Egypt. J. Chem. **Vol. 66**, No. 9. pp. 409 - 420 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Bioactive Constituents of Marine Soft Coral *Nephthea* sp. against Herpes simplex type I (HSV-1) and Coxsackie B4 (CoxB4) viruses; *In-Vitro* and *In Silico* studies

Nevine H. Hassan,[a] Seham S. El-Hawary,[b] Mahmoud Emam,[c] Mohamed A. Rabeh,[b,d],Abdullatif Bin Muhsinah [d], Yahya I. Asiri [e],Elsayed A.E. Hamed,[f] Usama Ramadan Abdelmohsen,[g,h*] and Nabil M. Selim[b*]

[a]*Pharmacognosy Department, Faculty of Pharmacy and Modern University for Technology andInformation, Cairo 11571, Egypt*
[b]*Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza 11562, Egypt*
[c]*Phytochemistry and Plant Systematics Department, National Research Centre, Dokki, Cairo12622, Egypt*
[d] *Pharmacognosy Department, College of Pharmacy, King Khalid University, Abha 61441,Saudi Arabia*
[e] *Pharmacology and Toxicology Department, College of Pharmacy, King Khalid University, Abha 61441, Saudi Arabia*
[f] *National Institute of Oceanography and Fisheries, Hurghada-Red Sea (NIOF) 84511, Egypt*
[g]*Pharmacognosy Department Faculty of Pharmacy, Minia University, 61519 Minia, Egypt*
[h.]*Pharmacognosy Department, Faculty of Pharmacy, Deraya University, 61111 New Minia, Egypt*

Abstract

The most catastrophic problem that affects living standards is viral infections. This work aims to discover and evaluate new antiviral agents from marine sources. Antiviral activities of *n*-hexane, dichloromethane, ethyl acetate, and butanol fractions of octocoral*Nephthea*sp. were examined *in vitro* against Herpes simplex type I (HSV-1) and Coxsackie B4 (CoxB4) viruses using MTT assay. *n*-hexane fraction was the most bioactive fraction with IC_{50} 30.23 ± 0.007µg/ml on both viruses. The metabolic profiling of the *n*-hexane fraction was investigated as the most potent fraction via the Ultra Performance Liquid Chromatography method joined to a quadrupole time-of-flight hybrid mass spectrometer (UPLC-Q/TOF-MS), which led to the identification of twelve secondary metabolites (diterpenoid, triterpene, steroids, and fatty acids). A molecular docking investigation was supported by using Molecular Operating Environment (MOE) software to prove the mechanism of action. The highest binding energy score was for lauric acid with -10.2157 kcal/mol toward the Thymidine Kinase (TK), and Chabrolohydroxybenzoquinone G toward 3C protease (3Cpro) enzyme with -16.5115 kcal/mol. Finally, the results were confirmed by the inhibitory effect of bioactive fraction in-vitro against TK and 3Cpro enzymes. Our results highlighted *Nephthea*sp. as a rich source of non-polar effective constituents that might be a promising candidate for controlling viral infections.

Keywords: Antiviral, Docking study, *Nephthea* sp., UPLC-Q/TOF-MS, Thymidine Kinase, 3C protease.

1. Introduction

Infectious diseases are a variety of illnesses or disorders caused by pathogenic microbes (bacteria, viruses, fungi, and parasites) that have a direct impact on human health [1]. Increasing episodes of such resistance among pathogenic viruses have encouraged the treatment with natural molecules and marines [2]. So, steps are taken to develop natural medicine that guarantees zero facet effects and a reliable cure from damaging viruses [3].

Herpes simplex virus is an important pathogen for humans, infects and replicates in cells at the location of entrance consequently the finding of unique anti-HSV drugs deserves a great struggle [4].Herpes Simplex Virus Type 1 HSV-1 is a nuclear replicating enveloped virus that is typically developed through direct contact with septic lesions or body fluids such as saliva [5]. It causes asymptomatic infections but can cause painful blisters or ulcers around the lips, in the eyes, on the

*Corresponding author e-mail: nabil.selim@pharma.cu.edu.eg(Nabil M. Selim) andusama.ramadan@mu.edu.eg (Usama Ramadan Abdelmohsen).
Received date 2022-12-11; revised date 2022-12-25; accepted date 2022-12-25
DOI: 10.21608/EJCHEM.2022.180047.7313

mucous membrane of the oral cavity, and in the genitals [6].

Coxsackie B4 virus (CoxB4) is a major human pathogen that normally causes slight self-resolving signs such as fever, rash, and upper respiratory illness. Human enterovirus replicates at a high rate in the pancreas [7]. Severe pancreatitis can cause a broad range of diseases such as septic meningitis,myocarditis, hepatitis,gastroenteritis, pneumonia, and sudden death in neonates [8]. Acyclovir and Ganciclovir are drugs used to treat HSV-1 and CoxB4 infections. However, viral resistance to acyclovir has emerged as a problem in the treatment of viral diseases, especially in the treatment of immunocompromised patients [9].

Surveying for natural medicine, obtained from marine soft coral having antiviral activity represent an important step in finding an alternative viral disease treatment [10]. Secondary metabolites are prevalent in the family Nephtheidae. Studies have revealed that the *Nephthea* genus produces a variety of secondary metabolites, including sesquiterpenes, terpenes, and steroids [11]. Moreover, biological investigations carried out on *Nephthea* sp. soft coral demonstrated their varied potential as anticancer [12,13], anti-inflammatory [14], and anti-dermatophyte [15] agents.

In this work, the antiviral activity of four sub-fractions of *Nephthea* sp. was evaluated. The secondary metabolites of the most active fraction from *Nephthea* sp. were annotated based on UPLC-Q/TOF-MS analysis. It is also the first report to display an in silico study where the identified compounds were docked into the active sites of Thymidine kinase and 3C protease, two molecular targets of HSV-1 and CoxB4 viruses, respectively. Furthermore, the inhibition potency of n-hexane fraction on the viral Thymidine kinase and 3C protease enzymes was determined to confirm the antiviral activity (**Scheme 1**).

2. Experimental

2.1.Chemicals

All solvents were used for marine extraction and fractionation in analytical grade (methanol, n-hexane, dichloromethane, ethyl acetate, and butanol) were purchased from (El-Gomhouria Co., Cairo, Egypt), and were used after further distillation. Methanol and Acetonitrile of HPLC-grade were procured from SDFCL Fine-Chem Limited in Mumbai, India.

2.2.Soft coral material

Snorkeling in the Red Sea's shores (Hurghada, Egypt) in January 2020 generated *Nephthea* sp. soft coral. Dr. El-Sayd Abed El-Aziz (Invertebrates Lab. department, National Institute of Oceanography and Fisheries "NIOF", Red Sea Branch, Egypt) graciously authenticated the specimen.The voucher specimen was prepared, kept, and registered in the herbarium section of the Pharmacognosy Department, Faculty of Pharmacy, Deraya University under the number (NS-19-1-2020).

2.2.1.Extraction and fractions preparations

The frozen marine organism (1 kg) was reduced into small segments and extracted numerous times with a 1:1 mixture of methanol (MeOH) and methylene chloride (CH_2Cl_2) till exhaustion then using a rotary vacuum evaporator (Buchi, G, Switzerland) to give a concentrated extract at 60˚C in a water bath to yield 40 g. It was stored at -5 ° C till biological and phytochemical examination in February 2021.The obtained concentrated extract was suspended in distilled H_2O and then fractionated successively, using *n*-hexane (*n*-hex.), dichloromethane (DCM), ethyl acetate (EtOAc) and butanol (BuOH).The solvents were evaporated and concentrated until dryness and under reduced pressure to yield the *n*-hexane fraction (*n*-hex -F) 18 g, the dichloromethane fraction (DCM-F) 1.5 g, the ethyl acetatefraction (EtOAc-F) 4 g, and the butyl alcohol fraction (Bu-F) 7 g. The four fractions were preserved at 4 ˚C for further investigation.

2.3. Antiviral Activity

2.3.1. Virus, strains, and cell culture conditions

Coxsackie B4 (CoxB4) and herpes simplex type 1 (HSV-I) viruses were obtained from Lab of Virology (Science Way for Scientific Research and Consultations, Microbiology and Immunology Department, Faculty of medicine for girls, Al-Azhar University, Egypt). Normal vero cell line (adherent kidney epithelial cells from Cercopithecusaethiops, CCL-81) was cultivated in RPMI 1640 medium (Gibco, Tunisia) enriched with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 g/mL) then incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.3.2. Determination of cytotoxicity on VERO cells

The maximum non-toxic concentration (MNTC) of the four fractions on VERO cells was determined by the MTT colorimetric assay. Different concentrations of the investigated samples were set, growth medium was pour out from 96-well microtiter plates when forming a confluent sheet of VERO cells, the cell monolayer was wash away two times with rinse media, double-fold dilutions of the investigated samples were made in minimum essential media, 0.1 mL of each dilution was tested in different wells, leaving three wells as controls, the plate was incubated at 37°C and examined frequency for up to 2 days. Cells were examined for physical signs of toxicity, such as partial or complete

monolayer loss, rounding, shrinkage, or cell granulation. MTT solution (5 mg/mL in PBS) was prepared (BIO BASIC CANADA INC). Each well received a 20 L MTT solution before being shaken at 150 rpm for 5 minutes to thoroughly mix the MTT into the media. Incubated at (37° C, 5% CO2) for 1-5 hours to allow MTT to be metabolized, then media was removed (dry plate on paper towels to remove residue if necessary). Additionally, formazan is re-suspended in 200 mL of DMSO and shaken at 150 rpm for five minutes to properly combine the formazan and solvent. At 560 nm, the optical density is calculated, and at 620 nm, the background is removed. Optical density and cell count ought to be closely connected [16,17].

2.3.3. MTT assay protocol

Using an MTT assay with 10,000 cells overlaid in 200 μL of medium per well in a 96-well plate, the antiviral activity was assessed. A nonlethal dilution of the investigated samples and the virus suspension were incubated at equal volumes (1:1 v/v) for one hour, 100 μL were added from the viral/sample suspension, and the mixture was shaken at 150 rpm for five minutes. Three wells were left empty for blank controls. The cells were then allowed to attach to the wells at (37°C, 5% CO_2) overnight. On the way to give the virus sufficient time to work, the viral/sample suspension was incubated at (37°C, 5% CO_2) for 1 day. Two milliliters or more of MTT solution at 5 mg/mL were prepared in PBS for each 96-well plate, and the 20 μL MTT solutions was added to each well and located on a shaking table at 150 rpm for five minutes to systematically mix the MTT into the media. The plate was then incubated at (37°C, 5% CO_2) for 1-5 hours to permit the MTT to be digested before the media was removed (dry plate on paper towels to eliminate rest if necessary). Formazan (MTT metabolic product) was re-suspended in 200 μL DMSO and shaken for 5 minutes at 150 rpm on a shaking table to methodically mix the formazan into the solvent. At 560 nm, the optical density (OD) was obtained, and the background was subtracted at 620 nm. The optical density should be proportional to the number of cells [17]. The following equation was used to calculate the antiviral activity of four determinations;

$$\text{Antiviral activity}(\%) = \frac{(\text{Optical density of treated cells} - \text{Optical density of virus control})}{(\text{Optical density of cell control} - \text{Optical density of virus control})} \times 100$$

2.4. Metabolomics profiling Study

In accordance with Abdelmohsen*et al*.,[18] metabolomics fingerprinting was performed on the *n*-hexane fraction of *Nephthea* sp. using an Acquity Ultra Performance Liquid Chromatography system connected to a Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, USA). Chromatographic separation was performed on a BEH C18 column (2.1×100 mm, 1.7µm particle size; Waters, Milford, USA) with a guard column (2.1×5 mm, 1.7µm particle size) using 0.1% formic acid in water (v/v) as solvent A and acetonitrile as solvent B over a period of six minutes at a flow rate of 0.3 mL min^{-1}. The injection volume was 2 µL, and the temperature of the column was 40°C. MS Convert software was used to separate the raw data into positive and negative ionization files. The files were then imported into MZ mine 2.10, data mining software, for peak picking, deconvolution, deisotoping, alignment, and formula prediction. MarinLit, the Dictionary of Natural Products, and Competitive Fragmentation Modeling for Metabolite Identification were the databases used for compound identification (CFM-ID) [19].

2.5. Molecular docking

Molecular Operating Environment (MOE, 2019.0102) software was used for all molecular modelling research. The partial charges were determined automatically, and all minimizations were designed out with MOE until an RMSD gradient of 0.1 kcal·$mol^{-1}Å^{-1}$ was reached with the MMFF94x force field [20].

From the protein data bank (https://www.rcsb.org/structure/3LD6), the X-ray crystallographic structure of thymidine kinase complexed with Ganciclovir (Gan) (PDB ID: 1KI2) and the structure of Coxsackie B4 virus 3c protease (PDB ID: 2ZU3) were both downloaded. Water molecules and non-binding ligands were excluded from each co-crystallized enzyme before the protein was organized for docking using the Protonate 3D protocol in MOE with the default options. The co-crystallized ligand (Gan) and HYDROLASE INHIBITOR (ZU) were used to describe the binding site for docking. In addition, for docking, the triangle Matcher placement method and the London dG scoring function were used.

2.6. Inhibition assay of Thymidine Kinase and 3C protease Enzymes

At the VACSERA Confirmatory Diagnostic Unit, the *n*-hexane fraction of *Nephthea* sp. was examined for inhibitory activity against Thymidine Kinase and 3C protease in comparison to Acyclovir as the reference drug (Cairo, Egypt). Thymidine Kinase was quantitatively measured using an enzyme-linked immunosorbent assay kit by adding 50 μL of each sample or standard to the relevant wells. The plate was then sealed and shaken at 400 revolutions per minute for 1 hour at room temperature. Each well received 100 μL of TMB Development Solution before being incubated for 10 minutes in the dark with a 400 rpm plate shaker. Keep track of the TMB Substrate's kinetic evolution as opposed to the endpoint reading at 450 nm. Recording the blue color development with time in

the ready-made microplate reader just after adding TMB Development solution [21].

The Fluorogenic 3C protease Assay Kit was used to determine the inhibition of 3C protease enzyme activity. In order to achieve a final DTT concentration of 1 mM, 0.5 M DTT was added to 3C Protease Assay Buffer before measuring the amount of fluorescence using a Tecan fluorescent microplate reader. On the ice was put 3C protease. The enzyme-containing tube was temporarily spined to retrieve the entire contents of the tube after the initial thaw. 3C protease should be divided into single-use aliquots. The remaining undiluted enzyme was stored in aliquots at -80°C. A 30 µL diluted 3C protease enzyme solution was added to each of the wells labelled "Positive Control", "Inhibitor Control," and "Test Sample." To the "Blank" wells, 30 µL Assay buffer (containing 1 mM DTT) was added. The inhibitor solution was made by pouring 10 µL of inhibitor into each well labelled "Test Sample." The reaction was started by adding 10 µL of the substrate solution to each and then incubated overnight at room temperature. The fluorescence intensity was measured at 360 nm, and emission detection was done at 460 nm. The intensity of fluorescence can also be measured kinetically [22].

2.7. Statistical Analysis

The obtained results were expressed in mean ± SE (n=3).

3.Results and Discussion

Unfortunately, treatment failures because of antiviral resistance have been recognized since the beginning of antiviral agents, such as Acyclovir for the treatment of herpes simplex (HSV) infections[23].Nature is enriched with powerful compounds which act as HSV-1 and CoxB4 inhibitors [24,25].Marine compounds can provide a novel strategy for developing therapeutic approaches. When compared to conventional treatment, they have traits like a great chemical variety, low production costs, and quite mild side effects [26].

3.1. Antiviral Activity

The antiviral potential of the tested fractions on HSV-1 and CoxB4 viruses was investigated using the MTT antiviral assay. According to Tables 1&2 and Figures 1&2, the n-hexane fraction of *Nephthea* sp. had the highest antiviral activity against the HSV-1 and CoxB4 viruses, with an IC_{50} of 30.23 ± 0.007 µg/mL compared to acyclovir 360.92 ± 0.011 µg/mL. Butanol fraction exhibited weak antiviral activity against HSV-1 with an IC_{50} of 316.78 ± 0.005 µg/mL, whereas dichloromethane exhibited the weakest antiviral activity against CoxB4 virus with an IC_{50} of 48.24 ± 0.004 µg/mL.

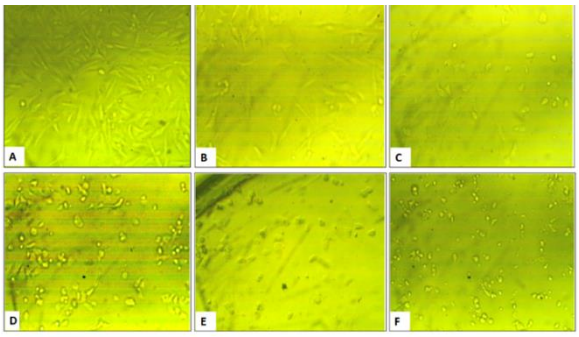

Fig 1. Microscopic images illustrate the effects of different fractions of *Nephthea* sp. on replication of HSV-1 virus; (A) Control Vero cell; (B) n-hex -F; (C) DCM-F; (D) HSV1 on Vero cell ;(E) EtOAc-F; (F) Bu-F.

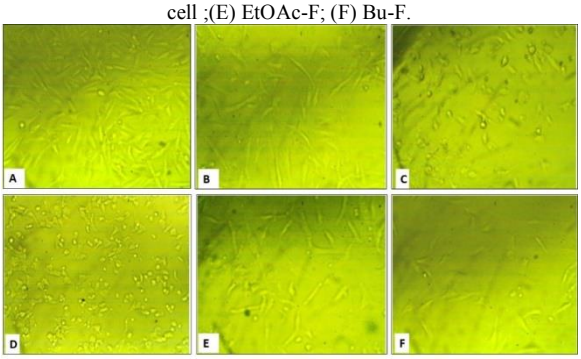

Fig 2. Microscopic images illustrate the effects of different fractions of *Nephthea* sp. on replication of Cox B4 virus; (A) Control Vero cell; (B) n-hex -F; (C) DCM-F; (D) HSV1 on Vero cell ;(E) EtOAc-F; (F) Bu-F.

Table 1
Cytotoxicity and MNTC (µg/ml) on Vero cell of *Nephthea* sp. fractions

Samples	MNTC (µg/ml)	Cytotoxicity on Vero cell (µg/ml)
n-hex- F	7.81	30.23 ± 0.007
DCM-F	7.81	48.24 ± 0.004
EtOAc-F	62.5	170.89 ± 0.003
BU-F	62.5	316.78 ± 0.005
Acyclovir	62.5	360.92 ± 0.011

*MNTC: Maximum Non-Toxic Concentration.

Table 2
Antiviral effect (%) of MNTC (µg/ml) of different *Nephthea* sp. fractions againstbHSV-1 and CoxB4 viruses

Samples	HSV1-virus	CoxB4-virus
n-hex- F	43.18	45.86
DCM-F	14.84	18.96
EtOAc-F	11.21	38.57
BU-F	1.36	34.03
Acyclovir	83.16	77.71

3.2. Metabolic profiling

The retention times, identities(Figures 3), observed molecular weight, and ionized mode for

metabolites are shown in Table 3 and figures4 for the UPLC–Q/TOF–MS profile of the *n*-hexane bio-active fraction. Utilizing macros, MZmine-based techniques, and online databases (Databases DNP and MarinLit) the recorded metabolites were tentatively identified [27] as mentioned in table 3;

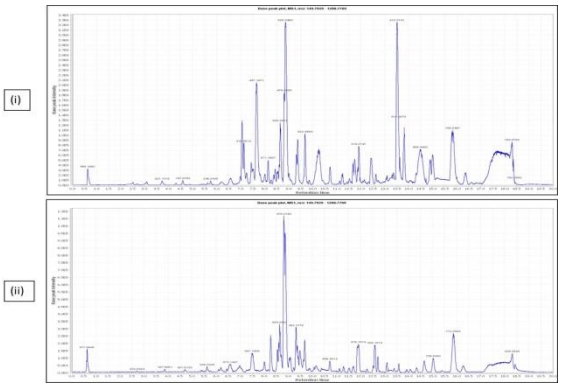

Fig 4. Total ion chromatogram of *n*-hexane bio-active fraction of *Nephthea* sp. recorded in (i) positive ionization mode and (ii) negative ionization mode.

Alkaloid, the mass ion of *m/z* 319.225 for the proposed formula $C_{18}H_{28}N_3O_2$ was recognized as clathriadic acid (5). Clathriadic acid that belongs to cyclic guanidine alkaloid is first published here from soft corals of the genus *Nephthea.*

Fat-soluble vitamin with expected chemical formula $C_{20}H_{28}O_2$'s mass ion of *m/z* 301.216 was also dereplicated as Retinoic acid (7).

Terpenes and sterols, the mass ions of *m/z* 343.227, 427.319, 487.285, 425.726, 487.306, 515.335, and 496.375 in agreement with the molecular formulas $C_{22}H_{30}O_3$, $C_{28}H_{42}O_3$, $C_{30}H_{48}O_5$, $C_{30}H_{50}O$, $C_{29}H_{44}O_6$, $C_{31}H_{46}O_6$, and $C_{29}H_{52}O_6$ were dereplicated as Cespitularin R (1), Chabrolosteroid B (2), Nebrosteroid O (3), Xestosterol (6), Chabrolohydroxybenzoquinone G (8), Nanjiol A (9), and Stigmastane-2,3,15,16,22,23-hexol (11), respectively.

Moreover, Fatty acids, owing to the detected mass ion of *m/z* 201.285 and the chemical formula $C_{12}H_{24}O_2$, another component was dereplicated as Lauric acid (4) a saturated fatty acid that was earlier identified from *Nephthea* sp.[15].

2-Hydroxy-19-hexacosenoic acid (12), another very long-chain fatty acid, was also identified by the chemical formula $C_{26}H_{50}O_3$ and the observed mass ion peak at *m/z* 409.367. It is noted that this chemical was previously described as one of the natural metabolites of the Nephtheidae soft corals, *Dendronephthya* sp.[37].

Anilide Fatty acid, stearic acid anilide (10), which was annotated from the mass ion of *m/z* 360.327 the corresponding molecular formula $C_{24}H_{41}NO$ was also described[15].

In previous studies, preliminary bioactivity tests using a CH_2Cl_2-MeOH (1:1) extract from the crushed *Nephthea* sp. organism revealed antiviral activity against the Vaccinia virus and Ranikhet disease virus (in vitro, 88% protection at 0.05 mg per ml and in vivo, 70% protection at 0.01 µg per ml, respectively).

Elshamy*et al*[38] reported that soft coral are distinguished by a subset of diterpenoids known as cespitularia, cespihypotin, cespitulactam, cespitularin, and cespitulons. The isolated cespitularinditerpenoids and secosteroids from marine exhibited cytotoxic activity.These compounds' antiviral activities were assessed by comparing them to the positive control, cyclosporine A [39]. The steroid Chabrolosteroid B and the oxygenated sterol Nebrosteroid O were previously obtained and formerly characterized by *Nephtheachabrolii*[30,31].

Additionally, Xestosterol was identified from some members within the Nephtheidae family [33].Meroditerpinoid and Polyoxygenated steroid;Chabrolohydroxybenzoquinone G and Nanjiol A both of them were previously reported from *Nephthea* sp. [40]. Also, Stigmastane-2,3,15,16,22,23-hexol, a triterpene that was previously identified from *Nephthea* sp. [35].

Moreover, Chabrolohydroxybenzoquinone G was isolated as aMeroditerpinoid mildly cytotoxic constituent of *N. chabrolii*[41].Furthermore, from acetone extract of the soft coral *Nephtheachabrolii*Nebrosteroid O was isolated and checked for antiviral activity against human cytomegalovirus (HCMV) by a human embryonic lung (HEL) cell line, but regrettably it lacked such activity[40].

According to Nitbani*et al*, the amphiphilic and lipophilic properties of the saturated fatty acid (lauric acid) offer a virucidal mechanism. The carboxylic group is responsible for the polar property of lauric acid and causes interaction of it with certain functional groups in the cell membrane which cause damage to the viral cell [42]. After being exposed for one minute at pH 7, lauric acid exhibited substantial virucidal effects against HSV-1 and HSV-2 [42].2-Hydroxy-19-hexacosenoic acid, another very long-chain fatty acid, is noted that this chemical was previously described as one of the natural metabolites of the Nephtheidae soft corals, *Dendronephthya* sp.[37].

3.3. Molecular Docking Analysis.

To promote scouting and get a greater idea about the possible targets affected by dereplicated compounds of *Nephthea* sp. to produce their antiviral activity [43],*in-silico* molecular docking simulations were performed with thymidine kinase (TK) and Coxsackie B4 virus 3c protease (3C pro). While, the examination of the binding interactions of

Ganciclovir to the active site of the thymidine kinase enzyme, it shows strong hydrogen bond interactions with Glu83, Gln125, Arg176, Glu 225 & His 58 Figure 5.

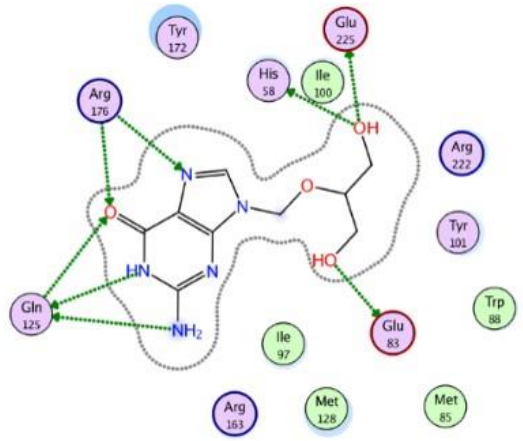

Fig 5.2D interactions of Gan within thymidine kinase active site

The docking setup was first validated by self-docking of the co-crystallized ligand (Gan) in the vicinity of the binding site of the enzyme, the docking score (S) was -9.4953 kcal/mol. and root means square deviation (RMSD) was 1.0015Å Figure 6.

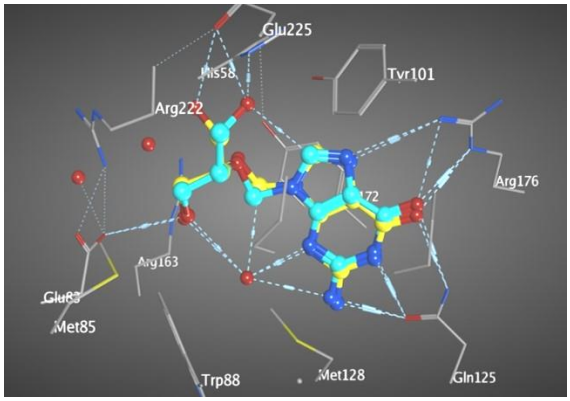

Fig6. 3D representation of the superimposition of the co-crystallized (yellow) and the docking pose (cyano) of Gan in the active site of the thymidine kinase enzyme

Moreover, the examination of the binding interactions of (ZU) to the active site of the enzyme, shows strong hydrogen bond interactions with Val162, Gly145, Asn165, His161, Gly164, and Thr142 Figure 7. Self-docking of the co-crystallized ligand (ZU) in the vicinity of the binding site of the 3C pro enzyme was set up with a score (S) -16.0335 kcal/mol. and root means square deviation (RMSD) was 0.9320 Å (Figure 8).

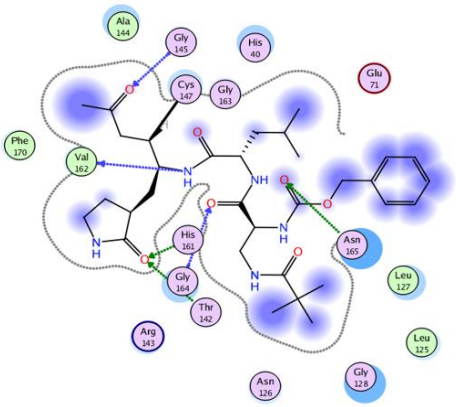

Fig 7.2D interactions of ZU within Coxsackie B4 virus 3c protease active site

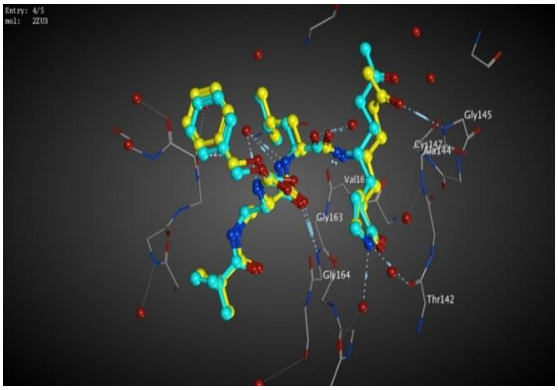

Fig 8.3D representation of the superimposition of the co-crystallized (yellow) and the docking pose (cyano) of ZU in the active site of Coxsackie B4 virus 3c protease enzyme

Lauric acid and Chabrolohydroxybenzoquinone G showed the highest binding energy score toward TK& 3Cpro by -10.2157 and -9.24406 kcal/mol, respectively Figures 9&10. Additionally, most of the remaining compounds were almost equal to Gan and ZU binding free energy, as listed in Table 4. Finally, the obtained *in silico* docking data showed how valid the covering of the dereplicated metabolites of *Nephthea* sp. within thymidine kinase (TK) and Coxsackie B4 virus 3c protease (3C pro) active sites, which could explain their antiviral activity toward HSV-1 and COX B4 viruses.

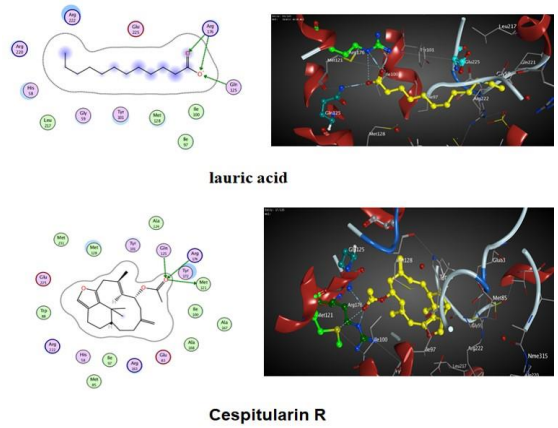

Fig 9.2D (left) & 3D (right) interactions between the highest two scoring metabolites and the amino acids of thymidine kinase active site

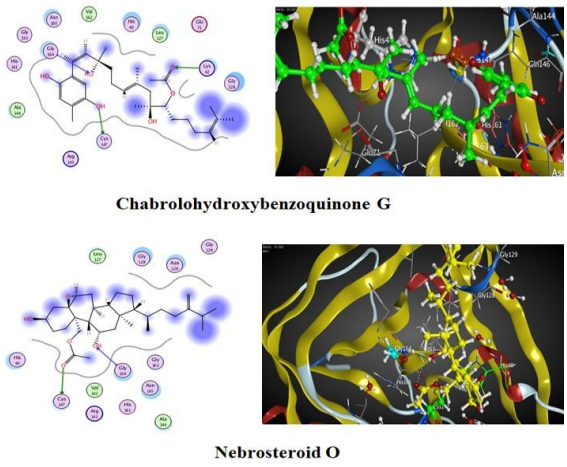

Fig 10. 2D (left) & 3D (right) interactions between the highest two scoring metabolites and the amino acids of the active site of 3c protease

Ultimately, the accomplished docking of our study predicts patterns of interactions between the identified metabolites of the biologically most active fraction *(n*-hexane) from *Nephthea* sp. with herpes simplex thymidine kinase and Coxsackie virus B4 3C protease, which were higher than those of the co-crystal inhibitors, additionally, the *in-vitro* inhibitory performance of the active fraction toward thymidine kinase and 3C protease enzymes and compatible with the previous literature may explain our findings and the existence of all structures together promoting and provide a supposed elucidation for the antiviral activity of the bioactive *n*-hexane fraction of marine soft coral *Nephthea* sp. [44].

3.4. Inhibition of viral Thymidine Kinase and 3C protease Enzymes

The primary function of Thymidine Kinase is the phosphorylation of thymidine to form deoxythymidine 5'-phosphate, dTMP, and is necessary for DNA replication of the HSV-1 virus [45]. The 3C protease, also known as Main Protease (M^{pro}), plays a vital role in processing the polyproteins that are translated from the viral RNA 3C protease inhibitors that can block viral replication and are promising potential drug candidates that could be used to treat patients suffering with the Coxsackie B4 virus infection. They are the important target of acyclovir drug and antiviral agents.

Acyclovir drug showed IC_{50} against3C pro and TK with 4.781±0.338 μg/ml and 280.5±18.8 pg/ml, respectively. In addition, the *n*-hexane fraction of *Nephthea* sp. showed inhibitory activity toward 3C pro and TK with IC_{50}= 9.685 ±1.818 μg/ml and 466.5±40.2 pg/ml, respectively as shown in (Figure 11).

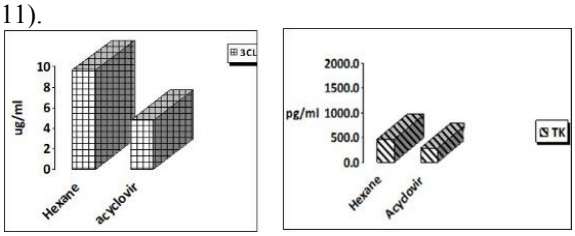

Fig 11. Inhibition activity (IC_{50}) of *n*-hexane fraction of *Nephthea*sp. against 3C protease (left) and Thymidine Kinase (right) enzymes

3. Conclusions

The current study focused on the antiviral potential of various fractions of the soft coral *Nephthea* sp., which demonstrated significant inhibitory activities against HSV-1 and CoxB4 viruses. *n*-hexane fraction exhibited the highest activity against tested viruses. Such implications are most likely underpinned by the availability of a variety of compounds, mostly sterols, terpenoids and fatty acids mined using UPLC-MS-based metabolomics for the active *n*-hexane fraction. Comparative docking screening of the identified metabolites revealed their ability to interact with the active sites of TK and 3C pro enzymes, indicated their likely contribution to *Nephthea* sp. antiviral potential as Thymidine kinase and 3C pro inhibitory molecules, particularly compounds (3) and (6) which were confirmed by the *in-vitro* inhibitory effect of *n*-hexane bioactive fraction of *Nephthea* sp. against TK and 3C pro enzymes. Furthermore, our findings supported the intriguing role of *Nephthea* sp. as an antiviral agent, which was addressed for the first time in marine invertebrates. The findings highlighted the potential of *Nephthea* sp. to complement the current therapeutic arsenal against viral infections, providing a good starting point for future research on the development of viral therapies using marine soft corals.

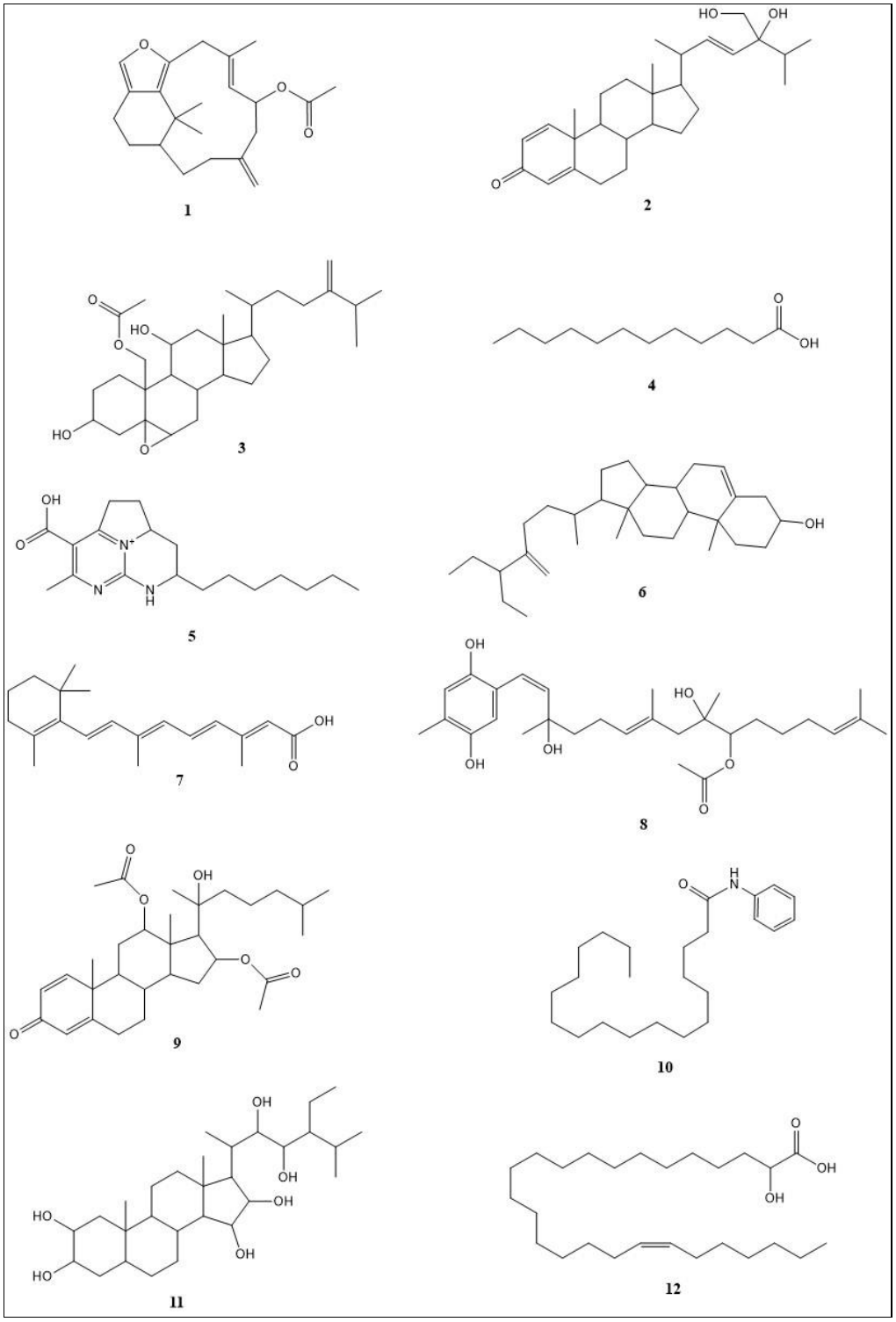

Fig 3. Chemical structures of the proposed compounds identified and dereplicated from the bio-active *n*-hexane fraction of *Nephthea* sp.

Table 3

Tentatively identified Secondary Metabolites identified of *Nephthea* sp. (using UPLC–Q/TOF–MS)

No	Proposed Compounds	Molecular formula	Rt. (min.)	Ionization mode	*M/Z*	Molecular weight	Δ Mass (ppm)	Chemical Class	References
1	Cespitularin R	$C_{22}H_{30}O_3$	7.21	+ve	343.2270	342.2197	0.79	Diterpinoid	[28,29]
2	Chabrolosteroid B	$C_{28}H_{42}O_3$	8.31	+ve	427.3196	426.3122	-2.59	Steroid	[30]
3	Nebrosteroid O	$C_{30}H_{48}O_5$	8.60	-ve	487.2850	488.2923	-1.40	Oxygenated steroid	[31]
4	Lauric acid	$C_{12}H_{24}O_2$	9.01	+ve	201.2166	200.3209	1.35	Saturated Fatty acid	[15]
5	Clathriadic acid	$C_{18}H_{28}N_3O_2$	9.13	+ve	319.2257	318.2177	-1.28	Cyclic guanidine alkaloid	[32]
6	Xestosterol	$C_{30}H_{50}O$	9.47	-ve	425.7269	426.7242	-0.94	Sterol	[33]
7	Retinoic acid	$C_{20}H_{28}O_2$	10.14	+ve	301.2167	300.2094	1.63	Fat-soluble vitamin	[15]
8	Chabrolohydroxybenzoquinone G	$C_{29}H_{44}O_6$	11.21	-ve	487.3060	488.3132	-1.007	Meroditerpinoid	[30]
9	Nanjiol A	$C_{31}H_{46}O_6$	12.76	+ve	515.3353	514.3279	-2.83	Polyoxygenated steroids	[34]
10	N-phenyl stearamide	$C_{24}H_{41}NO$	13.49	+ve	360.3279	359.3196	- 2.42	Anilide Fatty acid	[15]
11	Stigmastane-2,3,15,16,22,23-hexol	$C_{29}H_{52}O_6$	14.07	-ve	495.3683	496.3756	-1.58	Triterpene	[35]
12	2-Hydroxy-19-hexacosenoic acid	$C_{26}H_{50}O_3$	16.21	-ve	409.3676	410.3748	-2.67	Very long-chain fatty acid	[36]

+ve: positive, **-ve**: negative.

Table 4.
Docking Free Binging Energy Scores (kcal/mol) Results of Detected metabolites of UPLC–Q/TOF–MS of *Nephthea* sp. on the Binding sites of thymidine kinase of herpes simplex virus type I (PDB ID: 1KI2) and Coxsackie B4 virus 3c protease (PDB ID: 2ZU3)

	Compound	Thymidine Kinase (1KI2)					Coxsackievirus B4 3c protease (2ZU3)				
		a	b	c	d	e	a	b	c	d	e
Co-crystalized ligand	Ganciclovir	-9.49	GLN 125 GLU 83 HIS 58 GLU 225 GLN 125 GLN 125 ARG 176 ARG 176 ARG 176	N1 O4' O3' O3' N2 O6 O6 O6 N7	H-donor H-donor H-donor H-donor H-donor H-acceptor H-acceptor H-acceptor H-acceptor	2.97 3.37 2.99 3.08 3.01 3.31 2.83 3.45 3.76					
	HYDROLASE INHIBITOR (ZU)						-16.0335	VAL 162 ASN 165 GLY 164 THR 142 HIS 161 GLY 145	N O O O O O	H-donor H-acceptor H-acceptor H-acceptor H-acceptor H-acceptor	3.09 2.77 2.92 2.52 2.77 2.81
Identified Metabolites	2-Hydroxy-19-hexacosenoic acid	-5.61	MET 121 ARG 176 GLN 125 GLN 125 ARG 176 ARG 176	O O O O O O	H-donor H-acceptor H-acceptor H-acceptor Ionic Ionic	3.82 2.97 2.75 2.97 3.52 3.37	-9.6965	ARG 143 THR 142 HIS 161	O O O	H-donor H-acceptor H-acceptor	2.77 3.00 3.02
	Cespitularin R	-9.24	MET 121 GLN 125 ARG 176	O O O	H-donor H-acceptor H-acceptor	3.62 2.60 3.41	-8.7994	HIS 40	C	H-pi	4.45
	Chabrolohydroxy benzoquinone G	-4.04	GLU 225 ARG 176	O O	H-donor H-acceptor	2.35 3.49	-16.5115	CYS 147 GLY 164 LYS 42	O O O	H-donor H-acceptor H-acceptor	3.03 2.78 3.06

Chabrolosteroid B	-5.76	GLU 225 ARG 220 ALA 168 ARG 222	O O O O	H-donor H-donor H-acceptor H-acceptor	2.79 2.78 3.34 3.05	-11.2844	VAL 162	O	H-donor	3.13
Clathriadic acid	-6.04	ARG 176 ARG 176 GLU 225 ARG 176 ARG 176	O O N O O	H-acceptor H-acceptor Ionic Ionic Ionic	2.77 2.74 3.94 2.77 2.74	-9.1112	LYS 42 LYS 42 LYS 42	O O O	H-acceptor Ionic Ionic	3.60 3.60 3.64
Lauric acid	-10.21	ARG 176 ARG 176 GLN 125 ARG 176 ARG 176 ARG 176 ARG 176	O O O O O O O	H-acceptor H-acceptor H-acceptor H-acceptor Ionic Ionic Ionic	2.92 2.85 3.09 3.66 2.92 2.85 3.66	-7.9022	LYS 42 LYS 42 LYS 42 LYS 42 HIS 40	O O O O C	H-acceptor H-acceptor Ionic Ionic H-pi	3.25 3.09 3.25 3.09 3.99
Nanjiol A	-4.21	MET 121 GLU 225 GLN 125 ARG 176	O O O O	H-donor H-donor H-acceptor H-acceptor	3.82 2.89 2.61 3.19	-6.4094	HOH 389	O	H-acceptor	2.33
Nebrosteroid O	-4.07	GLN 125 ARG 176	O O	H-acceptor H-acceptor	2.84 3.27	-12.4029	CYS 147 GLY 164	O O	H-donor H-acceptor	3.28 2.93
Retinoic acid	-6.54	GLU 225 ARG 222 ARG 222 ARG 222	C O O O	H-donor H-acceptor Ionic Ionic	3.02 3.34 3.34 3.89	-8.6326	GLY 145 GLN 146	O O	H-acceptor H-acceptor	3.57 3.29
Stearic acid anilide	-5.80	ARG 222 ARG 222	O O	H-acceptor H-acceptor	2.96 3.05	-8.3833	GLY 164 GLY 164 HIS 40	N O C	H-donor H-acceptor H-pi	2.86 3.12 4.21
Stigmastane-2,3,15,16,22,23-hexol	-3.23	GLN 125 GLU 225 ARG 176	O O O	H-donor H-donor H-acceptor	2.50 2.30 2.86	-10.9926	GLY 164 GLY 164	O O	H-acceptor H-acceptor	3.32 3.18
Xestosterol	-3.48	GLN 125	O	H-donor	2.99	-7.4653	TYR 122	O	H-acceptor	3.18

a:Score (kcal/mol), **b**:Amino acids, **c**: Interacting groups, **d**:Type ofinteraction, **e**: Length

4. Conflicts of interest

The authors declare no conflict of interest.

5. Formatting of funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

6. Acknowledgment

We are grateful to Assistant Lecturer Rehab Hamed (Pharmaceutical Department, Faculty of Pharmacy, Modern University for Technology and Information, Egypt) for her kindly carrying out the Molecular docking study.

7. References

[1] K. Anderson, A.A. Cunningham, N.G. Patel, F.J. Morales, P.R. Epstein, P. Daszak, Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers, Trends in ecology & evolution, 19 (2004) 535-544.

[2] E. Noumi, M. Snoussi, A. Merghni, F. Nazzaro, G. Quindós, G. Akdamar, M. Mastouri, A. Al-Sieni, O. Ceylan, Phytochemical composition, anti-biofilm and anti-quorum sensing potential of fruit, stem and leaves of Salvadora persica L. methanolic extracts, Microbial Pathogenesis, 109 (2017) 169-176.

[3] H. Yuan, Q. Ma, L. Ye, G. Piao, The traditional medicine and modern medicine from natural products, Molecules, 21 (2016) 559.

[4] A. Astani, J. Reichling, P. Schnitzler, Comparative study on the antiviral activity of selected monoterpenes derived from essential oils, Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 24 (2010) 673-679.

[5] P.G. Arduino, S.R. Porter, Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features, Journal of oral pathology & medicine, 37 (2008) 107-121.

[6] M.A. Nasr-Eldin, A. Abdelhamid, D. Baraka, Antibiofilm and antiviral potential of leaf extracts from Moringa oleifera and rosemary (Rosmarinus officinalis Lam.), Egyptian Journal of Microbiology, 52 (2017) 129-139.

[7] D.J. Taylor, S.M. Hamid, A.M. Andres, H. Saadaeijahromi, H. Piplani, J.F. Germano, Y. Song, S. Sawaged, R. Feuer, S.J. Pandol, Antiviral effects of menthol on coxsackievirus B, Viruses, 12 (2020) 373.

[8] E.L. Tan, A.P.Y. Wong, C.L. Poh, Development of potential antiviral strategy against coxsackievirus B4, Virus Research, 150 (2010) 85-92.

[9] A. Karimi, M.-T. Moradi, M. Saeedi, S. Asgari, M. Rafieian-Kopaei, Antiviral activity of Quercus persica L.: high efficacy and low toxicity, Advanced biomedical research, 2 (2013).
[10] G.J. Elkin, J.J. Rojas, A. Martínez, Pharmacological Developments Obtained from Marine Natural Products and Current Pipeline Perspective, Natural Product Communications, 6 (2011) 1934578X1100600233.
[11] O.H. Abdelhafez, J.R. Fahim, S.Y. Desoukey, M.S. Kamel, U.R. Abdelmohsen, Recent updates on corals from Nephtheidae, Chemistry & biodiversity, 16 (2019) e1800692.
[12] O.H. Abdelhafez, J.R. Fahim, R.R. El Masri, M.A. Salem, S.Y. Desoukey, S. Ahmed, M.S. Kamel, S.M. Pimentel-Elardo, J.R. Nodwell, U.R. Abdelmohsen, Chemical and biological studies on the soft coral Nephtheasp, RSC Advances, 11 (2021) 23654-23663.
[13] O.H. Abdelhafez, J.R. Fahim, M.E. Rateb, C.J. Ngwa, G. Pradel, U.R. Abdelmohsen, S.Y. Desoukey, M.S. Kamel, Cytotoxic potential of Nephthea sp.-derived actinomycetes supported by metabolomics analysis, Natural Product Research, (2022) 1-6.
[14] O.H. Abdelhafez, T.F.S. Ali, J.R. Fahim, S.Y. Desoukey, S. Ahmed, F.A. Behery, M.S. Kamel, T.A. Gulder, U.R. Abdelmohsen, Anti-inflammatory potential of green synthesized silver nanoparticles of the soft coral Nephthea sp. supported by metabolomics analysis and docking studies, International Journal of Nanomedicine, 15 (2020) 5345.
[15] N.H. Hassan, S.S. El-Hawary, M. Emam, M.A. Rabeh, U.R. Abdelmohsen, N.M. Selim, Potential Inhibitors of CYP51 Enzyme in Dermatophytes by Red Sea Soft Coral Nephthea sp.: In Silico and Molecular Networking Studies, ACS Omega, (2022).
[16] E.G. Haggag, A.M. Elshamy, M.A. Rabeh, N.M. Gabr, M. Salem, K.A. Youssif, A. Samir, A.B. Muhsinah, A. Alsayari, U.R. Abdelmohsen, Antiviral potential of green synthesized silver nanoparticles of Lampranthuscoccineus and Malephoralutea, International journal of nanomedicine, 14 (2019) 6217.
[17] S.A. Zidan, M.A. Orabi, M.A. Mustafa, M. AAl-Hammady, M.S. Kamel, Anti-HSV-1 and hepatoprotective activities of the Soft coral Sarcophytonacutum from the red sea, Journal of Pharmacognosy and Phytochemistry, 5 (2016) 247.
[18] U.R. Abdelmohsen, C. Cheng, C. Viegelmann, T. Zhang, T. Grkovic, S. Ahmed, R.J. Quinn, U. Hentschel, R. Edrada-Ebel, Dereplication Strategies for Targeted Isolation of New AntitrypanosomalActinosporins A and B from a Marine Sponge Associated-Actinokineospora sp. EG49, Marine Drugs, 12 (2014) 1220-1244.
[19] D.J. Raheem, A.F. Tawfike, U.R. Abdelmohsen, R. Edrada-Ebel, V. Fitzsimmons-Thoss, Application of metabolomics and molecular networking in investigating the chemical profile and antitrypanosomal activity of British bluebells (Hyacinthoides non-scripta), Scientific reports, 9 (2019) 1-13.
[20] R.H. Abd El-Aleam, R.F. George, G.S. Hassan, H.M. Abdel-Rahman, Synthesis of 1, 2, 4-triazolo [1, 5-a] pyrimidine derivatives: Antimicrobial activity, DNA Gyrase inhibition and molecular docking, Bioorganic Chemistry, 94 (2020) 103411.
[21] J.K. Kumar, S. Holmgren, K.H. Levedahl, M. Höglund, P. Venge, S. Eriksson, AroCell TK 210 ELISA for determination of TK1 protein: age-related reference ranges and comparison with other TK1 assays, BioTechniques, 68 (2020) 334-341.
[22] V. Kumar, J.S. Shin, J.-J. Shie, K.B. Ku, C. Kim, Y.Y. Go, K.-F. Huang, M. Kim, P.-H. Liang, Identification and evaluation of potent Middle East respiratory syndrome coronavirus (MERS-CoV) 3CLPro inhibitors, Antiviral research, 141 (2017) 101-106.
[23] S. Chilukuri, T. Rosen, Management of acyclovir-resistant herpes simplex virus, Dermatologic clinics, 21 (2003) 311-320.
[24] J. Wu, H. Power, M. Miranda-Saksena, P. Valtchev, A. Schindeler, A.L. Cunningham, F. Dehghani, Identifying HSV-1 Inhibitors from Natural Compounds via Virtual Screening Targeting Surface Glycoprotein D, Pharmaceuticals, 15 (2022) 361.
[25] N.M. Fahmy, A.M. Abdel-Tawab, Isolation and characterization of marine sponge–associated Streptomyces sp. NMF6 strain producing secondary metabolite (s) possessing antimicrobial, antioxidant, anticancer, and antiviral activities, Journal of Genetic Engineering and Biotechnology, 19 (2021) 1-14.
[26] M. Asif, M. Saleem, H.S. Yaseen, A.H. Yehya, M. Saadullah, H.M. Zubair, C.E. Oon, P.M. Khaniabadi, S.H. Khalid, I.U. Khan, Potential role of marine species-derived bioactive agents in the management of SARS-CoV-2 infection, Future Microbiology, 16 (2021) 1289-1301.
[27] L. Macintyre, T. Zhang, C. Viegelmann, I. Juarez Martinez, C. Cheng, C. Dowdells, U.R. Abdelmohsen, C. Gernert, U. Hentschel, R. Edrada-Ebel, Metabolomic tools for secondary metabolite discovery from marine microbial symbionts, Marine drugs, 12 (2014) 3416-3448.
[28] W.-C. Chao, Studies on the secondary metabolites from the soft corals Nephtheachabroli and Cespitulariacaerulea, (2018).
[29] Y.-C. Shen, Y.-R. Wu, J.-J. Lin, K.-L. Lo, Y.-C. Kuo, A.T. Khalil, Eight new diterpenoids from soft coral Cespitularia hypotentaculata, Tetrahedron, 63 (2007) 10914-10920
[30] F. Amir, Y.C. Koay, W.S. Yam, Chemical constituents and biological properties of the marine soft coral Nephthea: a review (Part 2), Tropical Journal of Pharmaceutical Research, 11 (2012) 499-517.

[31] K.M. Allam, A.I. Khedr, A.E. Allam, M.S.A. Abdelkader, E.S. Elkhayat, M.A. Fouad, Chemical and Biological Diversity in Nephthea Soft Corals in the Current Decade: A Review, Journal of advanced Biomedical and Pharmaceutical Sciences, 4 (2021) 124-133.
[32] A.G. Tempone, P. Pieper, S.E. Borborema, F. Thevenard, J.H.G. Lago, S.L. Croft, E.A. Anderson, Marine alkaloids as bioactive agents against protozoal neglected tropical diseases and malaria, Natural product reports, (2021).
[33] D.J. Faulkner, Marine natural products, Natural product reports, 18 (2001) 1R-49R.
[34] Z.-Y. Shao, D.-Y. Zhu, Y.-W. Guo, Nanjiols A− C, New Steroids from the Chinese Soft Coral Nephthea b ayeri, Journal of natural products, 65 (2002) 1675-1677.
[35] F. Izzati, M.F. Warsito, A. Bayu, A. Prasetyoputri, A. Atikana, L. Sukmarini, S.I. Rahmawati, M.Y. Putra, Chemical diversity and biological activity of secondary metabolites isolated from Indonesian marine invertebrates, Molecules, 26 (2021) 1898.
[36] A.B. Imbs, D.A. Demidkova, T.N. Dautova, Lipids and fatty acids of cold-water soft corals and hydrocorals: a comparison with tropical species and implications for coral nutrition, Marine biology, 163 (2016) 1-12.
[37] A.B. Imbs, N.A. Latyshev, N.V. Zhukova, T.N. Dautova, Comparison of fatty acid compositions of azooxanthellateDendronephthya and zooxanthellate soft coral species, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 148 (2007) 314-321.
[38] A.I. Elshamy, M.I. Nassar, T.A. Mohamed, M.-E.F. Hegazy, Chemical and biological profile of Cespitularia species: A mini review, Journal of Advanced Research, 7 (2016) 209-224.
[39] A. Patra, A. Majumdar, Secondary metabolites of a soft coral (Nephthea sp.) of the Bay of Bengal, Arkivoc, 9 (2003) 133-139.
[40] S.-K. Wang, S.-Y. Puu, C.-Y. Duh, New steroids from the soft coral Nephtheachabrolii, Marine Drugs, 11 (2013) 571-580.
[41] M. Menna, C. Imperatore, F. D'Aniello, A. Aiello, Meroterpenes from marine invertebrates: Structures, occurrence, and ecological implications, Marine Drugs, 11 (2013) 1602-1643.
[42] Hilmarsson, H.; Kristmundsdottir, T.; Thormar, H., Virucidal activities of medium-and long-chain fatty alcohols, fatty acids and monoglycerides against herpes simplex virus types 1 and 2: comparison at different pH levels. *Apmis*2005, 113, (1), 58-65.
[43] B.K. Mahmoud, A.N.E. Hamed, M.N. Samy, U.R. Abdelmohsen, E.Z. Attia, M.A. Fawzy, R.H. Refaey, M.A. Salem, S.M. Pimentel-Elardo, J.R. Nodwell, Metabolomic profiling and biological investigation of Tabebuia Aurea (Silva Manso) leaves, family Bignoniaceae, Natural Product Research, 35 (2021) 4632-4637.
[44] H. Hilmarsson, T. Kristmundsdottir, H. Thormar, Virucidal activities of medium-and long-chain fatty alcohols, fatty acids and monoglycerides against herpes simplex virus types 1 and 2: comparison at different pH levels, Apmis, 113 (2005) 58-65.
[45] Y. Xie, L. Wu, M. Wang, A. Cheng, Q. Yang, Y. Wu, R. Jia, D. Zhu, X. Zhao, S. Chen, Alpha-herpesvirus thymidine kinase genes mediate viral virulence and are potential therapeutic targets, Frontiers in microbiology, 10 (2019) 941.