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Chemical and Biological Investigation of the Red Sea Sponge *Echinoclathria species*.

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Abstract

Aim:

The present study was aimed to chemically and biologically investigate the Red Sea sponge *Echinoclathria sp.* as a source of valuable biologically active secondary metabolites.

Materials and methods:

The methanolic extract of *Echinoclathria sp.* was chromatographed over successive silica gel columns to isolate the secondary metabolites. Their structures were clarified on the basis of spectroscopic techniques, including 1D and 2D-NMR, as well as mass spectrometry and chemical methods. The cytotoxic activity was evaluated via the Sulpho-Rhodamine-B (SRB) for the isolated compounds and was compared with standard Cisplatin and Doxorubicin.

Results:

Chemical investigation of the Red Sea sponge *Echinoclathria sp.*, led to the isolation of one new ceramide, namely ((R)-2'-hydroxy-*N*-((2S,3S,4R)-1,3,4-trihydroxy-icosan-2-yl) icosanamide) (**1**) along with six known compounds; 3-*β*-hydroxy-cholest-5-en-7-ketosterol (**2**), thymine (**3**), uracil (**4**), deoxythymidine (**5**), deoxyuridine (**6**) and cholesterol (**7**). Compounds **2**, **5**, **6** and **7** were reported for the first time from the genus. Compound 1 and 2 exhibited significant cytotoxicity against different human cell lines (Hep G2, MCF-7, Hela, and HCT-116). Moreover, compound **2** has been found to possess significant anti-inflammatory activity.

Conclusions:

The present study reveals the hidden cytotoxic and antiflammatory activities of compounds isolated from the Red Sea sponge *Echinoclathria sp***.** to put an emphasis on this organism potential medicinal uses.

Keywords: *Echinoclathria sp*.; ceramides; cytotoxicity; anti-inflammatory activity.

INTRODUCTION

Marine ecosystems are proven to be a unique source of chemical entities with a vast range of biological activities [1]. The Red Sea is a great source of these bioactive metabolites since it has high levels of marine biodiversity and great seasonal fluctuations of water temperatures, air and salinity [2]. Over the past 50 years, marine sponges have been a prominent source of a wide range of secondary metabolites. Many natural products from sponges possess complex and unique structures with a variety of interesting biological activities, some of them with good potential for medical applications [3-5]. Sponges of the genus *Echinoclathria* was found to be a source of potent cytotoxins [6]. On the other hand, genus *Echinoclathria* was previously proven to be a rich source of steroids [7, 8], pyridine alkaloids [9]. In this study, we report the isolation and structural elucidation of one new ceramide **1** together with six known compounds **2**, **3**, **4**, **5**, **6** and **7** from the Red Sea sponge *Echinoclathria sp*. Up to our knowledge; this is the first report of marine ceramides from this genus. Moreover, the *in vitro* growth inhibitory activity of compound **1** and **2** against different human cancer cell lines and the inflammatory activity of compound **2** were evaluated.

MATERIALS AND METHODS

General experimental procedures

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT-135 and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on a Varian AS 400 (Varian Inc., Palo Alto, CA, USA). IR spectra were recorded on a Bruker Tensor 27 (Bruker Corporation, Billerica, MA, USA). UV spectra were obtained on a Varian Cary 50 Bio UV–visible spectrophotometer. The UV spectra were recorded on a double beam Shimadzu UV–visible spectrophotometer (model UV-1601 PC, Kyoto City, Japan). IR spectra were recorded on Nicolit FT IR spectrophotometer (Nicolet Company, Nicolet, Canada) range 400–4000, USA. High resolution mass spectra were recorded using a Bruker BioApex (Bruker Corporation). Fatty acid methyl ester was identified using Hewlett Packard (HP) gas liquid chromatography, series 6890 equipped with flame ionisation detector. A capillary column (HP-INNOWAX, polyethylene glycol, 30 m ×530 µm, film thickness 1.00 µm) was used in separation of the fatty acid. The injector port temperature was set at 250°C (splitless mode) and a pressure of 14.81 psi and the detector cell at 275°C. The flow rate of the carrier gas (N_2) was 30 mL/min. The initial column temperature was 70°C and increased to 200°C by the rate of 4°C/ min, then isothermally for a total run time of 32.5 min. Pre-coated silica gel G-25 UV254 plates were used for thin layer chromatography (TLC) (20 cm × 20 cm) (E. Merck, Darmstadt, Germany). Silica gel Purasil 60A, 230–400 mesh was used for flash column chromatography (Whatman, Sanford, ME, USA).

Sponge collection and identification.

The sponge *Echinoclathria sp.* was collected from Safaga at the Egyptian Red Sea, air-dried and stored at low temperature (-24 ºC) until processed. Voucher specimens were deposited at the Zoological Museum of the University of Amsterdam under registration numbers ZMAPOR18964 and in the herbarium section of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt under registration number SAA-9.

Extraction and isolation

A sample (2 Kg, wet weight) of *Echinoclathria sp.* was defrosted and repeatedly extracted with methanol (3 × 5 L). The combined extracts were concentrated under vacuum to afford a reddish brown residue EC (150 g). This residue was subjected to vacuum liquid chromatography (VLC) on flash silica gel using *n*-hexane, DCM and MeOH successively, giving three fractions; EC-H (18.75 g), EC-D (41 g) and EC-M (90 g). The fraction eluted with DCM (100 %) was chromatographed over silica gel column using *n*-hexane: EtOAc gradient elution to give 8 sub-fractions (EC-D-1 ~ EC-D-8). Fraction eluted with *n*-hexane: EtOAc (90:10), EC-D-2 (2.8 g), was further chromatographed over SiO_2 column using $CHCl_3$: MeOH (97:3) isochratic elution to afford compound **1** (40 mg, white amorphous powder). Fraction EC-D-3 (6.1 g), which was eluted with *n*-hexane: EtOAc (85:15), was chromatographed over silica gel column and eluted with *n*-hexane: EtOAc (90:10) to yield **7** (70 mg, white needles). Fraction EC-D-4 (5.2 g), which was eluted with *n*-hexane: EtOAc (80:20), was subjected to repeated purification steps over silica gel column and eluted with *n*-hexane: EtOAc gradient elution to yield **2** (15 mg, white needles). The methanolic fraction EC-M (90 g) was subjected to chromatography on silica gel column, eluted with $CHCl_3$: MeOH: H_2O gradient elution of (1:0:0 ~ 6.5:3.5:0.5) to give 15 sub-fractions EC-M-1 ~ EC-M-15. Fraction EC-M-9 (1.7 g) was purified by Sephadex LH-20 column using $CHCl_3$: MeOH as eluent to yield **3** (8 mg, white solid, thymine) and **4** (4 mg, white solid, uracil). Fraction EC-M-12 (10.2 g) was subjected to silica gel column using $CHCl_3$: MeOH: H_2O gradient elution of (1:0:0 ~ 65:35:0.5) to afford 8 sub-fractions EC-M-12-1 ~ EC-M-12-8. Fraction EC-M-12-3 (1.1 g) was exposed to repeated purification steps on sephadex LH 20 column using $CHCl_3$: MeOH (1:1) as an eluent to give **5** (80 mg, white solid) and **6** (63 mg, white solid).

Ceramide hydrolysis.

The pure compound (5 mg) was heated with 5 mL of 1 M HCL in 15 mL of MeOH for 4 h at 90° C. The mixture was extracted with *n*-hexane, and the *n*-hexane layer was concentrated under vacuum to give the hydroxyl fatty acid methyl ester of **1**. The hydroxyl fatty acid methyl ester of **1** was separately subjected to Lemieux oxidation [15, 26, 27]. Thus, 0.023 mol/L aqueous $KMnO_4$, 0.09 mol/L $NaIO_4$ (2.0 mL), *t*-BuOH (1.0 mL), and 0.04 mol/L aqueous K_2CO_3 (0.5 mL) were slowly added to the hydroxyl fatty acid methyl ester of **1**. Then, the mixture was stirred for 24 h at room temperature, quenched with 2.5 mol/L H_2SO_4 (0.5 mL) and saturated aqueous Na_2SO_3, and then extracted with Et_2O (5×3 ml). The organic layer was dried over Na_2SO_3. Finally, the concentrated, dried residue was esterified with excess CH_2N_2 in Et_2O overnight. The resulting ester was used for GC-MS analysis.

Spectral data

((R)-2'-hydroxy-*N*-((2S,3S,4R)-1,3,4-trihydroxy-icosan-2-yl) icosanamide) (**1**). white amorphous powder; $[\alpha]_D$ +9° (c 0.91, $CHCl_3$+MeOH). HR-ESI-MS (positive ion mode) found *m/z* 656.6146 $[M+H]^+$ (calcd for $C_{40}H_{82}NO_5$); GCMS of FAME: 312 $[M]^+$. White substance; HR-ESI-MS (positive ion mode) *m/z* = 698.6694 $[M+H]^+$ (calcd. for $C_{43}H_{88}NO_5$: 698.6662); ^{1}H NMR (C_5D_5N, 400 MHz): δ_H 0.86 (6H, *t*, *J* = 6.8 Hz, H_3-20 and H_3-20'), 1.25 (62H, *m*, H_2-5-19 and H_2-4'-19'), 2.05 (2H, *m*, H_2-3') , 4.27 (1H, *m*, H-4), 4.35 (1H, *m*, H-3), 4.43 (2H,*m*, H_a-1), 4.49 (2H,*m*, H_b-1), 4.61 (1H, *m*, H-2'), 5.12 (1H, *m*, H-2) and 8.58 (1H, *d*, *J* = 8.8, NH); ^{13}C NMR (C_5D_5N, 100 MHz): δ_C 14.2 (C-20, C-20'), 22.5 (C-19, C-19'), 29.7 (C-6 and C-4'), 29.9 (C-16 and C-18), 31.7 (C-7-15 and C-5'-18'), 33.7 (C-5 and C-3'), 35.3 (C-17), 52.9 (C-2), 62.0 (C-1), 72.4 (C-2'), 72.9 (C-4), 76.7 (C-3) and 175.2 (C-1').

***In-vitro* evaluation of cytotoxic activity**

The cytotoxicity of the isolated compounds was measured by the Sulpho-Rhodamine-B (SRB) assay as described by [28]. This was performed on different human cell lines: liver carcinoma cell line (Hep G2), cervical carcinoma cell line (Hela), colon carcinoma cell line (HCT-116), breast carcinoma cell line (MCF-7), and prostate carcinoma cell line (PC-3) which were kindly provided by the National Cancer Institute (Kasr El Ainy Street, Cairo, Egypt). The cells were plated into 96-multiwell plates (104 cells/ well) for 24 hours before treatment with the pure compounds to allow attachment of cells to the plate's wall. Cells were routinely cultured in Dulbeco's Modified Eagle's Medium (DMEM). Different concentrations of the tested samples (0, 50, 100, 150 and 200 µg/ml in DMSO) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds under test for 48 hours, at 37ºC and in atmosphere of 5% CO_2. After 48 hours, the cells were fixed, washed and stained with Sulpho-Rhodamine-B stain. Excess stain was washed with acetic acid and the attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. Moreover, the IC_{50} (The dose that reduces survival to 50%) was calculated.

Anti-inflammatory assay

Using the carrageenan-induced rat paw oedema test as described by [29], thirty six male albino animals divided into six groups (each of six animals) were used. They were administered one single oral dose of the tested sample and the reference drug in specific doses. The negative control group received saline. One hour later all the animals had a subplanter injection of 0.1ml of 1% carrageenan solution in saline, in the right hind paw and 0.1% of saline in the left hind paw compared to Indomethacin (20 mg/Kg. b.wt.)

Thickness of the right hind paw (mm) was measured immediately before and 1, 2, 3 and 4 h post carrageenan injection with a micrometer caliber.

The percentage of oedema produced and that of oedema inhibition due to drug administration were, respectively calculated as follows:

Oedema = (Wt of right paw - Wt. of left paw) ×100/Wt. of left paw

% Oedema inhibition = (Mc – Mt) × 100/Mc

Where, Mc is the mean oedema in control rats and Mt is the mean oedema in drug-treated animals.

The statistical comparison of the difference between the control group and the treated sample was carried out using two-way ANOVA followed by Duncan's multiple range test. Results are expressed as mean ±SE (n=6).

RESULTS AND DISCUSSION

Chemistry

Compound **1** was isolated as a white amorphous powder, The high-resolution ESI-TOF mass spectrum of **1** showed a pseudo-molecular ion peak at *m/z* 656.6146 $[M+H]^+$, which in conjunction with the detailed analysis of the ^{13}C spectrum and DEPT, revealed a molecular formula of $C_{41}H_{83}NO_5$, representing one degree of unsaturation. The ^1H-NMR spectrum in (C_5D_5N, 400 MHz) showed resonance of an amide proton doublet at δ_H 8.58 (1H, *d*, *J*=8.8 Hz) and protons of a long methylene chain at δ_H 1.25 and the ^{13}C-NMR spectrum in (C_5D_5N, 100 MHz) showed a signal at δ_C 175.2 (C-1'). Also, the presence of carbons resonating at δ_C 62.0 (C-1), 76.7 (C-3) δ_H, 72.9 (C-4) and 72.4 (C-2') in the ^{13}C-NMR spectrum indicated the presence of hydroxyl methyl (CH_2O) and three oxymethine groups (CHO), respectively, which resonate in the ^1H-NMR spectrum a δ_H 4.35 (1H, *m*, H_a-1) and 4.49 (1H, *m*, H_b-1) for the hydroxyl methyl group, and at δ_H 4.61, 4.35 and 3.27 (each 1H, m) for the oxymethines. All the The aforementioned spectral data, in addition to the cluster of methylene groups centered at δ_H 1.25 (*m*)/ δ_C 31.7, two terminal methyl groups at δ_H 0.86 (6H, *t*, *J*= 6.8)/ δ_C 14.2, and a nitrogen bearing methine group at δ_C 52.9 (C-2) / δ_H 5.12 (1H, *m*, H-2) were in good agreement with those reported for phytosphingosine-type ceramides possessing a 2-hydroxyl fatty acyl moiety [10-14].

Analysis of the ^1H-^1H COSY, HMQC, and HMBC spectra led to the assignment of proton and carbon signals for **1**. The positions of the hydroxyl groups were confirmed by ^1H-^1H COSY correlation sbetween H_2-1/ H-2, H-2/H-3, H-3/H-4, H-4/H_2-5, and H2'/H_2-3' and also from HMBC correlations of H_2-1/C-2, H_2-1/C-3, H-3/C-4, H-3/C-5, H-4/C-2, H-4/C-3, H-2/C-1' and H-2'/C-1', leading to the assignment of C-1/C-2/C-3/C-4/C-1'/C-2'. **Fig. 1**

The length of the fatty acid and long chain base (LCB) were determined on the basis of the results of its methanolysis followed by GC-MS analysis of the methanolysis products [15]. GC-MS analysis of the fatty acid methyl ester of **1** was carried out after hydrolysis and afforded a molecular ion peak at *m/z* 312 $[M]^+$ in GC-MS spectrum. The relative configurations of C-2 (δ 52.9) and C-3 (δ 76.7) were predicted to hold a d-*erythro* stereochemistry at C-2 and C-3 as the naturally occurring phytosphingosine base, which was consistent with those reported for other (2*S*,3*R*,2′*R*) sphingosine moieties [16-18]. The absolute configuration at C-2, C-3, C-4 and C-2' were determined as 2S,3S,4R,2'R from the chemical shifts of H-2, H-3, H-4 and H-2', which were in good agreement with those reported in the literature and the optical rotation value [19]. Accordingly, the structure of **1** was assigned as R-2'-hydroxy-*N*-((2S,3S,4R)-1,3,4-trihydroxylcosan-2-yl) icosanamide. The known compounds were identified through the analysis of the spectroscopic data and comparison of their data with those in the literature as 3β-hydroxycholest-5-en-7-one (**2**) [20], thymine (**3**) [21], uracil (**4**) [21], thymidine (**5**) [23], deoxyuridine (**6**) [24], cholesterol (**7**) [25] as shown in figure 1.

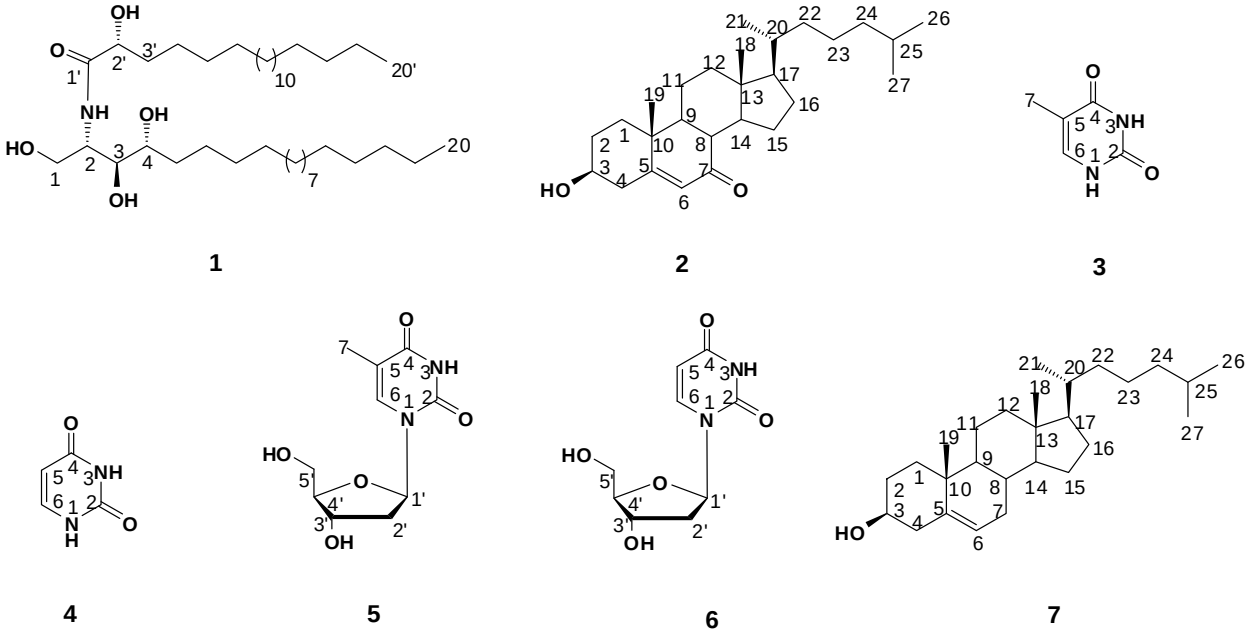

Figure1: Chemical structures of the isolated compounds.

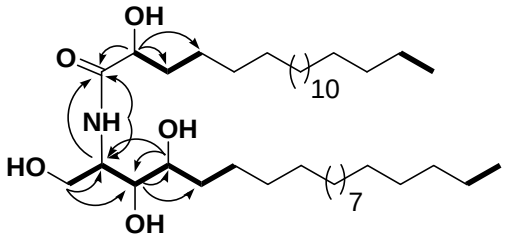

Fig.2. Important ^{1}H-^{1}H- COSY (bold) and HMBC (arrows) Correlations for Compound 1

Biological Activity

Cytotoxic activity

The cytotoxic activity was evaluated via a two-stage process, beginning with measurement of the sensitivity of compound **1** and **2** against a panel of four human cancer cell lines representing different tumor types, namely liver carcinoma cell line (Hep G2), cervical carcinoma cell line (Hela), colon carcinoma cell line (HCT-116) and breast carcinoma cell line (MCF-7) at a single dose of 100 µg/mL, followed by the evaluation of potential cytotoxicity at five different concentrations (0, 50, 100, 150 and 200 µg/ml in DMSO) against the most promising cell lines, corresponding to the maximum percentage of inhibition achieved at the single dose experiment. The initial screening effect i.e., sensitivity test indicated that, compound **1** displayed a significant inhibitory activity against breast carcinoma cell line (MCF-7) and liver carcinoma cell line (Hep G2) on the other hand, compound **2** displayed a significant inhibitory activity against (Hep G2), cervical carcinoma cell line (Hela), colon carcinoma cell line (HCT-116) **Fig.2.** Compound **1** displayed high potential cytotoxicity against HepG2 (IC_{50} 25.4 µM) and MCF-7 (IC_{50} 28.3 µM), while compound **2** displayed a significant inhibitory activity against Hep G2, Hela, and HCT-116 with IC_{50} 82.3, 78.5, 75.4 µM respectively. The inhibitory properties of these compounds are compared with standard Cisplatin and Doxorubicin (**Table. 1**).

Anti-inflammatory activity

The anti-inflammatory activity (**Table.2**) of **2** was evaluated on carrageenan-induced rat hind paw oedema model. Compound **2** (20 mg/kg) has been found to possess significant anti-inflammatory activity on the tested experimental model.

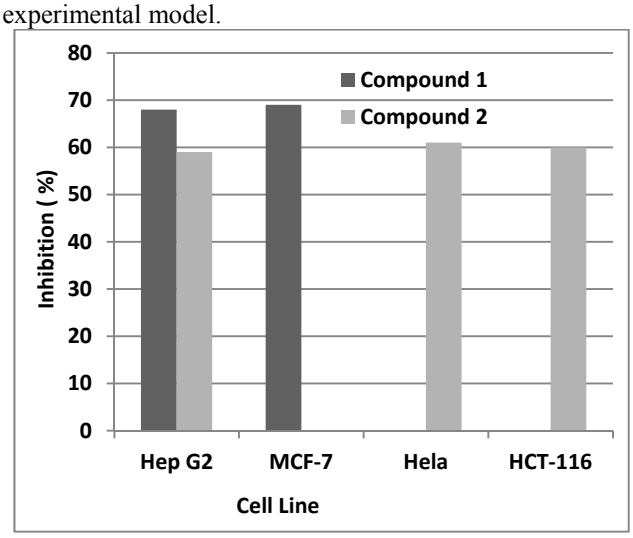

Fig.3. Sensitivity Test of Compound 1 and 2 against Different Human Carcinoma Cell Lines at 100 µg/mL.

Table 1: IC50 VALUES [µM] OF COMPOUNDS 1, 2 AND CISPLATIN AGAINST DIFFERENT HUMAN CELL LINES; $HepG_2$ and MCF-7, Hela AND HCT-116

Time (hour)	Zero	1		2		3		4	
Groups	Paw diameter [mm]	Paw diameter [mm]	Oedema thickness[mm]	Paw diameter [mm]	Oedema thickness [mm]	Paw diameter [mm]	Oedema thickness [mm]	Paw diameter [mm]	Oedema thickness [mm]
Control	3.61±0.09	4.93±0.07	1.32	4.03±0.13*	1.42	4.13±0.13*	1.52	4.27±0.8	1.66
Compound **2** 20 mg/kg	3.55±0.11	4.18*±0.22	0.63	4.00*±0.11	0.45	3.95*±0.07	0.4	3.90*±0.06	0.35
Indomethacin 20 mg/kg	3.00±0.09	3.4*+0.04	0.4	3.3*+0.06	0.3	3.2*±0.01	0.2	3.14*±0.01	0.14

Each data point represents the mean± SD of four independent experiments (significant differences at $p < 0.05$). NA, Not active

Table 2: EFFECT OF COMPOUND 2 AGAINST CARRAGEENAN INDUCED PAW OEDEMA IN RATS

Sample	Human cell lines			
	HepG2 IC_{50} [µM]	MCF-7 IC_{50} [µM]	Hela IC_{50} [µM]	HCT-116 IC_{50} [µM]
Compound 1	25.4±0.38	28.3 ±0.72	NA	NA
Compound 2	82.3±0.18	NA	78.5±0.21	75.4±0.21
Cisplatin	21.3 ±0.4	15.3 ±0.1	-	13.1±0.2
Doxorubicin	-	-	6.84±0.11	-

Drugs were orally administered 1hr prior to carrageenan injection. Oedema was induced in the rat right hind paw by S.C. injection of 0.1 ml of 1% carrageenan suspension in saline. Thickness of the right hind paw (mm) was measured immediately before and 1, 2, 3 and 4 h post carrageenan injection with a micrometer caliber. Results are expressed as mean ±SE (n=6).

The statistical comparison of difference between the control group and the treated groups was carried out using two-way ANOVA followed by Duncan's multiple range test.

* Significantly different from zero time at $p < 0.05$.

CONCLUSIONS

The present work led to the isolation of a new ceramide ((R)-2'-hydroxy-*N*-((2S,3S,4R)-1,3,4-trihydroxy-icosan-2-yl) icosanamide) (**1**) along with a known (3-*β*-hydroxy-cholest-5-en-7-ketosterol) (**2**) from the crude extract of *Echinoclathria sp*.. Compound **1** displayed high potential cytotoxicity against HepG2 (IC_{50} 25.4 µM) and MCF-7 (IC_{50} 28.3 µM), while compound **2** displayed a significant inhibitory activity against Hep G2, Hela, and HCT-116 with IC_{50} 82.3, 78.5, 75.4 µM respectively. Moreover, compound **2** (20 mg/kg) has been found to possess significant anti-inflammatory activity. Therefore, this sponge can be considered as a potential source of both anticancer and anti-inflammatory agents.

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