– 7 meters.

Marine Sponge materials

The marine sponge *Clathria arbuscula, Crella cyathophora,* and *Xenia macrspiculata* **Figure (1)** were gathered from the Red Sea- Hurghada by diving in the Red Sea at depth 1-7 m, GPS: 27°17'12.4"N, 33°46'38.8"E in May 2021 and identified by Professor Mohammed Abdel Latif Ezz El-Arab, National institute of oceanography and fisheries, Hurghada, Egypt. The sample was transferred to the laboratory in a plastic container containing seawater, cut into small pieces, and left to dry.

Figure (1).: General features of collected sponges and coral.

Marine sponge extracts

Samples of *Xenia macrspiculata*, *Clathria arbuscula*, and *Crella cyathophora* were cut into small pieces and used for preparing the methanol extract. Where 17.5, 16.44, and 29.65 g extracts were obtained from the extraction of 500 g fresh. Each extract was shaken for 3 hours at 150 rpm and 25°C than leaved in the dark place over night. Whatman sheets were then used for repeated extract filtration, and the residue was further extracted using the solvent for 3 days. The leftover methanol from each extract was evaporated at 40°C using a rotary evaporator according to the methods described by **[13]** with some modification.

Antioxidant activities analysis

DPPH radical scavenging activity.

As stated in **[14],** the evaluation of DPPH radical scavenging activity was carried out. A serial dilution was carried out on a 96-well plate to get the intended The extracts were prepared at final concentrations of 3.12- 12.5- 25- 50-and 100 µg/mL dry weight.. After mixing 100 µL of diluted samples with 100 µL of DPPH solution (200 µM), the mixture was left to incubate at 25°C for 30 minutes without light. To measure the absorbance at a Spectro wavelength of λ=515 nm, DMSO was employed as a control. Nine biological and technology equivalents' DPPH activity. The findings were presented as a percentage of the DPPH inhibition. The calibration curve was created Using the rate of inhibition of the Trolox solution.

Ferric reducing antioxidant potential (FRAP) assay.

In accordance with **[15].** methodology In a 96-well microplate, 280 µL of freshly made FRAP solution was combined with 20 µL of diluted samples, and the mixture was incubated for 30 minutes at 37°C in the dark. The absorbance of the samples was then measured at λ= 590 nm wavelength. The information was presented as (µM Trolox/g dry weight (DW).

Total phenolic content (TPC):

The total amount of phenolic compounds in extracts was determined with the Folin Ciocalteu reagent. All tests were carried out in triplicate and the total phenol was expressed as mg of gallic acid equivalents (GAE) per 100 g of extract. For this purpose, the calibration curve of gallic acid was drawn. One ml of standard solutions in concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared to draw the standard curve in methanol. A concentration of 1mg/ml of extract in methanol were also prepared in methanol and 0.5ml of the previous solution was introduced into test tubes and mixed with 2.5ml of a 10-fold dilute Folin Ciocalteu reagent and two ml of 7.5% sodium carbonate solution. The tubes were covered with para film and allowed to stand for 30 minutes at room temperature and the absorbance was read at 750 nm **[16]** .

Total flavonoid content (TFC):

The flavonoids content of each extract was measured based on the methods described by **[17] .** Briefly, 0.5ml of sample (5g/L) was mixed with 1.5ml of methanol and then 0.1 ml of 10% potassium acetate and 2.8 ml of distilled water were added. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. The

results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g extract). The standard curve was prepared by quercetin in different concentrations (5-50 mg/L).

Cell culture :

The MDA-MB-231, HUH-7, and NB4 cell lines, which correspond to breast cancer, hepatocellular carcinoma, and acute promyelocytic leukemia (APL) respectively, were obtained from Nawah Scientific Inc. situated in Mokatam, Cairo, Egypt. The MDA-MB-231 and HUH-7 cell lines were cultivated in a controlled environment with 5% (v/v) carbon dioxide at a temperature of 37 °C. The MDA-MB-231 and HUH-7 cell lines were cultured in DMEM basal media, while the NB4 cell line was cultivated in RPMI medium. The RPMI medium was fortified with streptomycin at a concentration of 100mg/ml, penicillin at a concentration of 100 units/ml, and heat-inactivated FBS at a concentration of 10% (VWR, Suwanee, GA, USA).

Cell viability assay

MDA-MB-231 and HUH-7 Cell lines.

The SRB assay was used to evaluate cellular viability. Aliquots of 100 µL cell suspension containing 5x103 cells were cultured in complete media for 24 hours in 96-well plates. Subsequently, the cells were provided with an additional 100 µL aliquot of medium containing extracts at different doses (10, 100, 100 µg/ml). Following a 72-hour exposure to the extract, the cells were immobilized by introducing 150 µL of a 10% TCA solution into the medium and allowing it to incubate for one hour at a temperature of 4°C. After the TCA solution was removed, the cells were rinsed five times with distilled water. The solution was left undisturbed at ambient temperature for 10 minutes in a dark environment after the addition of 70 µL aliquots of SRB solution (0.4%w/v). Following three rounds of washing with a 1% acetic acid solution, the plates were left to dry naturally overnight. The SRB dye, which was coupled to proteins, was dissolved in 150 µL of TRIS solution at a concentration of 10 mM. The absorbance at a wavelength of 540 nm was measured using a BMGLABTECH®-FLUOstar Omega microplate reader located in Ortenberg, Germany **[18], [19].**

NB4 (APL) cell line

Cell viability was assessed by WST-1 assay using Abcam® kit (ab155902 WST-1 Cell Proliferation Reagent). Aliquots of 50 µL cell suspension (3x103 cells) were seeded in 96- well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 50 µL media containing drugs at serial concentrations. After 48 h of drug exposure, cells were treated with 10 µL WST-1 reagent and the absorbance was measured after 1 h at 450 nm using a BMG LABTECH®- FLUOstar Omega microplate reader (Allmendgrün, Ortenberg). **[20-21].**

The cell growth inhibition rate was determined by calculating the percentage of cell viability, using the following formula: Cell viability (%) = (treated cells - blank cells)/(control cells - blank cells) × 100

Cell growth inhibition (%) = 100 - (% Cell viability).

Untargeted Liquid Chromatography-Mass Spectrum (UPLC-MS) metabolome analysis

Metabolic profiling was conducted using untargeted Liquid Chromatography-Mass Spectrometry

(LC-MS). As previously mentioned, the Q Exactive MS (Thermo) was utilized in conjunction with electrospray ionization mass spectrometry (ESI-MS) to analyze the samples. For the LC-MS analysis, each sample was treated with 0.2 ml of 80% methanol. The LC system consists of an ACQUITY UPLC HSS T3 column with dimensions of 1002.1mm and a particle size of 1.8 m, as well as an Ultimate 3000LC. The mobile phases used in the experiment were A, which consisted of a solution of 0.05% formic acid in water, and B, which was acetonitrile. The elution process used a gradient, with the proportion of B increasing over time. Specifically, from 0 to 1 minute, the proportion of B was 5%, from 1 to 12 minutes it increased from 5% to 95%, from 12 to 13.5 minutes it remained at 95%, and from 13.6 to 16.0 minutes it decreased back to 5%. The mobile phases were flowing at a rate of 0.3 mL/min, the column temperature was maintained at 40°C, and the sample manager temperature was kept at 4°C. The mass spectrometry parameters for electrospray ionization in positive (ESI+) and negative (ESI-) modes are as follows: The heater temperature is set at 300°C. The sheath gas flow rate is 45 arbs, the aux gas flow rate is 15 arbs, and the sweep gas flow rate is 1 arb. The spray voltages are 3.0 kV and 3.2 kV, respectively. The capillary temperature is 350°C. The S-Lens RF levels are 30% and 60%, respectively.

Metabolite Identification

The metabolite MS/MS spectra were obtained by using the following databases: METLIN [22], the Human Metabolome Database (www.hmdb.ca) [23], Mass bank [24], and MetFrag [25], according to the accepted criteria of metabolite categorization. The metabolic pathway map was created with the data from reference [26].

Statistical Analysis:

The results obtained in the present study were evaluated utilizing a One-Way ANOVA analysis. The results were presented as the average value plus or minus the standard deviation, with statistical significance determined at a threshold of $P < 0.05$ using Prism 7 software.

The raw data was collected using Compound Discover (3.0, Thermo) by taking into account the m/z value and retention period of the ion signals. The multivariate analysis used either ESI- or ESI+ ions. PCA was first used as an unsupervised method for data visualization and outlier detection. MetaboAnalyst used a heat map to do statistical analysis on the compounds under investigation. In addition, we used Sigma Plot (version 14.5) to produce somewhat consistent groupings of metabolites across the samples.

RESULTS

Chemical constituent screening of methanolic extracts from marine sponges

As represented in mg of gallic acid equivalent GAE/g extract and mg of quercin equivalent GAE/g extract, the total phenolic contents (TPC) and total flavonoid contents (TFC) of various types of extracts are displayed in Figure 1B respectively. TPC and TFC were 16.17± 0.38, 28.58± 2.31, 51.08± 0.14, 4.33±0.13, 3.19±0.06 and 5.74±0.17 in

the *clatharia* (SR), *crella*(S) and *xenia*(CS)respectively **Table(1) .** TPC of *xenia* has been found to be significantly ($p < 0.05$) higher than that of *clatharia* and *crella*. In recent years, many methods have been used to look at different samples because natural products contain many biological components that can act as antioxidants.

Table 1. Total phenolic compound and total flavonoids of *clatharia* , *crella* and *xenia* methanolic extracts.

Marine sponge and coral	Methanol 99.8%	
	Total phenolic compound (mg/g)	Flavonoids (mg/g)
Clathria	16.17*** ± 0.38	4.33**±0.13
Crella	28.58**± 2.31	3.19±0.06
Xenia	51.08***± 0.14	5.74***±0.17

a*: mg GAE/g of dry extracts b: mg QE/g of dry extracts. Each value is expressed as the mean ±SE (n=3). (**and***) are significant and highly significant respectively at $p<0.05$.

Antioxidant activity of methanolic extracts from marine sponges :

The antioxidant activity of marine sponge methanolic extracts was evaluated, and the findings are shown in **Table (2).** Different levels of DPPH and FRAP activity have been identified in all marine sponge methanolic extracts. The DPPH and FRAP activity were 31.66±4.63%, 39.63±2.50%, 36.69±1.94%, 497.5± 17.9µM TE/g DW, 222.3± 8±7.3, and 705.7±16.44µM TE/g DW in the *clathria*, *crella*, and *xenia* respectively. There was no statistically significant ($p < 0.05$) difference between the different methanolic extracts of marine sponges in the DPPH radical scavenging activity assay. Furthermore, it has been revealed that the FRAP activity of xenia is significantly ($p < 0.05$) higher than that of *clatharia* and *crella.*

Table (2): Total Antioxidant activity DPPH and FRAP Assay methanolic extracts of marine sponges *Clathria Crella* and *Xenia* .

Marine sponge	Methanol 99.8%	
	DPPH% inhibition	FRAP Trolox equivalent per mM sample (µM TE/ mg)
Clathria	31.66* ±4.63	497.5*** ± 17.9
Crella	39.63±2.50	222.3± 8±7.3
Xenia	36.69±1.94	705.7±16.44

Values are mean ± SD (standard deviation) . (** and ***) are significant and highly significant respectively at $p< 0.05$

Assessing the cytotoxic effects of methanolic extracts of marine sponges against various cancer cell lines

The percentage of MDA-MB 231, NB4-APL, and HUH-7 cell growth was assessed after being subjected to various extracts (*clatharia*, *crella*, and *xenia*) at concentrations of 1000 µg/ml for 72 hours and the results are displayed in **Table (3).** Methanolic extracts of marine sponges induced a statistically significant ($p < 0.05$) decrease in the percentage of cell proliferation in an extract-dependent manner. The MDA-MB 231, NB4-APL, and HUH-7 cell proliferation percentages after exposure to *clatharia*, *crella*, and *xenia* were 32.61 ± 1.72, 46.66 ± 1.13, 16.94 ± 0.14, 25.05 ± 0.18, 18.00 ± 1.18, 50.36 ± 1.12, 50.88 ± 1.98 and 44.94 ± 0.57, respectively. The *clatharia* from all of the marine sponge methanolic extracts displayed the highest reduction in MDA-MB 231 and NB4-APL cell proliferation by 67.39 and 83.06 % respectively when compared to the control cells. The IC_{50} of this extract determined by SRB assay was 518.67 and 627.25 µg/ml in MDA-MB 231 and NB4-APL cells respectively. Furthermore, the *xenia* induced the strongest reduction in HUH-7 cell growth inhibition by 55.06% compared to the control cells. The IC_{50} for xenia in HUH-7 was 859.95 µg/ml, but the IC_{50} for *clatharia* and *crella* in the same cells was higher than 1000 µg/ml. Likewise, the NB4-APL cell line exposed to *clatharia* had the lowest percentage of cell growth inhibition (16.94 ± 0.14%), whereas the HUH-7 cell line exposed to *crella* had the highest percentage of cell growth (50.88 ± 1.98%) **Table (3).**

Table (3): *In vitro* cytotoxic activity of marine sponge extracts against MDA-MB 231, NB4-APL and HUH-7 cancer cells in the SRB assay and WST-1

Cell line	Concentration (µg/ml)	Marine sponge extracts		
		clatharia	**crella**	**xenia**
		cell proliferation %	**cell proliferation %**	**cell proliferation %**
MDA-MB 231	Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
	1000	32.61±1.72[a]	46.66±1.13[b]	35.33±1.49[a]
NB4-APL	Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
	1000	16.94±0.14[a]	25.05±0.18[b]	18.00±1.18[a]
HUH-7	Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
	1000	50.36±1.12[b]	50.88±1.98[b]	44.94±0.57[a]

The tests were conducted using three biological replicates, with each replication being done three times, resulting in a total of nine runs (n = 9). The numbers are expressed as the mean values plus or minus the standard division of the mean (n = 3). Differences having a p-value less than 0.05 are denoted by distinct superscript letters within the same row.

Table (4): IC_{50} of marine sponge extracts against MDA-MB 231, NB4-APL and HUH-7 cancer cells in the SRB assay and WST-1.

Cell line	IC_{50} (µg/ml)	Marine sponge extracts		
		clatharia	*crella*	*Xenia*
MDA-MB 231		518.67	860.72	496.17
NB4-APL		627.25	652.00	373.83
HUH-7		>1000	>1000	859.95

Additionally, the percentage of cell proliferation in MDA-MB 231, NB4-APL, and HUH-7 cancer cells was calculated after 72 hours of incubation with various marine sponge extracts. The results are displayed in **Figure (2)**. The findings indicated that, NB4-APL cells were more susceptible to all the marine sponge extracts and recorded the highest percentage of cell proliferation which were 83.07 ± 0.14, 74.95 ± 0.18 and 82 ± 1.18 induced by *clatharia*, *crella* and xenia respectively. The MDA-MB 231 cells displayed a moderate susceptibility to all of the marine sponge extracts and had the moderate percentages of cell growth inhibition which were 67.39 ± 1.72, 53.34 ± 1.13, and 64.67 ± 1.49 % induced by *clatharia*, *crella*, and *xenia* respectively. Contrarily, the HUH-7 cells were the lowest susceptible to all of the marine sponge extracts and had the lowest percentages of cell growth inhibition which were 49.64 ± 1.12, 49.12 ± 1.98, and 55.07 ± 0.56. % induced by *clatharia*, *crella*, and *xenia*, respectively **Figure (2 A, B, C)**.

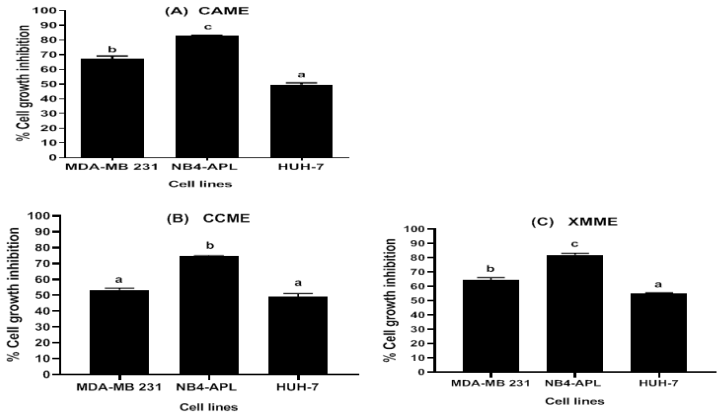

Figure (2): Cell growth inhibition of MDA-MB 231, NB4-APL and HUH-7 cancer cells induced by marine sponge methanolic extracts in the SRB assay. CAME= *Clathria arbuscula* methanolic extract CCME= *Crella cyathophora* methanolic extract, XMME= *Xenia macrspiculata* methanolic extract. The data are reported as the mean values plus or minus the standard error of the mean (SEM). Differences with a p-value less than 0.05 are denoted by distinct superscript letters.

DISCUSSION

Several marine taxa, including sea slugs, soft corals and sponges, have recently proven to be an important source of novel natural bioactive substances **[28,29,30].** Most natural compounds have been found in marine invertebrates, particularly marine sponges, from the beginning of Exploration of marine resources for the purpose of drug development in the field of natural products, with hundreds of compounds discovered from this source each year **[31,32].** The richest source of bioactive substances was found to be marine sponges. The bioactivities of three marine sponges, *Clathria*, *Crella* and *Xenia* were investigated in the current study using methanolic extracts. The chemical components of these extracts were investigated. All of the sponge extracts have antioxidant activity, as determined by DPPH radical scavenging activity and ferric ion reducing antioxidant power assay (FRAP). The TPC and TFC of several methanolic extracts of marine sponges were determined in the current investigation **Table (1**). In comparison to *Clathria*, and *crella*, the TPC of *xenia* has been determined to be higher. However, it has been demonstrated that *xenia* has a greater TFC than *clatharia* and *crella*, respectively. Similar research revealed that secondary metabolites Extracted from marine sponges obtained from the Red Sea had significant polyphenol contents, which indicated high tannin and flavonoid levels. The investigation of sponges has unveiled a diverse range of organic compounds with bioactive properties. These chemicals come from different groups, such as fatty acids, chromones, quinones, alkaloids, diketopiperazine, quinolones, anthraquinones, Benzoic acid derivatives, dihydropyridine, and trisindole. Additionally specified are terpenoids, macrolactam, ethers, carboxylic acid, and terpenes. These substances have anti-inflammatory, antimicrobial, anti-tumor, and antioxidant properties. **[33].**

Since the food and pharmaceutical sectors are constantly looking for ways to create natural, bioactive anti-aging and anti-carcinogenic substances that have observable health effects, antioxidant activity is now a popular subject. and the focus of intense research. According to **[34].** In the DPPH radical scavenging activity assay, there was no significant differences between the various methanolic extracts of marine sponges.

Comparing xenia to other marine sponge methanolic extracts the data shown FRAP assay revealed that it has the highest antioxidant activity **Table (2).** Recent research found that the methanolic extract of marine sponges had effective DPPH radical scavenging properties **[35].** The DPPH activity of extracts from Mediterranean Sea sponges exhibited good antioxidant activity**[36] .** Phenolic chemicals derived from sea Sponge tests have shown to possess antioxidant action. **[37].**

Marine sponges are a plentiful reservoir of uniquely structured natural chemicals, many of which have been proven to exhibit a wide range of biological actions in several living systems **[38].** Anticancer, antiviral, anti-inflammatory, and anti-Alzheimer activities have been discovered in the secondary metabolites of the sponge

Hyrtios aff. erectus, which was collected from the Red Sea in Egypt. **[39].** Similar research revealed that when marine sponge methanolic extracts were investigated against HT-29 cells using the MTT assay, they significantly reduced cell viability. The inhibitory effect is concentration-dependent and the extracts showed 35-78% inhibition. Both Spongia and Sigmadocia's methanolic extracts had IC_{50} values of 15.11 and 14.08 µg, respectively, indicating that they could be ideal candidates for the development of anticancer drugs **[40].** In the current investigation, methanolic extracts of marine sponges induced a statistically significant decrease in the percentage of cell proliferation in an extract dependent manner. The *clatharia* from all of the marine sponge methanolic extracts showed the greatest inhibition of MDA-MB 231 and NB4-APL cell proliferation **Table (3).** The high phenolic and flavonoid content of marine sponges may be responsible for their cytotoxic effect against various cancer cell lines in the present investigation Figure 1B. In fact, studies have shown that flavonoids and their metabolites affect the signaling pathways for PI 3-kinase, Akt/protein kinase B, tyrosine kinases, protein kinase C, and mitogen-activated protein kinase. By changing the phosphorylation status of target molecules and by regulating gene expression, inhibitory or stimulatory activities at these pathways are anticipated to have a substantial impact on cellular function **[41]** Evaluating the effectiveness of biomolecules as regulators of oxidative stress generally and in neurodegeneration requires a thorough understanding of the mechanisms by which flavonoids function, either as antioxidants or as modulators of cell signaling **[42].** Tyrosine kinase inhibition is a property of the flavonoid substances. As a result, the *clatharia* showed the highest levels of inhibitory activity in the growth of MDA-MB 231 and NB4-APL cells in the current investigation **Table (3).**

Metabolite profiling of marine sponges

There are 1,054 distinct compounds found in sea sponges. The compounds were allocated as follows: 793, 232, and 29 in the ESI+ (positive electrospray ionization), ESI- (negative electrospray ionization), and ESI+/ESI- modes (Supplementary Table x). Metabolite set enrichment analysis (MSEA) was used to identify the detected compounds using the Human Metabolome Database (HMDB) ID. This process identified the major chemical groups present in the observed metabolites. **Figure (4).** The chemical groups of the top 25 metabolite sets discovered in the chemical structure metabolite set collecting area are shown in the chart. **Figure (4B).** According to the bar chart, these are the top 25 chemical classes. Fatty acids and conjugates, organic dicarboxylic acids, purines, benzoic acids, indoles, pyrimidines, benzenes, phenylacetic acids, and pyridines were the key chemical groups with a higher p-value. The dynamic pie chart's colors indicate the proportion of compounds in each chemical category to all compounds. In Figure (4A). The group with the most was amino acids and peptides (red). Then came the isoprenoids (green), fatty acids and conjugates (purple), and benzamides (blue).

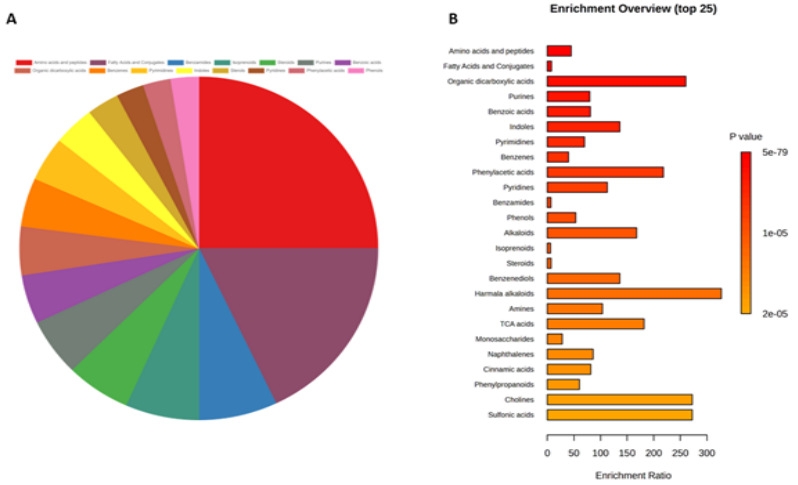

Figure 4: The chemical classification of marine sponge metabolites is shown using metabolite set enrichment analysis (MSEA) via an interactive pie chart (A) and a bar chart (B). The p-value is visually shown using color coding in the bar plot. The hues orange and red depict the high and low levels, respectively. The lines represent the enrichment ratio, which was computed by dividing the number of observed hits by the number of predicted hits. The interactive pie chart visually represents each chemical category using different colors, which are proportional to the total number of compounds.

The goal of PCA analysis is to find out how the molecule is different in the three sea sponges. The PCA plot **Figure (5A)**. showed that the first two parts, PC1 and PC2, described 100% of the variation in the metabolome makeup. The PC1 and PC2 were mostly linked to the variety of plant cells. The PCA scatter plot showed that the main metabolites found in SR were 2-Chloro-N, N-dimethyl-3-oxobutanamide, 4-Methyl-2-oxopentanoic acid, 3-Hydroxy-3-methyl glutaric acid, and cysteine sulfinic acid. 5,6-Dihydrouracil and SL 10:1; O/15:0; O, on the other hand, were the main molecules that helped CS tissue. **Figure (5B).**

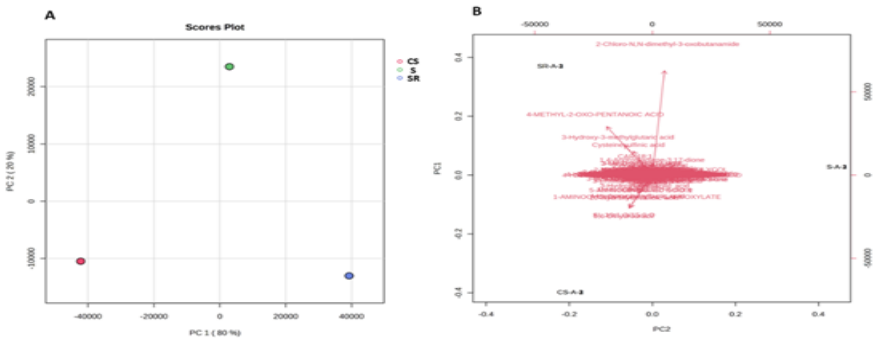

Figure 5. PCA was used to generate a 2D score plot (A) and biplot (B) displaying the metabolites obtained from marine sponges. The many concise abbreviations in the biplot relate to the scores of the observations, specifically referring to marine sponges. The vectors that are parallel relate to the variables (i.e., metabolites) that exhibit comparable response patterns. Xenia macrspiculata (CS) is represented by the color red, Crella cyathophora (S) is represented by the color green, and Clathria arbuscula (SR) is represented by the color purple.

Multivariate analysis of candidate metabolites, antioxidant and anticancer activities of marine sponges.

Based on VIP scores, PLS-DA was used to find molecules linked to sea sponges. Because of this, 32 different chemical parts (VIP > 1) were found in marine sponges' metabolites **Figure(6).** It was seen that organic acids, flavonoids, and phenolic acid made up a lot of the chemical groups in these biomarker substances found in sea sponges. The biomarker compounds were 2-Chloro-N,N-dimethyl-3-oxobutanamide,4-Methyl-2-oxo-pentanoic acid, 5,6-Dihydrouracil, 3-Hydroxy-3-methylglutaric acid, (E)-3-hydroxy-2-(2-hydroxypentadecanoylamino)dec-4-ene-1-sulfonic acid, Cysteinesulfinic acid, 10-Hydroxydecanoic acid, 1-Aminocyclopropane-1-carboxylate, 4-Hydroxyphenyllactic acid, [3-carboxy-2-[(Z)-octadec-11-enoyl]oxypropyl]-trimethylazanium, 1,4 Androstadiene-3,17-dione, 5-Aminolevulinic acid, 1H-Isoindole-1,3(2H)-dione, Olivetol, 3-Methylhistamine, 3-Ureidopropionic acid, Piperine, 3-Hydroxyanthranilic acid, 2-Oxobutyric acid, 2,4-Heptadienal, 2-Aminophenol, Nylidrin, 3-Methyloxyindole, Terbutaline, Cholecalciferol, Carnosine, 2-Hydroxybenzothiazole, Proscillaridin A, 3-(Trifluoromethyl)benzoic acid, Kojic acid, Indole-3-acetamide, and Acitretin. According to a prior research, the primary components of the phenolics found in marine plants were tannins, gallic acid, ellagic acid derivatives, flavonoids, and phenolic acid derivatives **[43].** Marine animals, sponges, and algae are the greatest sources of natural marine

chemicals, which have garnered significant interest due to their anticancer properties. The DPPH, FRAP, TPC, and TFC of marine plants were plotted alongside those 32 potential compounds to provide a more comprehensive understanding of the variations in metabolites, cytotoxicity, and antioxidant capabilities**[44].**Crude extracts of Martinique's marine sponges have been demonstrated to have possible antibacterial and cytotoxic effects on leukemia (MOLM14 and HL-60), glioblastoma (RES259), and breast cancer (MDA-MB231) **[45].**

The crude extract of N. magnifica demonstrated significant anticancer activity against breast cancer (Mcf-7) and colon cancer (caco-2) cell lines. **[46]** . The crude extracts of *C. siphonella* and *C. crassa* shown significant antiviral and anti-inflammatory effects, as well as a promising anticancer drug against colon and breast cancer **[47].**

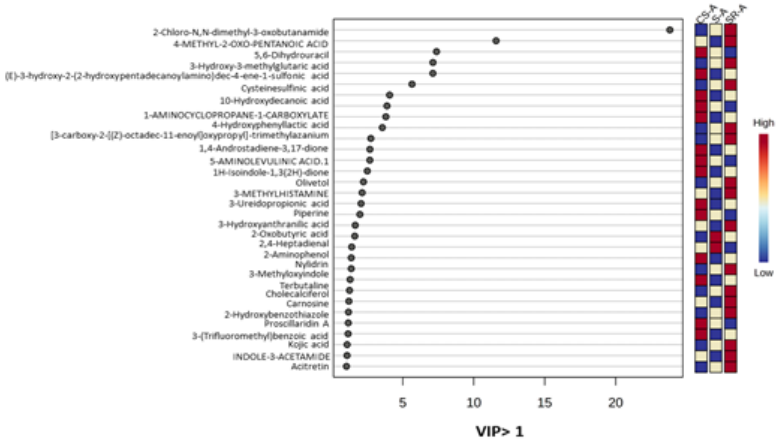

Figure 6. The sum of regression coefficients (coef.) of marine sponge candidate metabolites, weighted by their absolute values, when the VIP score is more than 1.0. The vibrant boxes on the right display the comparative concentrations of each metabolite in each group being studied.

In **Figure 7,** Each column corresponds to the three distinct marine plant tissues, whereas each row reflects the metabolites, antioxidant, and cytotoxicity activities of three duplicates. The plot utilizes the red and blue color scheme to symbolize varying levels of Levels of metabolites, as well as the antioxidant and cytotoxic properties. Upon analyzing the variance in color intensity across all samples, it was noted that the S sample exhibited the greatest levels of DPPH and TPC, while the CS tissue showed the highest levels of FRAP and TFC. Anticancer properties were detected for compound 'S' against MDA-MB-231, HUH-7, and NB-4 (APL) cancer cells. In addition, the levels of 3-

Hydroxy-3-methylglutaric acid and 2-Aminophenol were higher in the S tissue.

Research has shown that *Aaptos suberitoides* material can kill triple negative breast cancer cells. A sea sponge product called *Aaptos suberiotoides* was able to kill MDA-MB 231 cells with an IC_{50} value of 6 ppm **[48].**

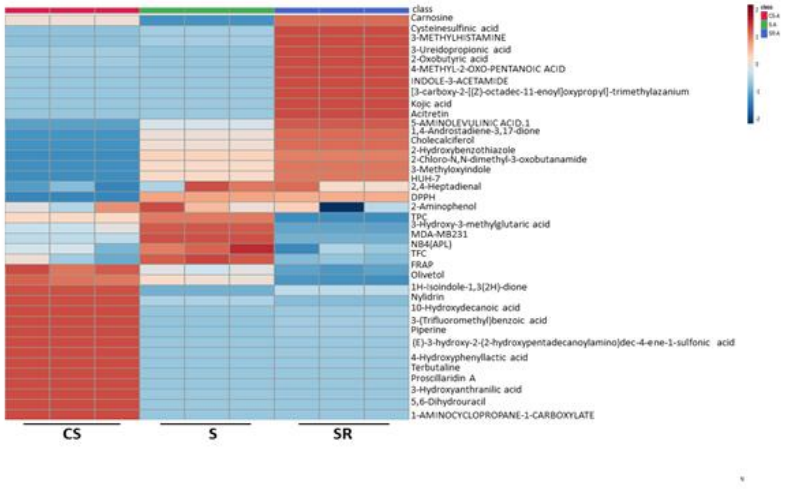

Figure 7 Displays a heatmap analysis of candidate metabolites (with a VIP score more than 1.0) that were found using partial least-squares discriminant analysis (PLS-DA). Additionally, it showcases the total phenolic and flavonoid content, as well as the anticancer and antioxidant properties, of marine sponges. The columns in the table correspond to several types of marine sponges, while the rows indicate the various metabolites, total phenolic content (TPC), total flavonoid content (TFC), anticancer characteristics, and antioxidant qualities. The plot's red and blue colors symbolize strong and weak intensities, with values ranging from -1 to +1. The intensity of the red color (ranging from +1 to +2 values) is directly proportional to the amount of metabolites, as well as the anticancer and antioxidant activities. Conversely, the intensity of the blue color (ranging from -1 to -2 values) is inversely proportional to the content of metabolites, as well as the anticancer and antioxidant activities.

This heatmap an investigation of Pearson's correlation coefficient was conducted to assess the relationship between marker chemicals and various antioxidant and anticancer properties. **Figure (8).** Cluster analysis suggested that DPPH and FRAP antioxidant activities had a comparable correlation pattern. In addition, the correlation between TPC and DPPH is higher than the correlation between FRAP and TPC. In contrast, the correlation between TFC and FRAP was higher than the correlation between DPPH and TFC. The MDA-MB-231 and NB4 anticancer activities were highly correlated with 3-Hydroxy-3-methylglutaric acid and 2-Aminophenol. HUH-7 anticancer activity

was highly correlated with 2,4-Heptadienal, 2-Chloro-N, N-dimethyl-3-oxobutanamide, 1,4 Androstadiene- 3, 17- dione, 3-Methyloxyindole, Cholecalciferol, and Carnosine. FRAP and DPPH antioxidant activities were highly correlated with 2-Aminophenol.

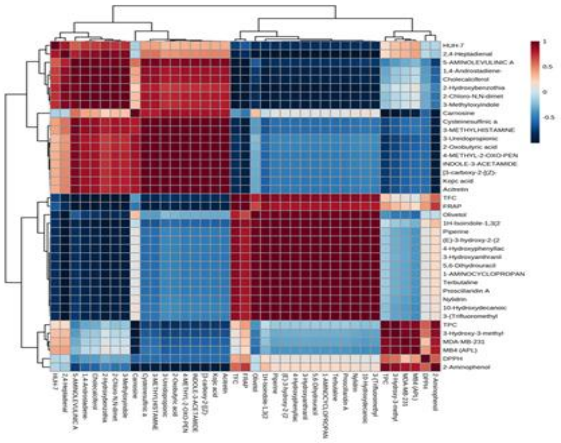

Figure 8 Generates a heatmap that visually represents the Pearson correlation values between candidate metabolites (with a VIP score greater than 1.0) and the total phenolic content (TPC), total flavonoid content (TFC), as well as the anticancer and antioxidant capacities of marine sponges. The correlation values vary from -1 to +1. Values around +1 suggest a robust positive correlation, whereas values nearing 0 indicate the lack of a linear connection between the variables. On the other hand, values close to -1 imply a significant negative correlation between the variables.

According to several publications, methanol extract of marine sponges (Demospongiae) was tested for a variety of bioactivities. The current results validate these findings. Repeated phytochemical analysis of the extract's chemical components disclosed the existence of triterpenoids, steroids, phenols, and alkaloids **[49] .**

These results came in accordance with **[50 ,51].** Antioxidants are substances or systems that prevent the production of free radicals, hence delaying the onset of autoxidation.

They find extensive use in the food, cosmetic, and medical sectors **[52]** a variety of cytotoxic substances, such as steroids, furanoditerpenes, sesquiterpenes, diterpenoids, terpenoids, and capnellenes, were identified from corals. Cembranolides make up the bulk of the isolated terpenoids. Among these, the species *Sinularia's* octocorals are abundant in terpenoid metabolites with cytotoxic potential. *Sinularin* is a terpenoid metabolite that has been isolated from the Chinese soft coral *Sinularia flexibilis*. Investigations into

the effects of sinularin on A2058 melanoma cells revealed that the drug dose-dependently suppressed cell growth (IC_{50}: 3.1 μg ml–1).

The ethanol extract of *Aaptos suberitoides* has been demonstrated to have cytotoxicity, anti-proliferation, and anti-migration action in trastuzumab-resistant HER2+ breast cancer cell lines, as well as an inhibitory effect on three-dimensional spheroid formation **[53].**

For in vitro evaluation of the cytotoxic effects of marine sponges, the following cancer cell lines were utilized: A549 (representing lung cancer), HeLa (representing cervical cancer), HepG2 (representing liver cancer), MCF7 (representing breast cancer), HL-60 (representing promyelocytic leukemia), HCT-116 and HCT-15 (representing colon cancer), PACA (representing pancreatic cancer), and KB (representing oral cancer) **[54].**

The comparatively more active sponges from a group of 26 were identified by a preliminary screening process using Three cell lines that are malignant: KB, HL-60, and HeLa. The sponges were assessed using hexane, ethyl acetate, and methanol extracts at 50 μg/mL using an MTT-based test. (Figure 1). On average, ethyl acetate extracts (56.7%) showed higher levels of cytotoxicity than hexanoic (44.2%) and methanolic (33.4%) extracts.

One of the recognized characteristics of cancer is the suppression of apoptosis. Cancer cells' capacity to prevent programmed cell death increases tumor development, encourages tumor cell dissemination to distant organs, and gives them resistance to cytotoxic anticancer drugs. Thus, bioactive substances that may stop the growth of cancer cells by triggering apoptotic pathways can be used **[55] .**

The cytotoxic effects of the crude methanol extract of *S. carteri* on cervical cancer were identified. Tissue derived from HeLa cells **[56].** The ethanol extract of *S. carteri* shows anti-cancer activity against breast cancer cells**. [57].** The findings regarding this research align with those of **[58],** who observed that the sponge extract exhibited significant antioxidant activity, as assessed by DPPH radical scavenging activity and total antioxidant activity. This activity is related to the high concentrations of phenolics, and flavonoids contained in certain sponge species. In addition, the ferric reduction ability of plasma (FRAP) of sponge extracts is given. This characteristic indicates that the antioxidant compounds act as electron donors and may reduce the oxidized byproducts of lipid peroxidation processes. They work as both primary and secondary antioxidants **[59] .**

These findings correlate with those of **[60],**who found that sea sponges had antioxidant properties utilizing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test and the ferric reducing antioxidant potential assay (FRAP). The marine methanol extract had the greatest DPPH activity and the highest FRAP activity (10.570.39 mm Fe2+/g extract). There is a strong relationship between antioxidant capability and total phenolic content.

4**. CONCLUSION**

In this work, we evaluated how flavonoids, total phenolics, anticancer ,antioxidant activity, potential, and untargeted metabolite profiles differed

across three different types of marine sponges. Untargeted metabolomics discovered 1,054 metabolites from several functional chemical groups, such as amino acids and peptides, fatty acids and conjugates, organic dicarboxylic acids, purines, benzoic acids, indoles, pyrimidines, benzenes, phenylacetic acids, and pyridines compounds. According to correlation study, each antioxidant capability of marine sponges is positively connected with a specific set of nutritional indicators. 2-Aminophenol and 3-Hydroxy-3-methylglutaric acid were identified as dietary indicators with enhanced antioxidant activity. Furthermore, 3-Hydroxy-3-methylglutaric acid and 2-Aminophenol were shown to have stronger anticancer activity. Our findings indicate that 2-Aminophenol has strong antioxidant and anticancer properties. This understanding is very valuable for individuals who want to use marine sponges to produce new medicinal medications.

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الملخص العربي:

تحديد ملامح المستقلبات والأنشطة المضادة للسرطان ومضادات الأكسدة في المختبر لثلاثة مستخلصات ميثانولية مختلفة من الإسفنج البحري من البحر الأحمر في مصر.

أحمد جمعة درويش – أسراء محمد مجاهد – حسنى محمد شفيق عبدالسلام – جمال فخرى عبد النعيم – سالى محمد عبد العزيز الشافعى

قسم الكيمياء الزراعية – كلية الزراعة – جامعة المنيا

تم جمع ثلاثة أنواع مختلفة من الاسفنج البحري (البحر الاحمر الغردقه-مصر) وهي Clathria arbuscula و Crella cyathophora و Xenia macrspiculataوتم عمل مستخلص ميثانولي لها لمعرفه ملامح الميتابوليتات وخصائصها المضادة للأكسدة والسرطان. تم استخدام اختبارات الفلافونويد والفينول الكلي و DPPHو FRAP للمضادات الأكسدة لتحديد أنواع مختلفة من المستخلصات الميثانولية للإسفنجات البحرية. كان تأثير مكافحة السرطان لخلايا السرطان MDA-MB 231 و HUH-7و NB4-APLيعتمد على الجرعة والمستخلص. وجد أن المحتوى الفينولي الكلي (TPC) لمستخلص Xenia macrspiculata الميثانولي أعلى من مستخلص Clathria arbuscula الميثانولي ومستخلص Crella cyathophora الميثانولي. بينما تبين أن إجمالي محتوى الفلافونويد (TFC) لمستخلص Xenia macrspiculata الميثانولي أعلى من مستخلص Clathria arbuscula الميثانولي ومستخلص Crella cyathophora الميثانولي. تم العثور على مستويات مختلفة من نشاط FRAP و DPPHفي جميع المستخلصات الميثانولية لاسفنج البحري. تم تحليل UPLC-TOF-MS للمستخلصات الثلاثة المختلفة. تم العثور على مجموع 1,054 ميتابوليت في تحليل الميتابولوم. كانت المجموعات الرئيسية للمواد الكيميائية هي الببتيدات والأحماض الأمينية والأحماض الدهنية والمركبات المرتبطة بها والأحماض العضويه ثنائيه الكربوكسيل والبيورينات والأحماض البنزويك والإندولات والبيريميدينات والبنزينات وأحماض الفينيل الأسيتيك ومركبات البيريدين. حدد التحليل الإحصائي متعدد المتغيرات 32 ميتابوليت، بما في ذلك 2 Chloro-N, N-dimethyl-3-oxobutanamideو4-Methyl-2-oxo-pentanoic acid و5,6Dihydrouracil و3 Hydroxy-3-methylglutaric acidو2-(E)-3-hydroxy-2-(hydroxypentadecanoylamino)dec-4-ene-1-sulfonic acidو10-Cysteine sulfinic acid و4 Aminocyclopropane-1-carboxylateو1Hydroxydecanoic acid-Hydroxyphenyllactic acid و3-carboxy-2-[(Z)-octadec-11-enoyl]oxypropyl]-trimethylazaniumو يمكن استخدام هذه المكتبة الواسعة من المستقلبات من الإسفنج البحري لاستهداف الإمكانات الطبيعية المضادة للسرطان بالتزامن مع الإجراءات المضادة للسرطان. سيستفيد كل من قطاعي الأدوية والتغذية بشكل كبير من هذا البحث .