Bioactive Pyrrole Alkaloids isolated from the Red Sea Marine Sponge *Stylissa carteri*

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Abstract: Fifteen pyrrole alkaloids were isolated from the Red Sea marine sponge *Stylissa carteri* and investigated for their biological activities. Four of them were dibrominated [(+) dibromophakelline, *Z*-3-bromohymenialdisine, (±) ageliferin and 3,4-dibromo-1*H*-pyrrole-2-carbamide], nine compounds were monobrominated [(-) clathramide C, agelongine, (+) manzacidin A, (-) 3-bromomanzacidin D, *Z*-spongiacidin D, *Z*-hymenialdisine, 2-debromostevensine, 2-bromoaldisine and 4-bromo-1*H*-pyrrole-2-carbamide)] and finally, two compounds were non-brominated derivatives viz., [(*E*-debromohymenialdisine and aldisine)]. The structure elucidation of the isolated compounds were based on 1D and 2D NMR spectroscopic and MS studies, as well as by comparison with the literature. *In-vitro*, *Z*-spongiacidin D exhibited a moderate activity on (ARK5, CDK2-CycA, CDK4/CycD1, VEGFR-2, SAK and PDGFR) protein kinases. Furthermore, *Z*-hymenialdisine displayed a moderate effect on (ARK5, VEGFR-2, SAK and PDGFR) protein kinases. While, (-) clathramide C showed a moderate activity on AURORA-A. Moreover, *Z*-3-bromohymenialdisine showed distinct inhibition of (AURORA-A, CDK4/CycD1, FAK, VEGFR-2, SAK and PDGFR) protein kinases. While, others showed only a marginal inhibitory activity e.g. agelongine and (+) manzacidin A. The inhibition of these prominent protein kinases suggests a potential use of these compounds as cytostatic drugs. In L5178Y cell lines, the most effective secondary metabolites were (+) dibromophakelline and *Z*-3-bromohymenialdisine. Finally, *Z*-hymenialdisine, *Z*-3-bromohymenialdisine and (±) ageliferin exhibited the highest cytotoxic activity on HCT116 cell lines.

Keywords: *Stylissa carteri*; Sponge; pyrrole alkaloids; protein kinase; cytotoxicity.

1 Introduction

Marine sponges (Phylum: Porifera) have attracted substantial research interest because of their ecological importance and their production of a wide range of bioactive compounds for pharmacological use [1,2]. The Red Sea is a unique and largely unexplored marine ecosystem, where its sponges have been studied during

the past two decades for their natural products and bioactive constituents, as well as for their ecological importance [3].

Stylissa carteri is one of the interesting sponges from the Red Sea. Several bromopyrrole alkaloids were isolated from it. A review demonstrated that these alkaloids showed promising biological activities, as hymenialdisine, first isolated in 1980 from the marine sponges of the genera Hymeniacidon, Acanthella, Axinella and Pseudaxinyssa. *S*. *carteri* is a well-known protein kinase inhibitor [4].

This sponge potently inhibited glycogen synthase kinase 3*β*, cyclin-dependent kinase 2 and cyclin-dependent kinase 5, whereas dibromocantharelline (another brominated sponge component) only displayed a significant inhibitory effect toward glycogen synthase kinase 3*β* with IC_{50} 3 µmol [5].

Moreover, 10-*E*-hymenialdisine and 10-*Z*-hymenialdisine showed a potent inhibition of RAF/MEK-1/MAPK cascade with IC_{50} values of 3 and 6 nM, respectively [6]. Both of these alkaloids also inhibited the growth of human LoVo tumor cells. Hymenialdisine competed with ATP for binding to distinct kinases, like cyclin-dependent kinases, glycogen synthase kinase-3*β* and casein kinase 1 [7]. Hymenialdisine inhibited interleukin-8 production in U937 cells by inhibition of nuclear factor-kappaB [8].

Furthermore, spongiacidin C (a pyrrole alkaloid was isolated from the marine sponge *Stylissa massa*) inhibited USP7, a deubiquitylating enzyme hydrolyzing the isopeptide bond at the C-terminus of ubiquitin. This potential cancer target was inhibited with IC_{50} 3.8 µM [9].

Finally, the brominated alkaloids *viz*., debromohymenialdisine, hymenialdisine and 3-bromohymenialdisine of *Axinella carteri* exhibited cytotoxic activities on L5178Y mouse lymphoma cell lines. They displayed ED_{50} values; 1.8, 3.9 and 3.9 µg/mL, respectively [10].

Therefore, this study aimed the investigation of the biological activities of the isolated alkaloids from *S. carteri viz*., *in-vitro* protein kinases activities and cytotoxic effects using two different cell lines.

2 Material and methods

2.1 Animal material

The sponge *Stylissa carteri* (syn. *Axinella carteri*) [Phylum: Porifera, Class: Demospongiae, Order: Halichondrida, Family: Dictyonellidae, Genus: *Stylissa*, Species: *S. carteri*] was collected in June 2006 at a depth of 12 m, from the Red Sea, Hurghada, Egypt. It was identified by Prof. van Soest, RWM, (Zoological Museum; Amsterdam, The Netherlands) for the identification of the sponge. It is a reddish orange flabellate sponge. The sponge material was immersed in ethanol immediately after collection. A voucher specimen was kept in ethanol under registration number ZMAPOR 19838 at the Zoological Museum, Amsterdam, The Netherlands.

2.2 Chromatography and spectroscopic analyses

2.2.1 Vacuum liquid chromatography (VLC)

It was performed on silica gel 60 (0.040-0.063 mm; Merck, Darmstadt, Germany). *n*-Hexane, CH_2Cl_2 and MeOH were used as mobile phases. Column chromatography was carried out on silica gel 60, sephadex LH-20 and reversed phase LiChroprep RP-18 (25-40 µm, Merck). For silica gel column chromatography, varying ratios of CH_2Cl_2/MeOH were used as mobile phases. For sephadex LH-20 column chromatography, the mobile phase was 100% MeOH. For RP-18 column chromatography, two mobile phases were used; either acetonitrile or acetonitrile/H_2O (3:7). TLC analysis was carried out using aluminum sheet precoated with silica gel 60 F254 (Merck, Darmstadt, Germany). The compounds were detected by their UV absorbance at 254 and 366 nm.

2.2.2 Analytical HPLC analysis

The samples were injected into a HPLC system equipped with a photodiode array detector (Dionex, Munich, Germany). The routine detection was at 235, 254, 280 and 340 nm. The separation column (125 X 4 mm ID) was prefilled with Eurosphere 100-5 C-18, 5 *µ*m (Knauer, Berlin, Germany) and flow rate 1 mL/min. The separation was achieved by applying a linear gradient from 90% H_2O (pH 2.0 Nanopure water using ortho-phosphoric acid 85% p.a., Merck) to 100% MeOH over 40 min.

2.2.3 Semi-preparative HPLC

The separations were done on a LaChrom-Merck Hitachi HPLC machine, pump L-7100, UV detector L-7400. The separation column (300 X 8 mm ID) was prefilled with Eurosphere 100-5 C-18, 5 *µ*m (Knauer, Berlin, Germany), flow rate 5 mL/min, UV detection at 280 nm). The compounds were eluted with a solvent system of nanopure H_2O/MeOH (gradient elution starting with a concentration of 10% MeOH and increasing the concentration in a linear manner within 25 min up to 60%).

2.2.4 Medium Pressure Liquid Chromatography (MPLC)

The separations were done on Büchi MPLC; Gradient molder with mixing chamber B-687, fraction collector: B-684, Column size: (ID 460 X 26 mm), was prefilled with reversed phase LiChroprep RP-18 (25-40 µm, Merck), flow rate 3 mL/min. The mobile phases were either H_2O/MeOH (7.5:2.5) or H_2O/MeOH gradient elution.
Mass spectra (ESI-MS) were recorded on a Thermo Finnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. HRFT-MS was recorded on a LTQ-FT-MS-Orbitrap (Thermo Finnigan, Bremen, Germany). Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter.

2.2.5 Spectroscopic analyses

1D and 2D NMR spectra were recorded at 300 °K on either a Bruker ARX-500 or AVANCE DMX-600 NMR spectrometer. Samples were dissolved in different deuterated solvents, whose choice was dependent on the solubility of each compound.

2.3 Extraction and isolation

The dried fine powder of *Stylissa carteri* (600 g) was extracted exhaustively with methanol (4x, 3 L each) and concentrated to yield (90 g) residue. The resulting extract was dissolved in the least amount of demineralized water and partitionated with EtOAc and *n*-BuOH, respectively. The extraction and fractionation were demonstrated in (Scheme 1a).

The *n*-BuOH-soluble material of *S*. *carteri* was concentrated under vacuum to afford 18 g. It was subjected to column chromatography on sephadex LH-20 using methanol as mobile phase. Four fractions were obtained (I to IV). Fraction (I, 3.05 g) was further subjected to reversed phase column chromatography (RP-18, 25-40 µm, Merck) using acetonitrile as a mobile phase to yield compounds **1** (6 mg). Furthermore, fraction (II, 3.58 g) was subjected to MPLC on reversed phase (RP-18, 25-40 µm, Merck) using H_2O/MeOH gradient elution to give three subfractions (Subfr. II-1 to II-3). Compound **2** (12 mg) was precipitated in pure form from subfraction (Subfr. II-1). On the other hand, subfraction (Subfr. II-2, 50 mg) was further submitted to column chromatography on reversed phase (RP-18, 25-40 µm, Merck) using acetonitrile/H_2O (3:7) as a mobile phase to give compounds **3** (5 mg) and **4** (2 mg), respectively. In addition to, subfraction (Subfr. II-3, 30 mg) was further purified on semi-preparative HPLC to obtain compound **5** (8 mg). Moreover, fraction (III, 4.9 g) was further subjected to MPLC using reversed phase (RP-18, 25-40 µm, Merck) and H_2O/MeOH (7.5:2.5) as an isocratic elution system to yield eight subfractions (from Subfr. III-1 to III-8). Subfraction (Subfr. III-3, 80 mg) was further purified using semi-preparative HPLC to afford three compounds **6** (3 mg), **7** (2 mg) in addition to **8** (4 mg), respectively. On the other hand, subfraction (Subfr. III-6, 30 mg) was submitted to further purification using semi-preparative HPLC to give compound **9** (5 mg). Furthermore, subfraction (Subfr. III-7, 30 mg) was subjected to semi-preparative HPLC to give compound **10** (7 mg). Finally, compound **11** (10 mg) was precipitated in pure form from subfraction (Subfr. III-8). Chromatographic fractionations of *n*-butanol fraction of *S. carteri* sponge were illustrated in (Scheme 1b).

While, the EtOAc soluble material of *S*. *carteri* was concentrated under vacuum to yield (12 g) residue. It was subjected to VLC on silica gel, using gradient elution consisting of different portions of *n*-Hexane/CH_2Cl_2 to CH_2Cl_2/MeOH. Elution started with 100% *n*-Hexane and the CH_2Cl_2 concentrations were increased gradually till 100% CH_2Cl_2 and then the MeOH concentrations were increased gradually till 100% MeOH. Ten fractions were obtained (from I to X). Compound **12** (3 mg) was precipitated as a pure substance from fraction (VII). Additionally, fraction (IX, 500 mg) was further subjected to silica gel column chromatography using CH_2Cl_2/MeOH in gradient elution manner to yield seven subfractions (from Subfr. IX-1 to IX-7). Subfraction (Subfr. IX-4, 110 mg) was further purified by reversed phase column chromatography (RP-18, 25-40 µm, Merck) using acetonitrile as a mobile phase to

afford three compounds; **13** (8 mg), **14** (6 mg) and **15** (2 mg), respectively. Chromatographic fractionations of *EtOAc* fraction of *S. carteri* sponge were shown in (Scheme 1c).

2.4 Protein kinase assay

Assays for the measurement of protein kinase activity were performed in 96-well FlashPlates (Perkin Elmer/NEN, Boston, MA, USA) in a 50 mL reaction volume. The reaction cocktail contained 20 mL assay buffer, 5 mL ATP solution (in demineralized water), 5 mL test compound (in 10% DMSO), 10 mL substrate and 10 mL purified recombinant protein kinase. The final concentration of ATP was 1 mM. The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 mM Na-orthovanadate, 1.2 mM DTT, 50 mg/mL PEG20000 and 1 mM [γ^{33}P]-ATP (approximately 5 * 10^5 cpm/well).

The following substrates were used: glycogen synthase kinase 3 (GSK3) (14-27): AKT1 serine-threonine kinase, tetra(LRRWSLG): AURORA serine/threonine kinases A and B, MEK kinase 1: B-RAF-VE kinase, Histone H1: Cyclin-dependent kinase (CDK2/CycA), Rb-CTF: Cyclin-dependent kinase 4 CDK4/CycD1, P53-CTM :anti-casein kinase 2 alpha (CK2alpha1), $Poly(Glu,Tyr)_{4:1}$: [epidermal growth factor receptor (EGFR), ephrin type B receptor 4 (EPHB4), ERBB2 receptor protein tyrosine kinase, focal adhesion kinase (FAK), insulin like growth factor 1 receptor (IGF1-R), SRC tyrosine kinase and vascular endothelial growth factor receptor (VEGF-R2)], Casein: Polo-like kinase PLK-1, $poly(Ala, Glu, Lys, Tyr)_{6:2:4:1}$: [INS-R and MET] and Casein: platelet derived growth factor (PDGFR-beta). Autophosphorylation was measured for ARK5 serine/threonine kinase, COT kinase and SAK kinase.

The assay for all enzymes contained 60 mM HEPES-NaOH (pH 7.5), 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 50 µg/mL PEG20000, 1 µM [γ-33P]-ATP. The reaction mixtures were incubated at 30 °C for 80 min and stopped with 50 µL 2% (v/v) H_3PO_4. The plates were aspirated and washed two times with 200 µL of 0.9% (w/v) NaCl or 200 µL H_2O. Incorporation of γ^{33}P was determined with a microplate scintillation counter (Microbeta Trilux, Wallac). All assays were performed with a Beckman Coulter/Sagian robotic system [11,12].

2.5 Cytotoxicity test

The cytotoxicity was determined by using two different cell lines:

2.5.1 L5178Y cell lines

They were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. An aliquot of 50 µL cell suspension (3750 cells) was pipetted into each cavity of a 96-well microtiter plate together with 50 µL of the compounds in EMEM (3 to 10 µg/mL) and incubated for 72 h (37 °C, 5% CO_2). A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 h (37 °C, 5% CO_2), the medium was centrifuged (15 min, 20 °C, 210 xg) with 200 µL DMSO and the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well spectrophotometer. The colour intensity is correlated with the number of viable cells [13,14]. All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% DMSO were included in the experiments.

2.5.2 HCT116 cell lines

They were evaluated according to Mosmann [14] with slight modifications [15].

2.6 Statistical analyses

All data are given as mean +/- SD. The significance of changes in the test responses was assessed using analysis of variance (GraphPad Prism: version 5.0, La Jolla, USA). Statistical significance was assessed by unpaired Student's (*t*) test, differences were considered to be significant at $p < 0.05$ and indicated as "*".

3 Results

3.1 Isolation of the pyrrole alkaloids

From the Red Sea sponge *S. carteri*, fifteen compounds were isolated. All isolated compounds were identified by comparison with different techniques of spectroscopy (UV, MS and NMR) data with those in the literature: (-) clathramide C (**1**) [16], agelongine (**2**) [17], (+) manzacidin A (**3**) [18], (-) 3-bromomanzacidin D (syn. *N*-methylmanzacidin C) (**4**) [19], (+) dibromophakelline (**5**) [20], *E*-debromohymenialdisine (**6**) [21], *Z*-spongiacidin D (syn. axinohydantoin) (**7**) [22], *Z*-hymenialdisine (**8**) [21], *Z*-3-bromohymenialdisine (syn. Spongiacidin A) (**9**) [21], 2-debromostevensine (syn. 2-debromoodiline) (**10**) [21], (±) ageliferin (**11**) [23], 3,4-dibromo-1*H*-pyrrole-2-carbamide (**12**) [24], aldisine (**13**) [25], 2-bromoaldisine (**14**) [25] and 4-bromo-1*H*-pyrrole-2-carbamide (**15**) [26-28]. The chemical structures are shown in (Figure 1).

3.1.1 (-) Clathramide C (1) [16]

Yellow residue.

UV (MeOH) λ_{max}: 221.5 and 277.2 nm.

$[\alpha]_D^{20}$-7.0^o (*c* 0.11, MeOH).

^{1}H-NMR (500 MHz, CD_3OD): 7.03 (1H, br s, H-2), 6.84 (1H, br s, H-4), 4.04 (1H, m, H-8), 2.28 (2H, m, H-9), 4.57 (1H, d, *J*=11.7 Hz, H-11a), 4.35 (1H, d, *J*=11.7 Hz, H-11b), 7.99 (1H, br s, H-13) and 1.46 (3H, s, H-15). ^{13}C-NMR (125 MHz, CD_3OD): 160.4 (C, C-6), 154.0 (CH, C-13), 125.6 (CH, C-2), 123.4 (C, C-5), 118.3 (CH, C-4), 98.2 (C, C-3), 67.2 (CH_2, C-11), 58.3 (C, C-10), 52.4 (CH, C-8), 32.1 (CH_2, C-9), 24.2 (CH_3, C-15) and C-16 not detected. ESI-MS: *m/z* 342 and 344 [M, 1:1] for $C_{12}H_{15}{}^{79}BrN_4O_3$.

3.1.2 Agelongine (2) [17]

Yellow residue.

UV (MeOH) λ_{max}: 217.1, 234.0 and 271.8 nm.

^{1}H-NMR (500 MHz, CD_3OD): 6.90 (1H, br s, H-2), 6.80 (1H, br s, H-4), 4.75 (2H, t, *J*=4.8 Hz, H-8), 5.03 (2H, t, *J*=4.5 Hz, H-9), 9.38 (1H, s, H-11), 8.97 (1H, d, *J*=8.0 Hz, H-13), 8.09 (1H, t, *J*=7.7 Hz, H-14) and 9.01 (1H, d, *J*=6.0 Hz, H-15). ^{13}C-NMR (125 MHz, CD_3OD): 166.7 (C, C-16), 160.4 (C, C-6), 147.4 (CH, C-11), 147.0 (CH, C-13),

146.5 (CH, C-15), 140.2 (C, C-12), 128.8 (CH, C-14), 125.6 (CH, C-2), 123.0 (C, C-5), 118.5 (CH, C-4), 98.2 (C, C-3), 63.6 (CH_2, C-8) and 61.8 (CH_2, C-9). ESI-MS: *m/z* 340 and 342 [M, 1:1] for $C_{13}H_{13}{}^{79}BrN_2O_4$.

3.1.3 (+) Manzacidin A (3) [18]

Yellow residue.

UV (MeOH) λ_{max}: 203.8 and 275.8 nm.

$[\alpha]_D^{20}$+11.6 ^{o} (*c* 0.67, MeOH).

^{1}H-NMR (600 MHz, DMSO-*d*$_6$): 12.56 (1H, br s, -N*H*-1), 7.22 (1H, br s, H-2), 6.90 (1H, br s, H-4), 4.27 (1H, d, *J*=11.0 Hz, H-8a), 4.21 (1H, d, *J*=11.0 Hz, H-8b), 2.12 (1H, dd, *J*=5.4, 14.2 Hz, H-10eq), 1.80 (1H, dd, *J*=10.7, 13.6 Hz, H-10ax), 4.36 (1H, d, *J*=11.0 Hz, H-11), 7.90 (1H, s, H-13), 9.55 (1H, br s, H-14) and 1.28 (3H, s, H-15). ^{13}C-NMR (150 MHz, CD_3OD): 174.2 (C, C-16), 160.4 (C, C-6), 151.3 (CH, C-13), 125.3 (CH, C-2), 123.4 (C, C-5), 118.3 (CH, C-4), 98.1 (C, C-3), 69.0 (CH_2, C-8), 54.0 (C, C-9), 51.6 (CH, C-11), 32.1 (CH_2, C-10) and 24.2 (CH_3, C-15). ESI-MS: *m/z* 343 and 345 [M, 1:1] for $C_{12}H_{14}{}^{79}BrN_3O_4$.

3.1.4 (-) 3-Bromomanzacidin D (N-Methylmanzacidin C) (4) [19]

Yellow residue.

UV (MeOH) λ_{max}: 218.1 and 276.3 nm.

$[\alpha]_D^{20}$ -3.4^o (*c* 0.3, $CHCl_3$).

^{1}H-NMR (600 MHz, CD_3OD): 7.04 (1H, d, *J*= 1.5 Hz, H-2), 6.85 (1H, d, *J*= 1.5 Hz, H-4), 4.58 (1H, d, *J*= 11.4 Hz, H-8a), 4.30 (1H, d, *J*= 12.3 Hz, H-8b), 2.63 (1H, dd, *J*= 5.7, 11.7 Hz, H-10eq), 1.94 (1H, dd, *J*= 11.4, 11.4 Hz, H-10ax), 4.13 (1H,dd, *J*= 5.0, 11.0 Hz, H-11), 8.00 (1H, br.s, H-13), 3.23 (3H, s, H-15) and 1.47 (3H, s, H-17). ^{13}C-NMR (150 MHz, CD_3OD): ^{13}C-NMR (150 MHz, CD_3OD): 174.3 (C, C-16), 160.7 (C, C-6), 154.0 (CH, C-13), 125.6 (CH, C-2), 123.4 (C, C-5), 118.5 (CH, C-4), 98.3 (C, C-3), 67.1 (CH_2, C-8), 58.3 (C, C-9), 52.4 (CH, C-11), 35.1 (CH_2, C-10), 36.9 (CH_3, C-15) and 21.4 (CH_3, C-17). ESI-MS: *m/z* 357 and 359 [M, 1:1] for $C_{13}H_{16}{}^{79}BrN_3O_4$.

3.1.5 (+) Dibromophakelline (5) [20]

Brown residue.

UV (MeOH) λ_{max}: 237.1 and 289.4 nm.

$[\alpha]_D^{20}$+3.0^o (*c* 0.11, MeOH).

^{1}H-NMR (500 MHz, CD_3OD): 7.01 (1H, s, H-3), 6.23 (1H, s, H-6), 2.42 (1H, m, H-11a), 2.43 (1H, m, H-11b), 2.16 (1H, m, H-12a), 2.19 (1H, m, H-12b), 3.63 (1H, m, H-13a) and 3.84 (1H, m, H-13b). ^{13}C-NMR (125 MHz, DMSO-*d*$_6$): 156.3 (C, C-15), 153.7 (C, C-8), 125.0 (C, C-4), 114.8 (CH, C-3), 106.1 (C, C-5), 102.0 (C, C-2), 82.4 (C, C-10), 68.2 (CH, C-6), 44.7 (CH_2, C-13), 38.5 (CH_2, C-11) and 19.0 (CH_2, C-12). ESI-MS: *m/z* 387, 389 and 391 [M, 1:2:1] for $C_{11}H_{11}{}^{79}Br_2N_5O$.

3.1.6 E-Debromohymenialdisine (6) [21]

Yellow amorphous powder.

UV (MeOH) λ_{max}: 212.0, 240.5 and 362.1 nm.

^{1}H-NMR (500 MHz, DMSO-*d*$_6$): 11.37 (1H, br s, -N*H*-1), 7.06 (1H,br s, H-2), 6.78 (1H, d, *J*=2.5 Hz, H-3), 7.76 (1H, br s, -N*H*-7), 3.19 (2H, t, *J*=5.1 Hz, H-8), (H-9) under solvent peak from ^{1}H-^{1}H COSY, 9.52 (2H, br s, -NH_2-14) and 10.40 (1H, br s, -N*H*-15). ESI-MS: *m/z* 245 (M) for $C_{11}H_{11}N_5O_2$.

3.1.7 Z-Spongiacidin D (syn. Axinohydantoin) (7) [22]

Yellow amorphous powder.

UV (MeOH) λ_{max}: 241.2, 259.8 and 360.1 nm.

^{1}H-NMR (500 MHz, DMSO-*d*$_6$): 12.16 (1H, br s, -N*H*-1), 7.00 (1H, s, H-3), 7.84 (1H, t, *J*=4.6 Hz, -N*H*-7), 3.19 (2H, br s, H-8), 3.19 (2H, br s, H-9), 9.58 (1H, br s, -N*H*-13) and 10.55 (1H, br s, -N*H*-15). ESI-MS: *m/z* 324 and 326 [M, 1:1] for $C_{11}H_9{}^{79}BrN_4O_3$.

3.1.8 Z-Hymenialdisine (8) [21]

Yellow amorphous powder.

UV (MeOH) λ_{max}: 210.1, 262.1 and 354.7 nm.

^{1}H-NMR (500 MHz, DMSO-*d*$_6$): 12.17 (1H, br s, -N*H*-1), 6.77 (1H, s, H-3), 7.93 (1H, t, *J*=4.3 Hz, -N*H*-7), 3.17 (2H, br s, H-8), 3.17 (2H, br s, H-9), 9.10 (2H, br s, -NH_2-14) and 10.56 (1H, br s, -N*H*-15). ESI-MS: *m/z* 323 and 325 [M, 1:1] for $C_{11}H_{10}{}^{79}BrN_5O_2$.

3.1.9 Z-3-Bromohymenialdisine (syn. Spongiacidin-A) (9) [21]

Yellow amorphous powder.

UV (MeOH) λ_{max}: 201.8, 271.6 and 330.1 nm.

^{1}H-NMR (500 MHz, DMSO-*d*$_6$): 13.06 (1H, br s, -N*H*-1), 7.87 (1H, br s, -N*H*-7), 3.18 (2H, br s, H-8), 3.18 (2H, br s, H-9), 8.81 (2H, br s, -NH_2-14) and 10.38 (1H, br s, -N*H*-15). ESI-MS: *m/z* 401, 403 and 405 [M, 1:2:1] for $C_{11}H_9{}^{79}Br_2N_5O_2$.

3.1.10 2-Debromostevensine (syn. 2-Debromoodiline) (10) [21]

Brown residue.

^{1}H-NMR (500 MHz, DMSO-d_6): 11.91 (1H, br s, -N*H*-1), 7.00 (1H, s, H-2), 8.50 (1H, t, *J*=5.7 Hz, -N*H*-7), 3.93 (2H, t, *J*=5.1 Hz, H-8), 6.16 (1H, t, *J*=4.8 Hz, H-9), 12.69 (1H, br s, -N*H*-12), 7.45 (2H, br s, -NH_2-13), 11.91 (1H, br s, -N*H*-14) and 6.87 (1H, s, H-15). ESI-MS: *m/z* 307 and 309 [M, 1:1] for $C_{11}H_{10}{}^{79}BrN_5O$.

3.1.11 (±) Ageliferin (11) [23]

Brown residue.

$[\alpha]_D^{20}$ 0^{o} (*c* 0.11, MeOH)

UV (MeOH) λ_{max}: 228.7 and 271.9 nm.

^{1}H-NMR (500 MHz, CD_3OD): 6.96 (1H, d, *J*=1.5 Hz, H-2), 6.96 (1H, d, *J*=1.5 Hz, H-2'), 6.85 (1H, d, *J*=1.3 Hz, H-4), 6.93 (1H, d, *J*=1.6 Hz, H-4'), 3.50 (1H, dd, *J*=14.5, 5.0 Hz, H-8a), 3.77 (1H, dd, *J*=14.5, 4.4 Hz, H-8b), 3.33 (1H, dd, *J*=13.6, 4.5 Hz, H-8'a), 3.64 (1H, dd, *J*=13.9, 3.2 Hz, H-8'b), 2.17 (1H, m, H-9), 2.27 (1H, m, H-9'), 3.83 (1H, d, *J*=7.3 Hz, H-10), 2.48 (1H, ddd, *J*=16.7, 7.9, 2.3 Hz, H-10'a), 2.78 (1H, ddd, *J*=16.7, 5.7, 1.5 Hz, H-10'b) and 6.79 (1H, br s, H-15). ^{13}C-NMR (125 MHz, CD_3OD): 163.2 (C, C-6), 162.9 (C, C-6'), 149.1 (C, C-13), 149.0 (C, C-13'), 127.6 (C, C-11), 127.3 (C, C-5), 127.2 (C, C-5'), 123.2 (CH, C-2), 123.1 (CH, C-2'), 122.9 (C, C-11'), 119.1 (C, C-15'), 114.3 (CH, C-4), 113.7 (CH, C-4'), 113.0 (CH, C-15), 97.7 (C, C-3), 97.5 (C, C-3'), 43.9 (CH, C-9), 42.7 (CH_2, C-8'), 40.1 (CH_2, C-8), 37.1 (CH, C-9'), 33.2 (CH, C-10) and 23.6 (CH_2, C-10'). ESI-MS: *m/z* 618, 620 and 622 [M, 1:2:1] for $C_{22}H_{24}{}^{79}Br_2N_{10}O_2$.

3.1.12 3,4-Dibromo-1H-pyrrole-2-carbamide (12) [24]

White amorphous powder.

UV (MeOH) λ_{max}: 234.0 and 276.2 nm.

^{1}H-NMR (500 MHz, DMSO-d_6): 12.62 (1H, br s, -N*H*-1), 6.90 (1H, d, *J*=2.5 Hz, H-5) and 7.58 & 7.17 (2H, br s, -NH_2). ESI-MS: *m/z* 266, 268 and 270 [M, 1:2:1] for $C_5H_4{}^{79}Br_2N_2O$.

3.1.13 Aldisine (13) [25]

Yellow residue.

UV (MeOH) λ_{max}: 220.5, 250.4 and 303.9 nm.

^{1}H-NMR (500 MHz, DMSO-d_6): 12.13 (1H, br s, -N*H*-1), 6.97 (1H, d, *J*=2.5 Hz, H-2), 6.53 (1H, d, *J*=2.5 Hz, H-3), 2.69 (2H, m, H-5), 3.34 (2H, m, H-6) and 8.30 (1H, br s, -N*H*-7). ^{13}C-NMR (125 MHz, DMSO-d_6): 194.3 (C, C-4), 162.2 (C, C-8), 127.9 (C, C-8a), 123.5 (C, C-3a), 122.3 (CH, C-2), 109.5 (CH, C-3), 43.5 (CH_2, C-6) and 36.5 (CH_2, C-5). EI-MS: *m/z* 164 (M) for $C_8H_8N_2O_2$.

3.1.14 2-Bromoaldisine (14) [25]

Yellow amorphous powder.

UV (MeOH) λ_{max}: 233.9 and 311.1 nm.

^{1}H-NMR (500 MHz, DMSO-d_6): 12.95 (1H, br s, -N*H*-1), 6.55 (1H, s, H-3), 2.69 (2H, m, H-5), 3.33 (2H, m, H-6) and 8.37 (1H, br s, -N*H*-7). ^{13}C-NMR (125 MHz, DMSO-d_6): 193.5 (C, C-4), 161.3 (C, C-8), 129.4 (C, C-8a), 124.6 (C, C-3a), 111.2 (CH, C-3), 105.2 (C, C-2), 43.4 (CH_2, C-6) and 36.3 (CH_2, C-5). EI-MS: *m/z* 242 and 244 (M, 1:1) for $C_8H_7{}^{79}BrN_2O_2$.

3.1.15 4-Bromo-1H-pyrrole-2-carbamide (15) [26-28]

Yellow residue.

UV (MeOH) λ_{max}: 232.9 and 269.9 nm.

^{1}H-NMR [500 MHz, $(CD_3)_2CO$)]: 10.90 (1H, br s, -N*H*-1), 6.84 (1H, dd, *J*=2.8, 1.5 Hz, H-3), 7.01 (1H, dd, *J*=2.5, 1.5 Hz, H-5) and 6.43 & 7.14 (2H, br s, -NH_2). ^{13}C-NMR (125 MHz, DMSO-d_6): 161.1 (C=O), 126.9 (C, C-2), 121.2 (CH, C-5), 112.0 (CH, C-3) and 94.8 (C, C-4). EI-MS: *m/z* 188 and 190 (M, 1:1) for $C_5H_5{}^{79}BrN_2O$.

3.2 Protein kinase inhibition

Protein kinases are sensitive targets for various pharmacological purposes, e.g. VEGFR-2 is an important target in cancer therapy. This kinase was inhibited by distinct brominated pyrrole derivatives from *S. carteri*: *Z*-spongiacidin D (**7**), *Z*-hymenialdisine (**8**), *Z*-3-bromohymenialdisine (**9**) as well as *E*-debromohymenialdisine (**6**) and 3,4-dibromo-1*H*-pyrrole-2-carbamide (**12**). *Z*-spongiacidin D (**7**) was shown to be the most active compound. It also inhibited AKT1, ARK5, AURORA-A, B-RAF-VE, CDK2/CycA, CDK4/CycD1, FAK, IGF1-R, SRC, VEGFR-2, COT, PLK-1, SAK and PDGFR-beta. A similar activity profile showed by *Z*-3-bromohymenialdisine (**9**), which inhibited AURORA-A, AURORA-B, CDK4/CycD1, FAK, SRC, VEGFR-2, COT, PLK1, SAK and PDGFR-beta. While, *Z*-

hymenialdisine (**8**) was able to inhibit the activity of AKT1, ARK5, CDK2-CycA, CDK4/CycD1, FAK, VEGFR-2, COT, PLK1, SAK and PDGFR-beta. But, (-) clathramide C (**1**), agelongine (**2**) (+) manzacidin A (**3**), *E*-Debromohymenialdisine (**6**) and 3,4-dibromo-1*H*-pyrrole-2-carbamide (**12**) showed only a slight protein kinase inhibition. Finally, all other compounds were not active. The protein kinase inhibitory profiles of the selected compounds are summarized in Table 1.

3.3 Cytotoxicity activity

All compounds were subjected to determine their *in-vitro* cytotoxicity employing L5178Y and HCT116 cell lines.

In the L5178Y cell lines, (+) dibromophakelline (**5**) and *Z*-3-bromohymenialdisine (**9**) showed cytotoxic activities with inhibition of growth 57.0% and 60.5%, respectively (10 µg/mL). While, the cytotoxicity of (-) clathramide C (**1**), *Z*-spongiacidin D (**7**), *Z*-hymenialdisine (**8**) and 3,4-dibromo-1*H*-pyrrole-2-carbamide (**12**), was not as prominent (growth inhibition of 25.3%, 36.7%, 37.0% and 38.4%, respectively). But, agelongine (**2**), (+) manzacidin A (**3**), (-) 3-bromomanzacidin D (**4**), *E*-debromohymenialdisine (**6**), 2-debromostevensine (**10**), (±) ageliferin (**11**), aldisine (**13**), 2-bromoaldisine (**14**) and 4-bromo-1*H*-pyrrole-2-carbamide (**15**) showed no significant cytotoxic activity in this cell lines. The results were demonstrated in Figure 2.

The cytotoxic activities were further analysed on HCT116 cell lines. In accordance with the results, which obtained in L5178Y cell lines, *Z*-3-bromohymenialdisine (**9**) and *Z*-hymenialdisine (**8**) exerted relatively high toxicity (significant effects at 25 µM after 24 h), but the other alkaloids, e.g. (+) dibromophakelline (**5**) showed no toxic effects. A relatively high toxicity was also caused by (±) ageliferin (**11**) and to a lesser extent, by *E*-debromohymenialdisine (**7**). All other compounds analyzed showed no significant cytotoxic effect up to concentrations of 50 µM. The results were illustrated in Figure 3.

4 Discussion

Marine sponges are of great pharmacological interest due to the diversity of their secondary metabolites. Although, the molecular mode of action of the most metabolites is still unclear, for a substantial number of compounds the mechanisms

by which they interfere with the pathogenesis of a wide range of diseases has been reported. Distinct metabolites possess antiviral, antitumor, anti-inflammatory, anti-oxidative, antibiotic or immunosuppressive activity. Due to these important biological activities, sponges have the potential to provide future drugs against diseases like cancer, malaria and inflammatory diseases [2,29].

This study was performed for the first time on the inhibitory effects of the compounds using 21 important protein kinases *in-vitro*. Despite the molecular similarity of distinct compounds (only marginal changes in structure), the effects of the compounds on protein kinase inhibition remained to be strongly dependent on the distinct structure. *Z*-spongiacidin D (**7**) exhibited the highest capacity to inhibit protein kinases. A potent inhibition of ARK5, CDK2/CycA, CDK4/CycD1, VEGFR-2, SAK and PDGFR-beta was detectable by *Z*-spongiacidin D (**7**). The structurally related, dibromonated compound *Z*-3-bromohymenialdisine (**9**) was also very potent, but with a slight different inhibition pattern of compound *Z*-spongiacidin D (**7**). The dibrominated compound *Z*-3-bromohymenialdisine (**9**), no inhibition of AKT1, ARK5, B-RAF-VE, CDK2/CycA and IGF1-R was detectable, but in case of AURORA-A and FAK a higher inhibitory effect was seen. The compounds *Z*-hymenialdisine (**8**) and *E*-debromohymenialdisine (**6**) were structurally similar to the previously mentioned compounds, but less potent. Since, *E*-debromohymenialdisine (**6**) is a non-brominated compound, but also possessed protein kinase inhibitory activity, it has to be concluded that this structural element is no necessity.

Protein kinase inhibition by hymenialdisine was already reported [4], especially kinases of the cell cycle seems to be affected. Also, it interacted with CDC2-Like Kinase 1 [30] and showed CHK1-modulatory effects of hymenialdisine analogues [31]. Moreover, it displayed an inhibition of checkpoint kinase by a hymenialdisine-derived analogue [32]. This was congruent with our finding of the inhibition of CDK2/CycA and CDK4/CycD1.

The effect of hymenialdisine/hymenialdisine analogues on 60 recombinant kinases was investigated systematically [33]. They reported eleven new targets like p90RSK, KDR, c-Kit, Fes, MAPK1, PAK2, PDK1, PKCθ, PKD2, Rsk1 and SGK. They also reported these analogues enhanced and/or dramatically altered selectivity relative to hymenialdisine. This result was also congruent to our findings; *Z*-spongiacidin D (**7**) and *Z*-3-bromohymenialdisine (**9**) showed a higher activity compared with *Z*-hymenialdisine (**8**). Our study reported an inhibition of ARK5 by *Z*-hymenialdisine, as well as VEGFR-2 and a weaker effect of this compound to inhibit

AKT1, CDK2/CycA, CDK4/CycD1, FAK, COT, PLK1, SAK and PDGFR-beta. To the best of our knowledge, no report about e.g. inhibition of AURORA-A and AURORA-B by hymenialdisine/hymenialdisine analogues exists in the literature.

This study was further performed to check the effect of protein kinases on the viability of tumor cells. In L5178Y cell lines, the potent compounds (+) dibromophakelline (**5**) and *Z*-3-bromohymenialdisine (**9**) showed a great inhibition of cell growth. While, *Z*-spongiacidin D (**7**), *Z*-hymenialdisine (**8**) and 3,4-Dibromo-1*H*-pyrrole-2-carbamide (**12**) also showed a moderate inhibition of cell growth. While, the most of compounds were not or only marginaly toxic. The dibrominated compounds *viz*., (+) Dibromophakelline (**5**) and *Z*-3-bromohymenialdisine (**9**) showed a comparable high toxicity. While, the dibrominated compound (±) ageliferin (**11**) showed very weak toxicity. This indicates that no structural activity relationship between brome atoms in these alkaloids and cytotoxicity.

On the other side, *Z*-hymenialdisine (**8**), *Z*-3-bromohymenialdisine (**9**) and (±) ageliferin (**11**) showed the highest cytotoxic effect on HCT116 cell lines. Consequently, this study demonstrated that the monobrominated compound *Z*-hymenialdisine (**8**) and the dibrominated compound *Z*-3-bromohymenialdisine (**9**) had cytotoxic effects against both of these cell lines. To the best of our knowledge, no reported toxicity of ageliferin existed in the literature. No correlation could be drawn to the kinase inhibition experiments and the assays concerning cytotoxicity.

5 Conclusion

Fifteen compounds, mostly monobrominated pyrrole alkaloids were isolated from *S. carteri* had protein kinase inhibitory activity and cytotoxicity. The most potent compounds were *Z*-spongiacidin D (**7**), *Z*-hymenialdisine (**8**) and *Z*-3-bromohymenialdisine (**9**). From the results of protein kinase inhibition and cytotoxicity displayed a good correlation, as distinct brominated pyrrole alkaloids effectively inhibited distinct protein kinases followed by a toxic mode of action. These compounds may be interesting for a potential use as pharmacological drugs. In conclusion, distinct pyrrole alkaloids isolated from *S. carteri* effectively inhibited distinct protein kinases *in-vitro* suggesting a potential use as pharmacological drugs.

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Conflict of interest statement: The authors report no declarations of interest.

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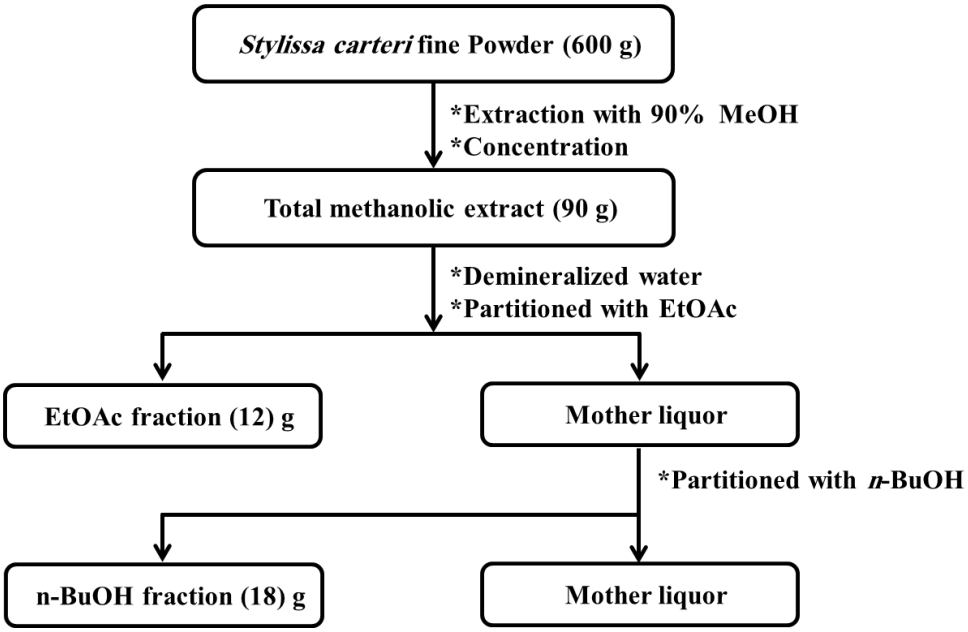
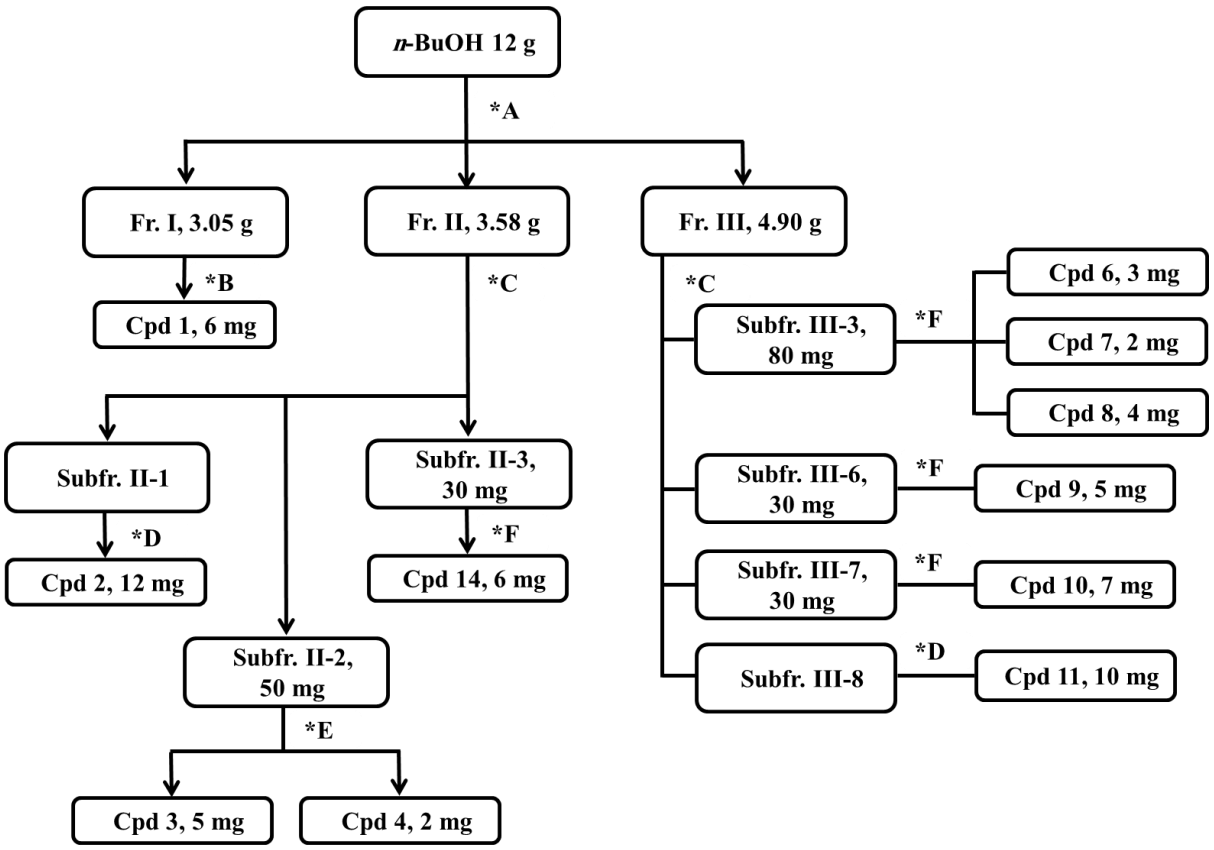
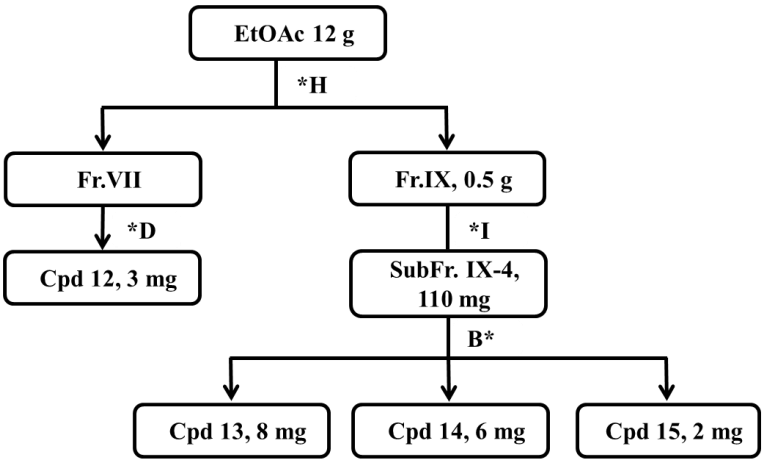


Scheme 1a. Extraction and fractionation of the total methanol extract of *S. carteri* sponge.



Scheme 1b. Chromatographic fractionation of *n*-butanol fraction of *S. carteri* sponge.



Scheme 1c. Chromatographic fractionation of EtOAc fraction of *S. carteri* sponge.

The chromatographic techniques abbreviations in the three schemes:
***A**: Column chromatography on sephadex LH-20 using MeOH as mobile phase.
***B**: Column chromatography on RP-18 using acetonitrile as a mobile phase.
***C**: MPLC on RP-18 using H_2O/MeOH gradient elution.
***D**: Precipitation by time.
***E**: Column chromatography on RP-18 using acetonitrile/H_2O (3:7) as a mobile phase.
***F**: Semi-preparative HPLC on RP-18 using nanopure H_2O/MeOH gradient elution.
***G**: MPLC on RP-18 using H_2O/MeOH (7.5:2.5) as a mobile phase.
***H**: VLC on silica gel, using gradient elution consisting of different portions of *n*-Hexane/CH_2Cl_2 to CH_2Cl_2/MeOH.
***I**: Column chromatography on Silica gel using CH_2Cl_2/MeOH gradient elution.

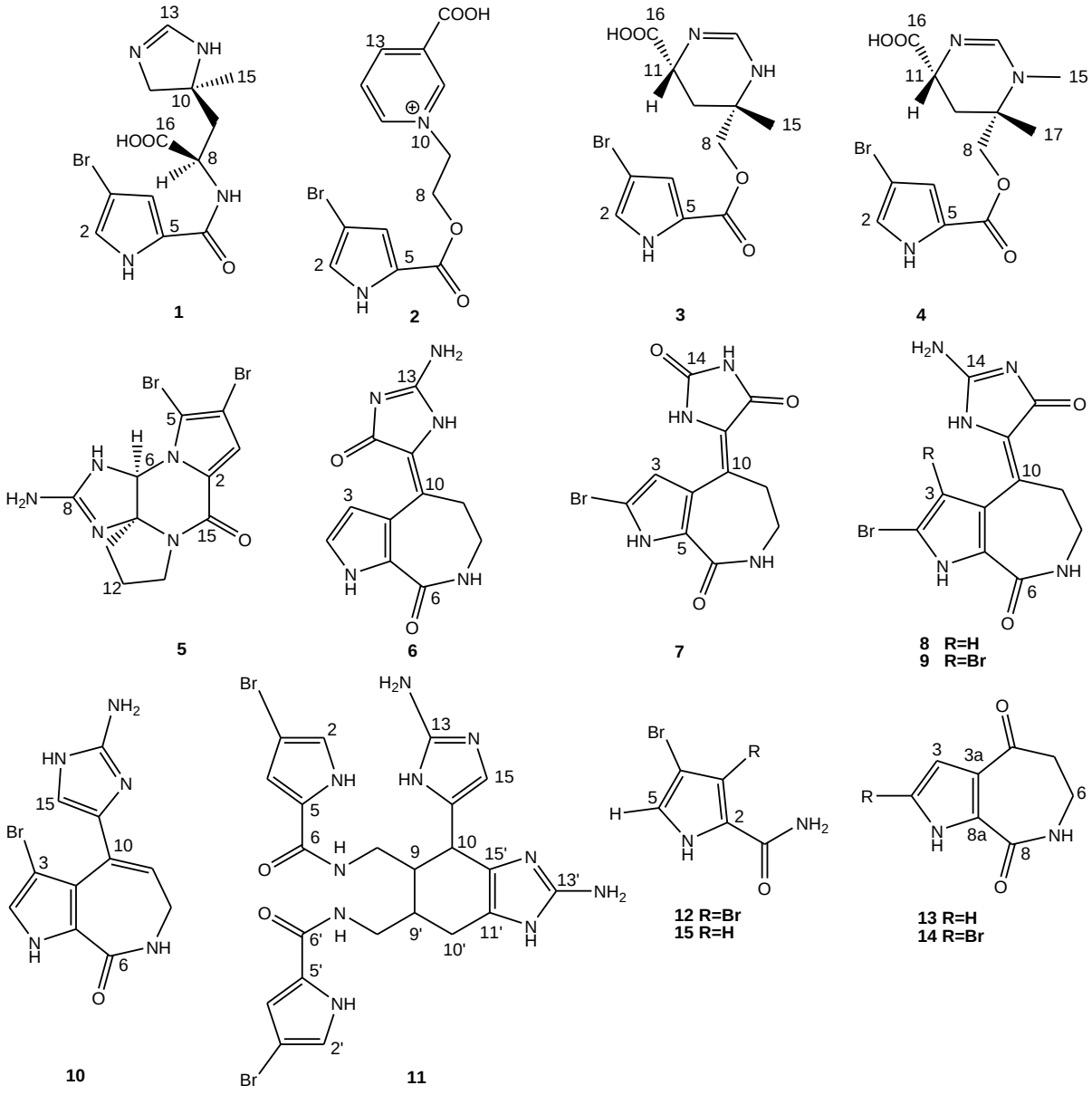


Figure 1: Chemical structures of the isolated compounds from *S. carteri* sponge.

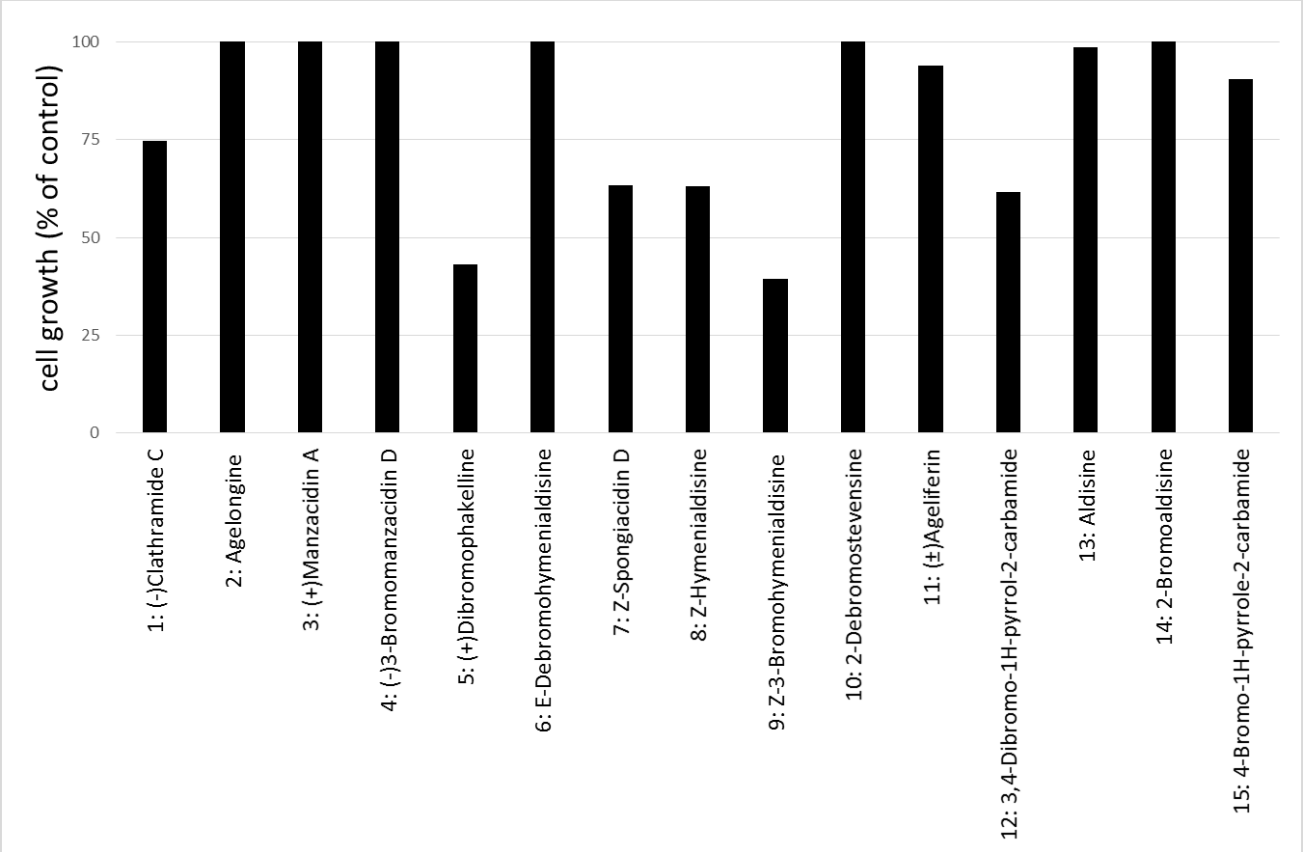


Figure 2: Cytotoxicity of the isolated compounds in L5178Y lymphoma cells from *S. carteri* sponge.

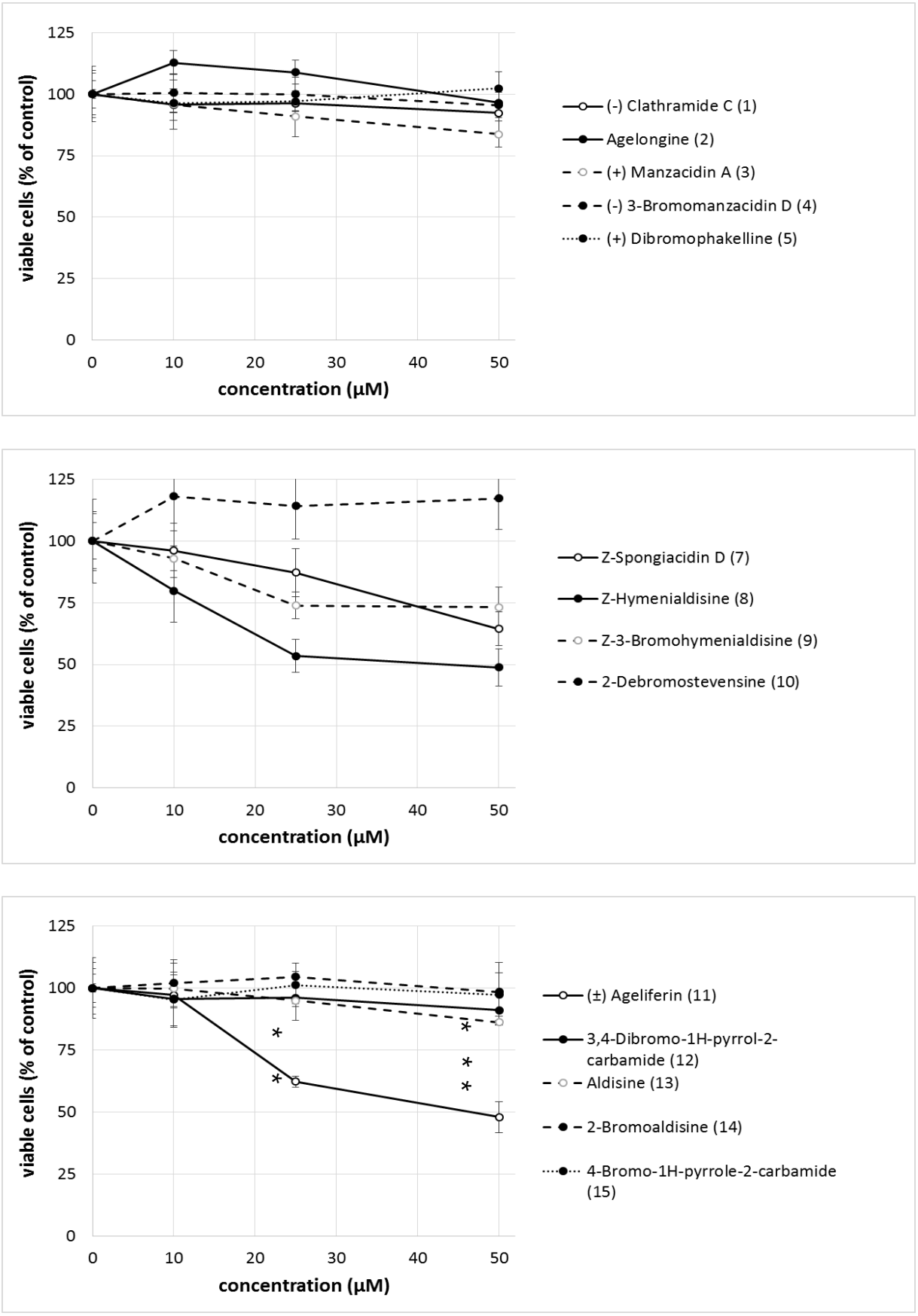


Figure 3: Cytotoxicity of the isolated compounds in HCT116 colon carcinoma cells from *S. carteri* sponge.

Table 1. Inhibition of protein kinase activity of the isolated compounds from *S. carteri* sponge.

Compound/Protein kinase	AKT1	ARK5	AURORA-A	AURORA-B	B-RAF-VE	CDK2-CycA	CDK4/CycD1	CK2-alpha1	EGFR	EPHB4	ERRB2	FAK	IGF1-R	SRC	VEGF-R2	COT	PLK1	SAK	INS-R	MET	PDGFR-beta
(-) Clathramide C (**1**)	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Agelongine (**2**)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(+) Manzacidin A (**3**)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E-Debromohymenialdisine (**6**)	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-	+
Z-Spongiacidin D (**7**)	+	++	+	-	+	++	++	-	-	-	-	+	+	+	++	+	+	++	-	-	++
Z-Hymenialdisine (**8**)	+	++	-	-	-	+	+	-	-	-	-	+	-	-	++	+	+	+	-	-	+
Z-3-Bromohymenialdisine (**9**)	-	-	++	+	-	-	++	-	-	-	-	++	-	+	++	+	+	++	-	-	++
3,4-Dibromo-1*H*-pyrrole-2-carbamide (**12**)	-	-	-	+	-	-	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-

++: Residual kinase activity > 20 % and ≤ 60 % . +: Residual kinase activity > 60 % and ≤ 80 %. -: Residual kinase activity > 80 %.
Activity on various protein kinases based on IC_{50} (µg/mL), Compound tested (concentration: 1 µg/mL).