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Brine shrimp cytotoxic activities of some methanolic extracts of marine algae and phytochemical analysis of green alga *Ulva lactuca* Linnaeus

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Abstract

Marine algae produce a wide range of pharmaceutically active metabolites. A total of 24 methanol extracts of algal species (8 green, 8 brown, and 8 red) collected from four different locations, namely, Arugambay, Hambantota, Krinda, and Tangalle, were evaluated for brine shrimp cytotoxicity. They were air-dried thoroughly and powdered using a grinder. Powdered algae were extracted three times with methyl alcohol. The methanol extracts of *Halimeda discoidea*, *Sargassum muticum*, *Dictyota kuenthi*, *Tricleocarpa fragilis*, *Galaxaura lapidescens* showed prominent activity (LC_{50} values ranged from 0.05 to 25.4 µg/mL) while *Cheilosporum acutilobum*, *Dictyota kuenthi* *Amphiroa anceps*, and *Amphiroa fragilissima* showed significant cytotoxic activity (LC_{50} values ranged from 66.15 to 350.1 µg/mL) among 24 extracts. Further, the chemical investigation of the methanol extract of *Ulva lactuca* Linnaeus led to the isolation of five compounds, phytol (**1**), oleic acid (**2**), sucrose (3), a triglyceride (**4**), and isofucosterol (**5**). As algae are highly abundant around Sri Lanka, some of them could be exploited for the isolation of anti-tumour compounds, while others could be exploited for the food in the Sri Lankan diet.

Keywords: Marine Algae, *Ulva lactuca* Linnaeus, Phytol, brine shrimp lethality bioassay, cytotoxicity

1. Introduction:

Marine algae are one of the most commonly utilized functional food and therapeutic agents in many parts of the world [1]. They are recognized for producing a variety of beneficial secondary metabolites, many of which show strong cytotoxicity, antiangiogenic, antioxidants, anti-inflammatory, enzyme inhibitors, anthelmintic, and antimicrobial activities [2-7].

The Sri Lankan coastal belt has a rich source of marine algal flora. The reported marine macroalgal flora of Sri Lanka comprises 396 species within 147 genera and 56 families [8]. Even though marine algae have medicinal properties and have potential food and nutraceutical possibilities, research on the chemistry and biological activities of these marine algae has not yet been fully investigated in Sri Lanka. Various value-added products are developed in overseas countries using seaweeds as a starting material. In our continuous research work on Sri Lankan marine algae and their endophytes [9, 10], methanol extracts of algae were investigated for brine shrimp cytotoxicity. This bioassay has been proven to be a safe, practical, and cost-aeffective approach for determining the bioactivity of fungal pathogenic mycotoxins [11] and seaweeds [3, 6]. *Ulva lactuca* contains a variety of chemical elements that are useful to the body. Furthermore, these compounds offer health

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and nutrition benefits for humans. It has been proven in earlier studies to be a source of anti-cancer [12], stimulated male infertility [13], and antioxidants [14]. By using various chromatographic techniques, the non-toxic methanolic extract of *Ulva lactuca* resulted in isolation of phytol (**1**), oleic acid (**2**), and sucrose (**3**). In our previous studies, a triglyceride with two linoleic acid units and one stearic acid unit (**4**), and isofucosterol (**5**) have been isolated from the same species [15]. In this paper, we describe the cytotoxicity of methanolic extracts of marine algae and the isolation and identification of pure compounds from *Ulva lactuca.*

2. Material and Methods

2.1. *Algae material*

In different seasons, algae were collected in plastic bags from various areas around the Sri Lankan coast. They were identified by direct comparison with the specimens and confirmed with the help of a taxonomist at the National Herbarium, Royal Botanic Gardens, Peradeniya, Sri Lanka. A voucher specimen was deposited by fixing it in 5% buffered formalin at the Natural Products Laboratory of the Institute of Fundamental Studies, Kandy, Sri Lanka. Each species was carefully rinsed in fresh water to remove salt, and epiphytes were dried in the shade.

2.2. *General procedure*

The melting point was determined with a Büchi 535 melting point equipment. The Bruker Avance-400 and 500 NMR spectrometers were used to record 1D and 2D NMR spectra in $CDCl_3$ and CD_3OD, respectively, at 400 and 500 MHz. The ^{13}C NMR tests were performed at 100 and 125 MHz on the same apparatus. Chemical shift (δ) were measured in parts per million (ppm) in comparison to tetramethylsilane (TMS). Column chromatography was carried out using silica gel (E-Merck, 230-400 mesh size).

2.3 Extraction and isolation procedure

In a grinder, dried seaweeds were ground and stored in plastic bags at room temperature until the assay carried out. Methanol (MeOH) was used three times using ultrasonicator to extract all the compounds from dry powder of algae (300 g). The extracts were combined, filtered through cotton wool, and dried on a rotary vacuum evaporator before being weighed. Plant material from *Ulva lactuca* (1.5 kg) was extracted with methanol using an ultrasonicator to obtain 200 g. The methanol extract (50 g) was chromatographed on a column of silica gel (350 g, Merck Kiselegel 60, 230-400 mesh ASTM) using *n*-hexane, ethyl acetate, MeOH and water as eluents to give seven major fractions F-1 to F-7 with their weights. F-1 (259 mg), F-2 (80 mg), F-3 (1.54 g), F-4 (6.3 g), F-5 (11.0 g), F-6 (6.4 g) and F-7 (14.0 g). Repeated silica gel (SiO_2) column chromatography of F-1 (eluent 0.5% ethyl acetate/hexane) yielded pure compound **4** (20 mg). Fraction F-2 was subjected to Preparative TLC using hexane:CH_2CI_2 (1:9) solvent system to give compound **1** (36 mg) as a pure compound. When 80 mg of F-3 was subjected to preparative TLC using CH_2CI_2 : MeOH (7:3) solvent system yielded fractions F-31 (33 mg) and F-32 (26 mg). Column chromatography of F-31 (33 mg) and F-32 (26 mg) yielded pure compound **5** (26 mg) and **2** (12 mg) respectively as pure compounds. Reverse phase column chromatography (RP-18, Merck, LiChroprep, 40-63 mm) of F-4

yielded three fractions F-41 (3.5 g), F-42 (1.2 g) and F-43 (150 mg). Further reverse phase column chromatography of F-42 yielded pure compound **3** (35 mg).

2.4 Micro-well cytotoxicity assay on brine shrimp (*Artemia salina*)

The toxicity of a methanol extract of seaweeds was assessed using a brine shrimp micro-well cytotoxicity assay [16]. Brine shrimp eggs (10 mg) were placed in a 250 mL beaker containing 150 mL of artificial seawater, and it was aerated and kept under continuous illumination (40 Watt yellow light bulb) and then they were incubated for 48 hours, i.e. till larvae become 2^{nd} instar nauplii stage. The methanol extract (2 mg) of algae were dissolved in dimethyl sulphoxide (DMSO) at a maximum concentration not exceeding 0.05 % and then diluted with artificial seawater (2 mL) and from that stock solution; 100, 50, 10 and 1 µL of solution were added to the wells of 96 micro-well plate in triplicates, where each well is 200 µL in volume. Above solutions will correspond to 1000, 500, 100 and 10 ppm in final concentrations. Then, 10 nauplii were counted and added to each well of 96 micro-well plate which contained seaweed extracts and volumed up to 200 µL by adding seawater. Number of surviving nauplii was counted after 24 hours of incubation at the room temperature (25 ^{o}C). In this experiment, the negative control was 0.05% DMSO in artificial seawater, while the positive control was 4-hydroxy-2-methylquinoline (Sigma-Aldrich H4, 360-1). United States Environment protection Agency (US EPA) Probit analyzer, version 1.5 was used to calculate the concentration that would kill 50% of the brine shrimps within 24 hours of exposure, i.e. the LC_{50} with 95% confidence intervals.

3. Results and Discussion

The cytotoxic activity of a total of 24 algal species was investigated. In the brine shrimp lethality assay, nine of these species had LC_{50} values of less than 1000 µg/mL. Many red algae species, including *Amphiroa anceps*, *Amphiroa fragilissima*, *Cheilosporum acutilobum*, *Galaxaura lapidescens*, and *Tricleocarpa fragilis*, have LC_{50} values of 225.1 µg/mL, 213.6 µg/mL, 66.1 µg/mL, 25.4 µg/mL, and 1.3 µg/mL, respectively. Among eight brown seaweeds, *Sargassum muticum*, *Dictyota kuenthi*, and *Dictyota dumosa* showed LC_{50} values of 4.7 µg/mL, 13.4 µg/mL, and 350.1 µg/mL, respectively. The highest activity was observed in the methanolic extract of green alga *Halimeda discoidea* with an LC_{50} of 0.05 µg/mL. This is due to the presence of cytotoxic compounds [17] (Table-1). However, methanolic extracts of some algae, including *Ulva lactuca*, did not show cytotoxic activity and showed LC_{50} > 1000 µg/mL. The cytotoxicity of hexane, CCI_4, and $CHCl_3$ fractions of methanolic extract of the seaweed *Sargassum tortile* has been described in previous investigations. In another investigation, brine shrimp death was produced by ethanolic extracts of *Sargassum swarizii* and *Sargassum binderi* at concentrations below 1000 µg/mL, and methanolic extracts of *Stoechospermum marginatum* and *Spatoglossum asperum* at concentrations of 443 and 415 µg/mL, respectively [4, 6]. Four species were found to cause brine shrimp death at concentrations lower than the positive control, 4-hydroxy-2-methylquinoline (30.15 µg/mL), in this study. These findings demonstrated that Sri Lankan seaweeds might be used to isolate anticancer chemicals (Table 1).

Table 1. Percent death of brine shrimp at different concentrations of methanolic extracts of algal species after 24 hours.

	Methanolic extracts	10 µg	100 µg	500 µg	1000 µg	LC_{50} (µg/mL)
	Green species					
1	*Ulva lactuca* Linnaeus	0	10	10	20	>1000
2	*Cladophora prolifera* (Roth) Kützing	10	20	30	30	>1000
3	*Valoniopsis pachynema*	10	20	30	60	>1000
4	*Codium fragile* (Suringar) Hariot	0	10	20	30	>1000
5	*Codium taylori P.silva*	0	0	10	10	>1000
6	*Halimeda discoidea* Decaisne	70	80	90	90	0.05
7	*Cladophoropsis sundanensis* Reinbold	0	10	10	20	>1000
8	*Chaetomorpha gracilisis*	0	10	20	30	>1000
	Brown species					
9	*Sargassum wightii* Greville	0	10	20	30	>1000
10	*Sargassum muticum*	30	40	90	90	4.72
11	*Sargassum fusiforme*	0	10	20	30	>1000
12	*Lessonaia fuscescens*	0	0	10	10	>1000
13	*Padina pavonica* (Linnaeus) Thivy	0	0	20	30	>1000
14	*Chnoospora minima* (K.Hering) Papenfuss	0	10	20	30	>1000
15	*Dictyota kuenthi*	40	40	70	80	13.4
16	*Dictyota dumosa*	10	20	40	60	350.1
	Red species					
17	*Amphiroa anceps*	10	30	60	60	225.1
18	*Amphiroa fragilissima*	10	30	60	60	213.6
19	*Cheilosporum acutilobum*	10	50	100	100	66.15
20	*Galaxaura lapidescens*	20	80	100	100	25.4
21	*Laurencia ceylanica*	10	30	60	60	>1000
22	*Gracilaria corniculata*	0	10	20	30	>1000
23	*Gracilaria multifarata*	10	10	20	30	>1000
24	*Tricleocarpa fragilis*	90	100	100	100	1.3
	7-hydroxy coumarin (positive control)	20	70	100	100	30.1

Repeated column chromatography on flash silica gel yielded five pure chemicals from the non-cytotoxic methanolic extract of *Ulva lactuca* Linnaeus. As described in the extraction and isolation technique, five pure compounds were isolated, and the structures of five of them were determined using NMR and Mass spectral data.

Phytol (1)

Compound 1 was separated as yellow oil with 20 carbon signals in ^{13}CNMR and DEPT spectrum due to 5 methyls, 10 methylenes, 4 methines, and a quaternary carbon. HMQC experimental data was used to assign all proton-carbon correlations. Two carbons were assigned as olefinic carbons, appearing at dc 123.3 and 140.0, and five carbons were ascribed as methyl carbons, appearing at δc 16.3, 19.91, 19.95, 22.8, and 22.9, respectively. Furthermore, a doublet at δ 0.84 (6H, J = 6.4 Hz) was identified as part of an isopropyl group that was connected with a methine proton that resonated at δ 1.48 in the 1H NMR spectrum. Another doublet was ascribed to two split methyl group at δ 0.82 (J = 6.0 Hz). The CIMS of the compound exhibited its molecular ions at m/z 295.0 ([M-H]$^+$) and 297.0 ([M+H]$^+$). According to the data above, the compound is (Z)-3,7,11,15-tetramethylhexadec-2-en-1-ol with the molecular formula $C_{20}H_{40}O$ (Figure-1).

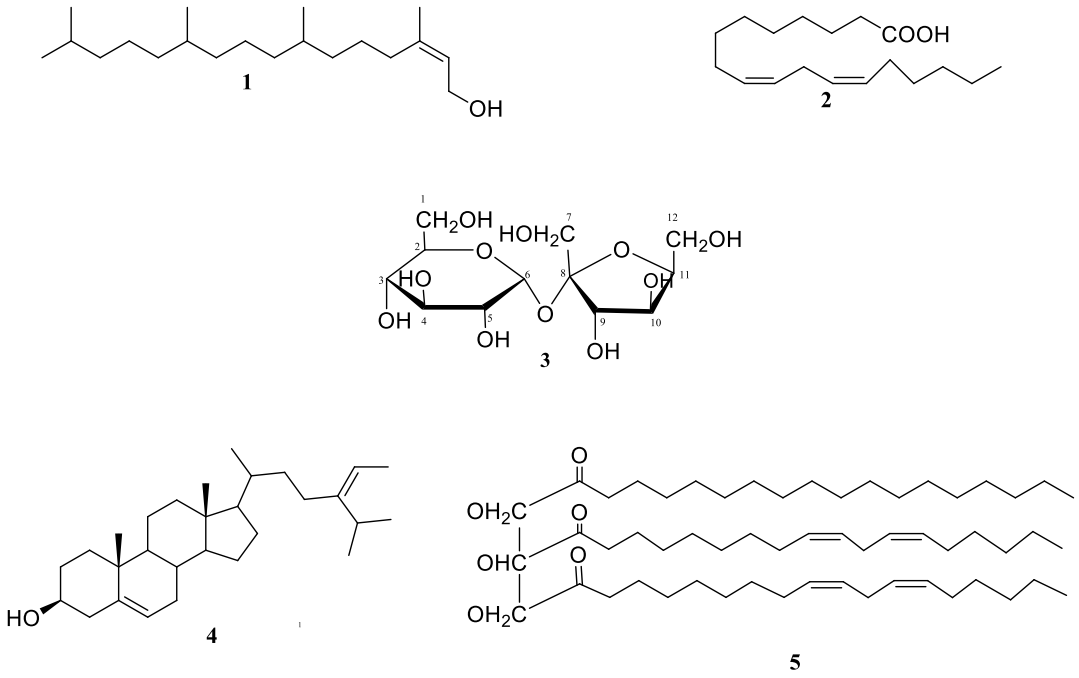

Figure -**1**. Structures of Compounds **1 - 5.**

Oleic Acid (2)

The compound **2** was isolated as a light-yellow powder. ^{13}C NMR and DEPT data showed 18 carbon signals due to 1 methyl, 14 methylenes, 2 methines and 1 quaternary carbon. All protons attached carbons were assigned by a HMQC experimental data. Of the 18 carbons, 1 quaternary carbon was ascribed to carbonyl carbons appeared at δ 179.3, two olefinic carbons appeared at δ 130.1 and 130.0,

and one methyl carbon at δ 14.3. ^{1}H NMR spectrum showed the presence of allylic (C=CH-C<u>H</u>$_2$), olefinic (C<u>H</u>=CH) and methyleni<u>c</u> (CH_2) protons appearing as multiplets at δ 1.9, δ 5.27 and δ 1.2-1.3 respectively in the alkyl chain. The structure of compound 2 was established as shown in Figure-1, having a molecular formula ($C_{18}H_{34}O_2$) using the above spectral data and by comparison with literature data.

Sucrose (3)

Three methylenes, eight methines, and one quaternary carbon atom were found in the ^{13}C NMR and DEPT spectra of compound 3, which was isolated as a hygroscopic semi solid. All protons were assigned by HMQC and HMBC experimental data. Of the 12 carbons, quaternary carbon was ascribed to oxy quaternary carbon (δc 106.3), 3 oxy methylenes (δc 65.24, 63.43, and 62.83) and 8 oxy methines. The ^{1}H NMR spectrum of 3 clearly showed a doublet at δc 6.22 (J = 4.0 Hz) indicating the presence of anomeric proton attached to carbon atom which resonated at δc 93.9. The COSY spectrum also indicated that this anomeric proton was coupled to the oxy methine proton at δ_H 4.21 (dd, J = 6.4 Hz, 10.0 Hz) attached to the carbon which resonated at δc 73.9. The connectivity between pyranose and furanose ring was established on the HMBC correlation of anomeric proton to oxy quaternary carbon atom of furanose ring. The compound's CIMS revealed quasi molecular ion peaks at m/z 341.0 ($[M-H]^+$) and 365.0 ($[M+Na]^+$). According to the details given above, compound 3 is sucrose, which has the molecular formula of $C_{12}H_{22}O_{11}$. The proposed structure was further confirmed by TLC comparisons of 3 with sucrose and its acetate derivatives.

4. Conclusion

According to the findings, *Ulva lactuca* methanol extract contains steroids, fatty acids such as linoleic acid and oleic acid, free sugars, and the acylic diterpenoid phytol. Conjugated linoleic acid is the precursor to linoleic acid, which is an essential fatty acid. The presence of linoleic acid and free sugars in *Ulva lactuca* highlights the nutritional value of this seaweed, which is popular in Far-East Asia. The availability of seaweeds is abundant in Sri Lanka and some could be used to isolate antitumor compounds, while others to use as food in the Sri Lankan diet.

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