RSC Advances

COMMUNICATION

Cite this: *RSC Adv.*, 2015, **5**, 51008

Received 24th April 2015
Accepted 2nd June 2015

DOI: 10.1039/c5ra07489d

www.rsc.org/advances

Isolation of stigmast-5,24-dien-3-ol from marine brown algae *Sargassum tenerrimum* and its antipredatory activity†

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In recent years many sterols with potent biological activity have been identified from marine sources. Here we report the isolation of stigmast-5,24-dien-3-ol (fucosterol) as a major metabolite from the bioactive hexane-fraction of *Sargassum tenerrimum* using different chromatographic techniques. Moreover, the chemical defense of brown algae *Sargassum tenerrimum* was investigated using feeding assay performed in an aquarium and in the field.

Macroalgae are classified into three higher taxa, brown (Phylum: Phaeophyta), red (Phylum: Rhodophyta) and green (Phylum: Chlorophyta), based on their pigmentation. Macroalgae, also known as seaweeds or sea vegetables, have been traditionally incorporated into Pacific and Asian foods for the last few years. These have recently become a popular addition to some Western diets and could be used as functional ingredients. Owing to the harsh environments (for example, changes of salinity, temperature, nutrients, UV-Vis irradiation, *etc.*) in which many macroalgae exist, they have developed effective defense mechanisms producing a great variety of secondary (biologically active) metabolites, which cannot be found in other organisms.[1] Macroalgae are harvested and used globally for many different applications, such as emulsifying agents, cosmetic formulations, medicine *etc.*[2] Moreover, the ecological significance of macroalgae is highlighted by the fact that they assist in supplying oxygen to the sea, acting as one of the primary producers in the marine food chain and also addressing various environmental stresses *i.e.*, defense against predators, tissue repair, holdfast adhesion, and protection against reactive species generated by oxidative processes.[3] Therefore, macroalgae need to adapt quickly to different biotic and abiotic stresses in ecosystem by means of chemical protection mechanisms such as creation of antioxidants to limit oxidation[4] and production of feeding-deterrents to prevent grazing.[5]

In the Phaeophyta (brown algae), *Dictyota* species have been extensively studied and are known to produce interesting natural products. Nevertheless, many of these metabolites had shown to possess broad-spectrum feeding deterrents against herbivores or show other biological activities.[6] For example, the few diterpenes such as pachydictyol A, dictyol E, dictyol B, dictyol B acetate, dictyol H, and an isolinerol/linearol mixture have been shown to be active anti-feedant metabolites.[7] Another class of metabolite widely found in Phaeophyta is terpenoid/steroids. It is always exciting to understand the ecological significance of these major constituents (in particular sterols) of seaweeds. In general, sterols are widely distributed throughout the majority of living cells. However, the principal sterols are synthesized differently depending on the organisms, *i.e.* cholesterol in animal; ergosterol in fungal cells, whereas fucosterol is predominantly found in brown seaweeds which constitute 83–97% of the sterol content.[8] Interestingly, stigmast-5,24-dien-3-ol (fucosterol) is widely utilized in biological activity studies, including antioxidant, hepatoprotective,[9] anti-inflammatory,[10] anticancer,[11] anti-fungal,[12] anti-diabetic,[13] antihistaminic, and anticholinergic.[14] Additionally, Vahouny *et al.* reported that fucosterol reduces the gastrointestinal absorption of cholesterol[15] and Hagiwara *et al.* observed that, fucosterol decreases angiotensin converting enzyme levels through the reduction of glucocorticoid receptors in endothelial cells.[16] The ecological significance of this sterol activity remains to be tested. Yet, this does not rule out possible grazer deterrence or chemical defense function of sterols in brown algae. In continuation of our research in marine natural product chemistry, herein we have reported the isolation of major metabolite from brown algae *Sargassum tenerrimum* and effect of their organic fractions on fish feeding. The main goal of present study was to examine the role of metabolites of *Sargassum tenerrimum*, as prospective anti-feedants.

A wide array of marine natural products has been identified from tropical algae and many of these metabolites deter

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c5ra07489d

herbivore from feeding on those algae in field as well as in laboratory aquarium. However, many non-polar compounds from algae have no effect on fish but it could have effect on other predators. Example, Teixeira *et al.* investigated[17] the ecological role of nonpolar metabolite *i.e.* cytotoxic hydroperoxysterol from Brazillian brown seaweeds *D. justii* & *S. schroederi* as defensive strategy against herbivory crabs. Later, Fenical *et al.* identified secosterol from octocoral *Pseudopterogorgia americana* which provide chemical defense to both coral and alga using feeding assay experiment.[18] Moreover, feeding deterrence of brown algae has received the most attention and phlorotannins were considered as defensive function in it. In present study, we had investigated *S. tenerrimum* for its metabolite contents and fish feeding. Crude extract, hexane and ethyl acetate fraction elicited a significant rejection of food pellets compared to control. In contrast, butanol fraction did not deter fish feeding in laboratory as well as in field experiment. Chemical analyses of these fractions (hexane and ethyl acetate) indicated the presence of sterol as major metabolite. The positive test for sterol in crude extract and fractions was confirmed by the development of silica gel TLC. The plate showed purple color spot after being sprayed with 10% H_2SO_4 solution followed by heating in oven. The BuOH fraction contains either amino acids or peptides (positive ninhydrin test). As consequence, we assumed that the deterrence of *S. tenerrimum* may be due to the effects of major metabolite *i.e.* sterol. Purification of hexane and ethyl acetate fraction by repeated gel permeation and column chromatography using sephadex and silica gel respectively yielded major compound identified as stigmast-5,24-dien-3-ol (**1**) as shown in Fig. 1. Compound **1** was isolated as a white solid that showed a molecular ion $[M + H]^+$ peak at *m*/*z* 413 in ESI-MS spectrum. The compound showed the IR absorption peaks at 3400 & 1600 cm^{-1} which were attributed to hydroxyl and olefinic group respectively. The ^1H NMR spectrum showed signals at δ 1.56 (d, $J = 6.8$ Hz, 3H), 1.02 (s, 3H), 1.01 (d, $J = 6.4$ Hz, 3H), 1.00 (d, $J = 6.8$ Hz, 3H), 0.96 (d, $J = 6.4$ Hz, 3H) and 0.76 (s, 3H) for 6 methyl groups indicating compound **1** as sterol core with vinylic methyl. The characteristic peak at δ 3.46 was attributed to oxygenated methine proton. Moreover, 2-olefinic proton signals at δ 5.34 ($J = 6.8$ Hz, 1H) and 5.17 (q, $J = 6.8$ Hz, 1H) indicated that this compound could be a stigmast-diene type sterol (Table 1, some of the proton values are not assigned as it is not possible to differentiate them). The ^{13}C NMR showed signals for 29 carbons. The peaks at δ 146.9 and 140.7 were attributed to 2 olefinic quaternary carbons, whereas 2 methine olefinic carbons appeared at δ 121.6 and 115.5. One oxygenated methine carbon appeared at δ 71.8 and 6 methyl carbon signals were seen at δ 22.2, 21.1, 19.4, 18.7, 13.2, 11.8. Finally, the isolated compound was identified as stigmast-5,24-dien-3-ol (**1**) and confirmed through the matching of physical and spectroscopic data with literature data.[19]

Table 1 ^1H and ^{13}C NMR spectroscopic data of compound **1** in $CDCl_3$

Position	δ_C, mult.	δ_H, mult.
1	37.25	
2	31.65	
3	71.8	3.52,m
4	42.29	
5	140.75	
6	121.7	5.36,d
7	31.9	
8	31.9	
9	50.12	
10	36.50	
11	21.07	
12	39.75	
13	42.34	
14	56.75	
15	24.32	
16	28.23	
17	55.77	
18	11.84	0.76,s
19	19.39	1.02,s
20	36.41	
21	18.74	1.01,d
22	35.21	
23	25.69	
24	146.99	
25	34.77	2.2,sep
26	22.13	0.96,d
27	22.24	1.0,d
28	115.5	5.19,q
29	13.16	1.56,d

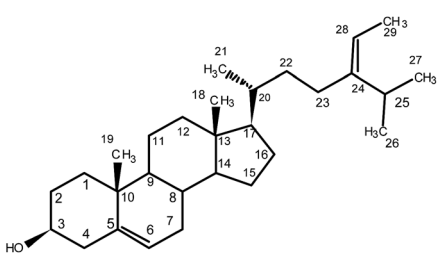

Fig. 1 Structure of isolated stigmast-5,24-dien-3-ol (**1**) from *S. tenerrimum*.

Recent studies documented that fucosterol exhibits cytotoxicity against breast and colon carcinoma cell lines *i.e.* proliferation of T47D cells, T47D and HT-29 cell.[11] Additionally, it is also known for its antidiabatic and antioxidant activity.[13] The antioxidant capacity of compounds has been related to the prevention of several diseases including cancer, coronary heart disorders, inflammatory disorders, ageing, and neurological degeneration in medicinal chemistry.[20] However, ecological significance of this reported activity remains to be subject of further studies. In general, the ecological roles of steroids remains unclear, although invertebrates produces this sterol for their reproduction, chemical signaling, defense or some time acts as a biosynthetic precursors to generate other toxic derivatives. Interestingly, crude extract, two fractions, and pure compound at natural concentration elicited significant rejection of artificial food pellets compared to control pellets in laboratory experiment (Fig. 2). Whereas, field experiment[21–23] showed almost similar results except ethyl acetate fraction, which showed more significance rejection compared to other

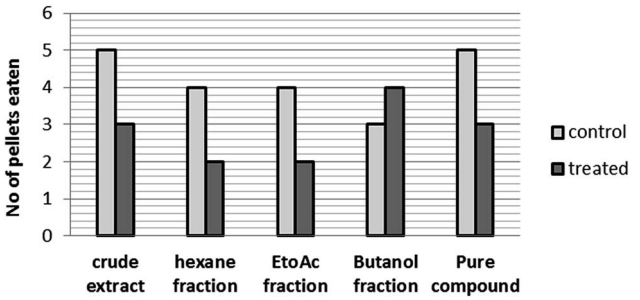

Fig. 2 Laboratory assay: consumption of food pellets containing crude organic extract, fractions & pure stigmast-5,24-dien-3-ol from *S. tenerrimum* by fishes. Extracts were considered deterrent if the number of pellets eaten were ≤3.

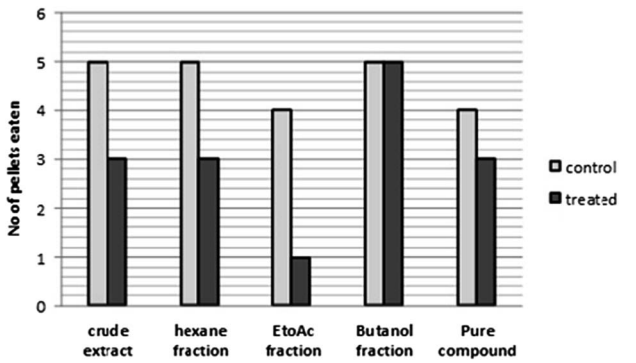

Fig. 3 Field assay: consumption of food pellets containing crude organic extract, fractions & pure fucosterol from *S. tenerrimum* by fishes. Extracts were considered deterrent if the number of pellets eaten were ≤3.

fractions (Fig. 3). In fact, these fractions of *S. tenerrimum* extract seemed to some extent inhibit grazing by rock pool fishes. The feeding effect could be due to two possible reasons either distasteful or toxic nature of stigmast-5,24-dien-3-ol (**1**). In first assay we verified that the crude extract inhibits activity of the rock pool fishes *Istigobio ornatus* and *Istiblennius dussumieri*. Additionally, fish feeding experiment with pure stigmast-5,24-dien-3-ol confirm the effect of deterrence towards feeding by *Istigobio ornatus* and *Istiblennius dussumieri* fishes. Thus we believe that, these metabolites produced by brown algae *S. tenerrimum* produces chemical defense and play ecological roles to protect themselves.

Conclusions

We have reported the isolation of the major metabolite fucosterol from *Sargassum tenerrimum* as a new source. According to the result reported herein, we proposed the ecological significance for the sterol found in this species of brown algae as a defensive chemical against fish feeding in particular *Istigobio ornatus* and *Istiblennius dussumieri* fishes. The probable reason for this observed deterrence could be the cytotoxicity or distasteful effect of fucosterol. Overall, fucosterol- a bioactive compound was found as an anti-feedant and seems to be involved in chemical defense mechanism of producing organism against rock pool fishes.

Acknowledgements

We would like to thank Dr P.A. Thomas for the identification of marine organisms. The authors wish to thank CSIR, OCEAN FINDER and European commission project FP7KBBE-2009-3-245137 for the financial support. MSM is grateful to UGC-BSR (India) (project no. F.30-102/2015/BSR) for start-up-grant and research funding.

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