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Antioxidant and Anticholinesterase Assets and Liquid Chromatography-Mass Spectrometry Preface of Various Fresh-Water and Marine Macroalgae

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ABSTRACT

The ethanol extracts from the fresh-water algae; *Chara hispida* L., *Cladophora glomerata* (Dilw.) Kütz, *C. fracta* (Dilw.) Kütz, *Spirogyra gratiana* Transeau, *Mougeotia* sp. (C.A. Agardh), *Vaucheria sessilis* (Vauch.) De Candolle, *Geminella mutabilis* (Breb.) Wille, the fresh-water plants; *Ranunculus rionii* Lagger and *Ceratophyllum demersum* L., as well as the marine algae; *Sciniaia furcellata* (Turn.) J. Agardh, *Dictyota dichotoma* (Huds.) Lam., *Padina vickersiae* Hoyt, *Halopteris scoparia* (L.) Sauvagau, and the sea grass; *Posidonia oceanica* (L.) Dell. were assessed *in vitro* for their antioxidant and anti-acetylcholinesterase (AChE) activities. Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity test at 0.125, 0.25, 0.5, 1.0, and 2.0 mg ml^{-1} and ferric-reducing antioxidant power assay (FRAP) at 0.5, 1.0, and 2.0 mg ml^{-1} concentrations. Total phenolic contents of the extracts were determined using Folin-Ciocalteau's reagent. The extracts were further analyzed qualitatively by LC-DAD-MS. *S. gratiana* had the best antioxidant activity, followed by *R. rionii*. LC-DAD-MS showed rich gallic acid and its ethyl ester contents in *S. gratiana*, while prosperous vitamin C content has been detected in *P. oceanica* for the first time in this study. *S. gratiana* possessed the highest inhibition (42.5±2.28%) at 2.0 mg ml^{-1} against AChE.

KEYWORDS: Algae, Aquatic plants, Antioxidant activity, Acetylcholinesterase inhibition, Total phenol content, LC-DAD-MS

INTRODUCTION

Antioxidants are the substances or nutrients which are able to prevent or slow the oxidative damage by scavenging free radicals created in the body during standard metabolic functions or introduced from the environment. Free radicals take part in several health conditions such as aging process, cancer, atherosclerosis, *etc.* (1–3). Antioxidants might be present in plant sources such as vitamins, minerals, flavonoids, phenolics, and carotenoids, *etc.* Synthetic and natural antioxidants are added as additives for prevention of food deterioration. However, more restrictions have been recently imposed on use of synthetic antioxidants due to their toxic effects (4,5). On the other hand, Alzheimer's disease (AD), the most common form of dementia, is associated with neurodegeneration and oxidative stress. One of the most accepted treatment strategies against AD has been acetylcholinesterase (AChE) inhibitors, a key enzyme which breaks down the neuromediator called "acetylcholine". Consequently, for any extract/compound, it is quite beneficial to possess both AChE inhibitory and antioxidative activities.

Seaweed refers to a general colloquial term to express macroscopic, benthic, marine algae and often consumed as food by people living in the Far East. In recent times, macroalgae have received an immense interest depending

on their low calorie and high vitamin, mineral, and dietary fiber ingredients (6). Therefore, in this study, we aimed to look into antioxidant and anti-acetylcholinesterase (AChE) activities of the ethanol extracts prepared from the following fresh-water algae; *Chara hispida* L., *Cladophora glomerata* (Dilw.) Kütz, *C. fracta* (Dilw.) Kütz, *Spirogyra gratiana* Transeau, *Mougeotia* sp. (C.A. Agardh), *Vaucheria sessilis* (Vauch.) De Candolle, *Geminella mutabilis* (Breb.) Wille, the fresh-water plants; *Ranunculus rionii* Lagger and *Ceratophyllum demersum* L., as well as the marine algae; *Sciniaia furcellata* (Turn.) J. Agardh, *Dictyota dichotoma* (Huds.) Lam., *Padina vickersiae* Hoyt, *Halopteris scoparia* (L.) Sauvagau, and the sea grass; *Posidonia oceanica* (L.) Dell. Their antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity test at 0.125, 0.25, 0.5, 1.0, and 2.0 mg ml^{-1} and ferric-reducing antioxidant power assay (FRAP) at 0.5, 1.0, and 2.0 mg ml^{-1} concentrations. Total phenol contents of the extracts were determined by Folin-Ciocalteau's reagent. Some phenolic acids, vitamin C, and carotenoids were identified in the extracts by LC-DAD-MS technique.

METHODS AND MATERIALS

Plant materials

Collection sites and dates of the algae samples are listed in Table 1. The materials were identified by one of us (Dr. Tahir Atici) and voucher specimens are preserved in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Extraction

Until the experimental procedures, the air-dried and powdered materials of all algae used [*Chara hispida* (CH), *Cladophora glomerata* (CG) Kütz, *C. fracta* (CF) Kütz, *Spirogyra gratiana* (SG), *Mougeotia* sp. (MO), *Vaucheria sessilis* (VS), *Geminella mutabilis* (GM), *Ranunculus rionii* (RR), *Ceratophyllum demersum* (CD), *Sciniaia furcellata* (SF), *Dictyota dichotoma* (DD), *Padina vickersiae* (PV), *Halopteris scoparia* (HS), and *Posidonia oceanica* (PO)] were kept in deep-freezer, and were weighed accurately (10 g) at the moment of the experiments after melting at room temperature and extracted with ethanol (250 ml × 2). Following evaporation of the ethanol under vacuum, the crude extracts were obtained. The yields of the extracts (w/w) are given as follows; CH: 2.8%, CG: 8.9%, CF: 1.6%, SG: 28.2%, MO: 4.3%; VS: 3.3%; GM: 5.7%; RR: 23.2%, CD: 16.5%, SF: 33.27%, DD: 3.0%, PV: 8.5%, HS: 6.1%, and PO: 9.8%.

DPPH radical scavenging assay

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by Blois's method (7). The samples were dissolved in ethanol (75%) and mixed with DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Gallic acid and butylated hydroxyanisol (BHA) were the references. Inhibition of free radical DPPH in percent (I%) was calculated as given below:

$I\% = [(A_{blank}/A_{sample}) / A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference.

Ferric-reducing antioxidant power assay (FRAP)

The iron-reducing power of the samples was tested using the assay by Lim and Quah (8). Briefly, 1 ml of samples was added to 2.5 ml of phosphate buffer (0.1 M, pH 6.6)

Table 1. Collection sites and dates of the algae samples

Materials	Family	Collection site	Collection date
Fresh-water algae			
C. hispida	Ulotrichaceae	Saldaa Lake	July, 2001
C. glomerata	Cladophoraceae	Beyşehir Lake	May, 1999
C. fracta	Cladophoraceae	Beyşehir Lake	May, 1999
S. gratiana	Zygnemataceae	Mogan Lake	April, 1999
Mougeotia sp.	Zygnemataceae	Sariyer Damn	May, 1999
V. sessilis	Vaucheriaceae	Mogan Lake	April, 1999
G. mutabilis	Ulotrichaceae	Artificial pond, Ankara	March, 2008
Fresh-water plants			
R. trichophyllus	Ranunculaceae	Mogan Lake	April, 1999
C. demersum	Ceratophyllaceae	Susuz Lake	April, 2008
Marine algae and sea grass			
S. furcellata	Galaxauraceae	Tekirdağ	September, 2000
D. dichotoma	Fucophyceae	Alanya	August, 1999
P. vickersiae	Dictyotaceae	Alanya	August, 1999
H. scoparia	Stypocaulaceae	Alanya	August, 1999
P. oceanica	Potamogetonaceae	Kuşadasi	August, 2000

and 2.5 ml of potassium ferricyanide (1%, w/v) and was incubated at 50°C for 20 min. Then 2.5 ml of 10% trichloroacetic acid (TCA) were added. 2.5 ml of this solution were mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%, w/v). After 30 min incubation, the absorbance was read at 700 nm. Analyses were triplicated. Gallic acid and BHA were used as the references tested at 0.125, 0.25, and 0.50 mg ml^{-1}. Increase in absorbance is commented as indicative of increased reducing power.

Total phenol contents (TPC) of the extracts

The concentration of total phenols in the extracts was determined by UV spectrophotometer using Folin-Ciocalteau's reagent (9). The absorbance was measured at 760 nm and the results obtained were expressed in mg of gallic acid (GA) *per* 100 mg of each extract (mg GA/100 mg extract).

Determination of AChE inhibitory activity

Anti-AChE activity was assayed by the spectrophotometric method of Ellman et al (10). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) was the enzyme source, while the substrate; acetylthiocholine iodide (Sigma, St. Louis, MO, USA) and 5,5'-dithio-bis(2-nitrobenzoic)acid (DTNB) were used for the measurement of the anti-AChE activity. All reagents and conditions were same as described in our previous publication (11). All of the experiments were performed in four parallel sets. Galanthamine purchased from Sigma (St. Louis, MO, USA) was the reference in this study.

LC-DAD-MS analysis

LC-DAD-MS analyses were performed using an Agilent Technologies 1200 series high pressure liquid chromatograph (HPLC), including a binary pump, vacuum degasser, autosampler, diode array detector, and coupled to an Agilent Technologies 1200 series Model VL single quadruple mass spectrometer equipped with an multimode ionization interface. Chromatographic separations were evaluated using Eclipse XDB-C18 column (15 cm × 4.6 mm, 5 µm) at room temperature. A mobile phase consisted of two eluents, (A) acetonitrile and (B) 40 mM formic acid. All solvents were filtered through a 0.45 µm Milipore filter before use and degassed in an ultrasonic bath.

Analysis of the extracts was carried out with gradient elution profile which is shown below:

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate
Initial	10	90	1 ml min^{-1}
10	100	0	1 ml min^{-1}
36	100	0	1 ml min^{-1}

The flow rate of 1.0 ml min^{-1}, and detection was at 270, 254, 280, and 330 nm. An injection volume of 10 µl was used. The system was controlled and data analysis was performed with Agilent ChemStation. The LC-MS instrumentation described here utilizes a quadruple MS system operating in SCAN mode. Full scan spectra from m/z 100 to 1000 were obtained in negative-ion mode. The API-ES process was used for mass spectral measurements. The negative-ion mass spectra of gentisic acid, vitamin C, astaxanthin, coumaric acid, beta-carotene, gallic acid ethyl ester, canthaxanthin, and benzoic acid were recorded in the total-ion monitoring mode using a series of fragmentor potentials to establish their fragmentation pattern. The mass spectrum consisted of the protonated molecular ion ($M+H$) at m/z 175 for vitamin C, m/z 153 for gentisic acid, m/z 595 for astaxanthin, m/z 163 for coumaric acid, m/z 535 for beta-carotene, m/z 197 for gallic acid ethyl ester, m/z 563 for canthaxanthin, and m/z 121 for benzoic acid. The fragmentor was set at 80 V for all compounds to observe the pseudomolecular ion. Spray chamber parameters were as follows: 5.0 l $minute^{-1}$ drying gas, 325°C drying gas temperature, 200°C vaporizer temperature, 60 psig. nebulizer pressure and 2000 V capillary voltage.

RESULTS

Antioxidant activity

The ethanol extracts of CH, CG, GF, SG, MO, VS, GM, RR, CD, SF, DD, PV, HS, and PO were assessed for their antioxidant capacity using DPPH radical scavenging activity test at 0.125, 0.25, 0.5, 1.0, and 2.0 mg ml^{-1} and FRAP assay at 0.5, 1.0, and 2.0 mg ml^{-1} (Tables 2 and 3). Only SG showed a high scavenging effect against DPPH having 54.8, 86.9, and 89.9% inhibitions at 0.5, 1.0, and 2.0 mg ml^{-1}, respectively. The marine algae were not effective in this assay, while RR had moderate activity with 40.4 and 68.3% at 1.0 and 2.0 mg ml^{-1}, respectively. FRAP of the extracts were observed to be quite low. Again, SG and RR were the most reactive in this test in comparison with rest of the extracts. The highest TPC was found to be possessed by the ethanol extract of SG (862 mg g^{-1} extract) and the fresh-water plants have been observed usually to have higher TPCs than the marine plants (Table 2).

AChE inhibition

In the anti-AChE assay, the extracts exerted insignificant inhibition against the enzyme at the tested concentrations, except for only SG whose inhibition was 39.1 and 42.5% at 1.0 and 2.0 mg ml^{-1}, respectively (Table 4).

Table 2. Percentage of inhibition±S.E.M.[b] against DPPH radical of the alga extracts

Extracts	Total phenol content[a]	Percentage of inhibition±S.E.M.[b] against DPPH radical				
		0.125 mg ml^{-1}	0.25 mg ml^{-1}	0.5 mg ml^{-1}	1.0 mg ml^{-1}	2.0 mg ml^{-1}
Fresh-water alga						
C. hispida	2.82±0.64	—[c]	—	—	2.2±0.74	2.6±0.39
C. glomerata	229.66±5.77	—	—	4.4±0.78	6.4±0.28	8.8±0.01
C. fracta	156.33±4.86	—	—	—	—	2.4±0.91
S. gratiana	862.1±3.10	—	—	54.8±1.25	86.9±1.14	89.9±0.53
Mougeotia sp.	286.33±4.41	—	—	—	5.7±0.92	9.2±0.09
V. sessilis	216.34±3.95	—	—	3.1±0.79	5.8±0.09	8.9±0.46
G. mutabilis	78.26±2.12	—	—	—	—	4.74±0.20
Fresh-water plants						
R. rionii	66.9±0.01	6.3±0.41	6.4±1.39	20.3±0.84	40.4±1.27	68.3±1.02
C. demersum	528.26±4.07	—	—	12.2±1.08	22.6±0.62	39.3±1.11
Marine algae and sea grass						
S. furcellata	—[d]	—	—	—	—	2.5±0.91
D. dichotoma	68.09±3.07	—	—	—	—	4.4±0.53
P. vickersiae	188.0±2.34	—	—	—	—	2.6±1.05
H. scoparia	46.9±0.12	—	—	—	—	3.6±0.72
P. oceanica	0.54±0.39	—	—	—	4.5±0.09	13.9±1.53
References						
Gallic acid	—	—	—	91.6±0.06	92.6±0.10	93.2±0.00
BHA	—	—	—-	77.9±0.48	81.6±1.67	82.9±0.68

[a]Expressed as gallic acid equivalent (mg g^{-1} extract),
[b]S.E.M.= Standard error mean,
[c]-= No activity,
[d]--= Not tested

Table 3. Ferric-reducing antioxidant power (FRAP) of the alga extracts

Extracts	Ferric-reducing antioxidant power (FRAP)±S.E.M[a]		
	0.25 mg ml−1	0.5 mg ml^{-1}	1.0 mg ml^{-1}
Fresh-water alga			
C. hispida	0.129±0.01	0.157±0.01	0.220±0.01
C. glomerata	0.150±0.01	0.219±0.01	0.342±0.04
C. fracta	0.070±0.01	0.092±0.01	0.125±0.02
S. gratiana	0.469±0.01	0.949±0.01	1.624±0.09
Mougeotia sp.	0.115±0.01	0.146±0.01	0.249±0.01
V. sessilis	0.090±0.01	0.111±0.01	0.161±0.01
G. mutabilis	0.107±0.03	0.199±0.01	0.265±0.01
Fresh-water plants			
R. rionii	0.268±0.01	0.494±0.06	1.039±0.04
C. demersum	0.175±0.01	0.329±0.02	0.609±0.02
Marine algae and sea grass			
S. furcellata	0.065±0.01	0.086±0.01	0.136±0.02
D. dichotoma	0.134±0.01	0.131±0.01	0.164±0.01
P. vickersiae	0.076±0.01	0.078±0.02	0.092±0.01
H. scoparia	0.066±0.01	0.085±0.01	0.122±0.01
P. oceanica	0.101±0.01	0.144±0.02	0.228±0.01
References	Concentrations for references (mg ml^{-1})		
	0.125	0.25	0.50
Gallic acid	1.585±0.01	3.569±0.02	3.677±0.02
BHA	0.973±0.05	1.470±0.01	2.595±0.44

[a]S.E.M.= Standard error mean

LC-DAD-MS analysis

Qualitative inspection of the ethanol extracts was carried out by LC-DAD-MS (Table 5). Chlorogenic, gentisic, gallic, coumaric, and ferulic, and benzoic acids, gallic acid ethyl ester, vitamin C as well as three carotenoids; asthaxanthin, beta-carotene, and canthaxanthin were detected in the extracts. Coumaric acid and vitamin C were observed in most of the algae, while gallic acid and its ethyl ester existed only in SG (Fig. 1). Astaxanthin was noticed in CH, MO, and RR, where beta-carotene was subsistent

Table 4. Acetylcholinesterase (AChE) inhibitory activity of the alga extracts

Extracts	Percentage of inhibition±S.E.M[a] against AChE		
	0.5 mg ml^{-1}	1.0 mg ml^{-1}	2.0 mg ml^{-1}
Fresh-water alga			
C. hispida	8.6±0.96	14.3±0.71	13.9±0.18
C. glomerata	8.8±1.04	10.3±0.94	12.2±0.43
C. fracta	—	—	3.6±0.66
S. gratiana	37.4±0.94	39.1±2.36	42.5±2.28
Mougeotia sp.	6.1±2.31	7.8±1.30	11.6±1.29
V. sessilis	—	—	—
G. mutabilis	7.8±2.09	12.8±1.49	15.9±0.30
Fresh-water plants			
R. rionii	—[b]	7.3±0.06	8.1±2.09
C. demersum	—	7.1±1.92	11.2±2.81
Marine algae and sea grass			
S. furcellata	10.9±0.57	13.9±1.65	22.1±1.28
D. dichotoma	6.0±1.27	9.2±1.58	13.5±0.58
P. vickersiae	7.9±2.17	8.8±0.53	10.2±1.27
H. scoparia	—	6.4±1.39	7.7±0.91
P. oceanica	—	8.8±1.05	11.6±3.06
Reference			
Galanthamine	—[c]	98.9±0.24	—

[a]S.E.M.= Standard error mean,
[b]—= No activity,
[c]—-= Not tested

Table 5. Phenolic acid, vitamin C, and carotenoid analysis of the algae extracts by LC—MS

Compounds Detected	Retention time (min)	EXTRACTS													
		CH	CG	CF	SG	MO	VS	GM	RR	CD	SF	DD	PV	HS	PO
Chlorojenic acid	3.42	—[a]	—	—	—	—	—	—	—	—	—	—	—	—	+[b]
Gentisic acid	3.51	—	—	+	—	—	—	—	+	+	—	—	—	—	+
Vitamin C	4.68	+	+	+	—	+	—	—	+	+	—	—	—	—	+
Gallic acid	7.35	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Gallic acid ethyl ester	7.62	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Benzoic acid	9.71	—	+	—	—	+	—	—	—	—	—	—	—	—	—
Ferulic acid	12.78	—	—	—	—	—	—	—	—	+	—	—	—	—	—
Astaxanthin	27.86	+	—	—	—	+	—	—	+	—	—	—	—	—	—
Beta—Carotene	29.40	—	—	—	—	—	—	—	—	+	—	—	—	—	—
Canthaxanthin	30.15	+	—	—	+	—	—	—	—	—	—	—	—	—	—
Coumaric acid	35.99	+	+	—	+	+	—	—	+	+	—	—	—	—	—

[a]= Not detected;
[b]= Detected

only in CD among the tested algae. Existence of vitamin C was revealed in a good number of the extracts, being the most prominent one as PO (Fig. 2).

There have been a limited number of reports on antioxidant activity of algae. Antioxidant activity is correlated with phenolic compounds and some lipophilic components. Antioxidant potential is affected by many factors and it is tough to explain this mechanism by only one method. Antioxidant properties of algae emerge generally as hydrogen donors, free radical scavengers, reducing agents, and metal chelators (12). In our screening, only SG and PO were superior to the other extracts in the antioxidant tests. Most of the seaweeds are known to contain dietary fiber and fiber has been reported to cause a decrease in anti-radical activity (13), which may be a part of the reason for lower anti-radical effect of the algal extracts, despite of their relatively good TPCs. Several other species of *Spirogyra* has been used as fish feeding and a topical antipyretic by the local people in Iberian peninsula (14,15) and reported to contain gallotannins, sterols, fat, mineral, and polysaccharides (16–19). Our analysis indicated existence of gallic acid and its ethyl ester by the prominent and abundant peaks in LC-MS chromatogram, together with coumaric acid and canthaxanthin (Fig. 1). We

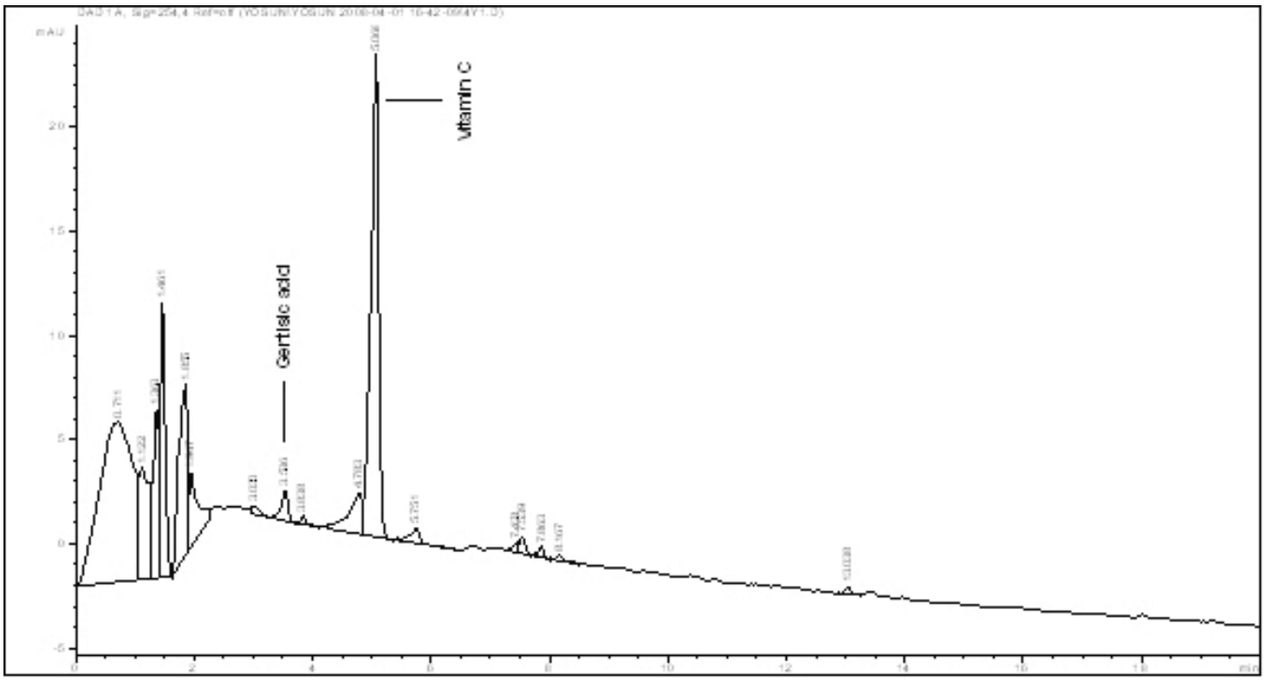

Figure 1. LC-DAD chromotogram of the ethanol extract of *P. oceanica* (PO)

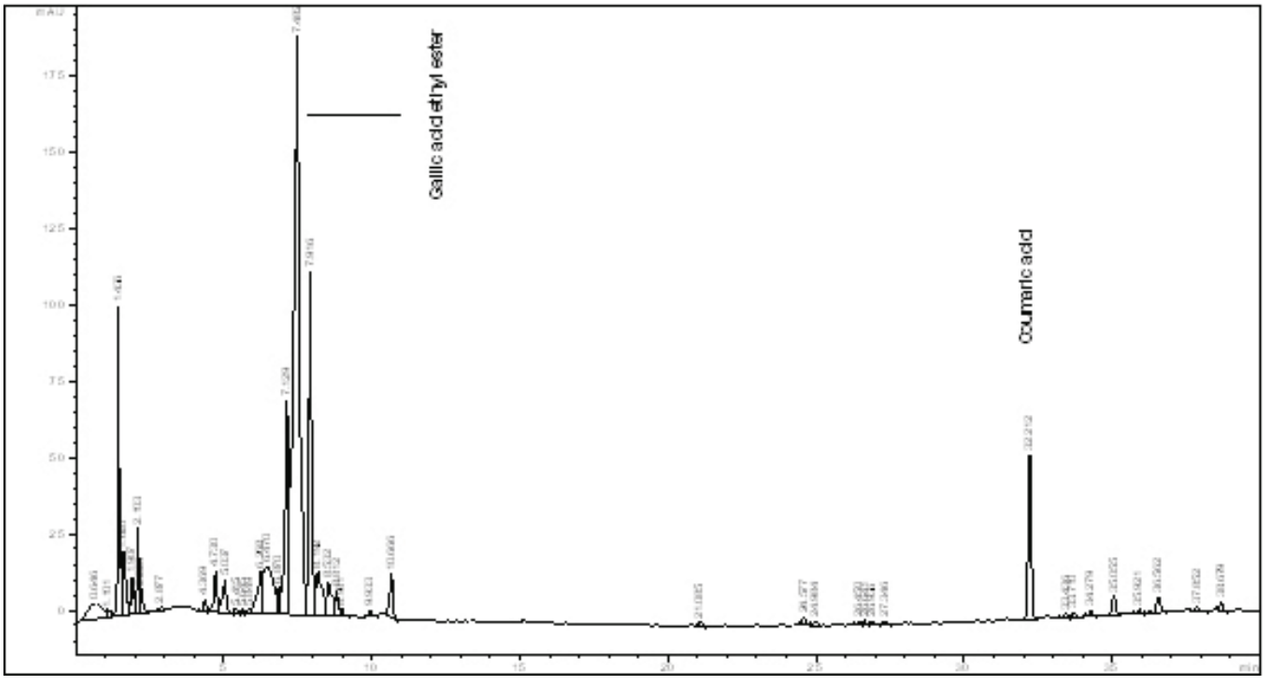

Figure 2. LC-DAD chromotogram of the ethanol extract of *S. gratiana* (SG)

have also shown very strong antioxidant activity of gallic acid, a natural phenol-type antioxidant, in two different methodologies (Tables 2 and 3) as also supported by various studies in seaweeds (20–22). Therefore, it may be considered that gallic acid and its ethyl ester might be the major contributors to its high antioxidant effect. Besides, canthaxanthin, a carotenoid detected in SG, has been recently reported with antioxidant effect (23–25). Despite of its relatively small amount, it might be co-contributor to antioxidant property of SG.

On the other hand, there has been a limited number reports on phytochemical ingredients of several *Ranunculus* species, that showed presence of flavonoids derivatives and anthocyanidins (26–29). Although phytochemistry of RR has not been investigated thus far, one can assume that similar compounds, flavonoids in particular, might exist in RR as well, which have probably the major effect on antioxidant activity of RR. Undoubtedly, gentisic and coumaric acids, vitamin C, and astaxanthin, detected by LC-MS for the first time in RR, also contribute to the activity. The extracts presented insignificant anti-AChE activity except for SG extract having a mild inhibition towards this enzyme (Table 2).

The present study elucidated for the first time antioxidant and anticholinesterase activity of the following algae; CH, CG, GF, SG, MO, VS, GM, RR, CD, SF, DD, PV, HS, and PO. SG possessed the best antioxidant activity, followed by RR. Rich gallic acid and its ethyl ester content of SG and prosperous vitamin C content of PO have been detected for the first time in this study. The results underline that SG and RR could be evaluated as the rich sources of antioxidants. Hence, our group proceeds to further analysis on SG and RR.

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