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The Screening of Nitrite Scavenging Effect of Marine Algae and Active Principles of *Ecklonia Stolonifera*

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The nitrite scavenging effect of methanol extracts of marine algae were evaluated to discover new natural nitrite scavengers. Among the tested seaweeds, *Ecklonia stolonifera*, an edible brown algae, showed the strongest scavenging effect. The MeOH extract was then sequentially partitioned into CH_2Cl_2, CH_2Cl_2 insoluble interface, EtOAc, *n*-BuOH, and H_2O layers. The EtOAc and *n*-BuOH fraction demonstrated high levels of nitrite-scavenging activity while the CH_2Cl_2, CH_2Cl_2 insoluble interface, and H_2O fractions were inactive. A column chromatography of the EtOAc fraction through silica gel and Sephadex LH-20 yielded phloroglucinol and a new compound tentatively named phlorotannin A. The nitrite scavenging activity of phloroglucinol (IC_{50}=3.9 μg/ml) was more potent than that of L-ascorbic acid (IC_{50}=65.0 μg/ml). However, phlorotannin A (IC_{50}=193.2 μg/ml) showed only low levels of activity. From the above results, it is possible to suggest that both the MeOH extract and their fractions and isolated phloroglucinol and phlorotannin A obtained from *E. stolonifera* may be applicable as scavengers of nitrite, which is a precursor for the formation of carcinogenic N-nitroso compounds.

Key words : seaweed, nitrite-scavenging activity, active principle, NMR, phloroglucinol, *Ecklonia stolonifera*

Introduction

Nitrite is used in many countries as deliberate food additives to stabilize the color of cured meats, contribute flavor, and protect against the danger of botulism (William, 1970; Roberts, 1975). In addition, nitrate is also used often as deliberate food additives (Seishi and Nakao, 1971). The potential toxicity of nitrate to animals has been ascribed to nitrite which is formed from nitrate by bacterial and salivary reduction either prior to ingestion or within the gastrointestinal tract (Hayashi and Watanabe, 1978).

Unlike nitrate, which is relatively inert, nitrite is very reactive, especially at low pH in its protonated form, nitrous acid (pKa=3.4). Nitrous acid can react both as a nitrosating agent and as an oxidizing agent.

Nitrite is intrinsically toxic. The exact lethal dose of nitrite in humans is not known, but it is estimated to be about 1 gram as sodium nitrite in adults (Gleason et al, 1969). The acute toxic effect of nitrite administration is the induction of infant methemoglobinemia. Nitrite reacts with oxyhemoglobin to convert it from its ferrous form to the ferric form (methemoglobin) that is unable to bind oxygen. The presence of a certain fraction of methemoglobin also distorts the oxygen dissociation curve of residual hemoglobin so that it transports oxygen less effectively (Peter, 1975).

Carcinogenic N-nitroso compounds are also produced by the acid-catalyzed reaction of nitrite with certain nitrogen compounds (Sen et al, 1969). Human may be exposed to N-nitroso compounds in tobacco products (Hoffmann and Hecht, 1985), contaminated air (Fine, 1982), water (Fine, 1982; Hartmetz and Slemrova, 1980), and food (Webb and Gough, 1980; Spiegelhalder et al, 1980). And humans may be exposed to N-nitroso compounds by the nitrosation of exogenous and endogenous amines in the stomach and possibly other tissues (Mirvish, 1975; Rounbehler et al, 1977). But direct evidence linking N-nitroso compounds with human cancer causation is still scant. Since the presence of nitrite is a prerequisite in the formation of N-nitroso compounds, any compound that could compete successfully with the secondary amine for the available nitrite would reduce the possibility of N-nitroso compound formation.

It was reported that certain compounds such as ascorbate (Mirvish et al, 1972; Archer et al, 1975; Fiddler et al, 1973; Fan and Tannenbaum, 1973; Gray and Dugan, 1975), erythrobate (Fiddler et al, 1973) and its esters

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(Sen et al, 1976; Pensabene et al, 1976), α-tocopherol (Fiddler et al, 1978; Mergenes et al, 1978), sorbic acid (Tanaka et al, 1978) and other reducing agents (sodium bisulfite, tannic acid, and thiols such as cysteine, 2-mercaptoethanol, and NADH) (Gray and Dugan, 1975; Hallet *et al*, 1980) which are endogenous to foodsfuffs or may be added to foods for preservative purposes, inhibit the formation of N-nitrosamines. Ascorbate and sorbic acid have been shown to react with nitrite to reduce the available nitrite in the nitrosation.

As already mentioned, N-nitroso compounds are formed by the interaction of nitrogenous compounds with nitrosating agents, the most important of which is acid nitrite. In this aspect, it may be worthwhile to begin on the nitrite scavenging effect reducing the formation of N-nitroso compounds.

In the present study, the methanol extracts of marine algae were screened for the nitrite scavenging effect, and the active principles of *Ecklonia stolonifera* which showed most potent activity were identified.

Materials and Methods

Algae material

All the seaweeds used in this experiment were collected at Tae Jong Dae, Pusan, in July, 1990. The algae were identified by a botanist Prof. H. G. Kim, Kangnung National University and voucher specimens were deposited in the author's laboratory (J. S. Choi). All the seaweeds were washed with fresh water and air-dried in the shade.

Reagents

Naphthylethylene-diamine-HCl, sulfanilamide and sodium nitrite were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Sulfanilamide reagent was prepared by adding 5 gram of sulfanilamide in a mixture of 50 mℓ conc. HCl and about 300 mℓ distilled water, and diluted to 500 mℓ with distilled water. N-(1-naphthyl)-ethylenediamide (0.5 gram) was dissolved in 500 mℓ of distilled water. All other chemicals used were reagent grade.

Apparatus

Melting points were determined on an Electrothermal digital micro melting point apparatus without correction. IR spectra were recorded on a Varian Techtron Model 635 spectrophotometer. The ^{1}H− and ^{13}C-NMR spectra were recorded on a Bruker AM 300 or Varian Unity 500 spectrometer with tetramethylsilane as the internal standard. Multiplicities of ^{1}H− and ^{13}C-NMR signals are indicated as s (singlet), d (doublet) and t (triplet). The phloroglucinol was run in DMSO-d_6, and phlorotannin A was run in CD_3OD. UV spectra were run with a Cecil 599 Universal automatic scanning spectrophotometer and fast atom bombardment mass spectrum (FAB-MS) was taken on a Kratos 25 MS RFA spectrometer.

Extraction and fractionation

All the seaweeds (50 g each) were extracted with hot MeOH to yield the methanol extract. The *Ecklonia stolonifera* (2.9 kg), which displayed significant scavenging activity, was further extracted three times with methanol, and the solvent was removed under reduced pressure to give a dark blue semisolid (500 g). Succesive partitioning yielded dichloromethane (80.3 g), dichloromethane insoluble interface (7.5 g), ethyl acetate (17 g), *n*-butanol (13.8 g), and water soluble (275.5 g) fractions, respectively.

Isolation of compound I (phloroglucinol) and compound II (phlorotannin A)

The ethyl acetate-soluble fraction was chromatographed over silica gel using a $CHCl_3$ - MeOH mixture and further separated by Sephadex LH-20 (solvent: MeOH) to yield the phloroglucinol and phlorotannin A in the order of polarity.

Properties of phloroglucinol and phlorotannin A

Phloroglucinol (I) : white hygroscopic powder; Mp 218 ℃; ^{1}H-NMR (DMSO-d_6, 300MHz) δ 8.88 (3H, s, phenolic OH), 5.66 (3H, s, aromatic H), ^{13}C-NMR (DMSO-d_6, 300 MHz) δ 168.8 (C-1,3,5), 104.1 (C-2,4,6)

Phlorotannin A (II) : white hygroscopic powder; ^{1}H-NMR (CD_3OD, 500MHz) δ : 5.98 (1H, d, J=2.0Hz), 5.95 (1H, d, J=2.0Hz), 6.00 (1H, d, J=2.0Hz), 6.02 (1H, d, J=2.0Hz), 6.14 (1H, s); FAB-MS (*m/z*) 369 (M^+−H); ^{13}C-NMR (CD_3OD, 500MHz) δ 95.7, 95.8, 99.0, 100.0, 124.5,

124.8, 127.9, 133.1, 139.3, 141.4, 143.5, 143.8, 147.0, 147.2, 154.4, 154.8

Assay of nitrite scavenging effect

The assay of nitrite scavenging effect was carried out according to the Standard Method (APHA, AWWA and WPCF, 1985), except for the addition of DMSO for sample preparation as shown in Fig. 1. To increase water solubility, many of the MeOH extracts were dissolved in 0.3% DMSO solution. Addition of DMSO below 0.5% concentration showed weak nitrite scavenging effect but did not affect the scavenging effect (<2%). Sample solutions and control solution, all containing 0.1 mM $NaNO_2$, were adjusted to the indicated pH with concentrated hydrochloric acid. Each mixture (10 mℓ) placed in a screw cap tube was incubated at 37℃ for 1 hr in a water bath. After incubation, the solution was reacted with sulfanilamide reagent (1mℓ) and naphthylethylene diamine reagent (1 mℓ). The absorbance of this solution was determined at 540 nm, and the remaining nitrite was calculated. The results were calculated by taking the mean of all triplicate values.

Results and Discussion

The nitrite scavenging effect of seaweeds

To find the nitrite scavengers from marine algae, the nitrite scavenging activity was evaluated by measuring the percentages of nitrite remaining after sample treatment. The control (absence of extracts) was taken as 100 %, and the percent intensity was calculated by spectrophotometry at 540 nm. The concentration for 50% scavenge is shown in Table I. As shown in Table I, some algal extracts such as *Hizikia fusiformis, Sargassum ringgoldianum, Ecklonia stolonifera,* and *Symphyocladia latiuscula* exhibited significant scavenging effects. The scavenging effect of these extracts were concentration dependent (Fig. 2). Among them, *E. stolonifera* showed the strongest effect. This crude MeOH extract showed scavenging activity almost comparable to that of L-ascorbic acid which is a well known nitrite scavenger. These results suggest that these algae extracts contained a certain nitrite sacvenging principle (s).

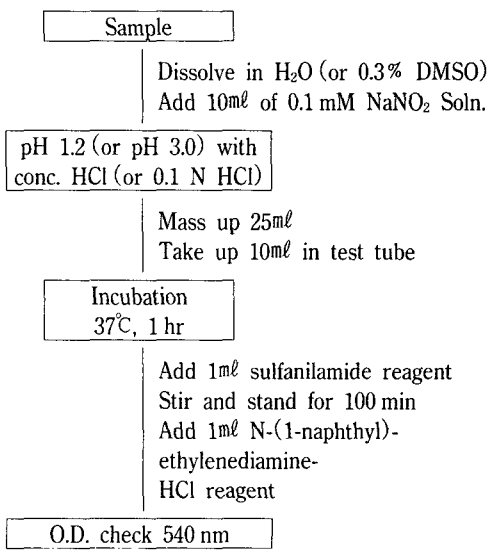

Fig. 1. Assay of nitrite scavenging effect.

The nitrite scavenging effect of the methanol extract and its subsequent fractions of *Ecklonia stolonifera*

The present study was also carried out to investigate

Table I. Nitrite scavenging effects of seaweeds at pH 1.2

Seaweeds	IC_{50}^{*} (mg/mℓ)
CHLOROPHYTA	
Enteromorpha crinita	>1.0
Ulva pertusa	>1.0
PHAEOPHYTA	
Hizikia fusiformis	0.31
Undaria pinnatifida	>1.0
Sargassum ringgoldianum	0.46
Sargassum miyabei	>1.0
Sargassum thunbergii	>1.0
Ecklonia stolonifera	0.08
RHODOPHYTA	
Gelidium amansii	>1.0
Carpopeltis cornea	>1.0
Chondrus crispus	>1.0
Pachymeniopsis elliptica	>1.0
Symphyocladia latiuscula	0.70
Grateloupia filicina	>1.0
Rhodymenia intricata	>1.0
Acrosorium flabellatum	>1.0
Gracilaria verrucosa	>1.0
Gigartina tenella	>1.0
Ascorbic acid	0.02

Values are means of triplicate experimental data.
*Amount required for 50% scavenging of nitrite.

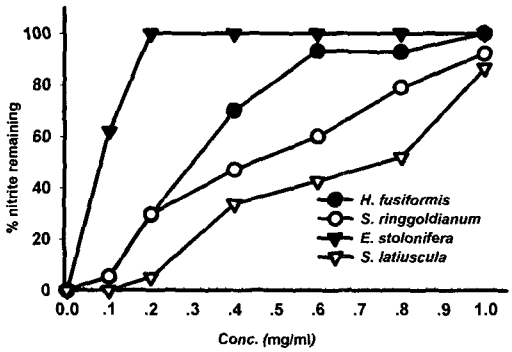

Fig. 2. The nitrite scavenging effect of MeOH extract obtained from *H. fusiformis*, *S. ringgoldianum*, *E. stolonifera*, and *S. latiuscula*.

the active principles from the MeOH extract of *Ecklonia stolonifera* which showed marked nitrite scavenging activity. The MeOH extract of *E. stolonifera* was partitioned into CH_2Cl_2, CH_2Cl_2 insoluble interface, EtOAc, *n*-BuOH, and H_2O layers, successively. And then, these solvent-soluble fractions were subjected to the measurement of nitrite scavenging activity. The nitrite scavenging effect of the MeOH extract and their fractions are shown in Table II. The nitrite scavenging effects of the MeOH extract and all other fractions obtained from the MeOH extract were observed in all cases except for the water fraction; and it was concentration dependent. The nitrite scavenging effects of the ethyl acetate and dichloromethane insoluble interface fraction were stronger than that of others. Their IC_{50} was 0.11 μg/mℓ and 0.20 μg/mℓ, respectively. This results suggest that the methanol extract, and the ethyl acetate, and the dichloromethane insoluble interface fraction of *E. stolonifera* are effective nitrite scavengers.

Table II. Nitrite scavenging effects of methanolic extract and subsequent fractions of *Ecklonia stolonifera*

Fractions	IC_{50}^{*} (μg)
MeOH extract	0.80
CH_2Cl_2 fraction	0.43
EtOAc fraction	0.11
BuOH fraction	0.40
H_2O fraction	-
Interface	0.20
L-Ascorbic acid	0.24

Values are means of triplicate experimental data.
*Amount required for 50% scavenging of nitrite.

Structures of compounds I and II

Compound I, a white hygroscopic powder, was suspected to be a phenolic compound according to its positive reaction to iron chloride. The IR spectrum of I showed a broad hydroxyl and aromatic absorption at 3300~3100 cm^{-1} and 1600 cm^{-1}, respectively. The ^{1}H-NMR spectrum of I showed signals of both aromatic (δ 5.66) and phenolic hydroxyl protons (δ 8.88). The signals at δ 8.88 disappeared in addition of D_2O. These data and ^{13}C-NMR (see materials and methods) indicated that I is a trisubstituted benzene derivative. Thus the structure of I was deduced to be phloroglucinol by the comparison of NMR spectral data with those reported in the literature.

Compound II, a yellow powder, was also recognized as a phenolic compound according to its positive reaction to ferric ferricyanide and bromocresol green. The psedomolecular ion at *m/z* 369[M^+−H] in the negative FAB-MS was consistent with the molecular formular $C_{18}H_{10}O_9$. The IR spectrum of II showed a broad hydroxyl absorption at 3350 cm^{-1} and aromatic ring absorptions at 1610 and 1510 cm^{-1}, respectively. The ^{1}H-NMR spectrum of II showed signals of both four *meta*-coupled aromatic protons[δ 5.98 (J=2.0 Hz), δ 5.95 (J=2.0Hz), δ 6.00 (2.0 Hz) and δ 6.02 (2.0 Hz)] and singlet aromatic proton[δ 6.14)]. These data and ^{13}C-NMR (see materials and methods) indicated that II is a dimeric phloroglucinol derivative and the chemical structure of II was proposed as shown in Fig. 3, and was given a trivial name, phlorotannin A. Although the phlorotannin derivatives were previously isolated from brown algaes such as *Analipus japonicus* (Glombitza and Zieprath, 1989), *Ecklonia kurome* (Fukuyama et al, 1989), *Sargassum spinuligerum* (Keusgen and Glombitza, 1995, Glombitza and Keusgen, 1995) as well as *Ecklonia stolonifera* (Taniguchi et al, 1991), this is the first example of its occurrence in natural sources.

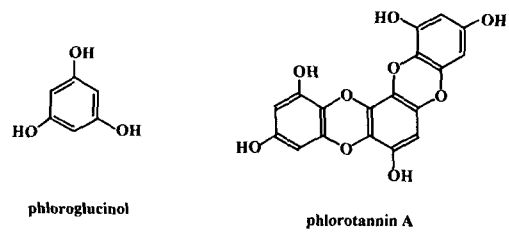

Fig. 3. Proposed structures of active principles.

The nitrite scavenging effect of active principles of *E. stolonifera*

The EtOAc fraction having the most scavenging effect was further purified to obtain active compounds I and II by repeated silica gel and gel filtration column chromatography. These compounds I and II were identified as phloroglucinol and phlorotannin A, respectively (*vide supra*). The effects of phloroglucinol, phlorotannin A, and L-ascorbic acid on the nitrite scavenge is shown in Fig. 4. The addition of phloroglucinol at a concentration of 2.50 to 20 μg/mℓ scavenged the nitrite by 22.6 to 100%. On the other hand, L-ascorbic acid scavenged the nitrite by 3.5 to 19.1% at the same concentrations. The nitrite scavenging effects of these compounds increased according to their respective concentrations. Their IC_{50} was 3.9 μg/mℓ and 65.0 μg//mℓ, respectively. The effects of phloroglucinol was about twenty times more active than that of L-ascorbic acid. These results suggest that phloroglucinol, as well as L-ascorbic acid, is an effective nitrite scavenger. However, the activity of phlorotannin A was weak.

Generally, algae has been used as a folk medicine in the curing of curare, gout, eczema, and gallstones, and as anthelmintics in Korea. Kim *et al.* (1987) reported nitrite scavenging activity of algal extracts. In particular, the methanol-soluble fractions extracted from *Codium fragile* and *Undaria pinnatifida* showed excellent scavenging activities. And they also found that the chloroform-soluble fraction of red algae, *Polyshiponia ulceolate* and *Enteromorpha compressa* possess a marked scavenging activity. Kim *et al.* (1996) also screened for nitrite scavenging activity of eight algal species *Laminaria japonica, Undaria pinnatifida, Codium fragile, Polyshiponia tenera, Sargassum fulvellum, Enteromorpha compressa, Ecklonia cava, and Ecklonia stolonifera.* The scavenging activity were higher in brown algae than in green and red algae. They also found that the brown algae family, Laminariaceae, *E. carva* and *E. stolonifera* that belong to genus *Ecklonia* showed a marked nitrite scavenging activity. Though the structure of active nitrite scavenging principles was not reported, they proposed the phenolic compounds as candidate because the extracts exhibited a higher reducing ability. These results consist with our present study that *E. stolonifera* has scavenging activity and phenolic compounds such as phloroglucinol and phlorotannin A are active principles. There was a report that phenolic compounds are effective in nitrite-scavenging (Kang, 1996).

Phloroglucinol has been isolated from many plants, for example, *Eucalyptus kino* and *Acacia arabica* (Dictionary of Natural Products, 1994), marine algaes, such as *Ecklonia stolonifera, E. cava, Cystophyllum hakodatense, Sargassum ringgoldianum, and Fucus vesiculosus* (Scheuer, 1981; Taniguchi et al., 1991). The antispasmodic effect and nitrite scavenging effect of phloroglucinol have been reported (Dctionary of Natural Products, 1994; Choi et al., 1989).

The findings of the present work indicate that the methanol extract of *E. stolonifera*, its subsequent fractions, and its component, phloroglucinol and phlorotannin A, might be useful as nitrite scavengers.

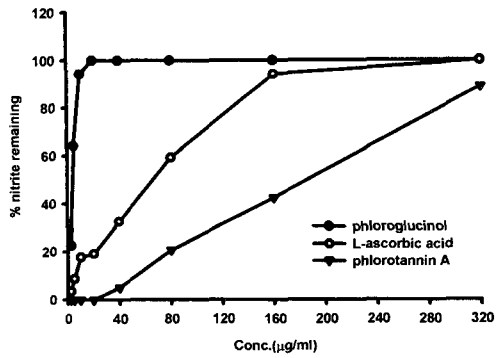

Fig. 4. The nitrite scavenging effect of phloroglucinol, phlorotannin A, and L-ascorbic acid at pH 1.2.

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