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NOTE

Fucoxanthin and pheophytin-*a* from the marine algae *Sargassum cinereum:* Isolation, characterization and their feeding deterrent activity on Shrimps

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Chemical investigation of the brown alga *Sargassum cinereum* collected from Goa coast, India led to the isolation of two bioactive pigments viz. pheophytin-*a* and fucoxanthin in a relatively good concentration. These pigments were known to possess several biological properties. Specifically, fucoxanthin is present in several micro- and macro-algae and known to exhibit a remarkable antioxidant, cytotoxic and, hypoglycemic activity. These pigments are being used in several health care products however; their feeding deterrent activity is not well understood. Here, we report the isolation of fucoxanthin and pheophytin-*a,* from this algal species by chromatographic techniques and their characterization on the basis of FTIR, UV-Vis, NMR and mass spectroscopic data. Additionally, feeding deterrent effects of the pigments on shrimps have been investigated by performing a feeding assay in an aquarium.

Keywords: Marine algae, fucoxanthin, pheophytin-*a*, feeding deterrence, spectroscopic data.

Introduction

Brown algae *Sargassum* sp. are widely found in different oceanic waters generally inhabit in shallow waters and coral reefs. They are rich source of several biological important metabolites including terpenoids[1], sterols[2,3], polysaccharides[4,5], and carotenoids[7–9]. Moreover, algae are the rich source of pigments such as fucoxanthin and pheophytin-*a*[10,11]. Fucoxanthin was first isolated from the marine seaweeds *Fucus*, *Dictyota,* and *Laminaria* sp.[12] and subsequently, isolated from several other seaweeds such as *Undaria pinnatifida suringar*[13], *Sargassum fusiformis*[14], and *Sargassum heterophyllum*[15]. Fucoxanthin showed a remarkable antioxidant activities[14], exhibited *in vitro* apoptosis on human cervical cell[16] as well as breast cancer cells[17], and also displayed anti-diabetic properties[18]. On the other hand, pheophytin-*a* showed inhibitory activity on human DNA topoisomerase II-α[19].

It is noteworthy that marine algae produced various secondary metabolites of which, some of them function as a chemical defense against natural enemies such as competitors, epiphytes, pathogenic bacteria, and harbivores[20–22]. For instance, *Dictyota* species have produced several natural products viz. dictyol E, dictyol B, dictyol B acetate, dictyol H[23,24] and pachydictyol A which were exhibited a broad spectrum of anti-feeding activities[24]. Moreover, certain diterpenes isolated from the marine algae *Canistrocarpus cervicornis* exhibited chemical defense property[25], whereas; the algal metabolites such as apakaochtodene A and B isolated from the alga *Portieria hornemannii* have shown feeding deterrent effects on its host *Aplysia parvula*[26]. In contrast, feeding deterrent effect of fucoxanthin and pheophytin-*a* is less understood although they represent the bulk of algal metabolites and exhibited several other biological properties. As a part of our ongoing work on marine natural products[27–31], herein, we wish to report isolation and spectroscopic characterization of fucoxanthin and pheophytin-*a* from the brown algae, *S. cinereum*. In addition, feeding deterrent activity of these pigments on shrimps has been investigated by performing feeding assay experiment in an aquarium.

Results and discussion

Isolation and spectroscopic characterization: Chemical investigation of *S. cinereum* collected from Goa coast led to the isolation of two pigments viz. pheophytin-*a* (**1**) and fucoxanthin (**2**) (Fig. 1). In this study, we found that pheophytin-*a* (**1**) and fucoxanthin (**2**) were present in good quantity from

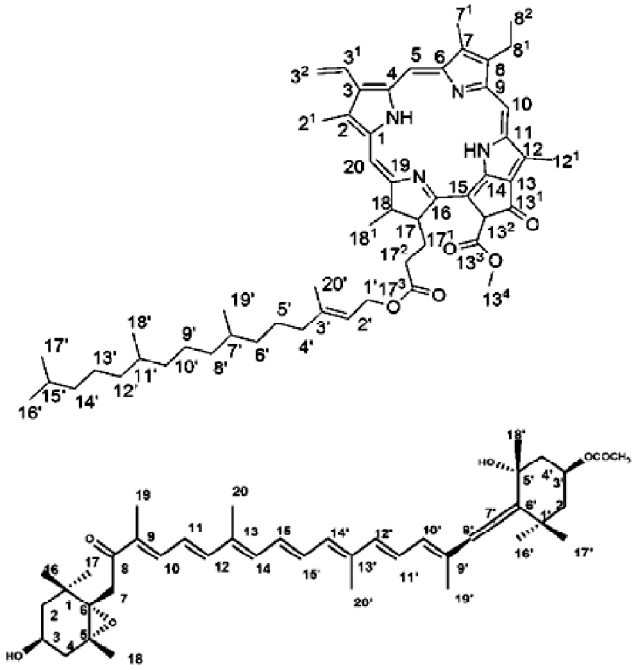

Fig. 1. Chemical structures of pheophytin-*a* (**1**) and fucoxanthin (**2**) isolated from *S. cinereum*.

a freshly collected wet alga of *S. cinereum* material yielding 0.1% pheophytin-*a* (**1**) and 0.04% of pure fucoxanthin (**2**), respectively. Compounds were isolated from the crude extract by successive column chromatography over silica gel and Sephadex LH-20. The identity of pheophytin-*a* (**1**)[10,32] and fucoxanthin (**2**)[12,33] were established by spectroscopic data (FT-IR, ^{1}H and ^{13}C NMR) and by comparison the values with those reported in the literature. The structure of pheophytin-*a* (**1**) consists of aporphyrin ring linked by a conjugated double bond and a phytyl group. UV-Vis spectrum of **1** in methanol displayed absorption peaks at λ_{max} 665, 608, 536 and 506 nm, respectively. IR spectrum of **1** showed absorption peaks at 3390 and 1739 cm^{-1} corresponding to -NH and ketone (>C=O) groups. The spectrum also showed absorption peaks at 1697 and 1622 cm^{-1} which are attributed to the carbonyl group of ester (C=O) and alkene (C=C) carbons. The ^{13}C NMR spectrum of pheophytin-*a* showed 55 distinct signals of which signals at δ169, 172 and 189 were assignable to the carbonyl carbons of porphyrin ring and phytyl group.

Fucoxanthin (**2**) was isolated as a red solid by successive column chromatography and its structure was established with the help of spectroscopic data. UV-Vis spectrum of fucoxanthin in methanol showed 6 absorption peaks in the region of λ_{max} 474–327 nm which are the characteristic of conjugated double bonds[34]. Fucoxanthin showed IR absorption peaks at 1928, 1735, 1656 and 3462 cm^{-1} that are attributed to allene, carbonyl ester, carbonyl, and hydroxyl groups, respectively.

The ^{13}C NMR spectrum of fucoxanthin (**2**) displayed peak for allenic carbon (C-7′) at δ 202 whereas, the signal for C=O (C-8) and C=O (C-32) were appeared at δ 197 and 170, respectively (see Experimental section). The ^{13}C NMR data for pheophytin-*a* (**1**)[10,31] and fucoxanthin (**2**)[13] are identical to those of the literature values. The mass spectral data of pheophytine-*a* (**1**) and fucoxanthin (**2**) showed peak at *m/z* 871.8482 and 659.0500, respectively corresponding to their $[M+H]^+$ ions. Optical rotation of fucoxanthin has remained unresolved and controversial. This is due to the fact that fucoxanthin shows strong light absorption thereby necessitating the use of concentration too low to register a reliable readings. In this present study, specific rotation of fucoxanthin measured in chloroform at 30°C showed $[\alpha]_D$ = +75.6° comparable to the earlier reported value (+72.5°)[34]. However, attempt to obtain specific rotation of pheophytin-*a*, was unsuccessful due to less stereo-centre and high molecular mass.

*Feeding assay of **1** and **2**:* Marine algae are known to produce feeding deterrent activity to prevent grazing[33]. They produce metabolites, such as polyphenol which exhibited chemical defense against the herbivorous snail *L. littorea*[36]. Recently, fucosterol was reported from the marine alga *Sargassum tenerrimum* and showed it has shown anti-predatory effects[3]. Further, Shaw *et al*. described feeding deterrent activity for a derivative of apofucoxanthin against copepods[37,38]. However, to the best of our knowledge, no study has carried out on the feeding deterrent effects of pheophytin-*a* (**1**) and fucoxanthin (**2**) on shrimps. In this study, the feeding deterrent effect of the compounds **1** and **2** have been investigated on shrimps by performing a feeding assay in an aquarium. The experiment was performed by feeding an equal number of compound adsorbed food pellets and normal food pellets to the tested fish that were acclimatized in brackish water in an aquarium for 3 days. Then the food pellets which were remained at the end of the experiment were counted. It was observed that the food pellets absorbed with compounds were significantly rejected by shrimps.

Interestingly, when the normal fish food pellet and the compound adsorbed fish food pellets (>10%) were fed to shrimps in an experimental tank, shrimps selectively consumed the normal fish food pellets indicating a significant deterrent activity of these compounds. The probable reason for rejection of compounds adsorbed fish food pellets by shrimps could be the distasteful effect of these pigments. Further, in order to understand the effect of concentration, the feeding experiment was performed in two separate tanks viz. control tank and experimental tank which were fed with normal fish food pellet and compound adsorbed fish food pellets of varying concentrations, respectively. As evident from Fig. 2, the number of adsorbed fish food pellets left behind (after the end of experiment) was increased with the increased in the concentration of the compound in fish food pellets. This result showed that deterrent activity depends on the concentration of the compound present in the fish food pellets.

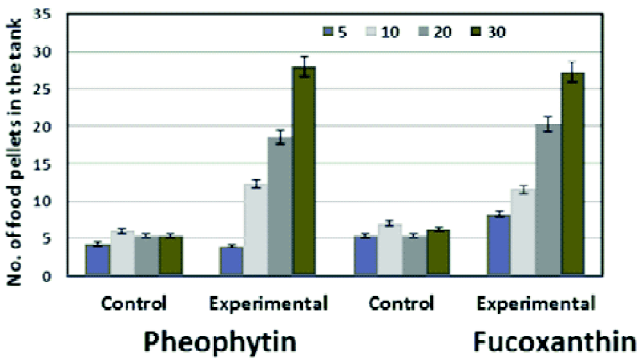

Fig. 2. Graph showing the number of pellets that remained in the tank at the end of experiment for varying concentration of pheophytin-*a* and fucoxanthin adsorbed fish food pellets. Concentrations were expressed in percentage (i.e. milligrams of compound adsorbed per 100 mg of fish food approximately).

Conclusion

This paper describes, isolation and characterization of pheophytin-*a* (**1**) and fucoxanthin (**2**) from the marine alga *Sargassum cinereum* collected from Anjuna, Goa Coach. Pheophytin-*a* (**1**) has been reported as a new source from this alga and characterized by spectroscopic data (^{1}H, ^{13}C NMR and ESI-MS). We found that both the fucoxanthin and pheophytin-*a*, was present in this alga in fairly good concentrations and the compounds showed feeding deterrent activity against shrimps. Pheophytin-*a* showed marginally higher deterrent activity than fucoxanthin. However, at lower concentrations (<5%) both the compounds **1** and **2** had no detrimental effect on shrimps. Further, the result showed that this edible alga may not effect on herbivores such as shrimps for consumption at low concentration but consumption at higher concentration might lead to deterrence on shrimps due to the presence of these pigments.

Experimental

Material and physical methods:

The brown algae *Sargassum cinereum* was collected from the intertidal region of Anjuna, Goa and a voucher specimen (NIOBOC-016) has been deposited at the National Institute of Oceanography, Goa. Solvents used for extraction and purification were of analytical grade and used as received. Optical rotation was measured in $CHCl_3$ on a Parkin-Elmer polarimeter. UV-Vis analysis was performed with a Perkin-Elmer 200 model and infrared spectra were recorded using a diffused reflection spectroscopy (DRS) assembly on a Shimadzu-8201PC spectrometer. NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer at 300.13 (^{1}H) and 75.47 MHz (^{13}C) with $SiMe_4$ as internal reference and coupling constants were given in Hertz. Mass spectral data analyses were performed on Waters UPLC-MS/MS (Xevo TQD) mass spectrometer. Column chromatography was performed on silica gel (60–120 mesh, Merck, Darmstadt, Germany or SRL Pvt. Ltd., India) and Sephadex LH-20.

Extraction and purification:

Freshly collected alga (500 g) was shocked in 2 litres of MeOH:$CHCl_3$ (1:1) for 3 days and filtered. The process was repeated three times to effect a complete extraction. The combined filtrate was concentrated in a rotary evaporator to give a crude extract of 40 g. Crude extract approx. 10 g was loaded over a silica gel column (60–120 mesh size, 70 cm×3 cm). The column was eluted with a gradient of the ethyl acetate-petroleum ether solvent system. The fraction eluted with 5%, 15% and 30% ethyl acetate and petroleum ether solvent mixture were collected separately and concentrated in a rotary evaporator giving 35 mg, 67 mg and 135 mg (165 mg) of fraction 1, fraction 2 and fraction 3, respectively. Each fraction was further purified on Sephadex LH-20 glass column, pre-equilibrated with $CHCl_3$-MeOH, yielding pure pheophytin-*a* **1** and fucoxanthin **2** in (49 mg) (128 mg).

Spectroscopic data of the compounds:

Pheophytin-*a* (**1**): Blackish green powder; UV-Visible λ_{max} (methanol): 665, 608, 536, 506, 407, 329; IR (Neat, cm^{-1}): 3390.86 (NH), 1739.79 (C=O keto), 1697.36 (C=O ester), 1622.13 (C=C); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.80–0.88 (m, 12H), 1.03–1.61 (m, 16H), 1.88 (m, 8H), 1.89 (m, 5H), 2.50–2.64 (m, 6H), 3.18 (s, 3H), 3.40 (s, 3H), 3.69 (m, 6H), 3.92 (s, 3H), 4.23 (m, 1H), 4.52 (m, 3H), 5.19 (t, 1H, *J* 6.9 Hz), 6.18 (t, 2H, *J* 11.5 Hz), 6.24 (d, 1H, *J* 17.8 Hz), 7.90 (dd, 1H, *J* 17.8, 11.5 Hz), 8.57 (s, 1H), 9.31 (s, 1H), 9.47 (s, 1H); ^{13}C NMR (75 MHz): (Porphyrin) δ 11.1 (C-7^1), 12.0 (C-2^1), 12.0 (C-12^1), 17.3 (C-8^2) 19.3 (C-8^1), 23.0 (C-16), 29.8 (C-17^1), 31.2 (C-17^2), 50.1 (C-18), 51.1 (C-17), 52.8 (C-13^4), 64.7 (C-13^2), 93.0 (C-20), 97.4 (C-5), 104.3 (C-10), 105.2 (C-15), 122.6 (C-3^2), 129.0 (C-3^1), 129.0 (C-13), 129.0 (C-12), 131.7 (C-2), 136.1 (C-7), 136.1 (C-3), 136.4 (C-4), 137.9 (C-11), 142.0 (C-1), 145.1 (C-8), 149.6 (C-14), 150.9 (C-9), 155.5 (C-6), 161.2 (C-16), 169.6 (C-13^3), 172.2 (C-19), 172.9 (C-17^3), 189.6 (C-13^1) (phytyl group): 17.3 (C-20′), 19.6 (C-19′), 19.7 (C-18′), 22.6 (C-17′), 22.7 (C-16′), 24.4 (C-9′), 24.7 (C-13′), 24.9 (C-5′), 27.9 (C-15′), 32.6 (C-11′), 32.7 (C-7′), 36.6 (C-6′), 37.2 (C-12′), 37.3 (C-8′), 39.3 (C-14′), 39.7 (C-4′), 61.4 (C-1′), 117.7 (C-2′), 142.8 (C-3′); ESI-MS: *m/z* 871.5737 $[M+H]^+$ calculated for $C_{55}H_{75}N_4O_5$ found 871. 8482.

Fucoxanthin (**2**): Red solid; UV-Visible λ_{max} (methanol): 474, 445, 422, 402, 367, 327; IR (Neat, cm^{-1}): 1928.82 (allene), 1735.93 (C=O ester), 1656.87 (C=O conjugated), 3462.22 (OH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.97 (s, 3H, Me-17), 1.04 (s, 3H, Me-16), 1.08 (s, 3H, Me-17′), 1.21 (s, 3H, H-18), 1.34 (s, 3H, H-18′), 1.36 (m, 1H, H-2 ax) 1.37 (s, 3H, Me-16′), 1.46 (m, 2H, H-2′ ax, H-2 eq), 1.59 (m, 1H, H-4′ ax), 1.75 (m, 1H, H-4 ax), 1.82 (s, 3H, H-19′), 1.95 (s, 3H, H-19), 2.00 (s, 7H, H-20, H-20′, H-2′ eq), 2.05 (s, 3H, C-3′ OAc), 2.35 (m, 2H, H-4 eq, H-4′eq), 2.61 (d, 1H, *J* 18.6 Hz, H-7), 3.66 (d, 1H, *J* 18.6 Hz, H-7), 3.81 (m, 1H, H-3), 4.95 (m, 1H) 5.38 (m, 1H, H-3′), 6.06 (s, 1H, H-8′), 6.14 (d, 1H, *J* 11.4 Hz, H-10′), 6.38 (m, 3H, H-14′, H-12′, H-14), 6.65 (m, 5H, H-11, H-12, H-15, H-15′, H-11′), 7.16 (d, 1H, *J* 10.2 Hz); ^{13}C NMR (75 MHz): δ 11.8 (C-19), 12.7 (C-20), 12.8 (C-20′), 13.9 (C-19′), 21.1 (C-18), 21.3 (Me, C-3′), 25.0 (C-16), 28.1 (C-17), 29.1 (C-16′), 31.2 (C-18′), 32.0 (C-17′), 35.1 (C-1′), 35.7 (C-1), 40.7 (C-7), 41.6 (C-4), 45.2 (C-4′), 45.4 (C-2′), 47.0 (C-2), 64.3 (C-3), 66.1 (C-5), 67.1 (C-6), 68.0 (C-3′), 72.6 (C-5′), 103.3 (C-8′), 117.4 (C-6′), 123.3 (C-11), 125.6 (C-11′), 128.5 (C-10′), 129.4 (C-15), 132.1 (C-14′), 132.4 (C-9′), 134.5 (C-9), 135.3 (C-13), 136.6 (C-14), 137.0 (C-12′), 138.0 (C-13′), 139.1 (C-10), 145.0 (C-12), 170.4 (C-3, C=O), 197.8 (C-8), 202.3 (C-7′); ESI-MS: *m/z* 659.4312 $[M+H]^+$ calculated for $C_{42}H_{59}O_6$ found 659.0500.

Procedure for feeding deterrent assay:

White shrimp *Penaeus indicus* of equal size were collected from a local fish farm of Goa and shrimps were acclimatized in brackish water in an aquarium for 3 days. Fish food pellets were obtained from the local market and compound-adsorbed fish food pellets were prepared as delineated here. A known weight of the compound was dissolved in acetone (2 mL) and then adsorbed on fish food pellets (50 mg, approx. 28 nos.) and kept for drying overnight. This process was repeated to facilitate complete adsorption of the compound in the fish food pellets. Following this procedure, compound adsorbed fish food pellets of varying concentration viz. 5%, 10%, 20%, 30% was prepared. In a typical experiment, shrimps fed with an equal number of normal fish food pellets and compound adsorbed fish food pellets in a tank. After 1 h the pellets remained behind was noted. Further, the feeding experiment of shrimp was also performed in two separate tanks viz. control and experimental tanks. Shrimps from the experimental tank were fed with the compound-adsorbed fish food pellets while the shrimps from the control tank were fed with normal fish food pellets. After 1 h the pellets remained behind in each of the tanks was noted. Compounds were considered deterrent if the number of food pellets (adsorbed with compounds) eaten was less than 10 pellets (which is determined by the number of pellets left behind at the end of experiment). The experiment was repeated twice using different concentrations of adsorbed fish food pellets.

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