

Evaluation of secondary metabolites from the red sea tunicate *Polyclinum Constellatum*

Abstract

Chemical investigation of the Red Sea tunicate *Polyclinum constellatum* afforded nine compounds, identified as thymidine (1), uridine (2), adenosine (3), inosine (4), 24-methylene cholesterol (5), dihydrocholesterol (6), cholesterol (7), oleic acid (8) and 1,3-palmityl-2-palmitoleoylglycerol (9). All compounds isolated for the first time from the genus *polyclinum*. Acetylation of compounds 1, 2, 3, 4 and 5 yielded thymidine 3',5'-diacetate (1-Ac), uridine 2',3',5'-triacetate (2-Ac), adenosine 2',3',5'-triacetate (3-Ac), inosine 2',3',5'-triacetate (2-Ac) and 24-methylene cholesterol-3-acetate (5-Ac), respectively. Compound 5 showed potent antitrypanocidal activity with IC₅₀ and IC₉₀ values of 3.39 and 6.69 µg/mL, comparable to α-difluoromethylornithine (3.58 and 8.53 µg/mL). The overall findings of the present study have shown that the Red Sea tunicate *Polyclinum constellatum* revealed different varieties of secondary metabolites with antiprotozoal and cytotoxic activities.

Keywords: red sea, tunicate, *polyclinum constellatum*, antimicrobial, antimalarial, antiprotozoal, cytotoxicity, acetylation of compounds, metabolites, cellulose, appendicularia, natural products

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Abbreviations: ¹H NMR, proton nuclear magnetic resonance; ¹³C NMR, carbon nuclear magnetic resonance; 2D, two dimension; δ, chemical shifts; s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; J, coupling constant; Hz, hertz; HRESIMS, high resolution electrospray ionization mass spectrometry; GC/MS, gas chromatographic mass spectrometry; m/z, mass to charge ratio; MeOH, methanol; EtOAc, ethyl acetate; CH₂Cl₂, methylene chloride; CHCl₃, chloroform; HCl, hydrochloric acid; DFMO, α-difluoromethylornithine; Ac, acetyl; min, minute(s); h, hour(s) THP-1, human monocytic cell line derived from an acute monocytic leukemia patient; IC₅₀, concentration that afford 50% inhibition; IC₉₀, concentration that afford 90% inhibition

Introduction

Tunicates are marine invertebrate animals (animals lacking a backbone) that are commonly found attached to rocks, they are three main groups, sessile ascidians, pelagic appendicularians and thaliaceans.¹ They are characterized by the possession of a tunic composed of cellulose.¹ Adult tunicates are filter feeders: the seawater enters a pharynx through an inhalating or oral siphon, in most cases set in motion by ciliary beating, food particles are trapped on a mucous net secreted by the endostyle, and the water and waste exit the body through an exhalating or atrial siphon.¹ Appendicularia, ascidians and some thaliaceans possess a tadpole-like larva with a notochord and metamorphose into sessile adults in the case of ascidians. Tunicates have reversible blood flow.¹ Natural products found in tunicate has began to attract many chemist's interests; alkaloids, carotenoids, macrolides and tunichromes.² The cytotoxic

and antibacterial activities of *Polyclinum indicum* and *Polyclinum madrasensis* extracts have been tested at various concentrations and showed the results of highest cytotoxicity assay conducted, indicating the presence of cytotoxic compounds in these ascidians.^{3,4} The crude extract of ascidian *Polyclinum madrasensis* showed hemolytic properties against human, cow, goat and chicken bloods that be considered a valuable source for secondary metabolites which could be of pharmaceutical interest.⁵ Polyclinal a sulfated polyhydroxy benzaldehyde has been isolated from extracts of the temperate colonial ascidian *Polyclinum planum*.⁶ The highest concentration of this metabolite was found in the zooid-rich outer layers of this ascidian suggesting that it may represent a potential chemical defense against predators.⁶ As part of our research dealing with the isolation and biological evaluation of active compounds from marine origin, the chemistry and the biological properties of the tunicate *Polyclinum constellatum* collected from the Red Sea was investigated, which led to the isolation of nine compounds (1-9), for the first time from the genus *polyclinum*.

Materials and methods

General experimental procedures

¹H-NMR, ¹³C-NMR and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on Bruker BioSpin Gm bH 400 and 500 spectrometers (Bruker, Rheinstetten, Germany). High resolution mass spectrometry were measured using a Bruker BioApex FT mass spectrometer (Bruker, Rheinstetten, Germany). GC/MS analysis was carried out using an HP 6890 series GC (Agilent

Technologies, Santa Clara, CA, USA), equipped with a split/splitless capillary injector, an HP 6890 series injector autosampler and an Agilent DB-5ms column (30m x 0.25mm x 0.25 μ m), interfaced to a HP 5973 mass selective detector (MSD). The injector temperature was 250°C, and 1 μ L of sample was injected in the splitless mode, with the splitless time set at 60s, the split flow set at 50mL/min and the septum purge valve set to close 60s after the injection occurred. The oven temperature was raised from 70 to 270°C (held for 20min) at a rate of 5°C/min, for a total run time of 60min; the transfer line temperature was 280°C. Chemicals for the pharmacological studies were purchased from Sigma-Aldrich (St. Louis, MO, USA) except the fetal bovine serum (Midwest Scientific, Valley Park, MO, USA) and the Bio-rad Bradford dye (Fisher Scientific, Pittsburg, PA, USA).

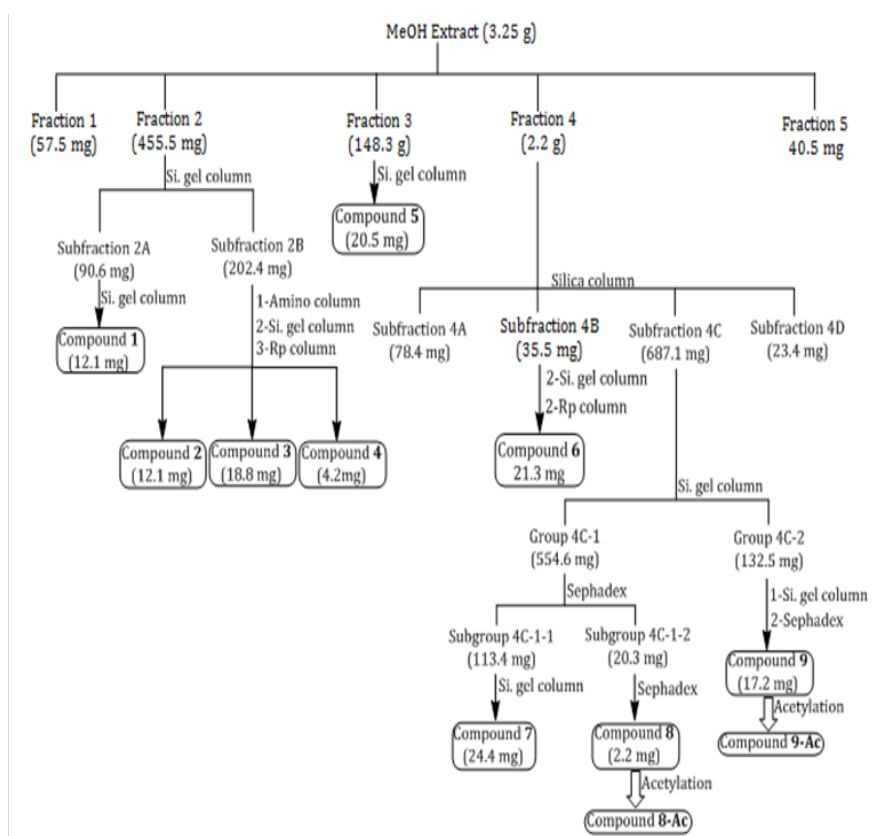
Tunicate material, collection and identification

The tunicate *Polyclinum constellatum* (coll. no. SAA-117) was collected in June, 2015 at depths between 10 and 20 m from the Egyptian Red Sea coast at Safaga. The sample was identified by Dr Tarek Temraz, Marine Science Department, Faculty of Science, Suez Canal University, Ismailia. The sample was directly frozen after collection and stored at -20 °C. A voucher specimen was kept at the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt, under a registration no. SAA-117.

Extraction and isolation

The fresh material (0.5kg) was frozen after collection. It was chopped while frozen into small pieces and extracted with MeOH (500mL x 3) at room temperature. The crude extract was concentrated till dryness under reduced pressure to give a dark green viscous crude extract (3.86g). A 3.25g of the extract was subjected to fractionation by vacuum liquid chromatography over a silica gel column (100g, 9cm x 30cm) eluted with hexanes/EtOAc (75:25, 0:100) followed

by each EtOAc/MeOH (50:50, 0:100) yielding 4 fractions. Fraction 2(455.5mg) was purified on silica gel column (20g, 34cm x 2cm) and eluted with hexanes/EtOAc in gradient manner from 5% to 20% to yield three subfractions (2A-2C). Subfraction 2A (90.6 mg) was further purified on silica gel column (5g, 25cm x 0.8cm) using hexanes/EtOAc (95:5) to give compound 9 (15.6mg). Subfraction 2B (202.4mg) was further purified on an amino column followed by silica gel column (8g, 25cm x 0.8cm) eluted with hexanes/EtOAc in gradient manner and a RP column (10g, 25cm x 0.8cm) using MeOH/H₂O (97.5:2.5) to yield compounds 5 (12.1mg), 6 (18.8mg) and 7 (4.2mg). Fraction 3(148.3g) was subjected to silica gel column (7g, 25cm x 0.8cm) eluted with CH₂Cl₂/MeOH (98:2) yielding compound 8(20.5mg). Fraction 4(2.2g) was subjected to silica gel column (80g, 46cm x 2.5cm) eluted with (CH₂Cl₂/MeOH, 98:2) to give four subfractions (4A-4D). Subfraction 4B(35.5mg) was subjected to silica gel column (2g, 25cm x 0.8cm) using CHCl₃/MeOH/H₂O (15:6:1) as a mobile phase followed by a RP column (10g, 25cm x 0.8cm) using MeOH/H₂O (50:50) to give compound 1 (21.3 mg). Subfraction 4C (687.1mg) was subjected to silica gel column (28 g, 34 cm x 2 cm) eluted with CHCl₃/MeOH/H₂O (15:6:1) to yield two fractions (4C-1 and 4C-2). Fraction 4C-1 (554.6mg) was subjected to sephadex LH-20 (100g, 46cm x 2.5cm) using MeOH as an eluent yielding two subfractions (4C-1-1 and 4C-1-2). Subfraction 4C-1-1 (113.4mg) was purified on a silica gel column (5g, 25cm x 0.8cm) using CHCl₃/MeOH/H₂O (15:6:1) as a mobile phase to yield compound 2(24.4mg). Subfraction 4C-1-2(20.3mg) was subjected to sephadex LH-20 (100g, 46cm x 2.5cm) using MeOH as an eluent to give compound 3(2.2mg). Fraction 4C-2(132.5mg) was subjected to silica gel column (6g, 25cm x 0.8cm) eluted with CHCl₃/MeOH/H₂O (15:6:1) followed by sephadex LH-20 (100g, 46cm x 2.5cm) to give compound 4(17.2mg) (Scheme 1).



Scheme 1 Isolation of the compounds 1-9 from the red sea tunicate polyclinum constellatum.

Acetylation of compounds 1, 2, 3, 4 and 5:

A 2mg of each compound was dissolved in 1mL of pyridine and then 2mL of acetic anhydride was added. The reaction mixture was kept at room temperature for 12h.⁷ The solvent was evaporated under vacuum to give compounds 1-Ac, 2-Ac, 3-Ac, 4-Ac and 5-Ac.

Esterification of compound 8:

Compound 8(4mg) was dissolved in 1mL of chloroform followed by addition of 1mL of 20 mM cupric acetate monohydrate in methanol and 5mL of 0.5N HCl in methanol. The mixture was stored for 60min at room temperature, and then extracted with 10mL of chloroform after addition of 10mL of water.⁸ The pooled chloroformic extract was washed with water and then evaporated to dryness under a flow of nitrogen to give the corresponding fatty acid methyl ester of compounds 8.

Acid hydrolysis of compound 9:

Compound 9(3mg) was added to 2mL of 1.2% HCl/MeOH and then stored for 20min at 45°C. The solution was extracted with hexanes (5mL). The fatty acid methyl esters in the hexanes layer were identified by GC/MS.⁹

In vitro antiprotozoal assay:

All compounds were tested for their antiprotozoal activities against *Leishmania donovani* promastigote, *L. donovani* axenic amastigotes, intracellular *L. donovani* amastigotes/THP1 cells and *Trypanosoma brucei*.¹⁰ The assays have been adapted to 384 well microplate format. In a 384 well micro-plate, the samples with appropriate dilution were added to the cultures of protozoan cells (2×10⁶ cell/mL). The plates were incubated at 26°C for 72h (37°C for axenic amastigotes and *T. brucei* trypomastigotes) and growth of the parasites in cultures were determined by Alamar Blue assay.^{10,11} The compounds were tested at concentrations ranging from 0.4-10µg/mL. The compounds were also tested against *L. donovani* intracellular amastigotes in THP1 cells employing a parasite-rescue and transformation assay.¹² Pentamidine and Amphoterecin B were tested as the standard antileishmanial agents. DFMO (α -difluoromethylornithine) was tested as a positive control for antitrypanosomal activity. IC₅₀ and IC₉₀ values were computed from dose-response curves using XLfit software.

In vitro antimicrobial assay:

All organisms used for the biological evaluation were obtained from the American Type Culture Collection (Manassas, VA, USA) and including the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria methicillin-resistant *S. aureus* ATCC 43300 (MRSA), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods as previously described.^{13,14} *M. intracellulare* was tested using a modified method according to the method described in the literature.¹⁵

In vitro Antimalarial Assay:

Antimalarial activity was determined against chloroquine sensitive (D6, Sierra Leone) and resistant (W2, Indo China) strains of *Plasmodium falciparum* by measuring plasmodial LDH activity as described earlier.¹³

Cytotoxicity evaluation:

The cytotoxicity of the compounds was determined towards four cancer cell lines (SK-MEL, KB, BT-549, SK-OV-3) and one noncancer kidney epithelial cell line (LLC-PK₁) according to a method described earlier.¹⁶

Results and discussion

Chemistry:

Compound 1 was isolated as needle crystals from methanol. A molecular formula of C₁₀H₁₄N₂O₅ was determined by HRESIMS at m/z 265.0875 ([M+Na]⁺, calcd for C₁₀H₁₄N₂O₅Na, 265.0800). From NMR spectroscopic data (Table 1) and comparison with the literature,¹⁷ compound 1 has a deoxyribose moiety attached to a pyrimidine nucleus with values for deoxyribose moiety at δ_H 7.04(1H, t)/ δ_C 85.8, δ_H 5.05(1H, m)/ δ_C 71.9, δ_H 4.48(1H, dd)/ δ_C 89.3, δ_H 4.25, 4.15(2H, dd)/ δ_C 62.8 and δ_H 2.67(2H, m)/ δ_C 41.9 while pyrimidine moiety values were at δ_H 8.17(2H, s)/ δ_C 137.1 and δ_H 1.88 (3H, s)/ δ_C 41.9 beside δ_C 165.5, δ_C 152.4 and δ_C 110.9. From the above spectroscopic data and comparing with literature,¹⁷ compound 1 was deduced as thymidine (Figure 1).

Compound 2 was isolated as yellowish amorphous powders. Its molecular formula (C₉H₁₂N₂O₆) was inferred from HRESIMS (negative ion mode) data [m/z 243.0675 ([M-H]⁻, calcd 243.0617)]. The NMR spectroscopic data (Table 1) is closely related to compound 1 except the presence of an olefinic proton at δ_H 5.65 (1H, d, $J=8$ Hz) instead of the methyl group at C-5 and additional hydroxyl group at C-2' (δ_H 4.92 (1H, m)/ δ_C 71.6). Comparing the ¹³C NMR chemical shifts with those reported in literature,¹⁸ compound 2 was identified as uridine (Figure 1).

Compound 3 was isolated as white amorphous powders. It has the molecular formula of C₁₀H₁₃N₅O₄ that was determined from the HRESIMS data [m/z 268.1053 [M+H]⁺, (calcd 268.1046)]. The analysis of NMR spectroscopic data (Table 1) showed the presence of the following; an oxygenated methylene group, six methines (four of them were found to be oxygenated, while two olefinic) and three quaternary carbons. From its spectroscopic data and comparing with literature,¹⁹ compound 3 was identified as adenosine (Figure 1).

Compound 4 was isolated as white crystals (MeOH). Its molecular formula C₁₀H₁₃N₅O₄ was deduced from HRESIMS (positive ion mode) data [m/z 269.0879 ([M+H]⁺, calcd for C₁₀H₁₃N₅O₄, 268.0886)] and (negative ion mode) [m/z 267.0822 ([M-H]⁻, calcd for C₁₀H₁₁N₅O₅, 267.0729)]. The NMR spectroscopic data (Table 1) of compound 4 were similar to compound 3 except that C-6 is attached to oxygen instead of nitrogen. By comparing its spectroscopic data with those reported in literature,¹⁷ compound 4 was identified as Inosine (Figure 1).

Compounds 5-9 were identified by comparison of their spectroscopic characteristics with those previously reported in the literature as 24-methylene cholesterol (5),^{20,21} dihydrocholesterol (6),²² cholesterol (7),²² oleic acid (8)²³ and 1,3-palmityl-2-palmitoleoylglycerol (9).^{24,25} Acetylation of compounds 1, 2, 3, 4 and 5 yielded thymidine 3',5'-diacetate (1-Ac), uridine 2',3',5'-triacetate (2-Ac), adenosine 2',3',5'-triacetate (3-Ac), inosine 2',3',5'-triacetate (2-Ac), cholesterol-3-acetate (5-Ac) and 24-methylene cholesterol-3-acetate (5-Ac), respectively (Figure 1).

Table 1 ¹H and ¹³C NMR data for compounds 1-4

	1		2		3		4	
Position	δX	δH	δX	δ	δX	δH	δX	δH
1	-	-	-	-	-	-	-	-
2	152.4	-	151.2	-	152.4	8.17, s	146.4	8.35, s
3	-	-	-	-	-	-	-	-
4	137.1	8.17, s	141.2	8.55, d(8.0)	149.1	-	148.1	-
5	110.9	-	102.2	5.65, d(8.0)	119.4	-	124.9	-
6	165.3	-	163.6	-	156.2	-	157.1	-
7	13.2	1.88, s	-	-	-	-	-	-
8	-	-	-	-	140	8.36, s	139.2	8.09, s
NH2	-	-	-	-	-	7.37, br.	-	-
1'	85.8	7.04, t(6.8)	88.1	5.78, t(5.2)	87.9	5.88, d(6.4)	87.9	5.88, d(2.0)
2'	41.2	2.67, m	70.3	3.98, m	73.5	4.61, m	74.6	4.50, m
3'	71.9	5.05, t(8.6)	74	4.04, t(8.6)	70.7	4.15, m	70.8	4.15, m
4'	89.3	4.49, m	85.3	3.85, m	85.9	3.97, m	86.1	3.96, m
5'	63.4	4.15, dd(3.0, 11.6)	61.3	3.55, dd(3.2, 12.0)	61.2	3.56, dd(3.1, 12.2)	61.7	3.57, dd(4, 12)
		4.25, dd(3.0, 11.6)		3.63, dd(3.2, 12.0)		3.68, dd(3.1, 12.2)		3.68, dd(4, 12)

¹H NMR(500 MHz) spectroscopic data [δ in ppm, mult.(J in Hz)]. ¹³C NMR(125 MHz) spectroscopic data(δ in ppm)

Antiprotozoal activity:

Antiprotozoal activity of all compounds was tested against *Leishmania donovani* Promastigote, *Leishmania donovani* Amastigote, *Leishmania donovani* Amastigote/THP1 and *Trypanosoma brucei*. 24-methylene cholesterol (5) showed promising antitrypanosomal activity with IC₅₀ and IC₉₀ values of 3.39, 6.69 μg/mL, which was comparable to DFMO (3.58, 8.53 μg/mL), the positive control (Table 2) (Figure 2). Other compounds tested did not show noticeable activity against the protozoan parasites. A recent study has reported 24-Methylenecyclopropane as new class of steroidal inhibitors-mechanism-based suicide substrates with promising activity against *T. Brucei*.²⁶ It would be interesting to investigate 24-methylene cholesterol as inhibitor of ergosterol biosynthesis as potential drug target.²⁷

Antimicrobial activity:

Antimicrobial activity of the compounds was determined against

bacterial strains, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare*, as well as against pathogenic fungi including *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* with no significant activity.

Antimalarial activity:

All the isolated compounds were tested against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum* by measuring plasmodial LDH activity but, none of the tested compounds showed any activity.

Cytotoxic activity:

All the isolated compounds were tested against SK-MEL, KB, BT-549, SK-OV-3 and LLC-PK₁. None of the compounds was cytotoxic to all the cancer cell lines up to the highest tested concentration of 25 μg/mL. However, compound 5 showed cytotoxicity to kidney cells with IC₅₀ value of 23 μg/mL.

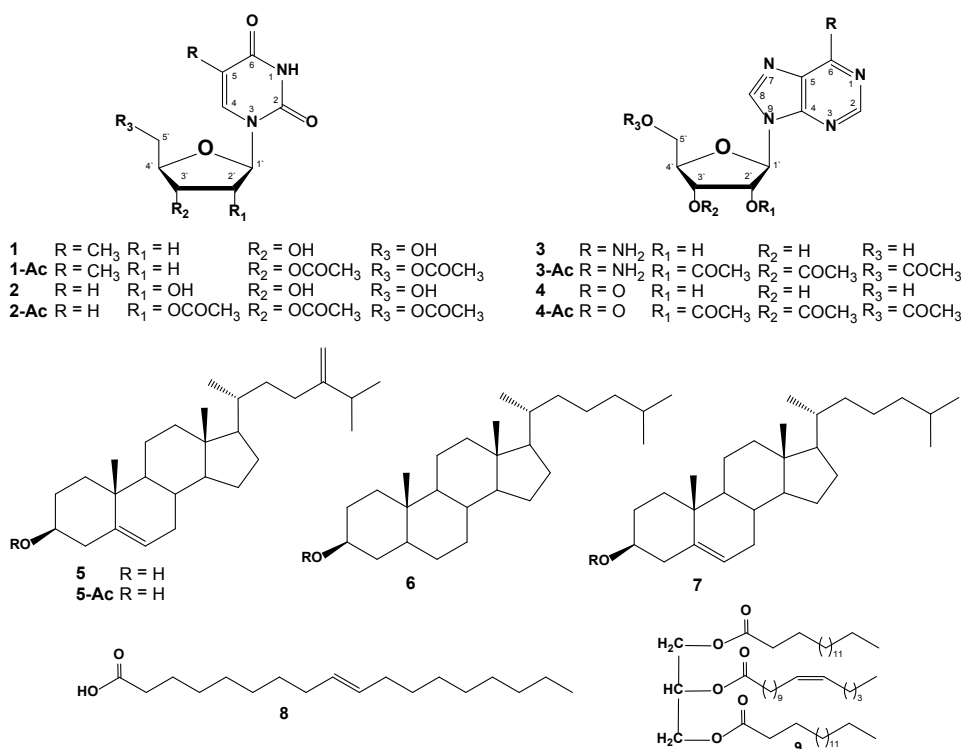


Figure 1 Chemical structures of the isolated compounds (1-9) and the acetylated compounds (1Ac-5Ac).

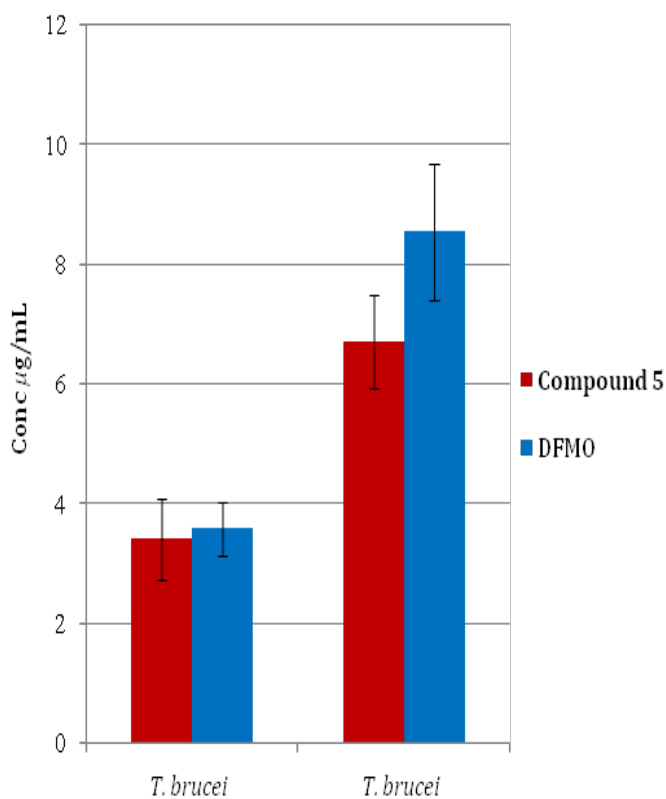


Figure 2 IC₅₀ and IC₉₀ values for *in vitro* activity of compound 5 against *Trypanosoma brucei*.

Table 2 IC₅₀ and IC₉₀ values for *in vitro* activity of compound 5 against *Trypanosoma brucei*

Trypanosoma brucei		
Compound	IC ₅₀	IC ₉₀
5	3.39±0.68**	6.69±0.78*
DFMO	3.58±0.45	8.53±1.15

IC₅₀ and IC₉₀ (µg/mL); DFMO, α-difluoromethylornithine; values are expressed as mean±S.E.M.(n=3); *p<0.05 significant when compared with the corresponding value of the standard group; **p>0.05 non significant when compared with the corresponding value of the standard group; done by independent Student's t-test.

Conclusion

Nine known compounds were isolated from the Red Sea tunicate *Polyclinum constellatum*. The structures were elucidated by spectroscopic analysis. The antimicrobial, antimalarial, antiprotozoal, and cytotoxic activities of the isolated compounds were evaluated. Compound 5 displayed good antitrypanocidal activity with IC₅₀ and IC₉₀ of 3.39 and 6.69µg/mL respectively, and cytotoxicity against LLC-PK₁ with IC₅₀ value of 23µg/mL.

Acknowledgments

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sample collection along the coasts of the Red Sea. Financial support was partially provided by the Egyptian government. Antimicrobial and antiprotozoal biological screenings at the NCNPR are partly supported by the cooperative scientific agreement with USDA-ARS (58-6408-2-0009).

Conflict of interest

Author declares that there is no conflict of interest.

References

1. Lemaire P, Piette J. Tunicates: exploring the sea shores and roaming the open ocean. A tribute to Thomas Huxley. *Open Biology*. 2015;5(6):150053.
2. Nakamura A, Ashino T, Yamamoto M. Structure determination of a very unusual peroxide from solitary ascidians, *Phallusia mammillata*, *Ascidia ahodori*, *styela pricata* and *halocynthia roretzi*. *Tetrahedron Letters*. 1991;32(34):4355–4358.
3. Rajesh RP, Ramasamy MS, Murugan A. Anticancer activity of the ascidian *polyclinum indicum* against cervical cancer cells (HeLa) mediated through apoptosis induction. *Med Chem*. 2010;6(6):396–405.
4. Bragadeeswaran S, Ganesan K, Sri Kumaran N, et al. Antibacterial and cytotoxic activities of ascidians *Polyclinum madrasensis* Sebastian, 1952 and *Phallusia nigra* Savigny, 1816 from Tuticorin Coast of India. *World Applied Sciences Journal*. 2010;9(12):1387–1391.
5. Bragadeeswaran S, Ganesan K, Sri Kumaran N. Hemolytic activities from ascidian *Polyclinum madrasensis* Sebastian, 1952 and *Phallusia nigra* Savigny, 1816 from Tuticorin coast of India. *Asian J Applied Sci*. 2011;4(6):630–639.
6. Lindquist N, Fenical W, Párkányi L, et al. Polyclinal, a new sulfated polyhydroxy benzaldehyde from the marine ascidian *Polyclinum planum*. *Cell Mol Life Sci*. 1991;47(5):503–504.
7. Mpetga JDS, Tene M, Wabo HK, et al. Cytotoxic cycloartanes from the fruits of *Caloncoba glauca*. *Phytochem Lett*. 2012;5(1):183–187.
8. Hoshi M, Williams M, Kishimoto Y. Esterification of fatty acids at room temperature by chloroform-methanolic HCl–cupric acetate. *J Lipid Res*. 1973;14(5):599–601.
9. Ichihara K, Fukubayashi, Y. Preparation of fatty acid methyl esters for gas-liquid chromatography. *J Lipid Res*. 2010;51(3):635–640.
10. Manda S, Khan S, Jain S, et al. Synthesis, antileishmanial and antitrypanosomal activities of N-substituted tetrahydro- β -carbolines. *Bioorg med chem lett*. 2014;24(15):3247–3250.
11. Ráz B, Iten M, Grether-Bühler Y, et al. The Alamar Blue® assay to determine drug sensitivity of African trypanosomes (*Tb rhodesiense* and *Tb gambiense*) in vitro. *Acta Trop*. 1997;68(2):139–147.
12. Jain SK, Sahu R, Walker LA, et al. A parasite rescue and transformation assay for antileishmanial screening against intracellular *Leishmania donovani* amastigotes in THP1 human acute monocytic leukemia cell line. *J Vis Exp*. 2012;70:e4054.
13. Radwan M, Wanas A, Fronczek F, et al. Polybrominated diphenyl ethers from the marine organisms *Lendenfeldia dendyi* and *Sinularia dura* with anti-MRsa activity. *Medicinal Chemistry Research*. 2015;24(9):3398–3404.
14. Samoylenko V, Jacob M, Khan S, et al. Antimicrobial, antiparasitic and cytotoxic spermine alkaloids from *Albizia schimperiana*. *Nat Prod Comm*. 2009;4(6):791–796.
15. Franzblau SG, Witzig RS, McLaughlin JC, et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. *J Clin Microbiol*. 1998;36(2):362–366.
16. Zhang Z, Ali Z, Khan SI, et al. Cytotoxic monacolins from red yeast rice, a Chinese medicine and food. *Food Chem*. 2016;202:262–268.
17. Jones AJ, Grant DM, Winkley MW, et al. Carbon-13 magnetic resonance. XVII. Pyrimidine and purine nucleosides. *J Am Chem Soc*. 1970;92(13):4079–4087.
18. Still IW, Plavac N, McKinnon DM, et al. Carbon-13 nuclear magnetic resonance spectra of N-, O-, and S-methylated uracil and thiouracil derivatives. *Can J Chem*. 1978;56(5):725–729.
19. Liu D, Wang S, Zhang L, et al. Chemical composition of *n*-BuOH extract of *Potentilla anserina* L. and its protective effect of EAhy926 endothelial cells under hypoxia. *Latin American Journal of Pharmacy*. 2011;30:1889–1894.
20. Sheu JH, Chang KC, Sung PJ, et al. Chemical Constituents of a Fomosan Soft Coral *Sinularia* sp. *J Chin Chem Soc*. 1999;46(2):253–257.
21. Shaker KH, Al-wahaibi LH. ¹³C-NMR of steroids from the soft coral *Litophyton arboreum*. *Int J Pharm Sci Rev Res*. 2016;36(1):149–152.
22. Wilson WK, Sumpter RM, Warren JJ, et al. Analysis of unsaturated C27 sterols by nuclear magnetic resonance spectroscopy. *J Lipid Res*. 1996;37(7):1529–1555.
23. Mohamed GA, Abd-Elrazek AE, Hassanean HA, et al. New fatty acids from the Red Sea sponge *Mycale euplectellioides*. *Nat Prod Res*. 2014;28(14):1082–1090.
24. Miranda-Vilela AL, Grisolia CK, Resck IS, et al. Characterization of the major nutritional components of *Caryocar brasiliense* fruit pulp by NMR spectroscopy. *Quim Nova*. 2009;32(9):2310–2313.
25. Cvačka J, Hovorka O, Jiroš P, et al. Analysis of triacylglycerols in fat body of bumblebees by chromatographic methods. *J Chromatogr A*. 2006;1101(1):226–237.
26. Miller MB, Patkar P, Singha UK, et al. 24-Methylenecyclopropane steroidal inhibitors: A Trojan horse in ergosterol biosynthesis that prevents growth of *Trypanosoma brucei*. *Biochim Biophys Acta*. 2017;1862(3):305–313.
27. Leaver DJ, Patkar P, Singha UK, et al. Fluorinated Sterols Are Suicide Inhibitors of Ergosterol Biosynthesis and Growth in *Trypanosoma brucei*. *Chem Biol*. 2015;22(10):1374–1383.