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Radical-scavenging activity of Aegean Sea marine algae

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Abstract

In the present work, thirteen algae from the Aegean Sea were examined for radical-scavenging activity (RSA) using the DPPH and chemiluminescence (CL) tests. Extracts of the brown alga *Taonia atomaria* exhibited the best RSA in comparison to the extracts of the other investigated species and approached the activity of powerful antioxidant standards. Column chromatography separation of *T. atomaria* extract, followed by preparative HPLC, resulted in the isolation of six metabolites, which were identified by spectral analyses (^{1}H NMR, ^{13}C NMR and MS). The isolated metabolites taondiol, isoepitaondiol, stypodiol, stypoldione, sargaquinone and sargaol were found to possess marked RSA.
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Keywords: Marine algae; Radical scavenging activity; Chemiluminescence; DPPH; *Taonia atomaria*

1. Introduction

In far east countries, seaweeds have been used in human diet since ancient times and have provided valuable nutrition for populations of these countries (Indergaard & Minsaas, 1991; Ito & Hori, 1999; Mabeau & Fleurence, 1993; Nisizawa, Noda, Kikuchi, & Watanabe, 1987). Nevertheless, in Western countries, consumption of seaweeds is limited (Ito & Hori, 1999; Mabeau & Fleurence, 1993). Based on the recent discoveries of many unique compounds in marine organisms (Blunt, Copp, Munro, Northcote, & Prinsep, 2005; Mayer & Lehmann, 2000), seaweeds are considered to be one of the richest sources of new bioactive metabolites (Shen, Hung, Prakash, & Wang, 2000), and their food value has been reassessed (Ito & Hori, 1999).

According to literature reports, the extract of the brown alga *T. atomaria* has exhibited the following activities: (a) anticancer activity due to the compounds stypodiol (Mayer & Lehmann, 2000) and stypoldione, which is an in vitro inhibitor of microtubule polymerization (Jacobs, Culver, Langdon, O'Brien, & White, 1985). Stypoldione shows relatively low toxicity and prolongs the survival of mice injected with tumor cells (O'Brien, White, Jacobs, Boder, & Wilson, 1984), (b) antifungal activity due to the compound taondiol (Wessels, Konig, & Wright, 1999), and (c) insecticidal activity, related to the compound isoepitaondiol (Gerwick & Fenical, 1981). In addition, the metabolite hydroxysargaquinone has shown cytotoxicity against P-388 lymphocytic cells.

Marine algae, like other photosynthesizing plants, are exposed to intense light and high oxygen concentrations that lead to the formation of free radicals and other strong oxidizing agents (Dykens, Shick, Benoit, Buettner, & Winston, 1992; Namaki, 1990). The absence of oxidative damage in their structural components (polyunsaturated fatty acids) (Matsukawa et al., 1997) and the stability during storage (Ramarathnam, Osawa, Ochi, & Kawakishi, 1995), suggests that their cells possess protective antioxidative systems (Jimenez-Escrig, Jimenez-Jimenez, Pulido, & Saura-Calixto, 2001).

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As of yet, only a few reports on the antioxidant activity of seaweeds have been published (Jimenez-Escrig et al., 2001). Since marine algae are rich in polysaccharides, minerals, proteins and vitamins, a documented antioxidant activity would elevate their value in the human diet and as food and pharmaceutical supplements (Yan, Nagata, & Fan, 1998).

In continuation of our studies toward the isolation of bioactive metabolites (Abatis et al., 2005; Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Bouskou, 2006; Kladi et al., 2005), we initiated an investigation for the evaluation of antioxidant properties of algal extracts from the Aegean Sea. The study focussed on species that are among the most abundant in the collection site and Aegean Sea, in general, and that could, in principle, become a natural source for the extraction of metabolites with antioxidant properties for use in cosmetics or food supplements.

2. Materials and methods

2.1. Plant material

Specimens of thirteen algae species were collected from the coastal areas of Kolimbari and Marathi, on the island of Crete, Greece. Identification of the algae was performed by Dr. Panos Panayotides at the Hellenic Centre for Marine Research. Specimens of the thirteen algae species are deposited at the Herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products with numbers ATPH MO226 – ATPH MO238 (with the order shown in Table 1).

2.2. Chemicals and reagents

Ascorbic acid, boric acid, dichloromethane, DPPH (2,2-diphenyl-1-picryhydrazyl radical), EDTA (ethylenediamine-tetraacetic acid), luminol (o-aminophthalyhydrazide), trolox and quercetin were purchased from Sigma- Aldrich (Germany). Cobalt (II) [$CoCl_2 \cdot 6H_2O$], 37% hydrochloric acid, silica gel and TLC were purchased from Merck (Germany). Ethyl acetate, ethyl ether and *n*-hexane were from Lab-Scan, Ireland and methanol from Riedel-de Haen, Germany. All solutions were freshly prepared before use. Distilled water was used throughout this study.

2.3. Extraction procedure

Wet tissues (15–20 g) from each of the 13 collected species were initially extracted with methanol and subsequently with dichloromethane. The obtained extracts were evaporated under vacuum at 30 °C. The residues were dissolved in methanol and used for the radical-scavenging activity tests.

2.4. Free radical-scavenging activity (DPPH)

A methanolic solution (50 μl) of each extract (5 different concentrations ranging from 0.5 to 30 mg/ml) was added to 1.95 ml of DPPH$^{\cdot}$ solution (6×10^{-5} M in methanol). The decrease of the DPPH concentration was monitored at 515 nm, using an HP 8452A diode-array spectrophotometer. When the reaction reached the steady state against a calibration curve, the quantity required to reduce the initial concentration (EC_{50} value) was calculated therefrom (Parejo, Codina, Petrakis, & Kefalas, 2000; Parejo, Petrakis, & Kefalas, 2000).

2.5. Hydroxyl radical-scavenging activity (CL)

Three dilutions of each sample were prepared, using methanol as a solvent. The concentration of the dilutions depended on the activity of the extract and ranged from 0.012 to 17 mg/ml. The chemiluminescence assays were carried out on a Jenway 6200 Fluorimeter (Leeds, U.K.), with the lamp off, using only the photomultiplier of the appara-

Table 1
RSA of the extracts examined by CL (IC_{50} expressed in mg extract/ml ± standard deviation) and DPPH (EC_{50}expressed in mg extract/g DPPH ± standard deviation)

Algae	Chemiluminescence (IC_{50} ± SD)		DPPH (EC_{50} ± SD)	
	MeOH extract	CH_2Cl_2 extract	MeOH extract	CH_2Cl_2 extract
Corralina elongata	$0.264 \pm 2 \times 10^{-4}$	$0.0729 \pm 4 \times 10^{-4}$	58.5 ± 0.04	25.6 ± 0.03
Laurencia obtusa	$0.335 \pm 3 \times 10^{-3}$	$0.0600 \pm 5 \times 10^{-4}$	65.9 ± 0.06	17.7 ± 0.05
Laurencia papillosa	$0.263 \pm 3 \times 10^{-3}$	$0.0450 \pm 2 \times 10^{-4}$	55.8 ± 0.03	12.4 ± 0.04
Peyssonnelia harveyana	$0.040 \pm 2 \times 10^{-4}$	$0.0050 \pm 5 \times 10^{-4}$	17.3 ± 0.02	4.90 ± 0.03
Rhodotamniella floridula	$0.248 \pm 3 \times 10^{-3}$	$0.0650 \pm 4 \times 10^{-4}$	53.1 ± 0.06	20.9 ± 0.01
Taonia atomaria	$0.017 \pm 1 \times 10^{-4}$	$0.0032 \pm 1 \times 10^{-4}$	5.10 ± 0.02	1.00 ± 0.01
Amphiroa sp.	$0.166 \pm 1 \times 10^{-3}$	$0.0120 \pm 1 \times 10^{-3}$	35.8 ± 0.03	7.70 ± 0.02
Halopteris scoparia	$0.225 \pm 2 \times 10^{-4}$	$0.0325 \pm 2 \times 10^{-4}$	45.5 ± 0.02	9.80 ± 0.04
Titanoderma bissoides	$0.659 \pm 5 \times 10^{-4}$	$0.1500 \pm 1 \times 10^{-4}$	100.3 ± 0.90	35.8 ± 0.07
Sargasum vulgare	$0.294 \pm 1 \times 10^{-4}$	$0.0510 \pm 5 \times 10^{-4}$	60.2 ± 0.01	15.6 ± 0.04
Dictyopteris membranacea	$0.078 \pm 8 \times 10^{-3}$	$0.0060 \pm 5 \times 10^{-3}$	25.4 ± 0.01	5.50 ± 0.03
Amphiroa cryptarthrodia	$0.008 \pm 1 \times 10^{-4}$	$0.0230 \pm 4 \times 10^{-4}$	36.8 ± 0.02	8.00 ± 0.08
Liagora sp.	$0.546 \pm 4 \times 10^{-4}$	$0.1250 \pm 1 \times 10^{-3}$	80.5 ± 0.04	30.9 ± 0.04

Measurements were performed in triplicate.

tus. 1 ml of boric acid buffer solution (0.05 M adjusted to pH 9 with 1 M NaOH) containing 0.2 mg/ml $CoCl_2 \cdot 6H_2O$ and 1 mg/ml EDTA was vortexed for 15 s with 100 μl of luminol solution (5.6×10^{-4} M) in boric acid (0.05 M adjusted to pH 9 with 1 M NaOH) in a test tube. Then, 25 μl of H_2O_2 (aqueous solution, 5.4×10^{-3} M) were added and the mixture was vortexed again for 30 s and quickly placed in a glass cuvette. The light intensity in the absence of the sample (I_0) was recorded when the plateau was reached (the lifetime of the plateau is 30 s). Immediately afterwards, 25 μl of the sample solution were added and mixed with a Pasteur pipette and the instantaneous decrease of the plateau was recorded (I).

The ratio I_0/I was plotted against mg of algae extract/ml for three dilutions of each extract and the quantity required to reduce I_0 by 50% (IC_{50} value) was calculated therefrom (Parejo et al., 2000).

2.6. Extraction and RSA guided fractionation of T. atomaria

A second large-scale extraction (130 g), aiming at the isolation of *T. atomaria* metabolites responsible for the radical scavenging activity, was performed, using mixtures of methanol and dichloromethane (1:3). The wet algae tissue was extracted three times (300 ml of solvent each time) for 24 h at room temperature and the combined extracts were concentrated under vacuum to afford 1.3 g of a dark brown oily residue.

Gravity chromatography separation was performed on silica gel (i.d. 4 cm, height 25 cm), with *n*-hexane and increasing volumes of EtOAc as the mobile phase. The gradient polarity was increased by steps of 10% EtOAc and eleven fractions (100 ml each) were collected and subsequently checked by TLC and by ^{1}H NMR. Since adequate separation was achieved, none of the fractions were combined. The weights of the fractions were: (1) 100% *n*-hexane = 2 mg, (2) 90% *n*-hexane: 10% EtOAc = 10 mg, (3) 80% *n*-hexane: 20% EtOAc = 10 mg, (4) 70% *n*-hexane: 30% EtOAc = 2 mg, (5) 60% *n*-hexane: 40% EtOAc = 10 mg, (6) 50% *n*-hexane: 50% EtOAc = 20 mg, (7) 40% *n*-hexane: 60% EtOAc = 50 mg, (8) 30% *n*-hexane: 70% EtOAc = 120 mg, (9) 20% *n*-hexane: 80% EtOAc = 110 mg, (10) 10% *n*-hexane: 90% EtOAc = 60 mg, (11) 100% EtOAc = 30 mg. The nine fractions that had adequate masses were submitted to DPPH and chemiluminescence tests.

Comparison of the chemical compositions of all fractions by ^{1}H NMR showed the more polar and more active fractions to contain the same sets of metabolites in varying ratios, besides chlorophylls that were much more abundant in the last fractions. Since fraction 8 had exhibited the highest RSA and was sufficient in quantity for further chromatographic separations, it was selected for preparative HPLC and isolation of metabolites. The chromatographic separation was performed on a Pharmacia LKB 2248 model (Uppsala, Sweden), with a flow rate of 2 ml/min at 300 nm wavelength on a Supelcosil SPLC-Si 25 cm × 10 mm, 5 um, column (Bellefonte, U.S.A.). The mobile phase was15% EtOAc and 85% *n*-hexane.

The major compounds were identified on the basis of their ^{1}H NMR and ^{13}C NMR spectra, recorded on a Bruker DRX 400 MHz spectrometer (Rheinstetten, Germany), as well as their mass spectra, obtained on a Hewlett–Packard 5973–6890 GC-MS system (Palo Alto, USA.) operating in EI mode, equipped with an HP 5MS 30 m × 0.25 mm × 0.25μm film thickness capillary column. The isolated compounds were submitted to the chemiluminescence and DPPH tests.

3. Results and discussion

3.1. Radical-scavenging activity (DPPH, CL) of Aegean Sea marine algae

Free radical-scavenging activity, expressed as EC_{50} (mg algae extract/g DPPH), is shown in Table 1. Free RSA ranged from 5.1 to 100 for the methanol extracts and from 1.0 to 35.8 for the dichloromethane extracts. Hydroxyl radical-scavenging activity, expressed as IC_{50} (mg algae extract/ml), ranged from 0.008 to 0.659 and from 0.003 to 0.150 for the methanol and dichloromethane extracts, respectively (Table 1). The lower IC_{50} and EC_{50} values of the dichloromethane extracts suggested that the less polar compounds were mainly responsible for the antioxidant activity.

Among the investigated algal extracts, those of *T. atomaria* were found to be the most active. More precisely, the methanol extract, according to the CL test was 60 times weaker than quercetin, 22 times weaker than trolox and 14 times weaker than ascorbic acid in terms of RSA. The dichloromethane extract of *T. atomaria*, which was found to be more powerful in terms of antioxidant activity, was 11 times weaker than quercetin, 4 times weaker than trolox and 2.6 times weaker than ascorbic acid.

In the DPPH test, *T. atomaria* methanol extract was 85 times weaker than quercetin, 43 times weaker than trolox and almost 20 times weaker than ascorbic acid, while the respective dichloromethane extract was shown to be 17 times weaker than quercetin, 8.5 times weaker than trolox and approximately 4.25 times weaker than ascorbic acid.

Table 2
RSA of chromatographic fractions examined by CL and DPPH tests

Fraction number	CL ($IC_{50} \pm$ SD)	DPPH ($EC_{50} \pm$ SD)
2	$0.0240 \pm 1 \times 10^{-4}$	5.10 ± 0.02
3	$0.0260 \pm 2 \times 10^{-4}$	4.90 ± 0.02
5	$0.0260 \pm 2 \times 10^{-4}$	5.30 ± 0.01
6	$0.0210 \pm 1 \times 10^{-4}$	4.00 ± 0.03
7	$0.0120 \pm 4 \times 10^{-4}$	0.41 ± 0.01
8	$0.0011 \pm 1 \times 10^{-4}$	0.21 ± 0.01
9	$0.0013 \pm 3 \times 10^{-4}$	0.26 ± 0.01
10	$0.0014 \pm 2 \times 10^{-4}$	0.30 ± 0.01
11	$0.0015 \pm 3 \times 10^{-4}$	0.28 ± 0.01

Measurements were performed in triplicate.

In an effort to identify the metabolites responsible for the antioxidant activity of *T. atomaria*, a second large scale extraction was performed and the extract was submitted to a RSA guided fractionation. The RSA of the fractions was examined by DPPH and CL tests (Table 2) and fraction 8 exhibited the highest activity, approaching the values of the three standards.

3.2. Isolation and radical-scavenging activity of T. atomaria constituents

Subsequently, fraction 8 was subjected to preparative HPLC, in order to isolate its constituents. The separations resulted in the isolation of six compounds, whose chemical structures were elucidated on the basis of their ^{1}H NMR, ^{13}C NMR and MS spectra. Comparison of the isolated metabolites spectral data with literature reports led to their identification as taondiol (Gonzalez, Darias, & Martin, 1971), isoepitaondiol (Rovirosa, Sepulveda, Quezada, & San Martin, 1992), stypodiol, stypoldione, sargaquinone (Gerwick & Fenical, 1981; Gerwick, Fenical, Fritsch, & Clardy, 1979) and sargaol (Numata et al., 1992). The isolated quantities were: 3.8 mg of taondiol, 1.9 mg of isoepitaondiol, 12.4 mg of stypodiol, 13.0 mg of stypoldione, 4.7 mg of sargaquinone and 3.3 mg of sargaol.

The RSA of the above-mentioned metabolites was found to be remarkable high (Table 3, Fig. 1). Taondiol and isoepitaondiol had higher RSA values than the others, with taondiol exhibiting the best RSA in CL and isoepi-

Table 3
RSA of the isolated metabolites and the standards (quercetin, trolox and ascorbic acid) examined by CL and DPPH tests

Compound	$IC_{50} \pm SD$	$EC_{50} \pm SD$
Stypodiol	$0.0010 \pm 1 \times 10^{-4}$	0.210 ± 0.002
Isoepitaondiol	$0.0005 \pm 3 \times 10^{-4}$	0.070 ± 0.001
Taondiol	$0.0004 \pm 2 \times 10^{-4}$	0.100 ± 0.001
Stypoldione	$0.0008 \pm 6 \times 10^{-4}$	0.180 ± 0.003
Sargaquinone	$0.0008 \pm 5 \times 10^{-4}$	0.200 ± 0.004
Sargaol	$0.0024 \pm 2 \times 10^{-4}$	0.200 ± 0.002
Quercetin	$0.0003 \pm 1 \times 10^{-4}$	0.060 ± 0.001
Trolox	$0.0007 \pm 3 \times 10^{-4}$	0.120 ± 0.001
Ascorbic acid	$0.0012 \pm 1 \times 10^{-4}$	0.250 ± 0.002

Measurements were performed in triplicate.

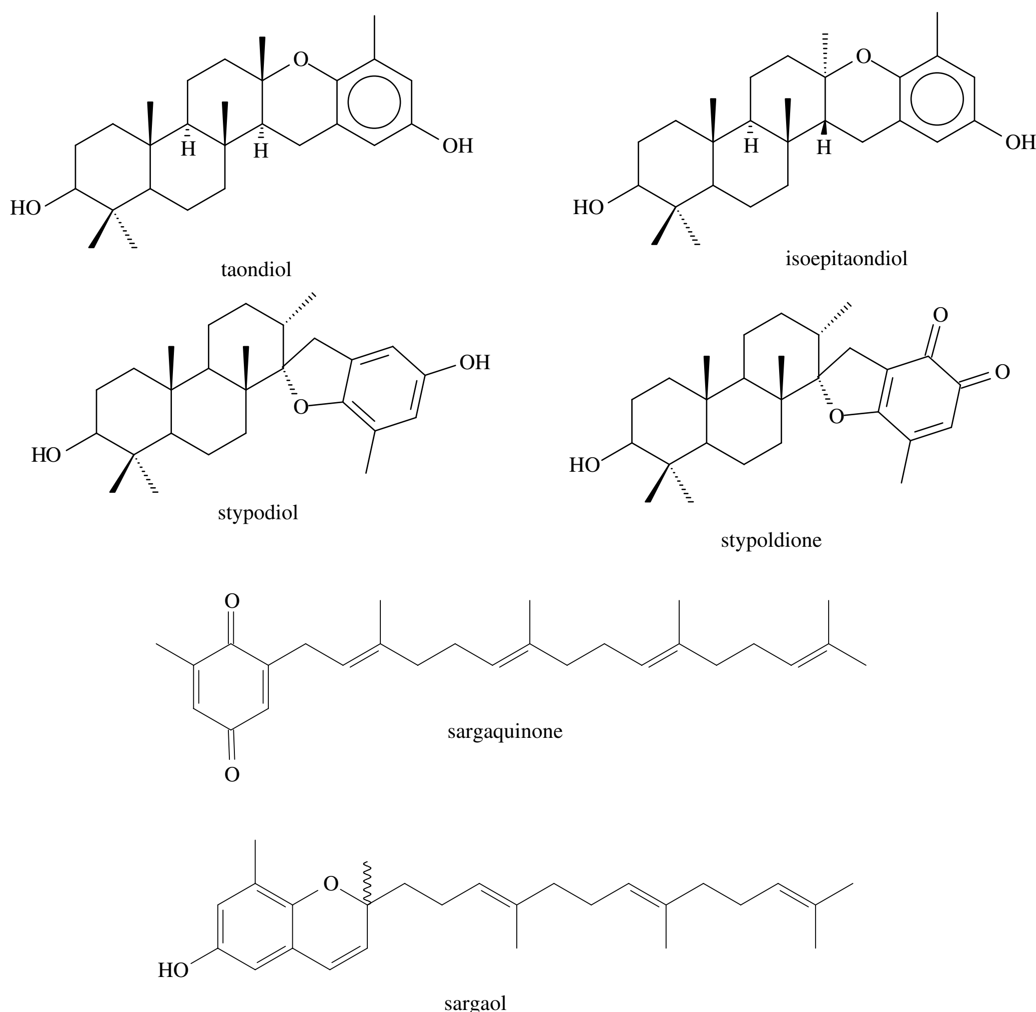

Fig. 1. Isolated metabolites of the brown alga *T. atomaria*.

taondiol in DPPH. More specifically, taondiol, the compound that manifested the highest RSA in the CL test, was 1.4 times weaker than quercetin, 1.7 times stronger than trolox and 3 times stronger than ascorbic acid. Isoepitaondiol, the compound that showed the highest RSA in the DPPH test, was 1.1 times weaker than quercetin, 1.7 times stronger than trolox and 3.4 times stronger than ascorbic acid.

4. Conclusion

The manifested antioxidant activity of some of the investigated alga species increases their importance as a potential new source of natural additives and nutritional supplements, especially when taking into account that there is an inverse relationship between the dietary intake of antioxidant-rich foods and incidences of human diseases (Lu & Foo, 2000).

This is the first report on the radical-scavenging activity of *T. atomaria* and the isolation, through a RSA guided separation protocol, of the metabolites responsible for its antioxidant properties. The remarkable RSA displayed by taondiol and epitaondiol, which were significantly more active than trolox and ascorbic acid, places them among promising future candidates as supplement agents in food, pharmaceutical and cosmetic industries.

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