Functionalized Alkylated Pyridines from the Red Sea Sponge *Callyspongia crassa* (Porifera, Callyspongiidae) as Antitumor Agents

Hajer Saeed Alorfi
Department of Chemistry, Faculty of Science, King Abdulaziz University, PO. Box 80203, Jeddah 21589, Saudi Arabia.
halorfi@kau.edu.sa*

(Received on 9th June 2017, accepted in revised form 26th May 2018)

Summary: The chloroform/methanol extract of the Red Sea sponge *Callyspongia crassa* has been subjected to fractionation and purification employing several chromatographic techniques including column and repeated preparative thin layer chromatography. Three new amino alkyl pyridine alkaloids, callyspongine A (**1**), B (**2**), and C (**3**) along with two known steroids, 24-methylenecholesterol (**4**) and 24-methylcholesterol (**5**) were isolated. Elucidation of chemical structures was achieved after extensive analysis of their spectroscopic data (1D and 2D NMR, UV, IR and MS) and comparison with reported compounds from the literature. Cytotoxicity of compounds **1**-**3** were tested against MCF-7 cell line. Interestingly, all compounds (**1**-**3**) showed good cytotoxic activity with IC_{50} ranged from 83.0 ± 0.074 to 110.9 ± 0.718 µM, employing lactate dehydrogenase (LDH) assay.

Keywords: Aminoalkyl pyridine alkaloids, Antimicrobial, Cytotoxicity, Marine sponges, *Callyspongia crassa*

Introduction

Marine sponges continue to be a hot spot for much of natural product researches. They are filter-feeding organisms and they suffer from the lack of natural defense. Therefore, they develop bioactive metabolites from marine environment [1]. Most discovered marine sponges have been reported as remarkable sources of bioactive compounds. In cancer research, natural compounds originated from marine sources are a rich story of aspiration to pharmacologists; e.g. eribulinmesylate, cytarabine and trabectedin with proven anticancer activity [2, 3]. Compounds that contain basic Nitrogen atom(s) are known as alkaloids. Alkaloids originated from marine organisms could be helpful against several diseases including cancer [4].

Natural pyridines were originally isolated from plants and later from insects, amphibians and marine animals, with astonishing roles in defense and as pheromones [5, 6]. Recently, the discovery and isolation of pyridine-based alkaloids from marine organisms have increased steadily [4]. The role of plants producing pyridine derivatives in folk medicine directed the attention of chemists towards studying the structure-activity relationship of these pyridine-containing compounds. Alkylated pyridine alkaloids, especially those having a long functionalized (NO_2, NH_2, NH_2O, C=C, alkyne,) chain alkyl at C-3 of the pyridine ring, are abundantly isolated from marine sponges of the order Haplosclerida, which allowed us to consider 3-alkyl pyridine compounds as chemotaxonomic markers for the systematic identification of haplosclerid members [6]. With broad spectrum of biological effects, including cytotoxicity, antibacterial, ichthyotoxicity, antifouling paints addition and enzyme inhibition [6-10], 3-alkylpyridine compounds retain their importance as target compounds for investigation and identification from their natural sources, aiming at isolation of new structures and/or new biological activity (s) [4-6].

Herein, a Red Sea sponge *Callyspongia crassa* (Order: Haplosclerida; Family: Callyspongiidae) was collected off the coast near Jeddah, and was investigated to identify its chemical content and further to discover antitumor activity related with these compound.

Experimental

General

Silica gel GF 254 (Merck, Darmstadt, Germany) was used for analytical thin layer chromatography (TLC). Preparative thin layer chromatography (PTLC) was performed on aluminum oxide plates (20 x 20 cm) of 250 µm thickness. Electron impact mass spectra were determined at 70 ev on a Kratos (Manchester, UK) MS-25 instrument. 1D and 2D NMR spectra were recorded by using Bruker (Karlsruhe, Germany) AVANCE II WM 400 MHz spectrometers and ^{13}C NMR at 100 MHz.Tetramethylsilane (TMS) was used as internal standard. Plates were sprayed with Dragendorff's reagent and with 50%-sulphuric acid in methanol and heated at 100 °C for 1-2 minutes.

*To whom all correspondence should be addressed.

Collection and processing of Callyspongia crassa

The marine sponge *Callyspongia crassa* had been collected using scuba diving at a depth of 10 to 20 m in November 2015, off the Red Sea Coast at Jeddah, Saudi Arabia (21°42′ 22.03″ N; 39°10′ 50.90″ E), and was identified by Prof. Dr. Mohsen El-Sherbiny (Faculty of Marine Sciences, King Abdulaziz University, Jeddah, Saudi Arabia). The collected animal was immediately frozen and lyophilized (VIRITIS Benchtop 4K Series, Model BT4KZL-105) until dryness and then extracted. A voucher specimen (SC-2015-17A) was deposited in the Faculty of Marine Sciences, KAU.

About 148.0 g of the dried sponge material was extracted three times by an equal mixture of $CHCl_3$ and MeOH at room temperature and the obtained extract was concentrated under vacuum to yield a brown gummy material (30.0 g).

Isolation and identification of compounds

The residue was homogenized with silica gel and then subjected to chromatography on a Si-gel open column (500 g, 75 × 2 cm) for separation of the chemical constituents; *n*-hexane was used as an eluent and the polarity was elevated by increasing amounts of EtOAc and then elution with $CHCl_3$ was elevated by increasing amounts of MeOH. Fractions of 50 ml were collected. The course of fractionation was monitored by TLC with the aid of both UV light and spray reagent. Preparative TLC (PTLC) was used to isolate individual compounds.

Callyspongine A (**1**)

The fraction eluted by $CHCl_3$: MeOH (8.5:1.5, v/v) (R_f= 0.2, 15.1 mg, based on dry wt.) was subjected to PTLC using a mixture of $CHCl_3$/MeOH (8.5:1.5). The band with R_f = 0.17 (orange spot with Dragendorff's reagent and brownish color with sulfuric acid raegent) gave a white powder (1.4 mg); mp 121-122 °C; $[\alpha]_D$ = +15.4 (MeOH; *c* = 0.02); UV λ_{max} (MeOH) 207, 257, 263, 268 nm; IR υ_{max} (neat) 3430, 3398, 1610, 1450, 812 cm^{-1}; HRESI-MS data *m/z* 292.2498 $[M]^+$ (calculated for $C_{18}H_{32}N_2O$ 292.2515); ^1H and ^{13}C NMR spectral data (Table-1).

Callyspongine B (**2**)

The fraction eluted by $CHCl_3$: MeOH (19: 1, v/v) (R_f= 0.27, 12.9 mg, based on dry wt.) was subjected to PTLC using a mixture of $CHCl_3$/MeOH (9.0:1.0). The band with R_f = 0.25 (orange spot with Dragendorff's reagent and brownish color with sulfuric acid raegent) gave an oily material (2.3 mg) $[\alpha]_D$ = +3.5 (MeOH; *c* = 0.02); λ_{max} (MeOH) 207, 259, 263, 267 nm; IR υ_{max} (neat) 3450, 3386, 1610, 1450, 812 cm^{-1}; HRESI-MS data *m/z*290.2704 $[M]^+$ (calculated for $C_{19}H_{34}N_2$ 290.2722); ; ^1H and ^{13}C NMR spectral data (Table-1).

Callyspongine C (**3**)

The fraction eluted by $CHCl_3$: MeOH (9.0:1.0, v/v) (R_f= 0.22, 8.1 mg, based on dry wt.) was subjected to PTLC using a mixture of $CHCl_3$/MeOH (8.5:1.5). The band with R_f = 0.35 (orange spot with Dragendorff's reagent and brownish color with sulfuric acid raegent) gave a white powder (1.3 mg); mp 99-101 °C; $[\alpha]_D$ = +19.4 (MeOH; *c* = 0.02); λ_{max} (MeOH) 207, 257, 263, 268 nm; IR υ_{max} (neat) 3430, 3398, 1611, 1452, 812 cm^{-1}; HRESI-MS data *m/z* 306.2653 $[M]^+$ (calculated for $C_{19}H_{34}N_2O$ 306.2671); ^1H and ^{13}C NMR spectral data (Table-1).

24-methylenecholesterol (**4**)

The fraction eluted by *n*-hexane: EtOAc (9.0:1.0, v/v) (R_f= 0.40, 15.0 mg) was subjected to PTLC using a mixture of *n*-hexane: EtOAc (9.0:1.0). The band with R_f = 0.36 (Reddish-brown spots with 50% sulfuric acid-methanol reagent) gave a white powder (3.9 mg). Compound **4** was identified by comparison of its spectral data with those in the literature [11]

24-methylcholesterol (**5**)

The fraction eluted by *n*-hexane: EtOAc (9.0:1.0, v/v) (R_f= 0.47, 15.0 mg) was subjected to PTLC using a mixture of *n*-hexane: EtOAc (9.0:1.0). The band with R_f = 0.43 (orange spots with 50% sulfuric acid-methanol reagent) gave a white powder (3.9 mg). Compound **5** was identified by comparison of its spectral data with those in the literature [11]

Biological activities

Cell line and culture conditions

The non-aggressive human mammary epithelial breast cancer cell line MCF-7 was purchased from VACSERA (Cairo, Egypt) and cultured in RPMI (Roswell Park Memorial Institute; Buffalo, New York, USA) medium supplemented with 100 µg/mL penicillin-streptomycin, 2.5 µg/mL fungizone, 10% heat-activated foetal calf serum, and 2 mM glutamine. Cells were allowed to grow at 37°C in a humidified atmosphere of 5% CO_2 and 95% air to form a monolayer. At 60 - 70% confluence, cells were subcultured; first they were washed with phosphate-buffered saline (PBS), then trypsinized with 3 mL of 0.25% trypsin in 0.03% EDTA, then washed with fresh medium and seeded at 1 x 10^4 cells/well in a 96-well microplate [12].

Table-1: ^{1}H and ^{13}C- NMR spectral data of **1-3**[a, b.]

	1		**2**		**3**	
Position	**δ(H)**	**δ(C)**	**δ(H)**	**δ(C)**	**δ(H)**	**δ(C)**
2	**9.15 (1H, s)**	**146.1**	**9.10 (1H, s)**	**146.2**	**9.08 (1H, s)**	**146.1**
3		**139.2**		**139.5**		**139.2**
4	**8.71 (1H, d, J= 4.8 Hz)**	**144.6**	**8.73 (1H, d, J= 5.4 Hz)**	**144.6**	**8.70 (1H, d, J= 4.8 Hz)**	**144.6**
5	**7.93 (1H, *d*, *J*= 6.8, 5.4 Hz)**	**127.2**	**7.93 (1H, *d*, *J*= 6.4, 5.4 Hz)**	**127.2**	**7.90 (1H, *d*, *J*= 6.4, 5.4 Hz)**	**1270**
6	**8.85 (1H, *d*, *J*= 8.0 Hz)**	**145.0**	**8.85 (1H, *d*, *J*= 6.4 Hz)**	**145.1**	**8.87 (1H, *d*, *J*= 8.0 Hz)**	**144.9**
7	**2.91 (2H, m)**	**33.6**	**2.67 (2H, m)**	**33.5**	**4.73 (1H, t, J= 7.2 Hz)**	**75.3**
8	**1.21-1.50 (2H, m)**	**28.1-31.9**	**1.21-1.50 (2H, m)**	**28.1-31.9**	**1.88 (2H, m)**	**22.1**
9-16	**1.21-1.50 (16H, m, H-9-H-16)**	**28.1-31.9**	**1.21-1.50 (16H, m, H-9-H-16)**	**28.1-31.9**	**1.21-1.50 (16H, m, H-9-H-16)**	**28.1-31.9**
17	**1.87 (1H, m, H-17)**	**31.4**	**1.21-1.50 (2H, m)**	**28.1-31.9**	**1.21-1.50 (2H, m)**	**28.1-31.9**
18	**2.93 (2H, *m*)**	**43.5**	**1.21-1.50 (2H, m)**	**28.1-31.9**	**1.21-1.50 (2H, m)**	**28.1-31.9**
19	**3.68 (1H, m, Ha) 3,53 (1H, dd, J= 14.0, 7.2 Hz, Hb)**	**66.6**	**1.91 (2H,m)**	**30.0**	**1.91 (2H, m)**	**31.5**
20			**2.90 (2H, m)**	**41.6**	**2.92 (2H, m)**	**41.5**

[a]) The solvent is $CDCl_3$. [b]) All assignments are based on 1D and 2D measurements (HMBC, HSQC, COSY

The reagent kit for the assay of LDH **(**lactate dehydrogenase) was purchased from Biorex Diagnostics (Antrim, UK). Methotrexate, 5-FU, and cisplatin were kindly supplied as a gift from the Oncology Center, Mansoura University, Mansoura, Egypt.

Table-2: IC_{50} [μM] of compounds **1**- **3** on MCF-7 by LDH Assay, after 72 h.

Tested compound	**IC_{50} [μM][a]**	**Positive control**	**IC_{50} [μM][a]**
1	**83.0 ± 0.074**	**Cisplatin**	**59.0 ± 0.045**
2	**110.9 ± 0.718**		
3	**96.4± 0.092**		

[a]Data shown are the mean ± SD of three experiments. The means were significantly different across the samples

Cell treatment

MCF-7 cells were treated with the isolated compounds at concentrations from 0 - 250 μg/mL. All chemicals were dissolved in RPMI medium and filtered through a membrane filter (0.2 μm) before cell treatment. LDH activities were measured at 1, 24, 48, and 72 h.

Cytotoxicity assay

Cytotoxicity was evaluated through monitoring the release of LDH into the medium. Fifty μl of the supernatant were drawn off from each cell culture well and assayed for LDH activity by measuring the absorbance at 340 nm [13].

Results and discussion

The sequential application of the chromatographic techniques including sephadex LH-20 and Si gel column and preparative thin layer chromatography of the chloroform-methanol extract of the Red Sea marine sponge *Callyspongia crassa* afforded three new functionalized alkylated pyridines**1, 2** and **3**, in 0.001%, 0.002% and 0.001% yield (based on dry weight of the sponge material), respectively, in addition to two known C-28 steroids, 24-methylenecholesterol (**3**) and 24-methylcholesterol (**4**) (Fig. 1).

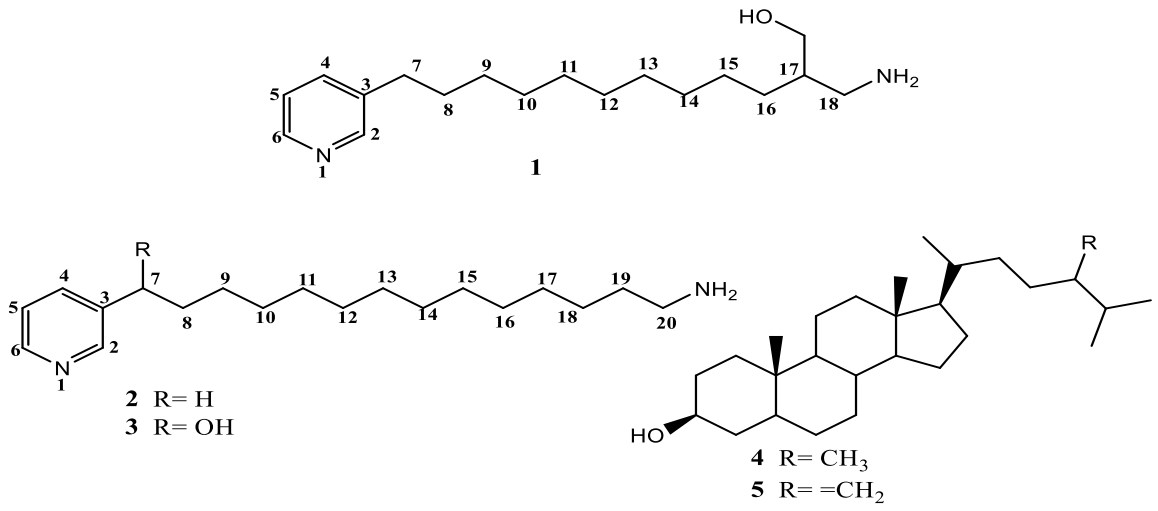

Fig. 1: Compounds isolated from *C. crassa*.

Compound **1** was isolated as a white powder material. The molecular formula was established as, $C_{18}H_{32}N_2O$, by HREIMS (*m*/*z* 292.2498), requires four degrees of unsaturation. The compound displayed a set of absorption bands at 207, 257, 263 and 268 nm in the UV spectrum, interpreted for aromatic compound. The IR spectrum assigned the presence of hydroxyl function, primary amine and pyridine ring [14] due to the presence of absorptions at 3430, 3398, 1610, 1450 and 812 cm^{-1}, respectively. ^{1}HNMR spectrum showed the presence of four aromatic protons resonating at δ_H 9.15 (s), 8.85 (d, *J*= 8.0 Hz), 8.71 (d, *J*= 4.8 Hz), and 7.93 (dd, *J*= 6.8, 5.4 Hz) ppm. The downfield chemical shift (δ_H) values in the aromatic region and the presence of one more nitrogen atom, together with the low and low coupling constant (*J*) values between the aromatic protons could refer to the presence of pyridine ring. The presence of five aromatic carbon signals in the ^{13}C NMR spectrum resonating at 146.1, 145.0, 144.6, 139.2 and 127.2 ppm, strengthen the previous conclusion that **1** is a pyridine-ring containing compound. Moreover, the aforementioned discussed multiplicities in the ^{1}H NMR data clearly indicate the presence of 3-substituted pyridine moiety (Table-1). The DEPT (Distortionless enhancement by polarization transfer) and ^{13}C NMR spectra indicated the presence of only one quaternary carbon atom resonating at δ_C 139.2 ppm, a number of signals due to methylene functions including two signals appeared at δ_H/δ_C2.93 (t, *J*= 7.2 Hz)/ 43.5 and at δ_H/δ_C3.68 (m) and 3.53 (dd, 14.0, 7.2 Hz) / 66.6, and no signal due to methyl function was observed. HSQC (Heteronuclear single quantum coherence) spectrum has been employed to associate all protons and carbons.^{1}H-^{1}H COSY (Correlation spectroscopy) spectrum showed the presence of two proton sequences: three protons representing a monosubstituted pyridine ring, where the proton at 7.93 (1H, dd) was correlated with both aromatic protons resonating at 8.85 (1H, d) and 8.71 (1H, d), this was first; the second one, represents a large sequence of protons started with an amino-bearing methylene protons resonating at δ_H 2.93 which is correlated with proton resonating at δ_H 1.87 as well as this proton is correlated with the oxygenated methylene protons at δ_H 3.68 and 3.53 and another methylene protons at δ_H 1.22-1.50. From the previous discussion and owing to no more unsaturation degrees, the compound should be 3-alkylated pyridine. The length of the side chain was determined from the molecular formula by subtracting of C_5H_4N to give $C_{13}H_{28}NO$. The HMBC (Heteronuclear multiple bond correlation) spectrum confirms the presence of 3-alkylpyridine and also showed the attachment of both the amino and the carbinol functions to the same methine group, hence the side chain is 2-hydroxymethylaminotrdecanyl group. The trivial name callyspongineA was given to compound **1 (**Fig. 2**)**.

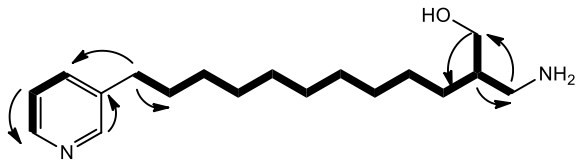

Fig. 2: Selected H-H COSY (▬) and HMBC correlations (↷) of compound **1**.

Compound **2** was isolated as an oily material. The molecular formula was established as,

$C_{19}H_{34}N_2$, by HREIMS (*m/z* 290.2704), requires four degrees of unsaturation. The compound displayed an absorption bands at 206, 258, 262, 268 nm, interpreted for aromatic compound. The IR spectrum assigned the presence of primary amine due to the presence of absorptions at 3390, 1608, 1445 and 812 cm^{-1}, supported by the absence of oxygen atom in the obtained molecular formula.^{1}HNMR spectrum showed the presence of four aromatic protons resonating at δ_H 9.10 (s), 8.87 (d, *J*= 6.4), 8.73 (d,*J*= 5.4), and 7.90 (dd, *J*= 6.4, 5.4) ppm. The downfield chemical shift (δ_H) values in the aromatic region and the presence of one more nitrogen atom, together with the low and coupling constant (*J*) values between the aromatic protons could refer to the presence of pyridine ring. The presence of five aromatic carbon signalsin the ^{13}C NMR spectrum resonating at 146.2, 145.1, 144.6, 139.5 and 127.3 ppm, strengthen the previous conclusion, that **2** is a pyridine-ring containing compound. Moreover, the aforementioned discussed multiplicities in the ^{1}H NMR spectrum clearly indicates the presence of 3-substituted pyridine moiety (Table 1). The DEPT and ^{13}C NMR spectra indicated the presence of only one quaternary carbon atom resonating at δ_C 139.2 ppm, a number of signals due to methylene functions including a signal appeared atδ_H/δ_C 2.90 (t, *J*= 7.2 Hz)/ 41.6 and no signal due to methyl function was observed. HSQC spectrum has been employed to associate all protons and carbons. ^{1}H-^{1}H COSY spectrum showed the presence of two proton sequences: three protons representing a monosubstituted pyridine ring, where the proton at 7.90 (1H, dd) is correlated with both aromatic protons resonating at 8.85 (1H, d) and 8.73 (1H, d), this was first, the second one represents a huge envelop of methylene protons started with a amino-bearing methylene. From the previous discussion and owing to no more unsaturation degrees, the compound should be 3-alkylated pyridine. The length of the side chain was determined from the molecular formula by subtracting of C_5H_4N to give $C_{14}H_{30}N$. Since **2** showed neither methine signals in ^{1}H NMR spectrum nor upward signals in the aliphatic region in the DEPT spectrum, Hence, the side-chain can be expressed as 1-amino tetradecylmoity. The HMBC spectrum undoubtedly assigned the location of the amino alkyl group at position 3, which achieved from the correlation between the methylene protons signal resonating at δ_H 2.67 and the carbon signals at 28.1 (C-8), 139.2 (C-3), 144.6 (C-4), and 146.1 (C-2). The trivial name callyspongine B was given to compound **2**.

Compound **3** was isolated as a white powder material. The molecular formula was established as, $C_{19}H_{34}N_2O$, by HREIMS (*m/z* 306.2653), requires four degrees of unsaturation. The compound displayed an absorption bands at 207, 259, 263 and 267 nm, interpreted for aromatic compound. The IR spectrum displayed absorptions due to primary amine, hydroxyl function, pyridine ring (3450, 3386, 1611, 1452 and 812 cm^{-1}) functionalities. Examining the ^{1}H,^{13}C, DEPT, HSQC and ^{1}H-^{1}H NMR spectral data of compound **3** showed similar features of compound **2** such as the presence of 3- substituted pyridine ring, amino group and C_{14} long chain hydrocarbon moietyin addition to the appearance of an absorption signal at δ_H/δ_C 4.73 (t, *J*= 7.2 Hz)/75.3. Such signal was deduced to be due to hydroxyl function owing to the absence of more unsaturation sites, no absorptions of carbonyl group or etheric linkage. The location of the hydroxyl group at C-7 was indicated by interpretation of its HMBC spectrum, where the proton resonating at 4.73 is correlated with the carbon signals at 22.1 (C-8), 139.2 (C-3), 144.6 (C-4), and 146.1 (C-2). The trivial name callyspongine C was given to compound **3**.

Compounds **4** and **5** were identified as 24 -methylenecholesterol and 24-methylcholesterol, respectively by comparing their spectral data with those published in literature [11].

Cytotoxicity activity of compounds **1-3** was tested against MCF-7 cell line. All compounds (**1-3**) showed good cytotoxic activity (Table-1). Compound **1** showed the highest cytotoxic activity with IC_{50} 83.0 ± 0.074 µM, then compound **3** 96.4 ± 0.092µM, and finally compound **2** displayed the lowest activity with 110.9 ± 0.718 µM, compared to cisplatin as the reference standard material IC_{50} 59.0 ± 0.045 µM. employing lactate dehydrogenase (LDH) assay.

Conclusion

The obtained results indicated the separation and identification of three new aminoalkyl pyridine alkaloids from the Red Sea sponge *Callyspongia crassa*. These compounds represent a major and biologically active class of alkaloids, the pyridine derivatives. The isolated metabolites displayed significant cytotoxic activity against human breast adrenocarcinoma cells (MCF-7) employing lactate dehydrogenase (LDH) assay. The current results could be used as a platform for either further investigation of this promising sponge material aiming at isolation of more active metabolites, or synthesis of similar derivatives for more potency of the biological assay.

Acknowledgments

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (G-254-247-38). The author, therefore, acknowledge with thank DSR for the technical and financial support. Also, the author would like to thank Prof. Mohsen El-Sherbiny for helping in sponge identification.

References

1. S. Kalimuth, J. Venkatesan and S. K. Kim, Marine derived bioactive compounds for breast and prostate cancer treatment: A review, *Current bioactive compounds*, **10**, 62 (2014).
2. S. Kalimuth, J. Venkatesan and S. K. Kim, Marine compounds and cancer treatment: 2017 updates, *Marine drugs*, **16**, 40 (2018).
3. J. Sperlich, R. Kerr and N. Teusch, The marine natural product pseudopterosin blocks cytokine release of triple-negative breast cancer and monocytic leukemia cells by inhibiting NF-κB signaling. *Marine Drugs*, **15**, 262 (2017).
4. K. S. Singh, and Majik, Bioactive Alkaloids from Marine Sponges, In Marine sponges: Chemicobiological and biomedical applications. ed. by: Pallela, R.; Ehrlich, H.Springer; New Delhi; India; 257 (2016).
5. M. J. Schneider, Pyridine and piperidine alkaloids. Alkaloids, *Chemical and Biological Perspectives*, **10**, 155 (1996).
6. T. Turk, K. Sepčić, I. Mancini and G. Guella, 3-Akylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use, *Studies in natural products chemistry*, **35**, 355 (2008).
7. G. Y. S. Wang, M. Kuramoto, D. Uemura, A. Yamada, K. Yamaguchi and K. Yazawa, Three novel anti-microfoulingnitroalkyl pyridine alkaloids from the okinawan marine sponge *Callyspongia* sp., *Tetrahedron Lett.*, , **37**, 1813 (1996).
8. S. B. Khan, H. Azhar ul, S. Perveen, N. Afza, A. Malik, S. A. Nawaz, M. R. Shah, M. I. Choudhary, Butyrylcholinesterase inhibitory guaianolides from *Amberboa ramosa*. *Arch. Pharmacal. Res***, 28** , 172 (2005).
9. F. Plisson, P. Prasad, X. Xiao, A. M. Piggott, X. C. Huang, Z. Khalil and R. J. Capon, Callyspongisines A-D: bromopyrrole alkaloids from an Australian marine sponge, *Callyspongia* sp., *Organic Biomolecular Chemistry*, **12**, 1579 (2014).
10. M. R. S. Rechana, v. Thankamani, Antibacterial and Antifouling activity of the Marine Sponge *Callyspongia diffusa* collected from south-west coast of India, *International Journal of Biotechnology and Biochemistry*, **12**, 33 (2016).
11. L. John Goad and T. Akihisa, Analysis of Sterols 1st. eds. Blackie Academic & Professional (1977).
12. A. Muhammad, I. Anis, A. Khan, B. P. Marasini, M. I. Choudhary, M. R. Shah, Biologically Active *C*-Alkylated Flavonoids from *Dodonaea viscose*, *Arch. Pharmacal. Res.*, **35**, 431 (2012).
13. R. Hilf, W. D. Rector and R. A. Orlando, Multiple molecular forms of lactate dehydrogenase and glucose 6-phosphate dehydrogenase in normal and abnormal human breast tissues, *Cancer*, **37**, 1825 (1976).
14. E. Quinoa and P. Crews, Niphatynes, methoxylamine pyridines from the marine sponge, *Niphates* sp., *Tetrahedron Letters.*, **28**, 2467 (1987).