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# Chemistry and Bioactivity studies of green alga *Ulva lactuca* L. and red alga *Amphiroa anceps* (Lamarck) Decaisne

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**Abstract**

Methanol extracts of common seaweeds *Ulva lactuca* and *Amphiroa anceps* were subjected to antibacterial, antifungal, antioxidant, cytotoxicity and seed germination inhibition assays. MeOH extract of *Amphiroa anceps* showed seed germination inhibition activity and cytotoxicity. However, no activity was observed in *Ulva lactuca* extract. Column chromatography of the methanol extract of *Amphiroa anceps* followed by PTLC gave sitosterol and stearic acid. Column chromatography of the MeOH extract of *Ulva lactuca* followed by PTLC gave several metabolites, and three of them were identified as (Z)-stigmasta-5, 24(28)-dien-3-ol, Oleic acid and a triglyceride with two linoleic acid units and one stearic acid unit.

**Introduction**

Marine algae (seaweeds) have been used as food, fodder and fertilizer since ancient times.[1-4] Seaweeds are the raw material for industrial production of agar, carrageenan and alginates.[5] Some substances extracted from marine algae have shown many pharmacological activities.[6-7] Even though Sri Lanka is surrounded by ocean, Sri Lankan marine algae remain largely unexplored, so that biomedical potentials of them remain largely unnoticed. Therefore, the present investigation is initiated with the hope of isolation and identification of biologically active and economically important compounds from Sri Lankan seaweeds.

The use of natural products as a source of medicines involves the search of new compounds from different origins. Marine organisms are an important source of biologically active compounds.[8] The marine macro algal flora of Sri Lanka comprise of 396 species within 147 genera and 56 families. This number is based on only a handful of sporadic accounts and the records on marine algal diversity of Sri Lanka are inadequately known.[9,10] Marine algae are known to produce an incredible diversity of secondary metabolites some of which show anticancer, cytotoxic, antimicrobial, anti-inflammatory, antiviral, anthelmintic, and insecticidal activities.[6,11]

Our ongoing research project is devoted to obtain bioactive and economically important compounds from marine algae/seaweeds collected from Sri Lankan coastal line.[12] MeOH extracts of the common seaweeds *Ulva lactuca* and red alga *Amphiroa anceps* were subjected to antibacterial, antifungal, antioxidant, cytotoxicity and seed germination inhibition assays. The MeOH extract of *Amphiroa anceps* was found to be cytotoxic towards the brine shrimp lethality bioassay and phytotoxic towards the lettuce seed germination bioassay. However, MeOH extract of *Ulva lactuca* did not show any activity. Cholesterol, isofucosterol, stigmast-5-en-3ß-ol,stigmasta, 5-22-dien-3ß-ol,22-dehydrocholesterol, ergosta-5(22)-dien-3ß-ol and ergost-5-en-3ß-ol and fucosterol have been reported from the extracts of *Ulva lactuca*.[1] In a previous communication we reported the isolation of (Z)-stigmasta-5,24(28)-dien-3-ol, oleic acid and a triglyceride with two linoleic acid molecules and one stearic acid molecule, from *Ulva lactuca*.[13]

**Experimental procedure**

*Algal material*

Red alga, *Amphiroa anceps* normally found at a depth of around 400m in the sea, was collected with the help of fishermen from Pottuvil in the Eastern part of Sri Lanka during August to December 2005.

The green alga, *Ulva lactuca*, was collected from the Arugambay shores, in the Eastern part of Sri Lanka in August 2005 by hand picking. Above seaweeds were identified by comparison with herbarium specimens at the Royal Botanic Gardens, Peradeniya, and specimens were preserved by fixing them in 3-5% buffered formalin salt water and kept at the Natural Products Chemistry laboratory, Institute of Fundamental Studies.

*Preparation of crude extracts*

Seaweeds were cleaned and washed several times with freshwater to remove salt, sand and other debris. Air-dried seaweeds were powdered using a grinder and were separately extracted for one hour with MeOH under sonication and filtered. MeOH in the filtrates was evaporated using a rotary evaporator to give crude extracts. Then the crude extracts were dried in a vacuum oven and tested for their bioactivities using bioassays given below.

**Seed germination inhibition bioassay**

Lettuce seeds (*Lactuca sativa* L.) were used in this test as they are inexpensive, easy to culture and require no upkeep between lettuce seeds experiments.[14] The germination rate in distilled water was examined at random before the experiment and found to be > 85%. Two 1000 ppm aqueous solutions of crude seaweed extracts were prepared separately at room temperature. All seeds were surface sterilized with 10% Clorox for 10 mins. Then they were washed 5 times (1 min/wash) with sterilized deionized water. Maximum germination of above surface sterilized seeds was observed following incubation in distilled water. Four replicates, each with 5 seeds were prepared for two extracts using sterile petridishes (90 mm) lined with sterile filter papers. Prepared aqueous extracts (5 ml) were added to each petridish. The positive control group was treated with 5 ml of 100 ppm Abscisic acid solution while the negative control was treated with deionized water. Prepared plates were then incubated in a growth chamber at 25 $^0$C in dark for 5 days. The effect of each extract was observed by calculating the percentage seed germination and by measuring radicle length to the nearest millimeter.

**Brine shrimp lethality bioassay**

The toxicity of extracts to brine shrimp *Artemia salina*[14] was determined using 7-hydroxy coumarin as the positive control. Concentrations of 1000, 500, 100 and 10 ppm of the seaweed extracts were used. Ten larvae were added to each concentration and the control in triplicate, and number of surviving larvae was counted after 24 hours of incubation. Finally the $LC_{50}$ values were calculated and compared with the the positive control using one-way ANOVA and turkey's pair wise comparison.

*Antifungal assay*[15]

TLC bioassay was used to check the antifungal activity of extracts and pure compounds against *Cladosporium*. In this method 10 ml of Czepak Dox Broth (CDB) was added to previously prepared *Cladosporium* cultures and was shaken well till the water turned cloudy. Then the spores were filtered into the spraying apparatus through a glass wool or muslin cloth. Concentration of the spore suspension was adjusted to 40-50 spores per field at X 400 under light microscope by adding prepared CDB medium. This spore suspension was sprayed on to previously spotted TLC/PTLC plates which were air dried for 6 hrs. Then the plates were incubated at room temperature in a moisture chamber for 2 days and observed for inhibition of growth.

Disc diffusion method which is commonly used for bacteria and also for yeast like fungi, was modified[16] in order to use for filamentous fungi like *Aspergillus*. In this method a liquid culture of *Aspergillus* on CDB was prepared by inoculating 7 days old fungus grown on Potato Dextrose Agar. Extracts (1mg) were dissolved in 100 µl of MeOH/ EtOAc and sterile disc papers (Diameter of 7 mm) were soaked with 20 µl of the prepared solution and allowed to dry completely. Meanwhile CDA medium was prepared, autoclaved and cooled to 45 $^0$C and then inoculated with the liquid culture of *Aspergillus* (0.5 ml of liquid culture for 25 ml of CDA medium). Then the medium was poured into sterilized

petridishes (20 ml per each) and left until solidified. After solidification, dried disc papers were placed on the inoculated medium, sealed with parafilm and kept for 24 hrs in a refrigerator at 4 $^0$C. Thereafter, the plates were transferred into an incubator (30 $^0$C). Readings were taken after 3 and 5 days. Diameters of the inhibition zones were measured along the two diameters at right angle to each other. Two replicates were used for each sample and benlate was used as the positive control.

**Isolation of pure compounds**

Powerded *Amphiroa anceps* (4.4 Kg) was extracted for one hour in MeOH under sonication to give 27.50 g of the crude dry extract. Above extract (26.5 g) was chromatographed on a column of silica gel (350 g, Merk Kiselegel 60,230-400 mesh ASTM) using n-hexane, EtOAc, MeOH and water as eluents to give nine major fractions F-1 (0.30 mg), F-2 (1.16 g), F-3 (0.65 g), F-4 (0.93 g), F-5 (0.24 g), F-6 (0.12 g), F-7 (0.23 g), F-8 (0.71 g) and F-9 (4.5 g). Repeated silica gel ($SiO_2$) column chromatography of F-2(0.20 g) and F-3 (0.20 g) (eluent 0.1% EtOAc/hexane) yielded pure compounds AA-1 (40 mg) and AA-2 (20 mg) respectively.

Powdered *Ulva lactuca* (1.0 Kg) was extracted for one hour in MeOH under sonication to give 200 g of the crude dry extract. Above extract (50 g) was chromatographed on a column of silica gel (350 g, Merk Kiselegel 60, 230-400 mesh ASTM) using n-hexane, EtOAc, MeOH and water as eluents. Further purification of some column fractions using PTLC and gravity column yielded seven pure compounds (UL-1 to UL-7), and the structures of three compounds were proposed using NMR and MS data.

**Results and discussion**

Compound AA-1 was isolated as a white crystalline compound (mp 138-140 $^0$C), and its $^1$H NMR spectrum indicated it to be a steroid. Comparison of NMR data with literature values and TLC comparison with an authentic sample confirmed compound AA-1 to be sitosterol. Mixed melting point (138-140 $^0$C) further confirmed the proposed structure for AA-1.

The compound AA-2 was isolated as a white crystalline powder with a melting point of 65-67 $^0$C. $^1$H NMR spectrum of AA-2 and its streaking nature on TLC plates indicated it to be a long chained fatty acid. $^{13}$C NMR and DEPT data of AA-2 showed 18 carbon signals due to one methyl, 16 methylenes, and one quaternary carbon. All proton attached carbons were assigned by a HMQC experimental data. Of the 18 carbons, one quaternary carbon was ascribed to a carbonyl carbon appeared at δ 179.3. The single methyl carbon appeared at δ 14.3 and the methylene carbon adjacent to the carbonyl carbon appeared at δ 34.2. The methylene carbon adjacent to the methyl carbon appeared at δ 32.1. In the $^1$HNMR spectrum of AA-2 single methyl group appeared as a triplet at δ 0.88 ($J$=6.8Hz). The methylene group adjacent to the carbonyl carbon appeared as a triplet at δ 2.35($J$=7.2Hz). Comparison of spectral data of AA-2 with literature values suggested AA-2 to be Stearic acid having molecular formula ($C_{18}H_{36}O_2$).[10]

The compound UL-1 was isolated from the methanol extract of *Ulva lactuca* as a yellow oil and its $^{13}$CNMR and DEPT spectra showed 57 carbon signals due to 3 methyl, 42 methylene, 9 methine and 3 quaternary carbon atoms. Of the 57 carbons, 3 quaternary carbons were ascribed for carbonyl carbons (δ 173.3 X 2, 172.9), 2 oxy methylene (δ 62.1 X 2) and one oxy methine (δ 68.9) for acyl substituted carbons, 8 olefinic carbons (δ 130.2, 130.0, 129.9, 129.8, 128.8, 128.3, 128 and 127.9) and 3 for methyl carbons (δ 14.1 X 2, 14.3). It is noted that the signals at δ 62.1 and δ 68.9 of $^{13}$CNMR spectrum are characteristic pattern for glycerol moiety. The $^1$HNMR spectrum of UL-1 clearly showed two double doublets at δ 4.15 ($J$=6.3, 12Hz) and δ 4.3 ($J$=6.3, 12Hz) indicating the presence of non-equivalent oxy methylene proton attached to carbon atoms, which resonated at δ 62.1. The COSY spectrum also indicated that those non-equivalent oxy methylene protons ($H_a^+$, $H_b^+$) were coupled to an oxy methine proton at δ 5.2 attached to the carbon which resonated at δ 69.8. Further $^1$HNMR spectrum of UL-1 showed the presence of allylic protons appeared as multiplets at δ 2.0 and 2.8, olefinic protons as multiplets at δ5.3-5.4 and methylene protons as multiplets at δ 1.2-1.3 in the acyl chains. The connectivity between glycerol and acyl moiety

was established on the HMBC correlation of $H_a'$ and $H_b'$ to C-1. All the protons of UL-1 were assigned by HMQC and HMBC experimental data. Considering above spectral data the structure of UL-1 was established as a triglyceride with two linoleic acid units and one stearic acid unit, having molecular formula ($C_{57}H_{102}O_6$) (see Fig-1).

The compound UL-3 was isolated from the methanol extract of *Ulva lactuca* as a white crystalline powder with a melting point of 136-137$^0$C. Its $^{13}$CNMR spectrum showed 29 carbon atoms. DEPT experiments indicated the presence of six methyls, ten $sp^3$ methylenes, seven $sp^1$ methynes, two $sp^2$ methynes, two $sp^3$ and two $sp^2$ quaternary carbons. Four of the 26 carbons were identified as olefinic carbons resonated at δ 116.4, 121.7, 140.8 and 145.8. The $^1$HNMR spectrum showed H under OH at C-3 resonated at δ 3.52 as a multiplet. Three methyl groups were observed as singlets at δ 0.68 and 0.94 and as a doublet at δ 1.0 ($J$=6.3Hz). A low field doublet appeared at δ 1.59 ($J$= *6.6*) was assigned for an allylic methyl group. An olefinic proton was appeared as a quartet at δ 5.11($J$=6.9). Further, the presence of another olefinic proton was detected as a distorted triplet at δ 5.3, which is a characteristic pattern of $\Delta^5$ – sterols.[2] The CIMS of UL-2 had $M^+$ of *m/z* 412 with *m/z* 413 $[M+1]^+$ as the base peak, consented with molecular formula $C_{29}H_{48}O$. Considering above spectral data and HMBC correlations the structure of UL-3 was confirmed as (Z)-stigmasta-5,24(28)-dien-3-ol (see Fig-1).

**Table 1: % Lettuce seed germination assay results**

| **Seaweed Extract** | ***% Seed Germination**** | ***Average radicle Length* (cm)*** |
|---|---|---|
| *Amphiroa* anceps | 35 | 1.47 ± 0.242 |
| Ulva lactuca | 95 | 1.09 ± 0.172 |
| Distilled water | 95 | 3.33 ± 0.172 |

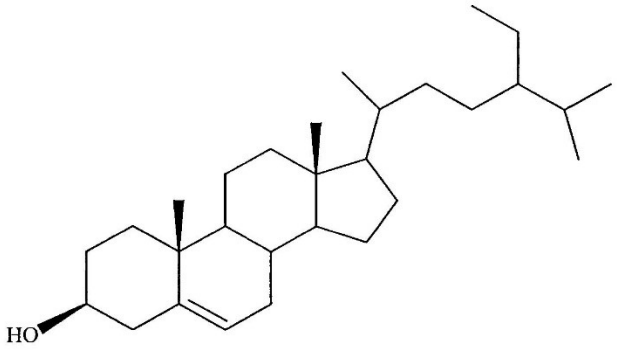
*Values are mean ± S.E.; n = 2; P< 0.001 vs. control; one-way ANOVA and Tukeys' Pair wise comparisons.

MeOH extract of *Amphiroa anceps* has shown significant difference in the average radicle length and % seed germination (35%) compared to the control (sterile distilled water). Therefore our observations suggest that the MeOH extract of *Amphiroa anceps* has a significant seed germination inhibition activity and could be used as a source of natural herbicide. Further studies are in progress with the hope of isolating the responsible allelochemical/s present in the seaweed extract, which has shown significant inhibitory activity.

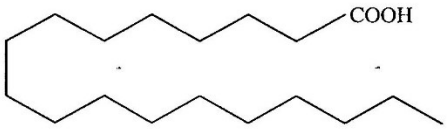
*Brine shrimp lethality bioassay*:

**Table 2: $LC_{50}$ values of different treatments.**

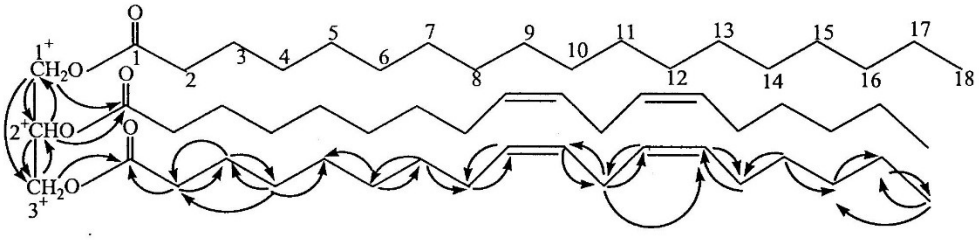
| **Seaweed Extract** | **$LC_{50}$ value (ppm)** |
|---|---|
| *Amphiroa anceps* | 10.5 |
| *Ulva lactuca* | >1000 |
| Positive control | 30.2 |



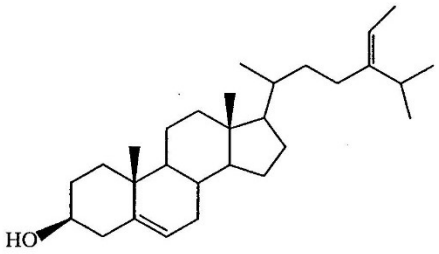

**Sitosterol (AA-1)**




**Steric acid (AA-2)**




**HMBC correlations of triglyceride (UL-1)**




**(Z)-stigmasta-5,24(28)-dien-3-ol (UL-3)**

**Figure-1**

*Seed germination bioassay*:

Results of brine shrimp lethality bioassay were estimated by probit software using mortality data. Lower $LC_{50}$ values (less then 1000 ppm), are an indication of cytotoxicity. *Amphiroa anceps* showed a significant level of cytotoxicity on brine shrimp when compared with the *Ulva lactuca* extract. The $LC_{50}$ value of *Amphiroa anceps* was found to be 10.5 ppm when compared with the positive control, 7-hydroxycoumarin, which gave the value of 30.2 ppm. $LC_{50}$ values of the MeOH extract of *Amphiroa anceps* showed a strong cytotoxicity on brine shrimps, which is an indication of potential anticancer activity and further studies on above extract is in progress.

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