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RESEARCH ARTICLE

Isolation and identification of antioxidant and hyaluronidase inhibitory compounds from *Ficus microcarpa* L. fil. bark

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Abstract

The aerial roots and bark of *Ficus microcarpa* L. fil. have been used as folk herbs for perspiration, alleviating fever, and relieving pain in Okinawa. The methanol extract of its bark showed high antioxidant and potential inhibitory activity against hyaluronidase. It was fractionated into hexane, ethyl acetate, butanol, and water fractions. As the ethyl acetate fraction exhibited the strongest activity, it was selected for further purification by repeated Sephadex LH-20 column chromatography and preparative HPLC. Seven compounds were isolated and identified as protocatechuic acid, chlorogenic acid, methyl chlorogenate, catechin, epicatechin, procyanidin B1, and procyanidin B3 by analysis of ESI-MS, UV, and ^{1}H- and ^{13}C-NMR spectra. All isolated compounds showed strong antioxidant activity when tested by all applied methods. Catechin, epicatechin, procyanidin B1, and procyanidin B3 exhibited excellent inhibitory activity against hyaluronidase. The results indicate that the extract of *F. microcarpa* bark may be utilized as a potential antioxidant and hyaluronidase inhibitor.

Keywords: Ficus microcarpa*; hyaluronidase inhibitor; antioxidant; phenolics*

Introduction

Ficus microcarpa L. fil. (Chinese banyan tree, Moraceae) is a popular ornamental plant in Ryukyu Island. Its dried leaves, aerial roots, and bark have been used as folk herbs for perspiration, alleviating fever, and relieving localized pain[1]. Chiang *et al.* reported that oleanonic acid, acetylbetulinic acid, betulonic acid, acetylursolic acid, ursolic acid, 3-oxofriedelan-28-oic acid, and 3β-acetoxy-25-hydroxylanosta-8,23-diene isolated from *F. microcarpa* showed significant cytotoxic activity against human nasopharyngeal carcinoma HONE-1, oral epidermoid carcinoma KB, and colorectal carcinoma HT29 cancer cell lines[2].

Hyaluronidases are a group of hydrolytic enzymes distributed throughout the animal kingdom and have high molecular weight hyaluronic acid (hyaluronan) as the predominant substrate[3,4]. Fragmented hyaluronic acid accumulated during tissue injury and degradation products of hyaluronic acid can stimulate the expression of inflammatory genes by a variety of immune cells at the injury site[5]. Inhibition of hyaluronidase (inhibition of hyaluronic acid degradation) therefore may be crucial in reducing disease progression and spread of venom/toxins and allergic disease[4]. Some typical anti-allergic drugs such as disodium cromoglycate (DSCG), transilist, liquiritigenin, and baicalein exhibit strong hyaluronidase inhibitory activity[6,7]. Many natural compounds, such as phenolics, flavonoids, and tannins, that are derived from plants have been intensively researched as hyaluronidase inhibitors[8,9].

Previously, we reported that the *F. microcarpa* bark extract exhibited excellent antioxidant and antibacterial activities that were correlated with its high level of phenolic compounds analyzed by gas chromatography-mass

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spectrometry (GC-MS) and high performance liquid chromatography (HPLC)[10]. However, many other bioactive phenolic compounds in *F. microcarpa* extract that cannot be identified by GC-MS are still unclear. In the present work, the inhibitory activity of *F. microcarpa* extract on hyaluronidase was investigated. Using bioassay-guided chromatographic fractionation, seven phenolic compounds were isolated, and these compounds were evaluated for hyaluronidase inhibitory and antioxidant activity.

Materials and methods

Chemicals

Hyaluronidase (type IV-S: from bovine testes), hyaluronic acid sodium salt, disodium cromoglycate (DSCG), potassium persulfate, 2,2-azinobis (3-ethylbenzothiazoline-6-fulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Chemical Co. Protocatechuic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

Plant material

F. microcarpa bark was collected from trees growing in the campus of the University of the Ryukyus, Okinawa, Japan in April 2006, and was identified by Professor Shinkichi Tawata (Faculty of Agriculture, University of the Ryukyus). A voucher specimen (061124) is deposited at the herbarium of the Faculty of Agriculture, University of the Ryukyus.

Preparation of the extracts

The ground fresh bark (2.36 kg) was macerated three times with methanol at room temperature. The combined methanol extracts were concentrated by low-pressure evaporation (<40°C) to reduce the volume, then distilled water was added and the aqueous solution was successively partitioned with hexane, ethyl acetate, and *n*-butanol. The obtained extracts, in addition to the aqueous solution, remaining after the extraction were filtered and dried under vacuum to give 33.8, 28.5, 49.8, and 60.4 g of hexane, ethyl acetate, butanol, and water fractions, respectively. As the ethyl acetate and butanol fractions showed higher hyaluronidase inhibitory and antioxidant activities compared with the other two fractions, they were subjected to further purification procedures (Figure 1).

Isolation procedures

Although the butanol fraction showed high hyaluronidase inhibitory activity, its chemical composition could not be investigated. By matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS and liquid chromatography (LC)-MS analysis (data not shown), it was confirmed that the butanol fraction contained rich-

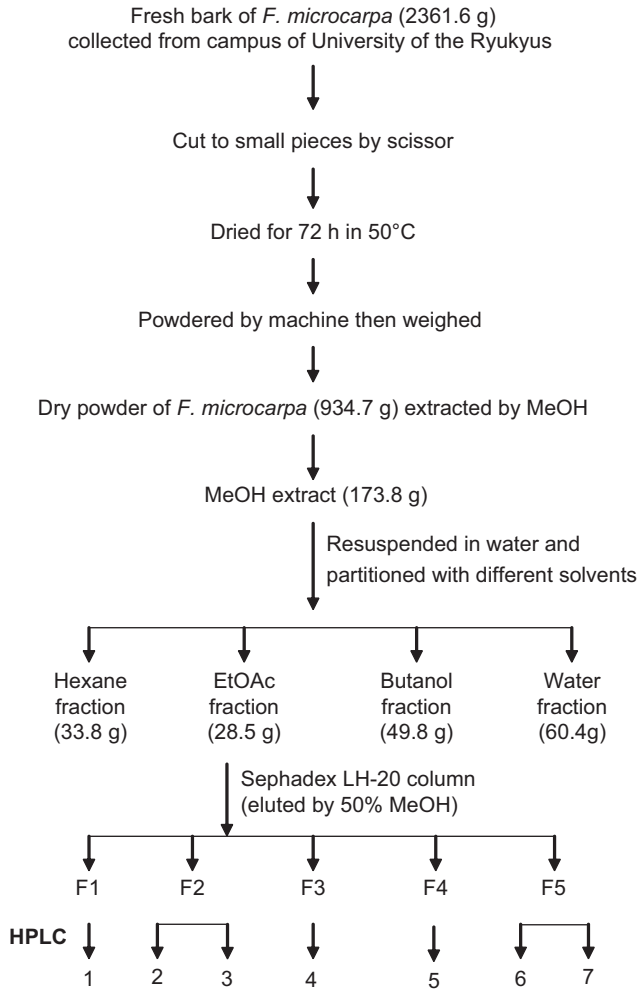

Figure 1. Separation scheme of bioactive compounds from *F. microcarpa*.

polymeric proanthocyanidin compounds (condensed tannins). Determination of the structures of tannins with higher degrees of polymerization is severely limited by the difficulty in separation and isolation of individual isomers from complex oligomeric mixtures. The complexity of chemical shifts in nuclear magnetic resonance (NMR) of higher oligomers makes the spectra difficult to interpret.

The ethyl acetate fraction (28 g) was dissolved in methanol:water (1:1, v/v, 150 mL), filtered through a 25 mm syringe filter (0.2 µm polyvinylidene fluoride (PVDF) filter medium; Whatman). One hundred fifty milliliters of fraction were divided three times, applied to a 140 cm × 3 cm i.d. glass column packed with Sephadex LH-20 (lipophilic Sephadex, bead size 25–100 µm; Sigma). Sephadex LH-20 was previously activated with 50% aqueous methanol. The column was eluted at a flow rate of 1.5 mL/min with 50% aqueous methanol and a fraction of 15 mL/tube was collected continuously with an SF-2120 fraction collector (Advantec Toyo Kalsha, Ltd., Japan). All collected fractions were analyzed by HPLC with a Synergi 4u Hydro-RP 80A column (150 mm × 4.60 mm; Phenomenex Co., USA) at 280 nm to check the polyphenol content. A gradient elution was performed with solvents A (water:acetic acid,

100:0.5, v/v) and B (methanol:acetonitrile, 3:1, v/v) as follows: 0–2 min, 5% B; 2–10 min, 5–25% B; 10–20 min, 25–40% B; 20–30 min, 40–50% B; 30–40min, 50–100% B; 40–45 min, 100% B; 45–50 min, 100–5% B. The flow rate was 0.8 mL/min and the injection volume was 5–10 μL. The fractions with similar retention time peaks were pooled, and all tubes were divided into five fractions (F1–F5) (Figure 2). This procedure was repeated twice to obtain sufficient amounts with yields varying from 125 to 300 mg. The fractions F1–F5 were further purified by preparative HPLC with a Shim-Pack PRC-ODS column (20 × 250 mm, Shimadzu Co.), under the buffer of methanol:acetonitr ile:water:acetic acid (30:9.5:60:0.5, v/v) at a flow rate of 7 mL/ min, and detected at 280 nm, and compounds **1–7** were isolated.

Instrumentation

Melting points were measured on a Büchi 535 instrument (Flawil, Switzerland). The ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on a Jeol JNM-ECA600 (Jeol, Japan) in CD_3OD. Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane (TMS) and the coupling constants are given in Hz. 2D NMR experiments (H-, C-correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC)) were obtained using standard pulse sequences. Electrospray ionization (ESI)-MS spectra were obtained using a Sciex API 2000 LC-MS/MS system (Applied Biosystems, Langen, Germany).

Estimation of hyaluronidase inhibitory activity

Hyaluronidase inhibitory activity of samples was determined spectrophotometically according to the methods introduced by Ito *et al.* and Lee and Kim, with minor modification[11,12]. Fifty microliters of bovine hyaluronidase (4000 units/mL) dissolved in 0.1 M acetate buffer (pH 4.0) were mixed with 100 μL of different concentrations of samples dissolved in acetate buffer, except the hexane fraction (first dissolved in a small amount of dimethylsulfoxide (DMSO), then dispersed in acetate buffer). After incubation at 37°C for 20 min, 100 μL of 1.25 mM calcium chloride was added to the reaction mixture to activate hyaluronidase, then the mixture was again incubated at 37°C for 20 min. The reaction was initiated by adding 0.25 mL hyaluronic acid sodium salt (0.8 mg/mL dissolved in acetate buffer). After incubation in a water bath at 37°C for 40 min, 100 μL of 0.4 N NaOH and 100 μL of 0.4 M potassium borate were added to the reaction mixture, then the mixture was boiled in a water bath for 3 min. After cooling on ice, 3 mL of *p*-dimethylaminobenzaldehyde (*p*-DAB) reagent (10 g of *p*-DAB dissolved in 12.5 mL of 10 N HCl and 87.5 mL acetic acid; before use, the reagent was diluted 10 times with acetic acid) was added to the reaction mixture, which was incubated at 37°C for 20 min again. After incubation, measurement was conducted at the absorbance of 585 nm against a blank (without hyaluronidase and test sample). The percent inhibition was calculated as follows:

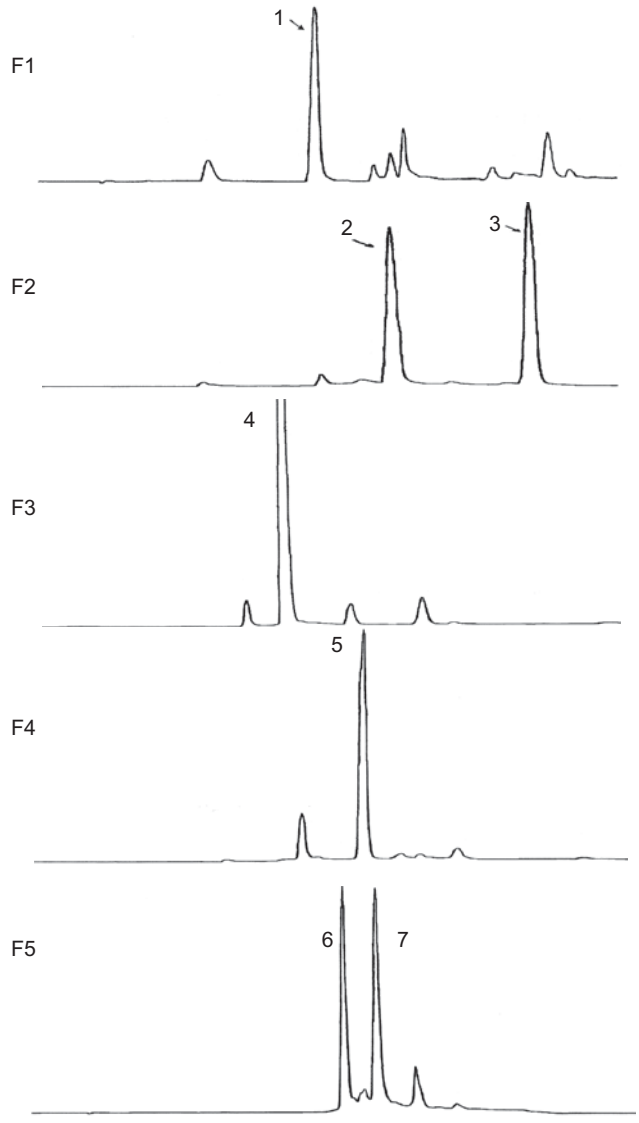

Figure 2. HPLC chromatograms as monitored by UV absorption at 280 nm for fractions F1–F5 in 50% methanol, as obtained by Sephadex LH-20 column chromatography of the ethyl acetate extract of *F. microcarpa* bark. The peaks numbered with Arabic numerals in the order of increasing retention time correspond to the compounds labeled with the numerals in Figure 3.

$$\text{Inhibition}\,(\%) = [A - (B - C)]/A \times 100\%$$

where A is the absorbance of the control (only acetate buffer, without test sample), B is the absorbance involving the test sample, and C is the absorbance of the test sample alone, without hyaluronidase.

DPPH radical scavenging assay

The DPPH free radical scavenging activity of extracts and isolated compounds was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method[13]. Different concentrations of sample and positive control Trolox in methanol solution (100 μL) were added to 3.9 mL of DPPH in methanol solution (0.1 mM). An equal amount of methanol was used as a blank. After incubation at room temperature for 30 min

in the dark, the absorbance was measured at 517 nm using a Shimadzu UV-Vis spectrophotometer (mini 1240). The activity of scavenging (%) was calculated using the following formula:

$$\text{DPPH radical scavenging}(\%) = [(OD_{control} - OD_{sample})/OD_{control}] \times 100\%$$

Total antioxidant activity (ABTS assay)

The $ABTS^+$ free radical scavenging activity of extracts and purified compounds was measured by the improved $ABTS^{\cdot+}$ method as described by Baltrusaityte *et al.*, with minor modification[14]. In this test, $ABTS^{\cdot+}$ radical cation was generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) after incubation at room temperature in a dark condition. Before use, the $ABTS^{\cdot+}$ solution was diluted with 80% ethanol to obtain an absorbance of 0.700 ± 0.050 at 734 nm; the spectrophotometer was preliminarily blanked with 80% ethanol. $ABTS^{\cdot+}$ solution (3.9 mL, absorbance of 0.700 ± 0.050) was added to 0.1 mL of test sample and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 6 min and the absorbance was immediately recorded at 734 nm. Appropriate solvent blanks were run in each assay and measurement was performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by the following formula:

$$ABTS^{\cdot+}\text{ radical scavenging}(\%) = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

Superoxide-radical scavenging activity by PMS-NADH system

The superoxide scavenging ability of extracts and compounds was assayed by the method of Lau *et al.* with minor modifications[15]. In the reaction mixture, 1.6 mL of phosphate buffer (0.1 M, pH 7.4) contained 105.6 μM β-nicotinamide adenine dinucleotide (NADH), 50 μM nitro blue tetrazolium (NBT), and sample in different concentrations dissolved in methanol. The reaction was initiated by adding 30 μM phenazine methosulfate (PMS) into the reaction mixture. Methanol was used as a control. After 10 min, the reaction mixture reached a stable color; the absorbance was measured at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[\frac{(OD_{control} - OD_{sample})}{OD_{control}}\right] \times 100\%$$

Results

Identification of the isolated compounds

Seven phenolic compounds were isolated from the ethyl acetate fraction by Sephadex LH-20 chromatography and preparative HPLC (Figure 3). Compound **1** (18 mg, retention time (t_R) = 6.5 min) was purified from F1, a white amorphous powder, MP: 197–199°C, ESI-MS: negative ion, *m/z*: 153, $[M - H]^-$. ^1H- and ^{13}C-NMR spectral analysis confirmed that compound **1** was identified as protocatechuic acid, compared with that of the authentic compound.

Compound **2** (28 mg, t_R = 10.8 min) was purified from F2, a white powder, MP: 204–206°C, ESI-MS: negative ion, *m/z*: 353.4, $[M - H]^-$; 375.3, $[M + Na]^-$, ^1H- and ^{13}C-NMR spectral data analysis confirmed that **2** was chlorogenic acid[16].

Compound **3** (18 mg, t_R = 15.2 min) was purified from F2, a white amorphous powder, MP: 202–204°C, ESI-MS: negative and positive ion, *m/z*: 367.1, $[M - H]^-$, 369.1, $[M + H]^+$, 391.2, $[M + Na]^+$, 759.3, $[2M + Na]^+$, UV λ_{Max} in methanol (nm): 329, 305, 243, 221. Spectra of ^1H- and ^{13}C-NMR of compound **3** were similar to those of compound **2**, but containing typical methoxy signal (δ_H 3. 70 and δ_C 53.0, OCH_3). The positions of methoxy and carbonyl ester substitution were determined by HMBC. Compound **3** was identified as methyl chlorogenate[17,18].

Compound **4** (22 mg, white amorphous powder, MP: 167–169°C, t_R = 12.8 min) was purified from F3, and compound **5** (19 mg, white powder, MP: 228–230°C, t_R = 14.9 min) was purified from F4 by preparative HPLC. Compounds **4** and **5** had the same ESI-MS data, *m/z*: 289.0, $[M - H]^-$; and the same MS/MS spectra, but different retention times. By analysis of ^1H-, ^{13}C-NMR, COSY, HMQC, and HMBC, compounds **4** and **5** were identified as catechin and epicatechin, respectively[19,20].

Compounds **6** (5.5 mg, t_R = 11.5 min) and **7** (6.3 mg, t_R = 12.9 min) were purified from fraction 5. Because the two compounds have similar retention times, purification processes were repeated three times by HPLC. Compounds **6** and **7** had the same ESI-MS data, *m/z*: 577.3, $[M - H]^-$, 579.4, $[M + H]^+$, 601.2, $[M + Na]^+$, and the same MS/MS spectra. Compounds **6** and 7 were hydrolyzed according to the following method: treatment of 0.5 mg compound with 0.1 N hydrochloric acid in ethanol (2 mL) at 60°C for 15 min. The result of analyzing the hydrolyzates of compounds **6** and **7** by HPLC with an ODSpak column (150 mm × 4.60 mm; Shodex, Co., Japan) showed that catechin and epicatechin were detected in the hydrolyzate of compound **6**, while only catechin was detected in the hydrolyzate of compound **7** (Figure 4). By analysis of ^1H-, ^{13}C-NMR again, compounds **6** and **7** were identified as procyanidin B1[21,22] and procyanidin B3[21,23,24], respectively.

Hyaluronidase inhibitory activity by the extracts

The hyaluronidase inhibitory activity of the methanol extract of *F. microcarpa* bark and its fractions is illustrated in Figure 5. The methanol extract exhibited inhibitory activity (3.4, 17.3, and 39.4%) against hyaluronidase at the concentration of 100, 200, and 400 μg/mL, respectively. Hexane, ethyl acetate, *n*-butanol, and water fractions were prepared from the methanol crude extract of *F. microcarpa* bark. Of these, the ethyl acetate and *n*-butanol fractions exhibited the stronger hyaluronidase inhibitory activity (83.7 and 81.4 %, respectively) at 400 μg/mL. From our previous study, the methanol extract of *F. microcarpa* bark as well as its ethyl acetate and

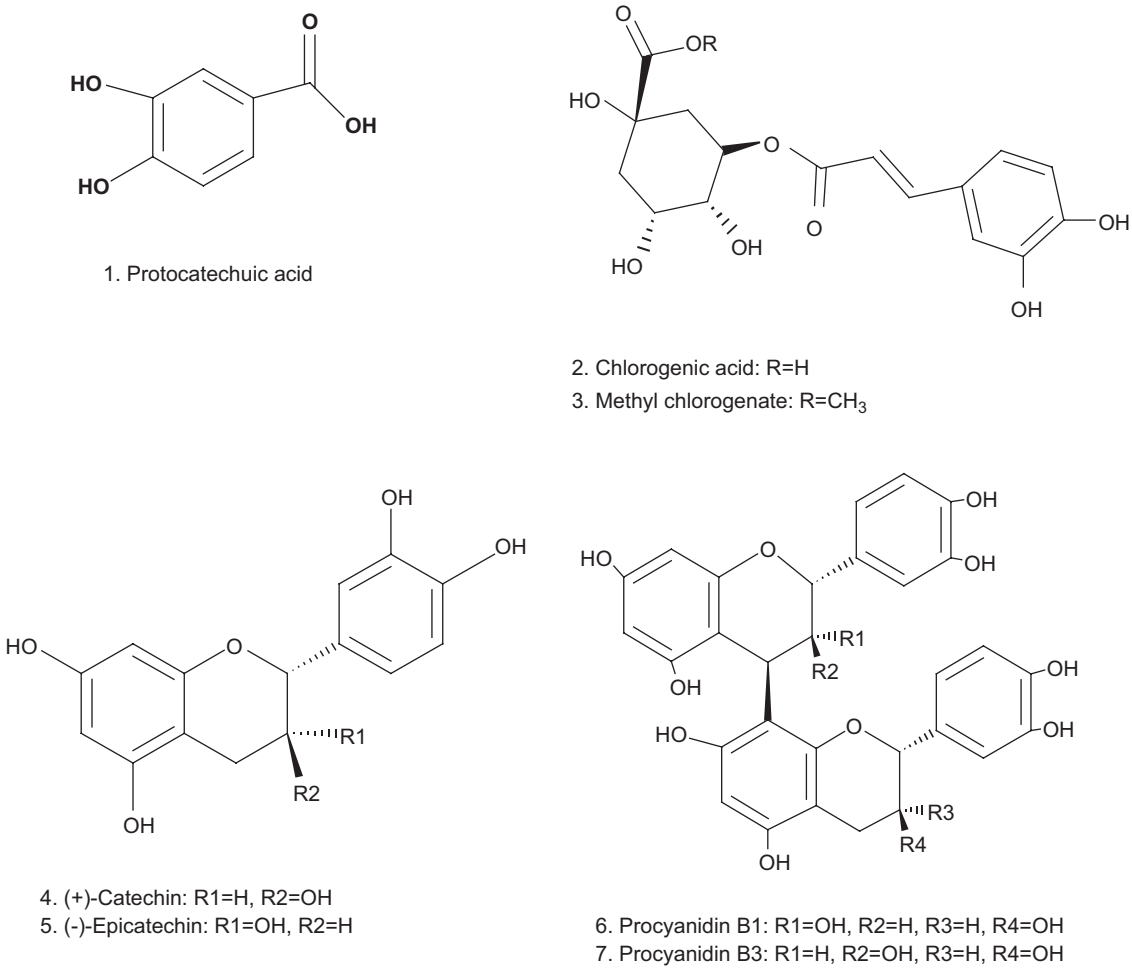

Figure 3. Chemical structures of isolated compounds from *F. microcarpa*.

n-butanol fractions showed strong antioxidant activity as assayed by different methods[10]. Since the ethyl acetate and *n*-butanol fractions had marked antioxidant and hyaluronidase inhibitory activities, they were subjected to further purification.

Hyaluronidase inhibitory activity by isolated compounds

Seven phenolic compounds were isolated form the ethyl acetate fraction by Sephadex LH-20 chromatography and preparative HPLC. The isolated compounds and anti-allergic drug DSCG (disodium cromoglycate, positive control) were evaluated for hyaluronidase inhibitory activity (Figure 6). For the assay, protocatechuic acid and methyl chlorogenate were only prepared at one concentration (500 μM); the other compounds were prepared at three different concentrations. Most compounds effectively inhibited hyaluronidase activity with increasing concentration. Protocatechuic acid and methyl chlorogenate showed minimal inhibitory activity at the concentration of 500 μM (6.3 and 2.2%, respectively). Chlorogenic acid also exhibited weak inhibitory activity (13.5%, 500 μM). However, catechin, epicatechin, procyanidin B1, and procyanidin B3 exhibited comparable inhibitory activity to DSCG (51.1%) against hyaluronidase at the concentration of 250 μM (29.9, 45.9, 50.1, and 28.9%, respectively). At the concentration of 125 μM, epicatechin (30.3%) and procyanidin B1 (33.5%) even showed higher activity than DSCG (22.9%).

Antioxidant activity of purified compounds

To evaluate the seven purified compounds and Trolox (positive control) for antioxidant potential, their DPPH, $ABTS^{+}$, and superoxide radical scavenging abilities were assayed. As presented in Table 1, all seven compounds exhibited high antioxidant activity in the three different evaluations. Among all compounds, catechin and epicatechin showed a superior DPPH (EC_{50}: 11.4 and 11.3 μM, respectively) and $ABTS^{+}$ radical scavenging activity (EC_{50}: 4.1 and 2.9 μM, respectively). However, the superoxide radicals were strongly inhibited by methyl chlorogenate (EC_{50}: 54.3 μM).

Discussion

The purpose of this research was to isolate and identify anti-allergic and antioxidant compounds from the bark of

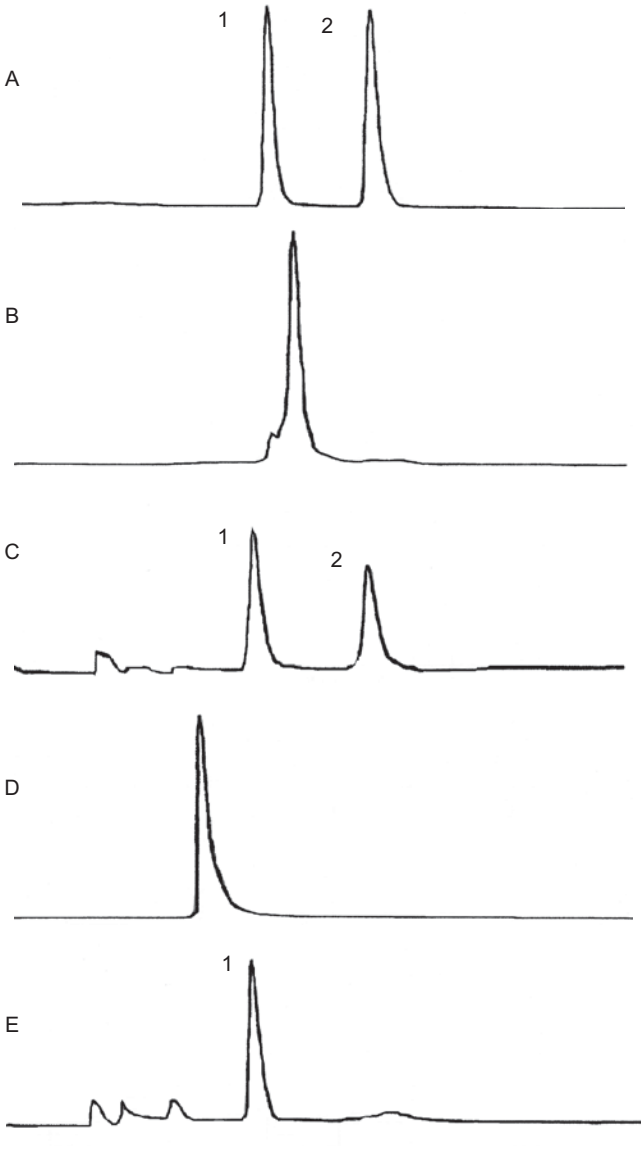

Figure 4. HPLC chromatograms as monitored by UV absorption at 280 nm (ODSpak column (150 mm × 4.60 mm; Shodex, Co.. Japan) for catechin, epicatechin, compounds **6**, **7**, and their hydrolyzates. (A) Standard of catechin and epicatechin (200 μg/mL); (B) compound **6**; (C) hydrolyzate of **6**; (D) compound **7**; (E) hydrolyzate of **7**. 1, catechin; 2, epicatechin.

F. microcarpa, which is well known as a medicinal herb for perspiration, alleviating fever, and relieving pain in Okinawa Island 1. Seven phenolic compounds, protocatechuic acid (**1**), chlorogenic acid (**2**), methyl chlorogenate (**3**), catechin (**4**), epicatechin (**5**), procyanidin B1 (**6**), and procyanidin B3 (**7**) were isolated by Sephadex LH-20 column chromatography and preparative HPLC. Compounds **1** and **4** were previously isolated from *F. microcarpa* by Kuo and Li[25]; however, as far as we know, compounds **2**, **3**, **5**, **6**, and **7** are reported for the first time from this plant. Komatsu *et al*. reported that contents of catechin and epicatechin as major components in green tea were about 4 mg and 10 mg/5 g powdered green tea[26], respectively. In our study, higher contents of catechin and epicatechin were recorded in *F. microcarpa* (33.3 mg and 19.4 mg/5 g powdered bark) compared with tea catechins. Chlorogenic acid was recorded in a high amount in *F. microcarpa* bark (6.7 mg/g DW bark) (Table 2). Protocatechuic acid, chlorogenic acid, methyl chlorogenate, catechin, epicatechin, procyanidin B1, and procyanidin B3

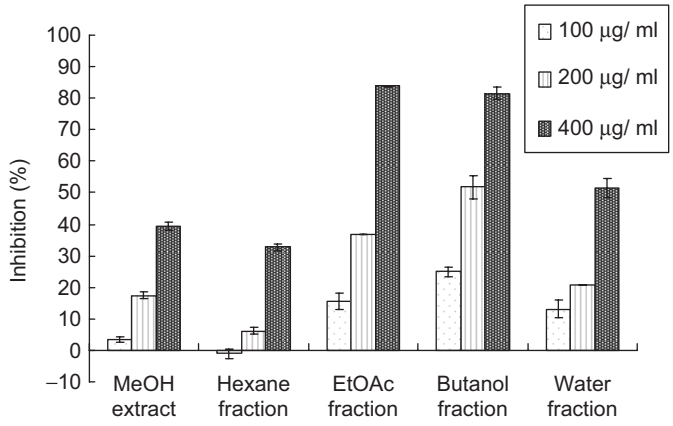

Figure 5. Hyaluronidase inhibitory activity by methanol extract and fractions of *F. microcarpa*. Each value is expressed as mean ± SD (n = 3), concentration of sample in assays is expressed as final concentration.

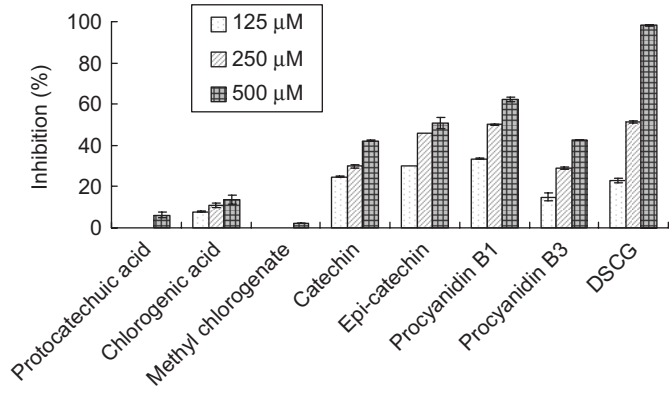

Figure 6. Hyaluronidase inhibitory activity by purified compounds of *F. microcarpa*. Each value is expressed as mean ± SD (n = 3), concentration of sample in assays is expressed as final concentration.

Table 1. Antioxidant activities of isolated compounds from *F. microcarpa*.

	Antioxidant activity (EC_{50}, μM)		
Compound	DPPH[a]	PMS-NADH[b]	ABTS[+c]
Protocatechuic acid	40.3 ± 1.4	279.2 ± 2.8	19.6 ± 1.3
Chlorogenic acid	61.6 ± 0.9	65.8 ± 2.0	28.2 ± 1.0
Methyl chlorogenate	31.9 ± 1.1	54.3 ± 1.4	16.0 ± 0.4
Catechin	11.4 ± 0.5	67.4 ± 1.8	4.1 ± 0.5
Epicatechin	11.3 ± 1.0	71.0 ± 1.4	2.9 ± 0.4
Procyanidin B1	12.4 ± 0.7	79.4 ± 0.9	—
Procyanidin B3	14.0 ± 0.4	80.5 ± 1.7	—
Trolox	17.6 ± 0.7	722.0 ± 3.4	2.0 ± 0.3

Note. —, not determined; each value is expressed as mean ± SD (n = 3). Concentration of sample in assays is expressed as final concentration.
[a]DPPH radical scavenging activity.
[b]Superoxide free radical scavenging activity.
[c]$ABTS^+$ free radical scavenging activity.

Table 2. Compound contents in *F. microcarpa* bark and ethyl acetate fraction.

Compound	Content (mg/g DW)	
	Bark[a]	Ethyl acetate fraction[b]
Catechin	6.7 ± 0.6	220 ± 1.9
Epicatechin	5.0 ± 0.9	60 ± 1.5
Chlorogenic acid	6.7 ± 0.4	101 ± 1.2
Methyl chlorogenate	—	—
Protocatechuic acid	—	—
Procyanidin B1	—	—
Procyanidin B3	—	—

Note. —, not measured. Each value is expressed as mean ± SD ($n = 3$).
[a]Compound content in dried powder of *F. microcarpa* bark.
[b]Compound content in dried ethyl acetate fraction.

are widely recognized to have antioxidant activity[27,28]. Our study revealed that these compounds are responsible for the antioxidant properties of *F. microcarpa*. Furthermore, the anti-allergic activity of chlorogenic acid, catechin, epicatechin, procyanidin B1, and procyanidin B3 has been under intensive investigation[11,29,30]. In our study, at the concentration of 500 μM, the hyaluronidase inhibitory activity of compounds **1–7** and positive control DSCG was in the following order: DSCG (98.2%) > procyanidin B1 (62.4%) > epicatechin (50.9%) > procyanidin B3 (42.7%) > catechin (42.25%) > chlorogenic acid (13.5%) > protocatechuic acid (6.3%) > methyl chlorogenate (2.2%). In contrast, at the concentration of 125 μM, the activity order was procyanidin B1 (33.5%) > epicatechin (30.3%) > catechin (24.7 %) > DSCG (22.9%) > procyanidin B3 (15.0%) > chlorogenic acid (10.8%). This result indicated that procyanidin B1, epicatechin, and catechin exhibited higher hyaluronidase inhibitory activity than did DSCG at a lower sample concentration, and the former were more sensitive against hyaluronidase than the latter.

The result indicated that isolated compounds **2**, **4**, **5**, **6**, and **7** showed both high antioxidant activity and high hyaluronidase inhibitory activity. Many researchers have also reported that some antioxidants showed high anti-allergic activity[11,31]. Active oxygen species such as superoxide radicals and hydroxyl radicals induce histamine release from mast cells. Therefore, by both scavenging of free radicals and suppression of chemical mediators from mast cells, allergic actions could be prevented.

In conclusion, seven phenolic compounds have been isolated and identified from the bark of *F. microcarpa*. Of these, five compounds were isolated for the first time. All these chemicals showed strong antioxidant and hyaluronidase inhibitory activities. Our results may provide a pharmacological explanation for the use of *F. microcarpa* in folk medicine in the Ryukyu Islands. Further investigation is needed to separate individual proanthocyanidin molecules from the butanol fraction of *F. microcarpa*.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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