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INHIBITORY SUBSTANCES FROM *MYRIOPHYLLUM BRASILIENSE* ON GROWTH OF BLUE-GREEN ALGAE

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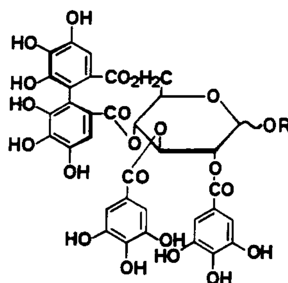
ABSTRACT.—Aqueous EtOH extracts of whole plants of parrotfeather (*Myriophyllum brasiliense*) showed a significant inhibitory activity on growth of the blue-green algae *Microcystis aeruginosa* and *Anabaena flos-aquae*. Bioassay-directed fractionation has led to the identification of the active substances as eugeniin [**1**], 1-desgalloyleugeniin [**2**], a mixture of epicatechin-3-gallate and catechin-3-gallate, gallic acid, quercetin, quercitrin, and avicularin.

Myriophyllum brasiliense Camb. (Haloragaceae) is an emerged aquatic perennial called parrotfeather. This plant originated from South America and is now widely distributed. *M. brasiliense* has strong procreative power and often becomes dominant in diverse aquatic systems. Several papers reported removal of nutrient salts by the plants of this genus (1–3). We have already investigated the ability of *M. brasiliense* to remove nutrient salts from eutrophicated water (4). In the course of that study, *M. brasiliense* was suspected to have inhibitory substances against blue-green algae living in water. The blue-green algae breed in eutrophicated water and often constitute water bloom, which causes the quality of water to become poor (5). Some species of these algae are known to contain cyclopeptides toxic to fish and mammals (6,7). Thus, it is important to find natural inhibitory compounds against the blue-green algae. In this paper, we report the isolation and characterization of anti-algal compounds from *M. brasiliense* and evaluation of their activities on the growth of blue-green algae.

RESULTS AND DISCUSSION

The inhibitory substances of *M. brasiliense* were purified by bioassay-directed fractionation. The whole plants of *M. brasiliense* were extracted with 70% aqueous EtOH. The extracts were concentrated and sequentially partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. Figure 1 shows the inhibitory activity of each fraction on the growth of *Microcystis aeruginosa*, which is one species of blue-green algae constituting water bloom. The EtOAc fraction has the most potent activity and the *n*-BuOH fraction the second most potent. The EtOAc fraction was chromatographed on Sephadex LH-20 to give five separated fractions. Each fraction had substantial activity, suggest-



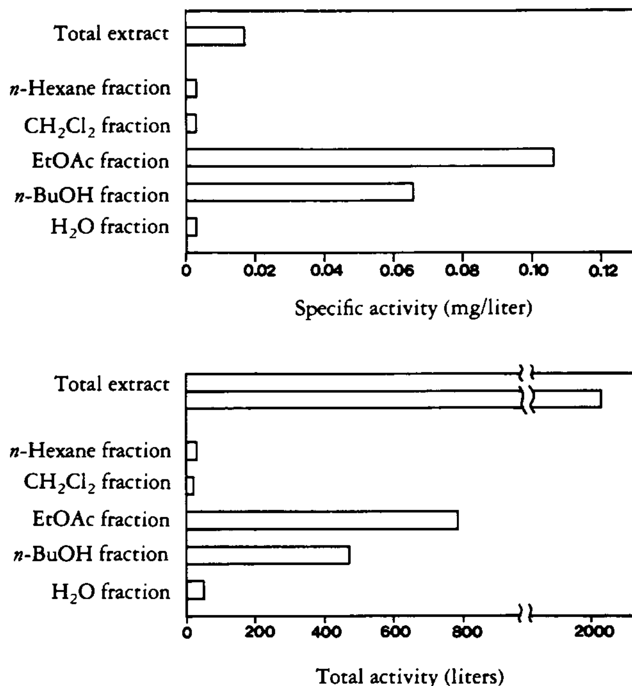


FIGURE 1. Distribution of inhibitory activities on growth of *Microcystis aeruginosa*. The inhibitory activity was expressed as follows: Specific activity (mg/liter) = $1/IC_{50}$ (mg/liter); total activity (liters) = specific activity (mg/liter) × weight of the fraction (mg).

ing that the active principle is not due to a single compound but to several substances. Each fraction was rechromatographed on Sephadex LH-20 and MCI gel CHP-20P, by which seven compounds were isolated as the active principles: eugeniin [**1**], 1-desgalloyleugeniin [**2**], a mixture of epicatechin-3-gallate and catechin-3-gallate, gallic acid, quercetin, quercitrin, and avicularin.

Eugeniin [**1**] exhibited the most potent activity among the isolated compounds. The concentration of **1** for 50% inhibition of growth (IC_{50}) of *M. aeruginosa* was found to be 1.6 μM (Figure 2, Table 1). 1-Desgalloyleugeniin [**2**] had the second most potent activity (IC_{50} = 3.7 μM). The flavonols, quercetin, quercitrin, and avicularin, showed less potent activity than the tannins (Figure 3, Table 1).

Compounds **1** and **2**, the hydrolyzable tannins, are composed of galloyl residues, a

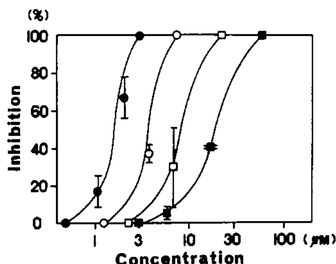


FIGURE 2. Inhibitory activities of tannins and gallic acid on growth of *Microcystis aeruginosa*. ●, Eugeniin [**1**]; ○, 1-Desgalloyleugeniin [**2**]; □, Epicatechin-3-gallate and catechin-3-gallate; ■, Gallic acid. Bars show the data of duplicate incubations.

TABLE 1. Inhibitory Activities of the Compounds Isolated from *Myriophyllum brasiliense* on Growth of *Microcystis aeruginosa* and *Anabena flos-aquae*.

Compound	Isolation yield (% of dry wt)	Growth inhibition (IC_{50}, μM)[a]	
		M. aeruginosa	*A. flos-aquae*
1	0.008	1.6	2.8
2	0.032	3.7	9.5
Mixture of epicatechin-3-gallate and catechin-3-gallate	0.009	8.2	6.0
Gallic acid	0.012	19	33
Quercetin	0.012	12	25
Quercitrin	0.21	9.0	45
Avicularin	0.026	180	28

[a]IC_{50} were determined from dose-response curves as described in Experimental.

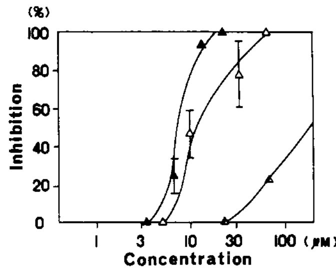


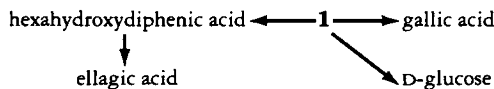
FIGURE 3. Inhibitory activities of flavonoids on growth of *Microcystis aeruginosa*. △, Quercetin; ▲, Quercitrin; ▲ Avicularin. Bars show the data of duplicate incubations.

hexahydroxydiphenoyl (HHDP) moiety, and glucose (Scheme 1). We therefore examined inhibitory activities of gallic acid, ellagic acid, and glucose to clarify which moiety is responsible for the activity. The activity of ellagic acid was almost equally as potent as that of gallic acid (Figure 4). However, glucose had no activity even in concentrations of up to 500 μM. These results indicate that galloyl and HHDP residues are both responsible for inhibitory activities of the hydrolyzable tannins **1** and **2**.

The isolated compounds showed inhibitory activity against the growth of *Anabaena flos-aquae*, another species of blue-green algae constituting water bloom, as well as against *M. aeruginosa* (Table 1).

The contents of gallotannin and proanthocyanidin in the culture water of *M. brasiliense* were determined after lyophilization as described previously (8). Approximately 0.003% of total gallotannin and proanthocyanidin in the plant was released into the medium during 10 days. This result suggested that the released tannins from the plant contribute at least partly to the inhibition of growth of the algae.

It has been already recognized that phenolic compounds from plants show a variety of biological activities (9–14), for example, antiviral action (9) and complexation with protein (10, 11). However, only a few papers reported the anti-algal activities of plant



SCHEME 1. Composition of hydrolyzable tannin **1**.

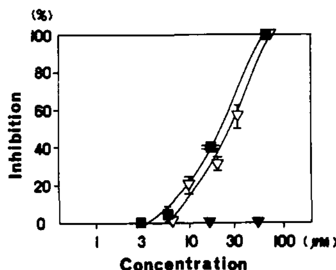


FIGURE 4. Inhibitory activities of ellagic acid, gallic acid, and glucose on growth of *Microcystis aeruginosa*. ▽ , Ellagic acid; ■, Gallic acid; ▼, Glucose. Bars show the data of duplicate incubations.

phenolics. Planas *et al.* (12) showed the inhibitory effect of the mixture of natural phenolics extracted from *Myriophyllum spicatum*, which belongs to the same genus as *M. brasiliense*, on the growth of blue-green and green algae. However, they did not isolate intact tannins and flavonoids as the active principles. We have isolated the tannins and the flavonoids as the anti-algal compounds from the aquatic plant. These activities are intensely interesting in relation to ecological roles of *M. brasiliense* in our environment. These inhibitory compounds might be applicable for improvement of water quality.

EXPERIMENTAL

PLANT MATERIAL.—The whole plants of *M. brasiliense* were collected in spring 1988, after cultivation in the greenhouse of the Medicinal Plant Gardens, Chiba University. The plants were dried at 50° in a paperchromatooven for 2 days.

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were taken with a JASCO DIP-181 digital polarimeter. Fabms were recorded on a JEOL HX-110. Eims were recorded on a Hitachi M-60. Ir were obtained on a Hitachi 260-10 infrared spectrophotometer in KBr disks. Uv were obtained on a Hitachi U-3200 spectrophotometer. ^{13}C and ^{1}H nmr were recorded on JNM-FX270, JEOL GSX 400, and GSX 500 with TMS as an internal standard, and chemical shifts are given in δ (ppm). The multiplicities of ^{13}C nmr were confirmed by the DEPT experiments. Fluorescence was recorded on a Hitachi MPF-4 fluorescence spectrophotometer. Tlc were performed on Si gel (Merck, Kieselgel $60F_{254}$) using CH_2Cl_2-MeOH (4:1) or C_6H_6-ethyl formate-HCO_2H (1:7:1) as eluents and uv light (254 nm) for visualization.

CULTURE OF BLUE-GREEN ALGAE.—Two species of blue-green algae were used: *M. aeruginosa* f. *aeruginosa* (strain number NIES-44) and *A. flos-aquae* f. *flos-aquae* (NIES-73), both from the National Institute for Environmental Studies, Tsukuba, Japan. These algae were cultured in sterilized CB medium (15) in glass tubes. The tubes were incubated at 25° under 2000 lux of fluorescent light for 16 h per day and were agitated once a day.

ANTI-ALGAE ASSAY.—The EtOH solution of the test compounds (10 μl) was added to the CB culture medium of algae (5 ml). The cell concentration of algae was equivalent to 150 μg per liter of chlorophyll a. After incubation for 24 h and 48 h, chlorophyll a in the algae was extracted with MeOH at 65° for 20 min. The concentration of chlorophyll a was determined fluorophotometrically (excitation 413 nm, emission 672 nm) as described previously (16). IC_{50}'s of the crude fraction or isolated compound were determined from dose-response curves obtained by at least four different concentrations of test compounds. In each concentration, duplicate incubations were carried out.

EXTRACTION AND FRACTIONATION OF PLANTS.—The whole dry plants of *M. brasiliense* (680 g) were extracted with 70% aqueous EtOH (5 liters × 3). The extracts were concentrated in vacuo to 1 liter and were partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH (each 500 ml × 3).

CHROMATOGRAPHY OF THE EtOAc FRACTION.—The EtOAc fraction (6.70 g) was chromatographed on Sephadex LH-20 (ø 3.0 × 20 cm) using MeOH as eluent. Fractions (25 ml) were combined mainly on the basis of tlc patterns: fraction 1 (0–125 ml, 0.19 g), fraction 2 (125–175 ml, 0.82 g), fraction 3 (175–250 ml, 1.52 g), fraction 4 (250–350 ml, 0.93 g), and fraction 5 (350–500 ml, 2.23 g). Each frac-

tion was rechromatographed on Sephadex LH-20 and MCI gel CHP-20P. Fraction 2 (400 mg) was subjected to Sephadex LH-20 with CH_2Cl_2-MeOH (1:1 to 0:1) and Me_2CO to afford gallic acid (13 mg) and quercitrin (150 mg). Fraction 3 (800 mg) was chromatographed repeatedly on Sephadex LH-20 with Me_2CO and with iPrOH to yield quercitrin (93 mg) and avicularin (20 mg). Fraction 4 (370 mg) was subjected to Sephadex LH-20 with Me_2CO and EtOH to afford quercetin (15 mg), catechin-3-gallate, and epicatechin-3-gallate (57 mg). Fraction 5 (370 mg) was chromatographed repeatedly on MCI gel CHP-20P CC with H_2O-MeOH (1:0 to 2:3) and with H_2O-MeOH (4:1 to 1:1) to yield **2** (16 mg). Compound **1** (65 mg) was purified from fraction 5 by repeated chromatography on MCI gel [H_2O-MeOH (1:0 to 2:3)] and Sephadex LH-20 (EtOH).

COMPOUND **1**.—A tan amorphous powder: mp 240–243°; $[\alpha]_D$ +63.7° (*c* = 0.35, MeOH) [lit. (9) +57.1°]; fabms $[M+Na]^+$ 961, $[M+2H]^+$ 940, $[M+H]^+$ 939, $[M+H-170]^+$ 769; ir ν max cm^{-1} 3400, 1720, 1620, 1450, 1350, 1200, 1020, 740. The data of uv, ^{13}C nmr, and ^1H nmr were the same as those reported in Wilkins and Bohm (17), Hatano *et al.* (18), and Nonaka *et al.* (19), respectively.

COMPOUND **2**.—A tan amorphous powder: mp 222–225°; $[\alpha]_D$ +126° (*c* = 0.20, MeOH); fabms $[M+Na]^+$ 809, $[M+H]^+$ 787, $[M+H-170]^+$ 617; ir ν max cm^{-1} 3400, 1720, 1620, 1450, 1350, 1210, 1040, 760. The data of uv, ^{13}C nmr, and ^1H nmr were the same as those reported in Wilkins and Bohm (17), Hatano *et al.* (18), and Wilkins and Bohm (17), respectively.

MIXTURE OF EPICATECHIN-3-GALLATE AND CATECHIN-3-GALLATE.—A tan amorphous powder: fabms $[M+H]^+$ 443; uv λ max (nm) (MeOH) 278; ^{13}C nmr (CD_3OD) epicatechin-3-gallate 167.6 (s, galloyl C=O), 157.9 (s, C-7), 157.9 (s, C-5), 157.3 (s, C-9), 146.3 (s, C-4′, -3″, -5″), 146.0 (s, C-3′), 139.8 (s, C-4″), 131.5 (s, C-1′), 121.5 (s, C-1″), 119.4 (d, C-2′), 116.0 (d, C-5′), 115.1 (d, C-6′), 110.3 (d, C-2″, -6″), 99.4 (s, C-10), 96.6 (d, C-6), 95.9 (d, C-8), 78.7 (d, C-2), 70.0 (d, C-3), 26.9 (t, C-4); ^{13}C nmr (CD_3OD) catechin-3-gallate 167.6 (s, galloyl C=O), 158.2 (s, C-7), 157.7 (s, C-5), 156.5 (s, C-9), 146.4 (s, C-4′, -3″, -5″), 146.3 (s, C-3′), 139.9 (s, C-4″), 131.6 (s, C-1′), 121.5 (s, C-1″), 119.3 (d, C-2′), 116.3 (d, C-5′), 114.5 (d, C-6′), 110.2 (d, C-2″, -6″), 99.7 (s, C-10), 96.5 (d, C-6), 95.6 (d, C-8), 79.7 (d, C-2), 71.2 (d, C-3), 24.4 (t, C-4). The data of ir and ^1H nmr were as reported by Haslam (20).

GALLIC ACID.—Colorless needles: ir ν max cm^{-1} 3400, 1670, 1610, 1540, 1440, 1330, 1270, 1030, 870; ^{13}C nmr as reported by Okuda (21).

QUERCETIN.—Yellow amorphous powder: mp >300° [lit. (22) 313°]; eims $[M]^+$ 302, 273, 245, 229, 153, 137, 128, 69; uv as reported by Markham (23); ir as reported by Briggs and Colebrook (24); ^{13}C nmr as reported by Breitmaier and Voelter (25).

QUERCITRIN.—Yellow amorphous powder: mp 178–180°; $[\alpha]_D$ −164.5° (*c* = 0.99, MeOH); fabms $[M+Na]^+$ 471, $[M+H]^+$ 449, $[\text{quercetin}+H]^+$ 303; ^{13}C nmr as reported by Breitmaier and Voelter (25).

AVICULARIN.—Yellow amorphous powder: mp 206–210° [lit. (22) 217°], $[\alpha]_D$ −145.5° (*c* = 0.20, MeOH); fabms $[M+H]^+$ 435, $[\text{quercetin}+H]^+$ 303; uv and ir as reported by Subramanian and Nair (26); ^{13}C nmr as reported by Markham *et al.* (27); ^1H nmr as reported by Fukunaga *et al.* (28).

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