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Phytochemical and biological investigation of *Litophyton arboreum*

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Abstract
Three known compounds including, uracil (1), 5-methyl uracil (2) and uridine (3) have been isolated from the soft coral *Litophyton arboreum* collected from the red sea. The isolated compounds were identified using spectroscopic techniques predominately ^{1}H-NMR and ^{13}C-NMR analyses, in addition to HR-ESI-MS. Moreover, compounds 1-3 were *in vitro* estimated for their cytotoxic activity against three human cancer cell lines (A549, MCF-7 and HepG2), and antileishmanial potential against *Leishmania major*.

Keywords: *Litophyton arboreum*, Nephtheidae, soft corals**,** cytotoxicity, antileishmanial

1. Introduction
Marine natural products have a diverse spectrum of biological effects, that are significant in the production of important compounds for development of drug [1]. Soft corals contain unique group of metabolites that showed great structure diversity with different bioactivities. Consequently, marine soft corals investigation will lead to invention of many chemically varied compounds with plentiful biological activities that could be used in pharmaceutical industry [2]. Soft corals of the Nephtheidae family, including twenty genera, are a rich source of medicinally active metabolites [3]. Steroids and terpenes are the most commonly described metabolites, and they have anticancer, anti-inflammatory, and antibacterial properties [4]. The genus *Litophyton* (Syn; *Nephthea* [5], is a well-known member of the Nephtheidae family, which is found primarily in the Indo-Pacific and Red Sea regions of the world [6]. The genus *Litophyton* has produced up to 250 bioactive compounds, the majority of which are sesquiterpenes, diterpenes, and polyhydroxylated steroids [7]. These secondary metabolites have been found to have interesting biological activity, particularly in the field of cancer treatment, where modest structural alterations affect potency and selectivity [7]. Only a few investigations on the chemical and biological properties of *L. arboreum* have been conducted [4, 8, 9]. So, *L. arboreum* has been studied for its phytochemical constituents and these studies have resulted in the discovery of different classes of secondary metabolites.

2. Materials and methods
2.1. Soft coral material
Litophyton arboreum soft coral was gathered via SCUBA diving technique in March 2018 at a depth of 10–15 m in front of the National Institute of Oceanography and Fisheries, Hurghada, Egypt's Red Sea. The sample was gathered and identified by Dr. Abdallah Alian, Department of Zoology, Faculty of Science, AL-Azhar University, Assiut-Branch, Assiut, Egypt. The material was kept frozen until the time of extraction. A voucher specimen (LA.5) was placed at the Department of Zoology, Faculty of Science, AL-Azhar University, Assiut-Branch, Assiut, Egypt.

2.2. General Experimental Procedures
^{1}H and ^{13}C-NMR spectra were measured on a JEOL spectrometer at 500 and 125 MHz, respectively. HR-ESI-MS data were noted on a Thermo Fisher Scientific LTQ Orbitrap XL spectrometer. Silica gel for column chromatography (70-230) was used for fine separation (E. Merck, Darmstadt, Germany). Silica gel 60 precoated plates F_{254} (E. Merck, Germany) were used for TLC detection. Reversed-phase (RP-C_{18}) silica gel purchased from Nacalai, Kyoto, Japan was utilized for reversed phase column chromatography separation. Inertsil ODS-3 column (GL Science, Tokyo, Japan) for HPLC analyses using refractive index detector (RID-6A, Shimadzu, Kyoto, Japan).

The Cancer cell lines (lung adenocarcinoma (A549), breast cancer (MCF-7), and hepatocellular carcinoma (HepG2)) were gained from the National Institute of Biomedical Innovation's Japanese Collection of Research Bioresources (JCRB) Cell Bank. The Institute of Tropical Medicine at Nagasaki University in Japan provides *Leishmania major*. Dimethyl sulfoxide, Dulbecco's modified Eagle′s medium, fetal bovine serum, medium 199, miltefosine, etoposide, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and kanamycin were obtained from Nacalai Tesque, Kyoto, Japan. Becton Dickinson provided the 96-well plates (Franklin Lakes, NJ, USA).

2.3. Extraction and isolation

Litophyton arboreum (~1.8 Kg wet wt.) were cut into small pieces and macerated in methanol until it was exhausted. The methanolic extract has been concentrated using reduced pressure to get a dried residue (38 g). The total methanolic extract has been fractionated using vacuum liquid column chromatography filled with silica gel. The elution was carried out with the solvents [*n*-hexane (3L), *n*-hexane-chloroform (1:1) (3L), chloroform (3L), EtOAc (3L), and MeOH (3L)], successively, produced *n*-hexane (1.0 g), *n*-hexane-chloroform (1:1) (9.0 g), chloroform (2.5 g), EtOAc (5.5 g), and MeOH (18.0 g) fractions.

The EtOAc fraction (5.5 g) was separated on a silica gel CC and developed initially with *n*-hexane then with *n*-hexane-EtOAc stepwise gradients with increasing EtOAc to 100%, followed by EtOAc-MeOH gradients with increasing MeOH to 100%, yielding 12 sub-fractions (F1~F12). The sub-fraction F7 (1.5 g) was chromatographed over reversed phase CC using MeOH-H_2O gradients with increasing MeOH to 100%, affording ten sub-fractions (F7-1~F7-10). The sub-fraction F7-1 (450 mg) was purified on HPLC using MeOH–H_2O (10:90) giving compounds 1 (5 mg), 2 (7 mg) and 3 (5.5 mg).

2.4. Evaluation of cytotoxicity

Cytotoxic activity was determined toward different cell lines, A549, MCF-7 and HepG2, using the colorimetric cell viability MTT method described by Samy *et al*., 2015 [10].

2.5. Evaluation of antileishmanial assay

The antileishmanial action was assessed using MTT colorimetric cell viability assay method described by Samy *et al*., 2014 [11].

3. Results and discussion

3.1. Identification of the isolated compounds (1-3)

The EtOAc fraction of the methanol extract of the soft coral *L. arboreum* was treated to normal and reversed-phase silica gel column chromatography, followed by purification using HPLC to give three compounds (1-3) as shown in Figure 1. Intensive spectroscopic examinations were used to identify their structures, as well as a comparison of their physical and chemical attributes to those previously described.

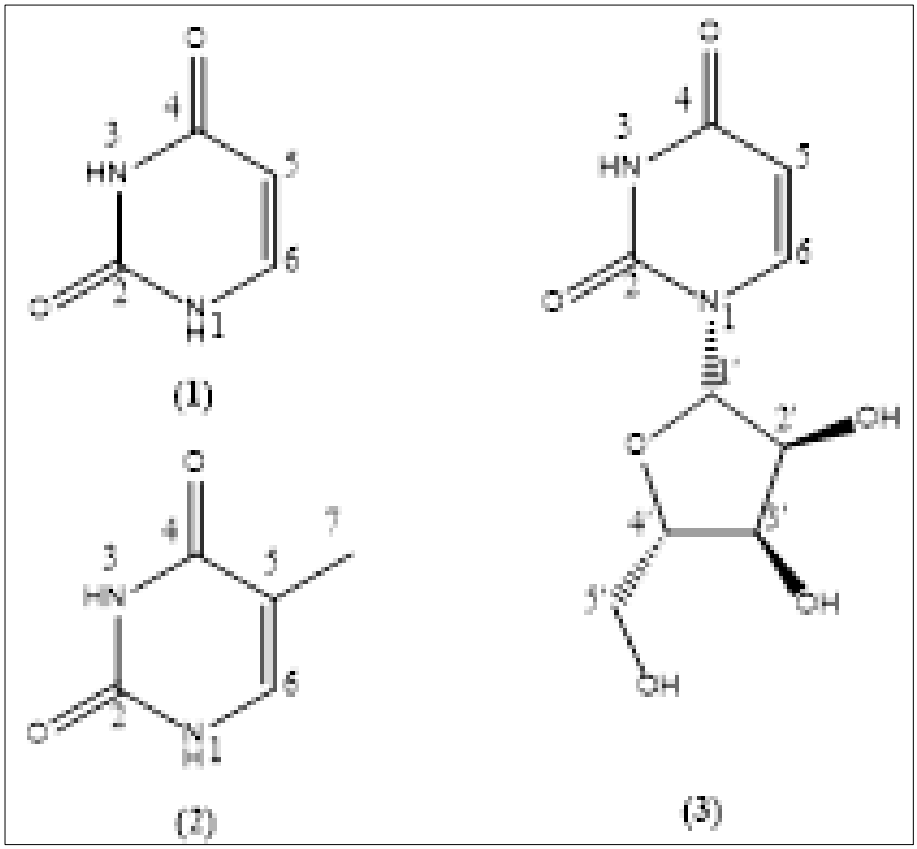

Fig 1: Structures of the isolated compounds **1–3** from *L. arboreum.*

Compound 1 was obtained as colourless needles (5 mg). It exhibited a molecular formula of $C_5H_6N_2O_2$ as determined by negative ion-mode HR-ESI-MS that exhibited molecular ion peak at *m/z* = 125.0361 $[M-H]^-$. ^{1}H-NMR spectrum (Table 1) showed two exchangeable imido broad singlet proton signals at d_H 10.98 and 10.79 were assignable for NH-3 and NH-1, respectively. In addition to the presence of two doublet proton signals at d_H 5.42 and 7.35 (*J*= 6.5 Hz) were assignable to H-5 and H-6, respectively. ^{13}C-NMR spectrum (Table 1) showed the presence of four carbon signals that attributed to imide carbonyl at d_c 164.3 and 151.5 were assignable for C-4 and C-2, respectively. In addition to two methine carbon signals at

100.2 and 142.2, respectively. The structure of compound 1 was identified as uracil [12].

Compound 2 was obtained as colourless needles (7 mg). It exhibited a molecular formula of $C_4H_4N_2O_2$ as determined by positive ion-mode HR-ESI-MS that showed molecular ion peak at m/z =113 $[M+H]^+$. ^{1}H-NMR spectrum (Table 1) showed two exchangeable imido broad singlet proton signals at d_H 10.97 and 10.56 were assignable for NH-3 and NH-1, respectively together with one methine singlet proton at d_H 7.22 was assignable for H-6, in addition to, one methyl singlet proton at d_H 1.71 was assignable for H_3-7. ^{13}C-NMR spectrum (Table 1) exposed the presence of five carbon signals that attributed to imide carbonyl at d_c 164.9 and 151.4 were assignable for C-4 and C-2, respectively, one quaternary carbon signal at d_c 107.6 was assignable for C-5, one methine carbon signal at d_c 137.7 was assignable for C-6, in addition to one methyl carbon signal at 11.8 was assignable for C-7. The structure of compound 2 was identified as 5-methyluracil [12].

Compound 3 was found as colourless needles (5.5 mg). It exhibited a molecular formula of $C_9H_{12}N_2O_6$ as determined by negative ion-mode HR-ESI-MS that showed molecular ion peak at *m/z* = 243.06 $[M-H]^-$. ^{1}H-NMR spectrum (Table 1) showed two doublet proton signals at d_H 5.58 and 7.82 (*J*= 7 Hz) were assignable to H-5 and H-6, respectively. In addition to the anomeric proton signal at δ_H 5.71(d, 1H, *J*= 4.5 Hz) characteristic for *b*-ribose moiety that confirmed through ^{13}C-NMR signals (Table 1) at δ_c 87.7 (C-1'), 73.6 (C-2'), 69.9 (C-3'), 84.9 (C-4'), 60.9 (C-5'). ^{13}C-NMR spectrum also revealed the presence of four carbon signals that attributed to imide carbonyl at d_c 163.2 and 150.8 were assignable for C-4 and C-2, respectively, two methine carbon signals at d_c 101.8 and 140.8, respectively was assignable for C-5 and C-6, respectively. The structure of compound 3 was identified as 1-*b*-D-ribofuranosyl uracil (uridine) [13].

Table 1: ^{1}H and ^{13}C-NMR spectroscopic data of compounds (1-3) (500 MHz and 125 MHz, respectively DMSO-d_6).

No.	Compound 1		Compound 2		Compound 3	
	d_C (m)	**d_H (m, *J* in Hz)**	**d_C (m)**	**d_H (m, *J* in Hz)**	**d_C (m)**	**d_H (m, *J* in Hz)**
2	151.5 (s)	-	151.4 (s)	-	150.8 (s)	-
4	164.3 (s)	-	164.9 (s)	-	163.2 (s)	-
5	100.2 (d)	5.42 (d, *J*=6.5)	107.6 (s)	-	101.8 (d)	5.58 (d, *J*=7)
6	142.2 (d)	7.35 (d, *J*=6.5)	137.7 (d)	7.22 (s)	140.8 (d)	7.82 (d, *J*=7)
7			11.8 (q)	1.71 (s)		
1’					87.7 (d)	5.71 (d, *J*=4.5)
2’					73.6 (d)	3.77 ~ 3.96 (m)
3’					69.9 (d)	
4’					84.9 (d)	
5’					60.9 (t)	
NH-1	-	10.79 (br s)	-	10.56 (br s)	-	-
NH-3	-	10.98 (br s)	-	10.97 (br s)	-	-

3.2. Cytotoxicity activity

The pure isolates obtained from the EtOAc fraction were examined against three cell lines including, A549, MCF-7 and HepG2. Compounds 1-3 showed no effect toward the examined cell lines (IC_{50}>100 µg/mL), comparing with the standard etoposide (IC_{50} 28.4 ± 4.5, 22.2 ± 4.2 and 20.2 ± 0.9 µg/mL, respectively).

3.3. Antileishmanial activity

The growth-inhibitory effects of the isolated compounds on the *L. major* promastigotes were evaluated. The results showed no antileishmanial activity (IC_{50}>100 µg/mL), comparing with that of the standard miltefosine with IC_{50} value of 7.7 ± 2.1 µg/mL.

4. Conclusion

Phytochemical investigation of the soft coral *Litophyton arboreum* resulted in isolation of three compounds that were reported in *L. arboretum* for first time. Therefore, *L. arboreum* was suggested to contain promising compounds for further chemical and biological investigation either from the EtOAc fraction or the remaining fractions.

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