

Isolation and Identification of Tyrosinase Inhibitors from marinealgae *Enteromorpha* sp.

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Abstract:

The extract of marine green algae *Enteromorpha* sp. was evaluated in vitro for inhibitory activity against mushroom tyrosinase enzyme. The principle active agents i.e. coumarin; 4-hydroxycoumarin (**1**) and two sterols; ergosta-5,7,22-trien-3 β -ol (**2**) & ergosterol peroxide (**3**) were isolated for the first time, from a crude methanol extract of *Enteromorpha* sp. showing anti-tyrosinase activity. Their structures were elucidated by IR, extensive NMR spectroscopy, LC-ESI-MS, Single crystal X-ray diffraction techniques. Thus, *Enteromorpha* sp. can be an alternative edible anti-tyrosinase agent.

Keywords: Marine natural products, anti-tyrosinase activity, *Enteromorpha* sp., Ulvaceae, marine algae

1. Introduction

Marine algae are chlorophyll-containing entities, made up of either a single cell or many cells and exist as colonies or as simple tissues. It is one of the essential part of marine ecosystem which perform a vital role of producing biomass in the ocean. In order to get rid of settling organisms, most of the marine algae produce chemically active metabolites in their vicinity.[1,2] As a result, marine algae have attracted attention as a source of wealthy biologically active secondary metabolites. Studies proved that marine algae display intriguing properties such as anti-cancer, antiallergic, anti-coagulant, anti-viral, anti-inflammatory, antiobesity, etc. along with anti-fouling abilities.[2] They are known to inherit metabolites like fatty acids, steroids, pyrroles, lipoproteins, amides, terpenoids, alkaloids, lactones, etc.[1] Besides being pharmacologically and ecologically significant, it is also rich in compounds with nutritional values (minerals, vitamins, proteins, carbohydrates, etc.).[3,4] Marine green macroalgae *Enteromorpha* sp. belonging to family Ulvaceae, is dissiminated worldwide, nurtures at tidal stretch and is excessively important in terms of its bio-medicinal and nutritional values.[5] It houses numerous bioactive secondary metabolites such as sterols, chlorophyll-related compounds,[6] phenolic polyketides,[7] α -pyridones,[8] polysaccharides[9], etc. Some of these compounds exhibit anti-inflammatory, quorum sensing inhibitory properties, etc.[8,10,11]. Hence, there is a need to explore this species for its therapeutic applications and further, discover the secondary metabolites responsible for those effects.

Tyrosinase enzyme, also known as polyphenol oxidase is a complex copper-holding enzyme, found in prokaryotes as well as in eukaryotes and bio-catalyzes the biosynthesis of melanin from tyrosine through a chain of oxidative reactions. Even though melanin plays an essential role in protecting the skin from getting damaged upon the exposure to harmful UV radiations; skin whitening is a classical issue in many human cultures. Moreover, undesirable enzymatic browning lowers the grade of commercially important fungi, fruits and vegetables. There are several synthetic, semi-synthetic and natural tyrosinase inhibitors derived from countless natural sources (plants, fungi, bacteria, etc.) reported in the literature.[12,13] As health safety is the primary motto of any food industry, it has drawn attention of scientific researchers towards exploiting naturally occurring sources for tyrosinase inhibitors as it would probably exhibit negligible toxic side-effects.[14].

In the quest of discovering an alternative natural source of tyrosinase inhibitors and in view of our research interest in marine natural products,[15–19] we were determined to evaluate the anti-tyrosinase property of crude methanol extract of *Enteromorpha* sp. Crude methanol extract of *Enteromorpha* sp. mildly inhibited the mushroom tyrosinase. This finding inspired us to further investigate the secondary metabolites which could be responsible for this interesting activity. In the

present study we are reporting isolation and characterization of major metabolites 4-hydroxycoumarin (**1**), ergosta-5,7,22-trien-3 β -ol (**2**), ergosterol peroxide (**3**) from the anti-tyrosinase active crude methanol extract of *Enteromorpha* sp. (**Figure 1**). Moreover, *Enteromorpha* sp. have not been evaluated for its anti-tyrosinase property till date. Also, this stands the first report on isolation of 4-hydroxycoumarin (**1**), ergosta-5,7,22-trien-3 β -ol (**2**), ergosterol peroxide (**3**) from *Enteromorpha* sp.

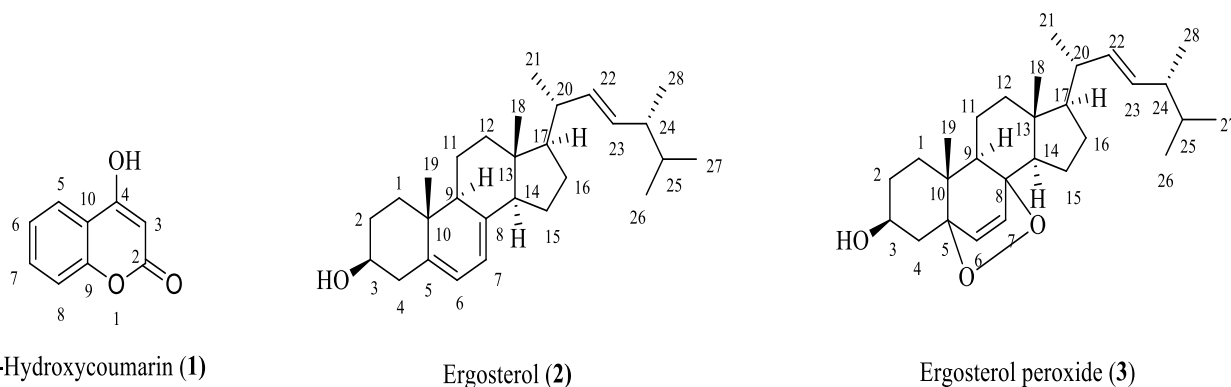


Figure 1. Structures of the secondary metabolites isolated from marine green algae *Enteromorpha* sp. in the present work.

2. Results And Discussion

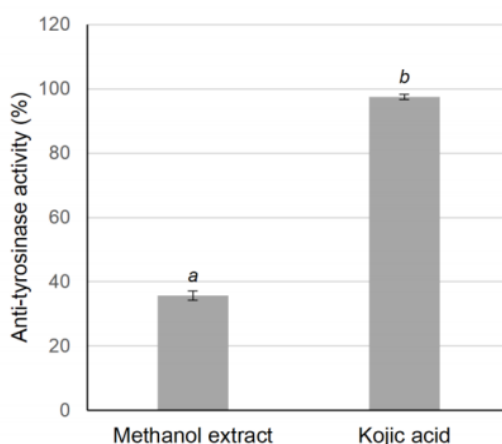


Figure 2. The in vitro anti-tyrosinase activity of crude methanol extract of *Enteromorpha* sp. at the concentration 0.6mg/mL for L-tyrosine. Error bars indicate standard deviation (SD) of the triplicates. Kojic acid was used as positive control. a,b Different superscripts are statistically significant at $p < 0.05$ as measured by post hoc Tukey's HSD test, performed by ANOVA. Comparisons between means were performed by student's t-test. The software used was IBM® SPSS Statistics v.23.0 program for Windows.

Primarily, crude methanol extract of *Enteromorpha sp.* with 35.70 ± 1.40 % inhibition (Mean \pm SD of three assays at the concentration 0.6 mg/mL for L-tyrosine) (**Figure 2**) was partitioned between hexane and ethyl acetate for further examination. The hexane fraction was subjected to silica gel column chromatography which delivered beige powder, appeared as a single spot on TLC (ethyl acetate/hexane 6:4). The ^1H NMR was recorded to obtain preliminary information. However, the data indicated the presence of trace amount of impurity with identical R_f value (indicated by TLC). Thus, this partially purified sample was subjected to multiple developments in ethyl acetate/hexane 3:7 as the mobile phase to increase the resolution. Finally, preparative TLC purification led to the isolation of 10 mg of beige powder. The molecular formula was established as $\text{C}_9\text{H}_7\text{O}_3$ from LC-ESI-MS data ($[\text{M} + \text{H}]^+$ 163.0386, calculated for $\text{C}_9\text{H}_7\text{O}_3$, 163.0395). Interestingly, NMR gave three ^1H signals corresponding to four protons in the aromatic region of spectrum and two aromatic quaternary ^{13}C signals (confirmed by DEPT data) which verifies the presence of 1,2-disubstituted aromatic ring. LC-ESI-MS data, together with NMR data indicated seven degree of unsaturation. The UV and IR analysis provides information about the presence of hydroxyl, carbonyl and aromatic skeleton; which accounts for six double bond equivalence and the left over one double bond equivalence must be due to a ring. Based on the above observations, coumarin scaffold was assigned for the isolated compound 1. However, we were not able to assign the position of the hydroxyl group (3- or 4-OH) on coumarin scaffold by ^1H , ^{13}C NMR and DEPT data.[20–22] Hence, we crystallized this beige powder from aqueous ethanol to obtain a transparent needle shaped crystal which was subjected to Single crystal X-ray diffraction studies to give the crystal structure of the compound 1 (**Figure 3**), confirming hydroxyl to be situated at 4th position on the coumarin core of 4-hydroxycoumarin (**1**). *Enteromorpha sp.* was found to be a new source for 4-hydroxycoumarin (**1**).

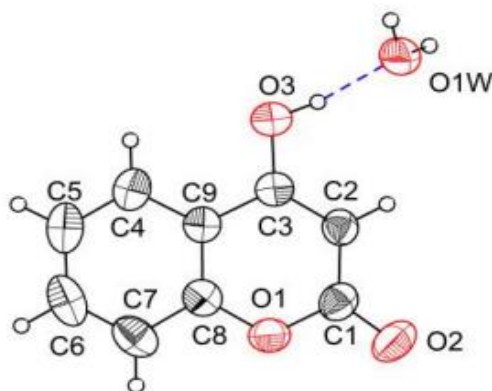


Figure 3. Crystal structure of 4-hydroxycoumarin (**1**). Thermal ellipsoids are drawn at the 50% probability level and H atoms are shown as spheres of arbitrary radii. Intramolecular H-bond is shown in broken line.

The TLC analysis of ethyl acetate fraction of *Enteromorpha* sp. showed intense coloured spot when the TLC plate was sprayed with 5% methanolic H₂SO₄ staining reagent. Hence, we targeted the purification of this spot on silica gel column (ethylacetate/hexane 3:7). For preliminary investigation, we recorded ¹H & ¹³C NMR of the partially purified compound **2** (brown sticky liquid). The ¹H NMR spectrum revealed characteristic pattern of steroid moiety with 28 ¹³C signals. The obtained sterol **2** was subjected to further purification by employing repetitive column chromatography techniques (silica gel and sephadex LH-20 gel column chromatography) to obtain brown solid. Sterol **2** displayed signals at δ_{H} 5.21 (t, J = 6.3 Hz) & δ_{C} 135.6, 132.0 due to double bond in side chain and δ_{H} 5.57-5.60 (m), δ_{H} 5.39-5.41 (m) and δ_{C} 141.3, 139.8, 119.6, 116.3 due to the conjugated double bonds. Another feature of NMR spectrum of sterol **2** was the signals at δ_{H} 3.60-3.70 (m) and δ_{C} 70.4 which were attributed to the oxygenated methine. Finally, based on NMR data and comparison with the reported data,[23–27] ergosta-5,7,22- trien-3 β -ol structure was assigned to compound **2**. In the process of purifying ergosta-5,7,22-trien-3 β -ol (**2**), another sterol **3** was obtained which appeared to be white powder. Captivatingly, the ¹H NMR spectrum of sterol **3** appeared almost similar to that of sterol **2**; except, the double bond proton signals shifted to δ_{H} 6.51 (d, J = 8.4 Hz) and δ_{H} 6.25 (d, J = 8.4 Hz) from δ_{H} 5.57-5.60 (m) and δ_{H} 5.39-5.41 (m). These proton shifts indicated the addition of oxygen molecule across the conjugated diene of sterol **2**. Sterol **3** was thus proved to be ergosterol peroxide (**3**). The data matched with the reported literature.[23–27] Ergosta-5,7,22- trien-3 β -ol (**2**) and ergosterol peroxide (**3**) were extracted for the very first time from *Enteromorpha* sp.

4-Hydroxycoumarin (**1**) and related compounds have been isolated from many plant species.[28] Recent studies documented that, 4-hydroxycoumarin (**1**) and its derivatives exhibit wide spectrum of pharmacological activities such as anti-arthritis, anti-inflammatory, anti-viral, anti-bacterial, anti-pyretic, anti-cancer etc. and have been resourcefully used in certain cardiac conditions, pulmonary embolism and as anti-coagulants, etc.[29–32] However, the ecological significance of the same remains to be a subject of further studies. Although, 4-hydroxycoumarin do not possess tyrosinase inhibitory activity as reported by Asthana, S. et al. [33] but, the presence of 4-hydroxycoumarin in algae is ecologically significant as coumarins aid in scavenging the harmful radicals generated during oxidative stress, protection from phytopathogens [34] and may also deter herbivores from feeding on these algae in the ocean. Ergosta-5,7,22- trien-3 β -ol (**2**) and ergosterol peroxide (**3**) have been isolated from plentiful fungal species.[35–38] Moreover, ergosterol peroxide (**3**) was first reported in 1947 as a natural product from fungus *Aspergillus fumigatus*.[39] It's still a puzzle whether ergosterol peroxide (**3**) is really a metabolite or just an artifact. Some fungi were known to

contain coloured photosensitizers which could be responsible for the oxidation of ergosta-5,7,22-trien-3 β -ol to ergosterol peroxide or ergosterol peroxide may be a product of photo-oxidation during the isolation procedure.[40,41] Ergosta-5,7,22-trien-3 β -ol (**2**) is a vitamin D₂ precursor and an essential part of the cell membrane in fungi, and hence; is a biologically significant molecule. Ergosta-5,7,22-trien-3 β -ol (**2**) and its analogues have been studied to possess anti-fungal activity, anti-tumor activity, immunosuppressive activities, etc.[42– 44] On the other hand, ergosterol peroxide (**3**) is well-known to inhibit breast cancer,[45] cervical cancer,[45] colon cancer,[46] lung cancer,[47] etc. According to Taofiq, O. et al. cosmoceutical formulation of ergosta-5,7,22-trien-3 β -ol inhibited tyrosinase enzyme, however; its activity decreased over the period of storage time.[48] Whereas, Mukaiyama, T. revealed that ergosta-5,7,22-trien-3 β -ol did not remarkably suppress the melanogenesis in mouse melanoma cell line (B16 10F7) but, ergosterol peroxide (**3**) could significantly lower the melanin formation in B16 10F7.[49] Thus, we believe that these bioactive metabolites (**1-3**) produced by algae is responsible for its chemical defence and play ecological roles in its protection. Overall, our results indicate that methanol extract of *Enteromorpha* sp. finds a remarkable opportunity in the development of an alternative edible source[4] of tyrosinase inhibitors, which can be incorporated in the diet of humans and animals[4].

Experimental Section

General remarks: Melting points (uncorrected) were determined in open capillary using Thiele melting point tube. Thin-layer chromatography was performed with Kieselgel 60 F₂₅₄ (Merck aluminum support plates) eluted in solvent system: ethyl acetate/hexane. Spots were developed in UV (365 nm), 5% methanolic H₂SO₄ (heated at 110°C, 10 min). The absorbances at 475 nm were recorded on Shimadzu UV2450 double beam spectrophotometer. Infrared data was recorded in the region between 4000 to 400 cm⁻¹ on Shimadzu IR-Prestige-21 instrument. Column chromatography was performed with sephadex LH-20 and silica gel 60-200 mesh size as packing material. ¹H NMR, ¹³C NMR and DEPT spectra were recorded at room temperature on Bruker instrument (400 MHz for ¹H and 100 MHz for ¹³C or 300 MHz for ¹H and 75 MHz for ¹³C), chemical shifts are recorded in ppm relative to tetramethylsilane (TMS) as the internal standard. The mass spectra was recorded on liquid chromatography-electrospray ionisation mass spectrometry (LC-ESI-MS). Crystal structure data was collected on Bruker D8 Quest Eco model diffractometer using graphite monochromated Mo-K α radiation ($\lambda = 0.71073 \text{ \AA}$) at 293 K. The structural refinement was done by full matrix least square against F² using all data (SHELXL). Crystallographic data for the structure of compound 1 reported herein have been deposited with the Cambridge Crystallographic Data Centre as Supplementary Publication No. 1919099. Copies of the data can be obtained free of charge, on

application to CCDC. (email:deposit@ccdc.cam.ac.uk; <https://www.ccdc.cam.ac.uk>). All the chemicals used in this study were of reagent grade and used as received without any further purification. Mushroom tyrosinase (*Agaricus bisporus*) (EC 1.14.18.1) was purchased from Sigma-Aldrich. L-tyrosine (purity $\geq 98\%$) was purchased from Sigma-Aldrich and kojic acid (purity $\geq 98\%$) was procured from Merck.

Collection of algae and extraction:

The algal species *Enteromorpha* sp. was collected from the coast of Rameswaram, Tamil Nadu, India. They were frozen as soon as possible and transferred to the laboratory. The freshly collected organisms were initially freeze dried. They were homogenized in a blender with little water and exhaustively extracted at room temperature with MeOH (1 Lit. x 3). The combined extracts were filtered and concentrated under vacuum on a rotary evaporator at low temperature to give crude methanolic extract. Methanolic extract (~15 g) of the algae *Enteromorpha* sp. was fractionated with hexane (100 ml x 3), ethyl acetate (100 ml x 3).

Isolation and purification:

The hexane fraction (832 mg) was chromatographed initially on series of silica gel column (60-200 mesh) eluting with ethyl acetate in hexane. Further purification was carried out on preparative TLC (ethyl acetate/hexane 6:4). This resulted in the purification of 10 mg 4-hydroxycoumarin (**1**). The purification of ethyl acetate fraction (282 mg) by repetitive silica gel column chromatography (60-200 mesh) with ethyl acetate/hexane 3:7 and further by Sephadex LH20 column with methanol/chloroform 1:1 as eluting solvent yielded 11 mg ergosta-5,7,22-trien-3 β -ol (**2**) and 6 mg ergosterol peroxide (**3**).

4-Hydroxycoumarin (1): beige solid; mp 215-216 °C (reported 213-215 °C)[20–22]; IR ν_{\max} cm^{-1} : 3377, 2885, 2719, 2576, 1612, 1550; ^1H NMR (MeOH- d_4 , 400 MHz) : δ_{H} 7.81-7.83 (1H, m), 7.52-7.57 (1H, m), 7.24-7.28 (2H, m), 5.55 (1H, s, H-D exchange)[50]; ^{13}C NMR (MeOH- d_4 , 100 MHz): δ_{C} 168.7, 166.3, 155.2, 134.0, 125.4, 124.6, 117.6, 117.4, 91.6; LC-ESI-MS: $[\text{M} + \text{H}]^+$ 163.0386 (calculated for $\text{C}_9\text{H}_7\text{O}_3$, 163.0395)

Ergosta-5,7,22-trien-3 β -ol (2): brown solid; mp 155-156 °C (reported 160 °C);[24] IR ν_{\max} cm^{-1} : 3432, 2957, 2870; ^1H NMR (CDCl_3 , 300 MHz) : δ_{H} 5.57-5.60 (1H, m), 5.39-5.41 (1H, m), 5.21 (2H, t, J = 6.3 Hz), 3.60-3.70 (1H, m), 2.44- 2.52 (1H, m), 2.22-2.36 (1H, m), 1.99-2.09 (4H, m), 1.84-1.92 (3H, m), 1.45-1.53 (5H, m), 1.23-1.37 (6H, m), 1.05 (3H, d, J = 6.6 Hz), 0.96 (3H, s), 0.93 (3H, d, J = 6.9 Hz), 0.81-0.86 (6H, m), 0.64 (3H, s); ^{13}C NMR (CDCl_3 , 75 MHz): δ_{C} 141.3, 139.8, 135.6,

132.0, 119.6, 116.3, 70.4, 55.7, 54.8, 46.2, 42.8, 40.8, 40.4, 39.1, 38.4, 37.0, 33.1, 32.0, 28.3, 23.0, 21.1, 19.9, 19.6, 17.6, 16.3, 12.0.

Ergosterol peroxide (3): white solid; ^1H NMR (CDCl_3 , 300 MHz): δ_{H} 6.51 (1H, d, $J = 8.4$ Hz), 6.25 (1H, d, $J = 8.4$ Hz), 5.10-5.27 (2H, m), 3.94-4.02 (1H, m); ^{13}C NMR (CDCl_3 , 75 MHz): δ_{C} 135.4, 135.2, 132.1, 130.1, 82.1, 79.4, 66.4, 56.2, 51.7, 51.1, 44.6, 42.8, 39.7, 39.3, 37.0, 36.9, 34.7, 33.1, 30.1, 28.6, 23.4, 20.9, 20.6, 19.9, 19.6, 18.2, 17.5, 12.9.

Method for anti-tyrosinase activity:

Tyrosinase inhibition assay was performed as described by Ko et al.[51] with modifications. Crude methanol extract of *Enteromorpha* sp. was dissolved in DMSO to get final concentration of 0.6 mg/mL. Kojic acid was used as the positive control. Briefly, 20 μL of mushroom tyrosinase (1000 U/mL) was pre-incubated with 120 μL the test sample in 50 mM phosphate buffer (pH 6.9) for 5 minutes at 30°C. Then, 2 mM L-tyrosine (300 μL) was added to each reaction mixture and incubated at 30°C for 30 minutes. The enzyme reaction was monitored by measuring the absorbance at 475 nm. The percent inhibition of tyrosinase reaction was calculated as follows: Percent Inhibition = $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$; where, A_{control} is the absorbance without the test sample in the DMSO and A_{sample} is the absorbance with the test sample in DMSO.

Conclusion:

In summary, marine algae are the primary producers in the marine ecosystem and have evolved with fascinating biochemical processes to compete for space, which serves them as a perfect source of biologically active secondary metabolites. The isolation of 4-hydroxycoumarin (**1**), ergosta-5,7,22-trien-3 β -ol (**2**) and ergosterol peroxide (**3**) from the methanol extract of *Enteromorpha* sp., exhibiting mushroom tyrosinase inhibition, wherein; the hydroxyl in compound 1 was assigned to be present on 4th position on the coumarin scaffold using Single crystal X-ray diffraction technique is the highlight of this study. Ergosta-5,7,22-trien-3 β -ol (**2**) and ergosterol peroxide (**3**) has been previously reported as potent anti-tyrosinase inhibitors which perfectly explains the anti-tyrosinase property of methanolic extract of *Enteromorpha* sp. There is a wide scope for studying the mechanism of inhibition by these secondary metabolites and to explore its ecological significance. This study unlocks the future scope of using methanolic extract of edible *Enteromorpha* sp. as a source of natural tyrosinase inhibitors, which can assist in dealing with various diseases caused by excessive melanin production as well as unwanted pigmentation/browning in crops, vegetables, fruits & other foodstuff.

Conflict Of Interest

The authors declare that there is no conflicts of interest.

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Supplementary Material

^1H , ^{13}C , DEPT spectra of the compounds 1, 2 and 3; crystallographic data & structure refinement parameters, bond distances and angles, a view of unit cell packing & the H-bonding situation around the lattice water OW1 of 1 are available as Supporting Information.

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