

**Studies on antioxidant, antibacterial and anti-inflammatory
active compounds from medicinal plants**

[薬用植物由来の抗酸化、抗菌及び抗炎症活性物質に関する研究]

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Abstract

Three Chinese and thirteen Okinawan traditional medicinal plants were selected in this study for their common use on anticancer, anti-arthritis, and anti-inflammatory purposes. However, the mechanisms of disease treatments and active components concerning most of these plants have not yet been clearly elucidated. Inhibitory activities against cyclooxygenase (COX), collagenase 3 (MMP-13) and hyaluronidase are often used to evaluate anti-tumor and anti-inflammatory abilities in some plant extracts or compounds. The anti-oxidative activity has been confirmed contributing kinds of cancer and inflammatory preventions because of its multiple functional roles. The objectives of this study were to evaluate the inflammatory related enzymes inhibitory and antioxidant activities of the selected herbs and some active compounds were identified or purified by activity-guided methods.

Sophora. subprostrata showed the highest COX inhibitory and antioxidant activities among three Chinese herbs, therefore it was selected for further isolating bioactive compounds. Two alkaloids matrine and oxymatrine were isolated and identified from aerial portion of *S. subprostrata*. The IC₅₀ values of matrine against COX-1 and COX-2 were 7.8 and 47 µg/ml, respectively, while oxymatrine exhibited 52.5 and 102.2 µg/ml values. This was the first report on COX inhibitory activity of matrine and oxymatrine *in vitro*, thus it may provide certain scientific supports for the medicinal use of these two compounds from *S. subprostrata* for inflammatory disorders.

As results of screening MMP-13 inhibitory and antioxidant activities in Okinawan herbs, *Curcuma longa*, *Ocimum basilicum* and *Curcuma aromatica* extracts showed high inhibitory effect on MMP-13 with IC₅₀ values of 27.8, 81.7 and 85.8 µg/ml, respectively. The chemical compositions of these three plant extracts were analyzed by

LC-MS. Curcumin and rosmarinic acid were identified and quantified in the extracts of *C. longa*, *C. aromatica*, and *O. basilicum*. Both curcumin and rosmarinic acid exhibited high MMP-13 inhibitory activity with IC₅₀ of 3.6 and 2.9 μM, respectively. *Ficus microcarpa* exerted the strongest antioxidant activity among the thirteen plants tested, so it was selected for further separation. The high antioxidant and antibacterial activities were found in ethyl acetate fraction of *F. microcarpa* bark and aerial root extracts. Twelve phenolic compounds were identified in this fraction through GC-MS and HPLC analyses. Methanol extract of *F. microcarpa* bark also showed potential inhibitory activity against hyaluronidase. Activity-guided further purification was performed by repeated Sephadex LH-20 column and preparative HPLC. Seven compounds were isolated and identified as protocatechuic acid, chlorogenic acid, methyl chlorogenate, catechin, epicatechin, procyanidin B1 and procyanidin B3 by analyses of ESI/MS, ¹H and ¹³C-NMR. Except protocatechuic acid and catechin, other five compounds were reported for the first time in *F. microcarpa*. All isolated compounds showed strong antioxidant activity when tested by three different methods. Catechin, epicatechin, procyanidin B1 and procyanidin B3 exhibited excellent inhibitory activity against hyaluronidase.

In summary, the results provided certainly pharmacological explanation of using *C. longa*, *C. aromatica*, *O. basilicum* and *F. microcarpa* in the folk medicine of the Ryukyu Islands. The findings suggested that the extract of *F. microcarpa* might be utilized as a potential source of natural antioxidant, antibacterial and hyaluronidase inhibitor in functional food industry.

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CHAPTER 1

General introduction

1.1. Antioxidant and anti-inflammatory activities of medicinal plants

The anti-oxidative activity has been confirmed contributing kinds of cancer and inflammatory preventions for its multiple functional roles. The production of oxidants is a typical event associated with aerobic metabolism. When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species or free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are generated (Kris-Etherton *et al.*, 2004). Accumulation of the free radicals in body organs or tissues can cause oxidative damage to biomolecules and membranes of cell, eventually leading to many chronic diseases, such as inflammatory, cancer, diabetes, aging, cardiac dysfunction and other degenerative diseases (Wang *et al.*, 2004). In the last 50 years, antioxidant and anti-inflammatory activities of extracts from medicinal or food plants have been extensively investigated. Many pharmacological studies have shown that extracts of some antioxidant plant possess anti-inflammatory, anti-allergic, anti-tumor, anti-bacterial, anti-mutagenic and anti-viral activities to a greater or lesser extent. Researchers reported that intake of fruits, vegetables and other foods having high antioxidant activity has been associated with reduced risks of cancer, cardiovascular disease, diabetes and other diseases (Kris-Etherton *et al.*, 2004). Trouillas *et al.* (2003) investigated the antioxidant, anti-inflammatory and antiproliferative properties of sixteen French herbal tea and found some herbs exhibited high antioxidant, anti-inflammatory and antiproliferative activities. Antioxidant activities in twenty traditional anti-inflammatory herbs extracts were investigated. The results suggested that the anti-inflammatory activities of these extracts could be explained, at least in part, by their antioxidant properties (Schinella *et al.*, 2002). Free radicals liberated from phagocyte cells are important in inflammatory processes, because they are implicated in

the activation of nuclear factor κ B, which induces the transcription of inflammatory cytokines and cyclooxygenase 2 (Winrow *et al.*, 1993).

1.2. Anti-inflammatory related enzymes

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages, mast cells, platelets, etc. Many enzymes have been reported as mediators of inflammation and seem to be involved in both acute and chronic inflammatory disorders (Heras *et al.*, 2001). It is known that cyclooxygenase (COX), nitric oxide synthase (iNOS), 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX), matrix metalloproteinases (MMPs) and hyaluronidases are the common mediators in this process. Because some substances such as leukotriene, prostaglandin, and human neutrophil elastase (HNE) release by related enzymes are all increased by neutrophil stimulation in a variety of inflammations and hypersensitivity-based human diseases (Manyatepek and Hoffmann, 1995; Surh *et al.*, 2001; Bemstein *et al.*, 1994), the blocking of these enzymes might explain the putative antiphlogistic activity of extracts prescribed for these disorders in folk medicine.

It is well known that cyclooxygenase (COX) is the rate-limiting enzyme for the conversion of arachidonic acid to prostaglandin H_2 (PGH₂). There are two isoforms of COX, named as COX-1 and COX-2. Although both isoforms are involved in the formation of prostaglandin (PG) endoperoxides, they are likely to have fundamentally different biological roles. COX-1 is constitutively expressed in most mammalian tissues and is thought to be involved in maintaining physiological functions. COX-2 is expressed at low levels in normal tissue, but it is strongly induced by inflammatory

mediators. Enhanced expression of COX-2, but not COX-1, has been observed in many different types of tumors and transformed cells (Seibert *et al.*, 1997; Lawrence *et al.*, 1999).

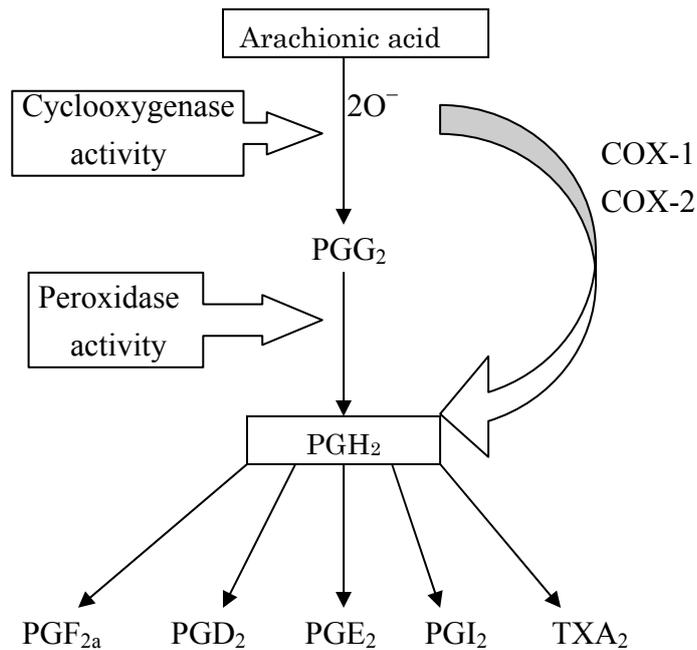


Fig. 1. Schematic diagram for the conversion of arachidonic acid to eicosanoids via the cyclooxygenase pathway.

Prostaglandins are formed by the oxidative cyclization of the central five carbons of arachidonic acid. Cyclooxygenase is a key regulatory enzyme, catalyzing the conversion of arachidonic acid to prostaglandin H₂ (PGH₂) (**Fig. 1**). This occurs via a two-step process due to the bifunctional nature of the enzyme. First, two molecules of oxygen are introduced into arachidonate, forming the bicyclic peroxide intermediate, prostaglandin G₂ (PGG₂). The second step requires the diffusion of PGG₂ to a distinct reactive site located on the other side of the molecule where PGG₂ is converted to freely diffusible PGH₂. PGH₂ is the immediate substrate for a series of cell-specific prostaglandin and

thromboxane synthases. It can be converted to PGE₂, PGD₂, PGF_{2α}, PGI₂ and thromboxane A₂ (TXA₂), which exit the cell and interact with receptors in an autocrine or paracrine fashion. These products play critical roles in numerous biological processes, including regulation of immune function, kidney development, reproductive biology and mucosal integrity of the gastrointestinal tract (Caughey *et al.*, 2001).

Matrix metalloproteinases (MMPs) comprise a family of secreted and membrane-bound endopeptidases which hydrolyze extracellular matrix proteins. Based on their preferred substrates and on structural features, MMPs can be divided into collagenases, gelatinases, stromelysins and membrane-type matrix metalloproteinases (Jiang *et al.*, 1992; Hooper, 1994). Collagenases are important proteolytic tools for extracellular matrix remodeling during organ development and tissue regeneration. Chronic activation of collagenases results in an excessive degradation of extracellular matrix components and is believed to contribute to many pathological conditions such as tumor progression, osteoarthritis, rheumatoid arthritis and many inflammatory diseases (Clark and Parker 2003; Ala-aho and Kähäri 2005; Blavier *et al.*, 2006; Deryugina and Quigley, 2006). As one of important enzyme of MMPs family, collagenase 3 (MMP-13) was reported to be involved in the development and metastasis of breast and lung carcinomas. Additionally, this enzyme plays an important role in degenerative bone diseases such as rheumatoid arthritis and osteoarthritis (Tardif *et al.* 2004 ; Burrage *et al.* 2007). Preclinical studies have provided compelling evidence that inhibition of MMPs would be therapeutic for inflammatory, malignant, arthritis and degenerative diseases (Murphy and Docherty, 1992; Skiles *et al.*, 2001). In recent years, several highly selective synthetic MMP-13 inhibitors have been tested for their effects against growth and invasion of malignant tumors and for therapeutic of osteoarthritis

and rheumatoid arthritis (Ala-aho *et al.*, 2005; Burrage *et al.*, 2007).

Hyaluronidases are a group of hydrolytic enzymes distributed throughout the animal kingdom and high molecular weight hyaluronic acid (hyaluronan) is used for the substrate (Watson, 1993; Girish and Kemparaju, 2007). 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 (Jiang *et al.*, 2007). Inhibition of hyaluronidase (inhibition of hyaluronic acid degradation) therefore may be crucial in reducing disease progression and spread of venom/toxin and allergic diseases (Girish and Kemparaju, 2007). Some typical anti-allergic drugs such as disodium cromoglycate (DSCG), transilist, liquiritigenin, and baicalein exhibited strong hyaluronidase inhibitory activity (Yingprasertchai *et al.*, 2003; Kakegawa *et al.*, 1992). Many natural compounds, such as phenolics, flavenoids and tannins that derived from plant as hyaluronidase inhibitors have been intensively researched (Hertel *et al.*, 2006; Mio and Stern, 2002).

1.3. Medicinal plants in Okinawa

Ryukyu Islands consist of more than 100 islands and locate in subtropical region of Japan, that have rich plant diversity and both tropical and temperate plants allow growing or being cultivated. In addition, the Black Current flows near the Ryukyu Islands and carries various species of Malayan plant to these islands (Hatusima, 1975). It was estimated that about 300 herb species have traditionally been used for disease treatment and pharmaceutical purposes. Various kinds of herbs naturally grow in Okinawa so as to be called “Okinawa is a treasure spot for herbs”. Local researches believe that Okinawa herbs contained abundant functional substances, such as

antioxidant, antibacterial and anti-inflammatory compounds because these plants are grown under strong sunshine. The inhabitants of Ryukyu have traditionally utilized many kinds of medicinal plants and characteristic Okinawan foods. Some researchers have indicated that the intake of typical Okinawan medicinal and edible plants contributes to the longevity of the local people. For example, in Okinawa it's been accustomed to drink many kinds of teas which are prepared by some herbs. *Orthosiphon aristatus* (BL.) Miq., *Houttuynia cordata* Thunb. and *Plantago asiatica* L. are common herbs that are sold in Okinawan herb store as tea materials. Mr Inafuku, the director of Ryukyu Bio-Resource Development Co., Ltd., explains that “Okinawa is called a ‘treasure trove of medicinal herbs’ thanks to its favorable geographical conditions and medicinal herbs has long been eaten in Okinawa, not just for their expected medicinal properties, but also as vegetables. With the keywords being ‘healthy’ and ‘long-living,’ Okinawa is the home of many health-food product developments, including teas, drinks, and supplements made from medicinal herbs” (Tamura, 2005).

Many researchers have focused on biological activities of Okinawan herbs and various active substances have been isolated and identified. Aqueous extracts from *Psidium guajava* L. and *Limonium wrightii*, two medicinal plants grown in Okinawa, have cardioprotective effects against myocardial ischemia-reperfusion injury in isolated rat hearts, primarily through their radical-scavenging actions (Yamashiro, 2003). Kinoshita *et al.*, (2007) reported that the antioxidant and hepatoprotective actions of *Terminalia catappa* L. collected from Okinawan Islands were evaluated *in vitro* and *in vivo* using leaves extract and isolated antioxidant. Chebulagic acid and corilagin were isolated from this plant and showed strong free radical scavenging effect. Corilagin also was protective against GalN/LPS-induced liver injury through suppression of oxidative

stress and apoptosis. The leaf of *Alpinia zerumbet* is a material for preparing traditional Okinawan food “Muchi”. Previous researchers of our laboratory found that the extract from this leaves showed high antioxidant activity and attributed to its higher phenolic content (Elzaawely *et al.*, 2007).

Traditional herbs have been used as pharmaceutical and dietary therapies for long times. A number of herbs and many relevant prescriptions have been screened and used for treating and preventing various tumors and inflammations during long-term folk practice. Nowadays, medicinal plants are still widely practiced particularly in the countryside and remote mountainous regions, and even in the major cities of many Asian countries, especially in China. Three published monographs about Okinawan herbs summarized and documented more than 300 species of traditional Okinawan herbs on the basis of long-term practical experience for many kinds of diseases. Most of the documented herbal medicines have been verified in clinical reports and experimental researches to be effective for treatment and prevention of such as inflammatory, tumors, ulcers diseases to a greater or lesser extent. In this study, the selected medicinal plants are commonly used as herbal medicines for anticancer and arthritis, analgesic, antipyretic and anti-inflammatory purposes in China or Okinawa.

In this thesis, **Chapter 2** reports cyclooxygenase inhibitory and antioxidant activities by methanol extracts of three Chinese medicinal plants and purified two alkaloids: matrine and oxymatrine from *S. subprostrata*. Screening for MMP-13 inhibitory and antioxidant activities of 13 Okinawan medicinal plants are described in **Chapter 3** and some high MMP-13 inhibitory compounds were determined and quantified by LC-MS; the highest antioxidant activity among the 13 medicinal plants was found in *F. microcarpa* extract. **Chapters 4** and **5** report the antioxidant and

antibacterial activities of bark, fruits, leaves and aerial roots of *F. microcarpa* extracts and analysis of components in their extracts by GC-MS and HPLC. **Chapter 6** gives the results of isolation and identification of antioxidant and antiallergic compounds from *F. microcarpa* bark and seven compounds were isolated and most were first reported from this plant. Finally, a general conclusion is summarized in **Chapter 7**.

CHAPTER 2

**Study on cyclooxygenase inhibitory and antioxidant activities
of three Chinese medicinal plants: *Sophora subprostrata*,
Hemsleya ambillis and *Hedyotis diffusa***

Abstract

S. subprostrata, *H. ambilis* and *H. diffusa* are traditional Chinese herbs for anti-inflammatory, anti-tumor and antipyretic. The purposes of present study were to evaluate the COX inhibitory and antioxidant activities of methanol extracts from the three herbs; to isolate and identify major components of herb extracts that showed higher bioactivity. Methanol extracts of *S. subprostrata* showed the highest COX inhibitory and antioxidant activities among three herbs. The two alkaloids were isolated from chloroform fraction of the aerial portion of *S. subprostrata* by activity-guided chromatographic fractionation. The structures of compounds were analyzed by LC-MS, LC-MS/MS, ¹H NMR, and ¹³C NMR and they were identified as matrine and oxymatrine. The results indicated that matrine and oxymatrine had high inhibitory activity against the COX (matrine, IC₅₀: 7.8 and 47 μg/ml against COX-1 and COX-2, respectively; oxymatrine, IC₅₀: 52.5 and 102.2 μg/ml against COX-1 and COX-2, respectively). Matrine and oxymatrine also showed high antioxidative activities measured by superoxide radicals scavenging (EC₅₀: 7.5 μg/ml, 6.2 μg/ml, respectively). This is the first report on COX inhibitory and anti-oxidant activities of matrine and oxymatrine *in vitro*. Thus it provided certain scientific supports for the medicinal use of these two compounds from *S. subprostrata* for inflammatory disorders.

Keywords: *Sophora subprostrata*; cyclooxygenase inhibition; antioxidant; matrine; oxymatrine

2.1. Introduction

Sophora subprostrata CHUN et T. CHEN (Chinese drug: Shan-Dou-Gen, Leguminosae) has been traditionally used as Chinese medicinal plant in southern China. It belongs to the category of antipyretic and toxin removing herbs, whose roots have been used to treat pain, fever, peptic ulcers, inflammation and coughing (Jiang, 1982). Moreover, certain alkaloids isolated from *S. subprostrata* have been confirmed to have some pharmacological effects, such as anti-pyretic (Cho *et al.*, 1986), anti-tumoral (Kojima *et al.*, 1970), and anti-nociceptive effects (Kamei *et al.*, 1997). As an important Chinese drug, extensive research about this herb has been performed in the past 50 years, and a number of constituents have already been isolated from this plant, including alkaloids (Shibata *et al.*, 1962), flavonoids (Kyogoku *et al.*, 1973) saponins (Sakamoto *et al.*, 1992) and polysaccharides (Qun *et al.*, 2003).

Hemsleya amabilis is a herb widely distributed in China. It has been used in the treatment of many infectious illnesses. In recent years, it has been used clinically to treat cancers and demonstrated an ability to greatly inhibit tumor development and growth with limited side effects (Kasai *et al.*, 1988).

Hedyotis diffusa is used to alleviate fever and toxin, to disperse painful swellings in the throat, mouth, and gums, to clear lung heat, and to alleviate constipation. It is also recorded as a herb to reduce inflammation to treat baldness and bites of snakes.

COXs play an important role in the synthesis of prostaglandin from arachidonic acid and have been widely used to evaluate the anti-inflammatory activities of natural products (Noreen *et al.*, 1998; Kuo *et al.*, 2004). There are two different forms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in various tissues, while COX-2

is induced in response to cytokines, growth factors and tumor promoters in inflammation. Many plant tissues contain a wide variety of compounds with anti-oxidant activity, such as flavonoids, lignans, alkaloids, terpenes and phenolics (Cai *et al.*, 2004). In addition to the protective effects of the endogenous antioxidant defense system, natural products with antioxidant activity could retard the oxidative damage of a tissue by increasing those defenses (Ng *et al.*, 2000). Some studies about the relationship of anti-oxidant and anti-inflammatory have shown that oxygen free radicals produced in inflammation and anti-oxidant compound may block arachidonic acid metabolism by inhibiting lipoxygenase activity, or may serve as a scavenger of reactive free radicals which are produced in arachidonic acid metabolism (Trouillas *et al.*, 2003).

So far, little information on the *in vitro* COX inhibitory and anti-oxidant activities of *S. subprostrata* extracts has been reported. The purpose of present study was to isolate and identify major components from this herb by COX inhibitory activity-guided chromatographic fractionation methods. Two kinds of alkaloids, matrine and oxymatrine were purified from the active CHCl₃ fraction of methanolic extract of *S. subprostrata*.

2.2. Materials and methods

2.2.1. Instruments

Melting points were determined on a BUCHI 535 (BUCHI Co.). ¹H-NMR and ¹³C-NMR spectra (ppm, J in Hz) were obtained using a JNM-A500 NMR spectrometer (JEOL) at 500 MHz and 125 MHz, respectively, with TMS as an internal standard.

2.2.2. Chemicals

COX (ovine) inhibitor screening assay kits (catalog No. 560101) and aspirin (catalog No. 70260) were purchased from Funakoshi Co., Japan, Cayman Chemical Company. Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Nakarai Tesque, Japan, Sigma, Germany. Trolox, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3(2)-*t*-butyl-4-hydroxyanisole (BHA) and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

2.2.3. Plant materials

The dried aerial parts of *S. subprostrata*, *H. amabilis* and *H. diffusa* used in this study were purchased (July 2003) from the Herb Store in Jiang Kou, Guizhou province, China. The plant identification was confirmed by Dr. Xu Shengguo (Central South Forestry University, China).

2.2.4. Extraction, isolation and identification

Dried powders (10 g) of *S. subprostrata*, *H. ambilis* and *H. diffusa* had been separately extracted with methanol for 12 h at room temperature. After solvents were removed under vacuum, and the dried methanol extracts were re-dissolved in methanol for measuring: (1) COX inhibitory activities by product assay; (2) antioxidant activities by DPPH free radical scavenging and PMS-NADH system.

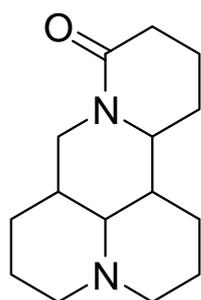
The dried aerial parts of *S. subprostrata* (235 g) were pulverized and extracted with methanol (3×900 ml, 72 h) at room temperature. The isolated crude extract was filtrated and then the solvent was removed at 40°C under a vacuum and freeze-dried to get 38.4 g. Dried crude extract was suspended in water (400 ml) and successively partitioned

with hexane, CHCl₃ and EtOAc. The obtained extract, in addition to the water solution remaining after extraction, was filtered and concentrated under reduced pressure to get 1.1, 5.1, 0.26 and 31.3 g of hexane, CHCl₃, EtOAc and water extracts, respectively.

The chloroform extract (5.1 g) was added with benzene (3×100 ml) to obtain the benzene-soluble fraction (1.9 g) and benzene-insoluble fraction (3.1 g). Because benzene-soluble fraction showed much higher COX inhibitory activity than benzene-insoluble fraction, so an aliquot of the benzene-soluble fraction (105 mg, dissolved in 10 ml 50% methanol) was applied to a C18 Sep-Pak column (Vac 35 cc, 10 g; Waters) previously wet with 50 ml methanol and equilibrated with 50 ml of 50% methanol. Then 50%, 75%, 90% and absolute methanol (120 ml of each) were used in succession to elute the column. Four fractions were collected and named as fractions A1 (40 mg), A2 (16 mg), A3 (34 mg), and A4 (8 mg), respectively. Then, COX inhibitory and anti-oxidant activities were evaluated for these fractions. A1 and A2 fractions showed a high activity, and were subjected to preparative HPLC purification.

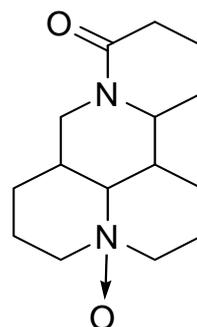
Fractions A1 and A2, were further purified by preparative HPLC using a Shimadzu Preparative HPLC equipped with SCL-10Avp System controller, LC-20AT pump, SPD-20A UV-VIS detector, FRC-10A fraction collector and Rheodyne Injector Model 7725i with 100 µl sample loop. Separation was achieved with a cosmosil Preparative C-18 AR column (Nacalai Tesque Company) (20×250 mm). The mobile phase consisted of acetonitrile : 0.01 M KH₂PO₄ : triethylamine in a ratio of 20 : 8 : 0.01 (v/v/v). Two main peaks with retention times 21.6 (compound 1) and 25.6 min (compound 2) were collected with a 3.0 ml/min of flow rate and 208 nm detection. The pooled fractions were concentrated and then CHCl₃ was added into these factions to extract the aimed compounds. Compounds isolation scheme is showed in **Fig. 2**. The two compounds

have been identified as matrine (**compound 1**) and oxymatrine (**compound 2**) by comparison with previously published data. Matrine is a colorless glassy solid; mp: 76°C; UV: (MeOH) λ_{\max} 204 nm; MS (positive ion): m/z 249.4 [M+H]. Oxymatrine is a colorless glassy solid; mp: 162-163°C; UV: (MeOH) λ_{\max} 194 nm; MS (positive ion): m/z 265.4 [M+H]. $^1\text{H-NMR}$ (600 MHz, CDCl_3) and $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) of matrine and oxymatrine also matched with published data (Chen, 2000). Structures of the two compounds are showed in **Fig. 1**.



Matrine: $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}$

(1)



Oxymatrine: $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}_2$

(2)

Fig. 1. Structures of matrine (1) and oxymatrine (2) isolated from *S. subprostrata*.

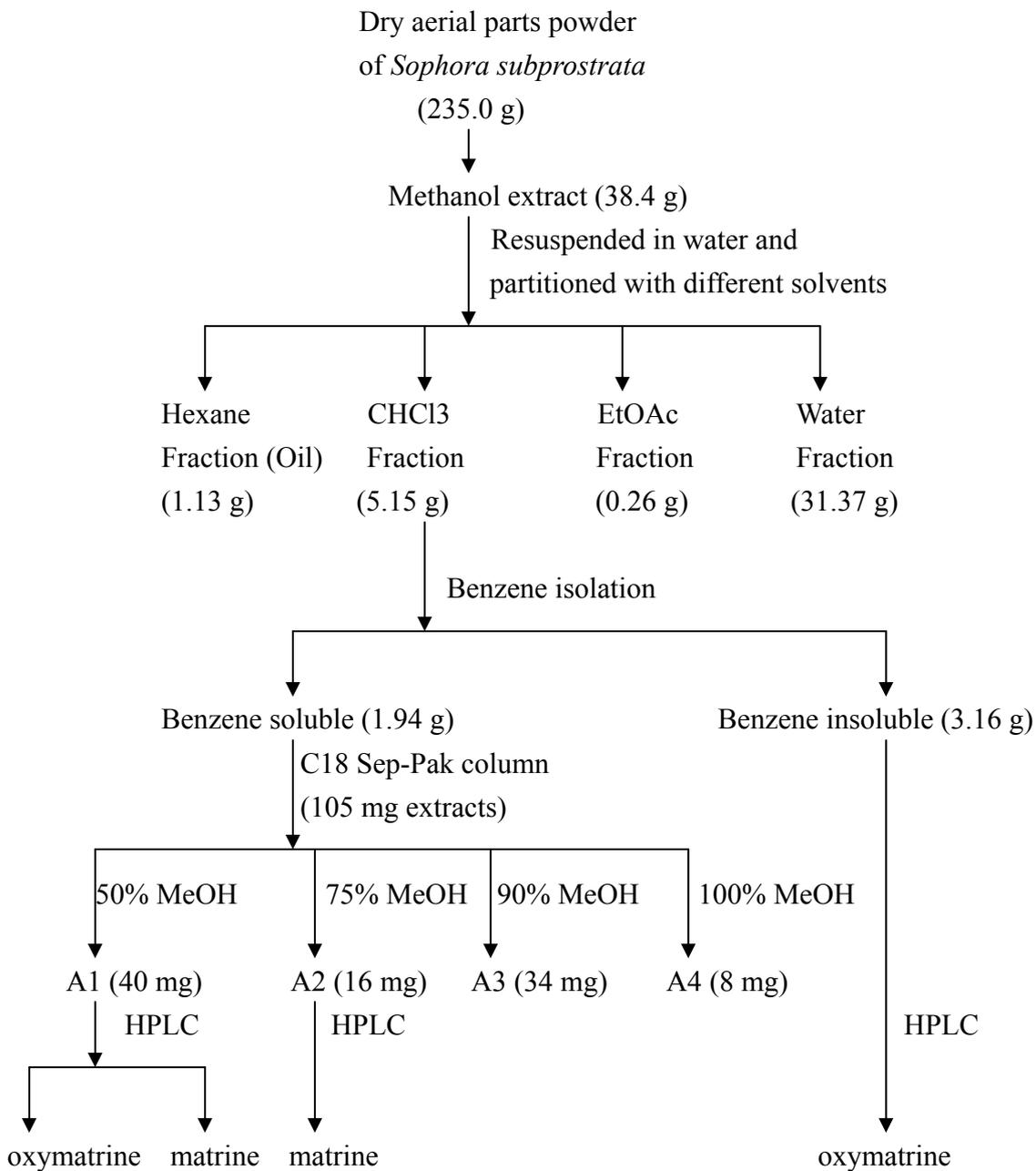


Fig. 2. Extraction scheme of antioxidant and COX enzyme inhibitory compounds from *Sophora subprostrata*

2.2.5. LC/MS spectrometer

LC/MS spectra were obtained using a Sciex API 2000 LC-MS/MS System (Model Sciex API 2000, Applied Biosystems, Langen, Germany) coupled to Agilent 1100 LC Binary pump equipped with Agilent 1100 Thermo Auto-sampler, Agilent 1100 Column Oven and Agilent 1100 Diode Array Detector in combination with SYNERGI 4 u MAX-RP 80 A C18 reverse phase column (150×4.6 mm, Phenomenex Company, USA.). Five µl samples were injected for analysis. Draw speed and eject speed were 200, and 200 µl/min, respectively. UV detector spectral was recorded between 190 nm – 400 nm with a 2 nm step width. Flow rate was 200 µl/min, solvent A = ultra-pure water and solvent B = HPLC grade MeOH. Gradient program: 0 min (95% A, 5% B), 2 min (85% A, 15% B), 10 min (50% A, 50% B), 20 min (30% A, 70% B), 25 min (20% A, 80% B), 30 min (10% A, 90% B), 35 min (5% A, 95% B), 40 min (0% A, 100% B), 45 min (0% A, 100% B) and 50 min (95% A, 5% B).

Mass spectra were obtained in an ion spray voltage of 5000 V (positive mode) and a temperature of 450 °C using a Turbolon spray ion source. Spectra were recorded between m/z 100-900 with a scan duration of 2 s/scan and an interscan time of 0.1 s. Spectra were processed using Biosystems/MDS SCIEX instruments Analyst Software (version: Analyst 1.4).

2.2.6. Determination of COX inhibitory activity

2.2.6.1 Product assay

The ability of the extracts from *S. subprostrata*, *H. ambillis*, and *H. diffusa* matrine and purified compounds matrine, oxymatrine to inhibit ovine COX-1 and COX-2 was determined using a COX (ovine) inhibitor screening assay kit according to the

manufacturer's instructions. Briefly, ten μl of different concentrations of test samples were added to a series of reaction buffer solutions (960 μl , 0.1 M Tris-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 μl) enzyme in the presence of heme (10 μl). After solutions were incubated for 5 min at 37°C, the reaction was initiated by adding 10 μl substrate solution (arachidonic acid, 100 μM). The reaction was stopped by the addition of 50 μl of 1 M HCl after 2 min, and then saturated stannous chloride solution was added to reduce PGH_2 to PGF_{2a} , which was measured by enzyme immunoassay. All treatments were carried out in 3 replications. The inhibitory activity was calculated by comparing with a plotted standard response curve. The concentration of the test compound causing 50% inhibition (IC_{50} , μM) was calculated from the concentration-inhibition response curve.

2.2.6.2 Peroxidase assay

The ability of the extracts to inhibit ovine COX-1 and COX-2 was determined using a colorimetric COX inhibitor screening assay kit according to the manufacturer's instructions. The colorimetric COX (ovine) inhibitor-screening assay utilizes the peroxidase component of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N,N-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm. In briefly, mixture consisted of 220 μl of 0.1 M Tris-HCl buffer (pH 8.0) containing Hematin (1.13 μM), TMPD (80 μM), COX-1 or COX-2 (40 nM), and different concentration extracts. positive control was carried out using aspirin or NS-398. Reaction was started by adding 10 μl of 100 μM arachidonic acid, then the absorbance was read at 590 nm after incubating for 5 minute at 25°C using a 96 microplate reader (Benchmark plus microplate 170-6930j1, BIO-RAD Company).

2.2.7. DPPH radical scavenging activity

Free radical scavenging activity of extracts was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Saha *et al.*, 2004). Different concentrations of extracts and positive control BHA in methanol solution (100 μ l) were added to 4 ml of DPPH 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 UV spectrophotometer (JASCO Ubest-50). Activity of scavenging (%) was calculated using the following formula:

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

2.2.8. PMS-NADH system for scavenging superoxide radicals

The superoxide scavenging ability of extracts was assayed by the method of Lau *et al.* (2002) with minor modifications. In the reaction mixture, 1.6 ml of phosphate buffer (0.1 M, pH 7.4) contained 105.6 μ M β -nicotinamide adenine dinucleotide (NADH), 50 μ M nitro blue tetrazolium (NBT) and samples in different concentrations dissolved in methanol. The reaction was initiated by adding 30 μ M phenazin 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 superoxide radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}}] \times 100\%$$

2.2.9. Statistical analysis

Data were analyzed by SAS version 6.12 using ANOVA with the least significant difference (LSD) at the 0.05 probability level.

2.3. Results

2.3.1. COX inhibitory activity of methanol extracts from *S. subprostrata*, *H. ambillis*, and *H. diffusa*

COX inhibitory activity of methanol extracts of *S. subprostrata*, *H. ambillis*, and *H. diffusa* was evaluated using colorimetric COX inhibitor screening assay kit (peroxidase assay) and enzyme immunoassay (EIA) kit (product assay). **Fig. 3** shows the values of COX-1 inhibition by three crude extracts as compared with the positive control aspirin or indomethacin. *S. subprostrata* extract indicated higher inhibitory activity than two other samples in the peroxidase assay and the product assay. In the peroxidase assay, methanol extracts (45.5 µg/ml) of *S. subprostrata*, *H. diffusa* and *H. ambillis* showed 15.4%, 7% and 4% inhibition of COX-1, respectively. Aspirin used as positive control showed 30.6% COX-1 inhibitory activity at concentration of 0.76 mM. In the product assay, methanol extracts (10 µg/ml) of *S. subprostrata*, *H. diffusa* and *H. ambillis* showed 17.4%, 1.2% and 12.8% inhibition of COX-1, respectively. When COX-2 inhibitory activity was measured in the product assay for extracts of three herbs at the concentration of 10 µg/ml, all showed very negligible inhibitory activity (data not shown).

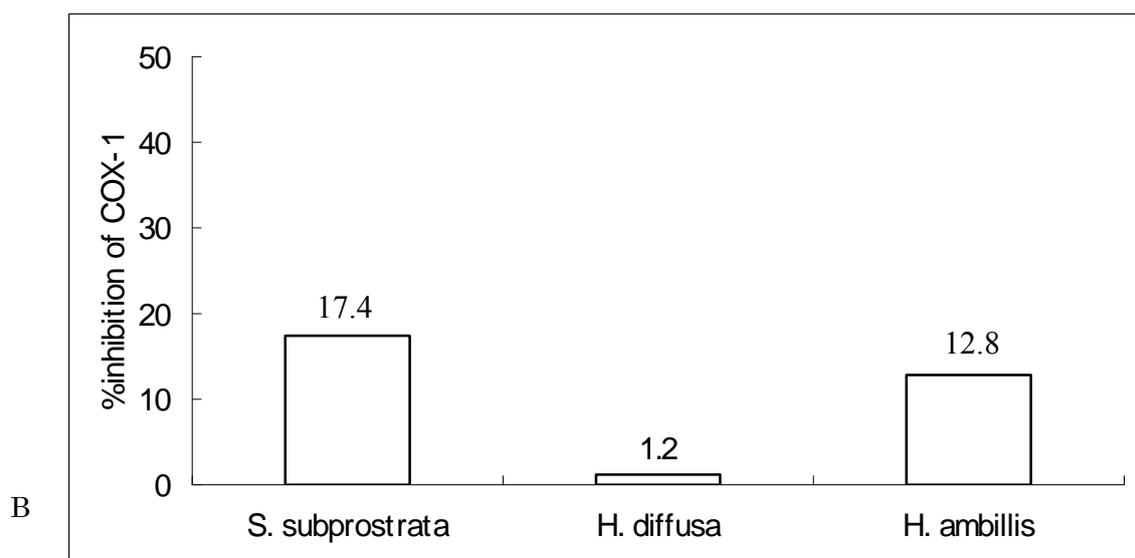
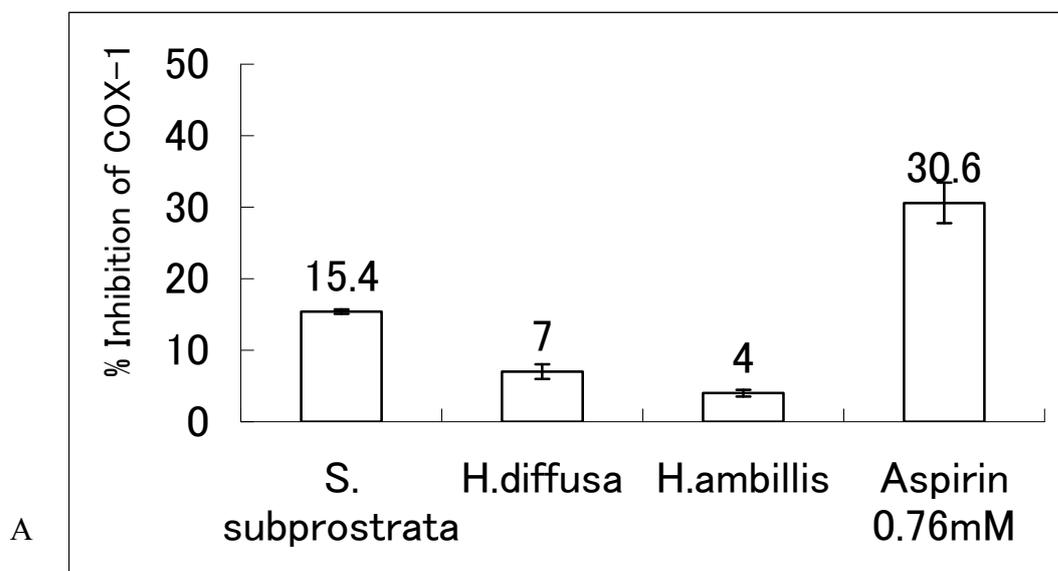


Fig. 3. *In vitro* COX-1 inhibitory activities assay by methanol extracts of *S. subprostrata*, *H. ambilllis* and *H. diffusa*, A: peroxidase assay by Colorimetric COX inhibitor-screening assay kit, sample concentration: 45.5 μ g/ml, positive control aspirin concentration: 0.76mM; B: product assay by COX (ovine) inhibitor screening (EIA) kit, sample concentration: 10 μ g/ml; Data points represent means \pm S. D. (n=3).

2.3.2. Antioxidant activity of methanol extracts of *S. subprostrata*, *H. ambillis*, and *H. diffusa*

As shown in **Fig. 4**, among the methanol extracts of three samples, at concentration of 25 µg/ml, *S. subprostrata* extract showed the highest DPPH free radical scavenging activity (15.6%), followed by *H. diffusa* extract (4.7%), and *H. ambillis* showed very negligible activity (2.6%). *S. subprostrata* extract scavenge DPPH radicals activity in a concentration-dependent manner with an EC₅₀ value of 130.5 µg/ml. The superoxide radicals were generated in a PMS-NADH system and assayed by the reduction of NBT. **Fig. 5** shows the superoxide radical scavenging ability of methanol extracts of three samples. *S. subprostrata* extract showed the highest activity (36.3%) at the concentration of 62.5 µg/ml among three samples. *H. diffusa* and *H. ambillis* extracts all showed minus values (-6.8% and -0.8%, respectively). Positive control ascorbic acid at the concentration of 2 mg/ml showed 31.2% scavenging activity and BHA at 31.25 µg/ml showed negligible scavenging activity (3.7%).

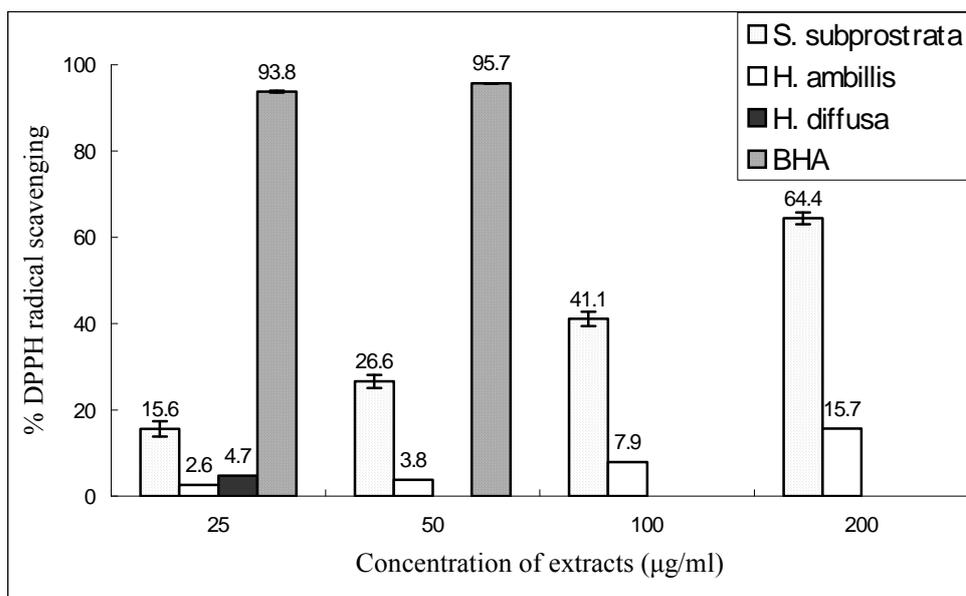


Fig. 4. *In vitro* anti-oxidative test of DPPH system for *S. subprostrata*, *H. ambilis* and *H. diffusa* methanol extracts and positive control BHA. Data points represent means \pm S. D. (n=3).

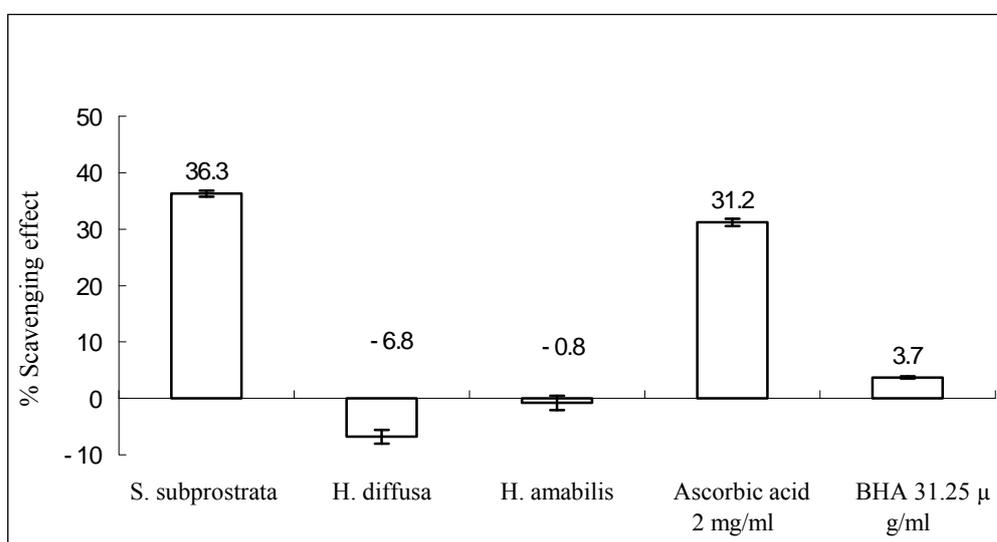


Fig. 5. *In vitro* anti-oxidative test of PMS-NADH system for *S. subprostrata*, *H. ambilis* and *H. diffusa* methanol extracts and positive control ascorbic acid and BHA. Sample concentration: 62.5 µg/ml. Data points represent means \pm S. D. (n=3).

2.3.3. COX inhibitory and antioxidant activities of methanol extract and sub-fractions from S. subprostrata

The methanol extract of *S. subprostrata* was separated into hexane, CHCl₃, EtOAc and water fractions. Then, they were subjected to COX inhibitory, DPPH radical scavenging and PMS-NADH system anti-oxidant assays. The COX inhibitory and anti-oxidant effect of the MeOH extract and the fractionated extracts are shown in **Fig. 6, 7 and 8**. These results indicated that the MeOH extract exhibited potential COX-1 and COX-2 inhibitory activity and anti-oxidant effects. At the adjusted concentration, the CHCl₃ fraction showed the highest COX-1 and COX-2 inhibition (41.1% and 30.8%, respectively) among all fractions. CHCl₃ fraction also exhibited higher DPPH radical scavenging activity (EC₅₀ 44.4 µg/ml) and superoxide radical scavenging activity (53.3%) compared with hexane, EtOAc and water fractions. The results suggested that COX inhibitory and antioxidant components more contained in CHCl₃ fraction

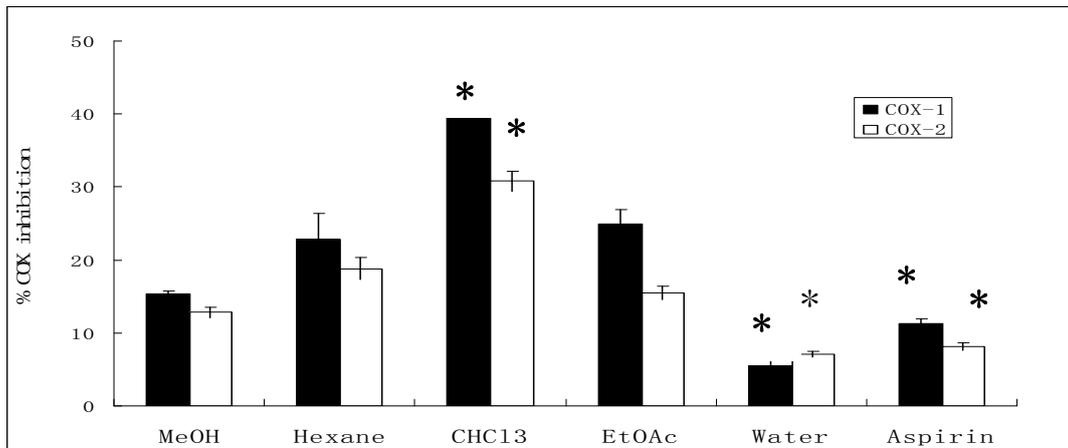


Fig. 6. Inhibitory activity of the extracts of *S. subprostrata* against COX.

Effect of methanol extract and sub-fractions of *S. subprostrata* for COX-1 and COX-2 inhibitory activities, samples concentration: 45.5 $\mu\text{g/ml}$. Aspirin was used as a positive control in the assay, the values represent means \pm S. D. (n=3), * $p < 0.05$ vs. methanol extract.

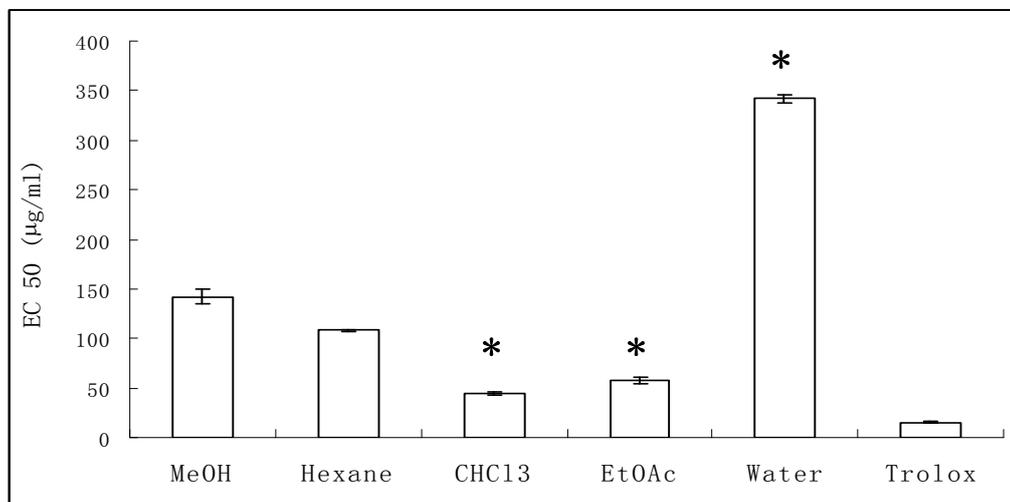


Fig. 7. DPPH radical scavenging activity (EC_{50}) of the extracts of *S. subprostrata*.

Values represent means \pm S. D. (n=3), * $p < 0.05$ vs. methanol extract.

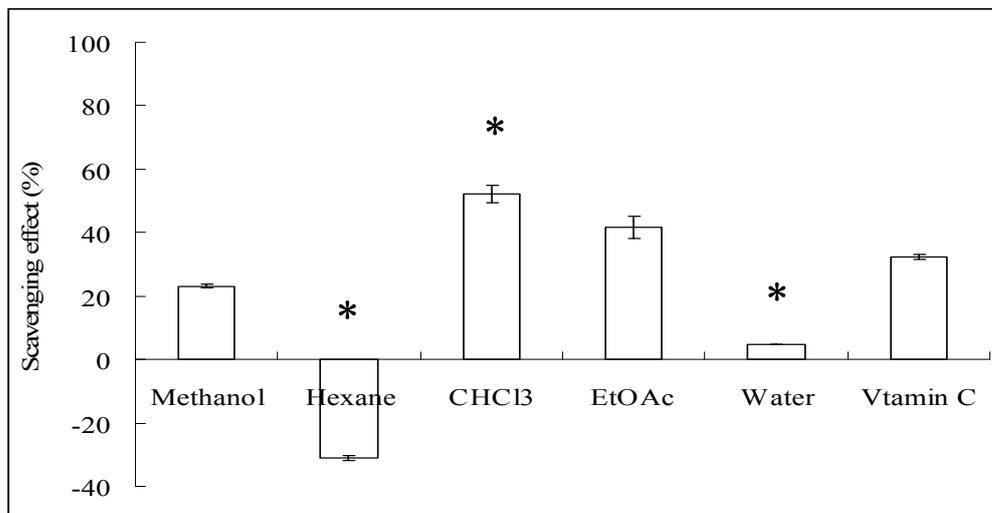


Fig. 8. PMS-NADH system anti-oxidant activity of the extracts of *S. subprostrata*.

Samples concentration: 30 µg/ml, the values represent means ± S. D. (n=3), * $p < 0.05$ vs. methanol extract.

2.3.4. Chemical composition in the extracts of *S. subprostrata* by LC/MS

LC/MS analysis of the methanol extract and sub-fractions of *S. subprostrata* resulted in the identification of two compounds, matrine and oxymatrine. LC/MS chromatograph of methanol extract is showed in **Fig. 9**. According to the analysis results of different fractions by LC/MS, the content of matrine and oxymatrine were gradually increased following COX inhibitory activity-directed chromatographic fractionation. As shown in **Table 1**, the matrine and oxymatrine contained in methanol extract were 2.7 and 11.6%, respectively, and in the CHCl₃ fraction were 12.0 and 60.1%, respectively. The contents of matrine and oxymatrine in the CHCl₃ fraction were much higher than in the methanol extract. The amount of matrine contained in benzene-soluble fraction was increased to 35.8%, but the amount of oxymatrine was

decreased to 38.4%. The matrine composition in the A2 fraction was approximately 53.0%. Based on the results of preliminary COX inhibitory and antioxidant activity tests, these activities might be due to the presence of matrine and oxymatrine and we also can deduce matrine exhibited higher COX inhibitory activity than oxymatrine. These two compounds were purified from the A1, A2 fractions by successive preparative HPLC.

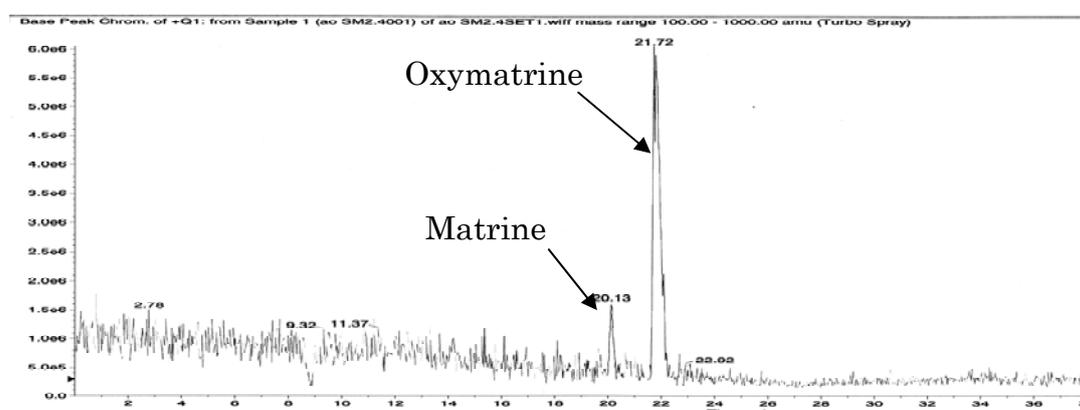


Fig. 9. LC/MS chromatograph of *S. subprostrata* methanol extract.

Table 1 Matrine and oxymatrine composition in extracts of *S. subprostrata* by LC/MS.

Extracts	Composition (%)	
	Matrine (20.5-21.0 min)	Oxymatrine (21.5-22.7 min)
Methanol extract	2.7	11.6
CHCl ₃ fraction	12.0	60.1
Benzene-soluble fraction	35.8	38.4
A1 fraction	38.7	18.2
A2 fraction	53.0	6.8

Matrine or oxymatrine in methanol extract or fractions were expressed by percentage of dried extracts. Data was calculated by using Biosystems/MDS SCIEX instruments Analyst Software (version: Analyst 1.4) which installed in LC/MS instrument.

2.3.5. COX inhibitory activities of matrine and oxymatrine

Isolated matrine and oxymatrine from *S. subprostrata* were tested for COX-1 and COX-2 inhibitory activities. The concentrations of each of the two compounds assayed were 365.1, 182.6 and 91.3 μM for the assays. These two compounds both showed concentration-dependence in COX-1 and COX-2 inhibition assays. The inhibition of COX activities (IC_{50}) for the two compounds is shown in Table 2. Standard anti-inflammatory compounds, aspirin and NS-398, were used as positive controls, where aspirin showed minor inhibitory activity against COX-1, and COX-2 (IC_{50} 1.04 and 2.23 mM, respectively), but NS-398 showed excellent COX-2 inhibitory activity (IC_{50} 5.6 μM). Matrine showed high COX-1 inhibitory activity (IC_{50} 31.3 μM) and moderate COX-2 inhibitory activity (IC_{50} 188.5 μM). Oxymatrine had weaker inhibitory activities against COX-1 (IC_{50} 197.8 μM) and COX-2 (IC_{50} 385.1 μM)

compared with matrine. Both matrine and oxymatrine showed higher COX-1 inhibitory activity than COX-2 inhibitory activity.

2.3.6. Anti-oxidant activity of matrine and oxymatrine

To examine the anti-oxidant activity effects of the isolated matrine and oxymatrine, the PMS-NADH system superoxide radicals scavenging methods were used. As shown in **Table 2**, matrine and oxymatrine exhibited moderate anti-oxidant activities when assayed by radical scavenging (EC_{50} 192.5 and 275.8 μ M, respectively), while EC_{50} of positive control Trolox was 126.6 μ M.

Table 2 COX inhibitory and antioxidant activity of matrine and oxymatrine

Samples	COX inhibitory activity		PMS-NADH assay
	IC_{50} (μ M)		EC_{50} (μ M)
	COX-1	COX-2	
Oxymatrine	197.8 \pm 2.4b	385.1 \pm 2.7b	275.8 \pm 3.5a
Matrine	31.3 \pm 0.6c	188.5 \pm 3.7c	192.5 \pm 2.3b
Aspirin	1041.1 \pm 8.5a	2228.1 \pm 18.4a	NT
NS-398	NT	5.6 \pm 0.1d	NT
Trolox	NT	NT	126.6 \pm 1.1c

Values represent means \pm S. D. (n=3); Values sharing the same letter in the same column are not significantly different ($P < 0.05$); NT: not tested.

2.4. Discussion and conclusion

Shibata and Nishikawa (1962) reported that matrine and oxymatrine were first

isolated from the roots of *S. subprostrata*. An intensive investigation into the pharmacology and clinical applications of these two alkaloids had been performed in the past and still remained one of the focal points of medical research. The main clinical applications of matrine and oxymatrine were treatment of tumor (Kojima *et al.*, 1970), viral hepatitis (Wang, 2000; Zheng *et al.* 2005), ocular inflammation (Chuang *et al.* 1987), ulcer (Yamazaki, 2000), cardiovascular and nociceptive diseases (Wei *et al.*, 1985; Kamei *et al.*, 1997). Matrine was also reported to be used as a non-steroidal anti-inflammatory drug (NSAIDs) (Kamei *et al.*, 1997). Hong *et al.* (2002) have reported that the methanol extract of *S. subprostrata* showed COX-2 and iNOS inhibitory activities; however they did not isolate active compounds. One research showed that matrine exhibited selective inhibitory effect on functional activity of COX-2 in the HT-29 cell line (Huang *et al.*, 2005). These results are accordance with our study. Matrine and oxymatrine were reported contained in many leguminous plants. Some researchers isolated these compounds from the root of *Sophora. flavescens*, the aerial parts of *Sophora. alopecuroides* (Yamazaki *et al.*, 2000; Ohmiya *et al.*, 2000). However, there are few reports about these alkaloids isolated from the aerial parts of *S. subprostrata*. The mechanism of anti-inflammatory activity of matrine and oxymatrine was not well understood. Therefore, it is necessary to clarify the COX inhibitory effect by these compounds. this study reports the COX-1 and COX-2 inhibitory activities *in vitro* of matrine and oxymatrine isolated from *S. subprostrata* for the first time, and also give some biological effects of its superoxide radical scavenging activities. The findings provide some scientific supports for the traditional use of *S. subprostrata* for controlling inflammatory disease.

CHAPTER 3

Screening of medicinal plant extracts for MMP-13 inhibitory and antioxidant activities

Abstract

The methanol extracts of thirteen medicinal plants from Okinawa were screened for matrix metalloproteinase-13 (MMP-13) inhibitory and antioxidant activities. MMP-13 inhibitory activity was measured by using a MMP-13 inhibitor assay kit, and antioxidant activities were performed by DPPH radical scavenging, superoxide radical scavenging, reducing power and metal chelating ability methods. Among the tested plants, *Curcuma longa*, *Curcuma aromatica* and *Ocimum basilicum* showed high inhibitory effect on MMP-13, with IC₅₀ values of 27.8, 85.8 and 81.7 µg/ml, respectively. The chemical compositions of these three extracts were analyzed by LC-MS. Curcumin was identified and quantified in extracts of *C. longa* and *C. aromatica* (58.6 and 28.7 mg/g extract, respectively). Rosmarinic acid was identified in extract of *O. basilicum* (47.3 mg/g extract). Both curcumin and rosmarinic acid exhibited excellent MMP-13 inhibitory activity (IC₅₀: 3.6 and 2.9 µM, respectively). On the other hand, the extracts of *Ficus microcarpa* and *Smilax sebeana* exerted strong antioxidant activities in all applied methods. These two plants and *C. longa* possessed high phenolic content that was analyzed using Folin-Ciocalteu's reagent. The results shown here may provide some scientific evidence for the use of several medicinal plants from Okinawa, for treating tumours, inflammatory diseases and arthritis.

Keywords: Matrix metalloproteinase-13 inhibition; antioxidant activity; medicinal plants; curcumin; rosmarinic acid

3.1. Introduction

Medicinal plants have been traditionally used for pharmaceutical and dietary therapy in long history. A number of herbs and many relevant prescriptions have been screened and used for treating and preventing various tumors and inflammations as folk practices. Nowadays medicinal plants were still widely practiced particularly in the countryside and remote mountainous regions, and even in the urban areas of many Asian countries.

Matrix metalloproteinases (MMPs) comprise a family of secreted and membrane-bound endopeptidases which hydrolyze extracellular matrix proteins. Based on their preferred substrates and on structural features, MMPs can be divided into collagenases, gelatinases, stromelysins and membrane-type matrix metalloproteinases (Jiang *et al.*, 1992; Hooper, 1994). Collagenases are important proteolytic tools for extracellular matrix remodeling during organ development and tissue regeneration. Chronic activation of collagenases results in an excessive degradation of extracellular matrix components and has been believed to contribute to many pathological conditions, such as tumor progression, osteoarthritis, rheumatoid arthritis and many inflammatory diseases (Clark *et al.*, 2003; Ala-aho *et al.*, 2005; Blavier *et al.*, 2006; Deryugina *et al.*, 2006). As one of important enzyme of MMPs family, collagenase 3 (MMP-13) has been reported to be involved in the development and metastasis of breast and lung carcinomas. In addition, this enzyme plays an important role in degenerative bone diseases such as rheumatoid arthritis and osteoarthritis (Tardif *et al.*, 2004 ; Burrage *et al.*, 2007). Preclinical studies have provided compelling evidence that inhibition of MMPs would be therapeutic for inflammatory, malignant, arthritis and degenerative diseases (Murphy *et al.*, 1992; Skiles *et al.*, 2001). In recent years, several highly selective synthetic MMP-13 inhibitors have been tested for their effects against growth

and invasion of malignant tumors and for therapeutic of osteoarthritis and rheumatoid arthritis (Ala-aho *et al.*, 2005; Burrage *et al.*, 2007). This study was carried out in the aim of finding some molecules or a group of molecules that can be used as MMP-13 inhibitors without the toxicity of the synthesized chemical compounds.

Antioxidant activity was among the first links between chemical reactions and biological activity. Many compounds, such as phenolics, alkaloids, flavonoids, lignans and terpenes were well known to possess antioxidant activity, which were proportional to other biological activity, including enzyme inhibition, anti-tumor, anti-ulcer, anti-viral hepatitis (Badami *et al.*, 2003; Ajaikumar *et al.*, 2005; Fragopoulou *et al.*, 2007).

Okinawa located in subtropical region of Japan, which has rich plant diversity. It was estimated that about 300 plant species have traditionally been used for disease treatment and pharmaceutical purpose (Hatushima *et al.*, 1976; Yosikawa, 1983 and Tawata *et al.*, 1985). In this study 13 medicinal plant species which being the well popular in Okinawa were selected for their efficacies against MMP-13 inhibitory activity, antioxidant activity and phenolic content. Identification and quantification of potent bioactive compounds from these plants were also performed.

3.2. Materials and Methods

3.2.1. Plant material

Thirteen folk herbs associated with anticancer, anti-arthritis and anti-inflammatory activities as described by Yosikawa (1983) and Tawata *et al.* (1985) were selected in this study. *Pentarrhizidium orientale* Hayata, *Alpinia zerumbet* (Pers.) B.L., *Zingiber officinale* Rosc., *Curcuma longa* L., *Curcuma aromatica* Salisd., *Catharanthus roseus* G. Don, *Orthosiphon aristatus* (BL.) Miq., *Elfvigia applanata* Karst, *Houttuynia cordata*

Thunb., *Plantago asiatica* L., *Ocimum basilicum* L. were purchased at a Naha herbalist, Okinawa, Japan, except *Smilax sebeana* Miq. and *Ficus microcarpa* L. f., which were collected in the campus of University of the Ryukyus in November of 2005. The voucher specimens have been deposited in Faculty of Agriculture, University of the Ryukyus. The scientific names and medicinally used parts were listed in **Table 1**.

Table 1. The name, family, medicinally used parts and yield of methanol extracts of selected 13 medicinal plants in Okinawa

Scientific name	Family	Medicinally used parts	Yield (w/w, %)
<i>Pentarrhizidium orientale</i> Hayata	<i>Trichomanaceae</i>	Leaves	13.5
<i>Alpinia zerumbet</i> (Pers.) B.L.	<i>Zingiberaceae</i>	Rhizome	14.0
<i>Zingiber officinale</i> Rosc.	<i>Zingiberaceae</i>	Rhizome	25.8
<i>Curcuma longa</i> L.	<i>Zingiberaceae</i>	Rhizome	11.9
<i>Curcuma aromatica</i> Salisd.	<i>Zingiberaceae</i>	Rhizome	16.2
<i>Catharanthus roseus</i> G. Don	<i>Apocynaceae</i>	Whole plant	16.0
<i>Orthosiphon aristatus</i> (BL.) Miq.	<i>Lamiaceae</i>	Leaves	11.7
<i>Elfvigia applanata</i> Karst	<i>Polyporaceae</i>	Whole plant	3.9
<i>Houttuynia cordata</i> Thunb.	<i>Saururaceae</i>	Whole plant	8.7
<i>Plantago asiatica</i> L.	<i>Plantaginaceae</i>	Whole plant	16.5
<i>Smilax sebeana</i> Miq.	<i>Smilacaceae</i>	Root	11.3
<i>Ficus microcarpa</i> L. f.	<i>Moraceae</i>	Bark and aerial root	10.2
<i>Ocimum basilicum</i> L.	<i>Labiatae</i>	Leaves	14.9

3.2.2. Preparation of the methanol extract

The samples were ground to fine powder and passed through a sieve (24 mesh), then dried to constant weight in desiccator at 40°C. Six grams of each sample were extracted with 35 ml of 100% methanol for 12 h at room temperature with shaking. The plant

materials were extracted twice in the same conditions. The methanol extracts obtained from each sample were filtered and dried under vacuum, then re-dissolved in methanol and stored under refrigeration for further analysis. The quantity of extracts was shown in **Table 1**.

3.2.3. Solvents and reagents

Nitro blue tetrazolium (NBT), curcumin, rosmarinic acid, phenazine methosulfate (PMS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), *N*-Hydroxy-1-(4-methoxyphenyl)sulfonyl-4-(biphenylcarbonyl)piperazine-2-carboxamide (CBC) and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Sigma Chemicals. Gallic acid, Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and all solvents at analytical grade were purchased from Wako Pure Chemical Industries, Japan.

3.2.4. MMP-13 inhibitory assay

The MMP-13 inhibitory activity of samples was determined by a MMP-13 inhibitor assay kit (Chondrex, Inc., Redmond, WA, USA, distributed by IWAI Chemicals Company, Japan, catalog # 3003). The human CBC was used as a positive control. A designate reaction was performed in the 96-well microtiter plate according to the manufacturer's protocol. The assay procedure was separated into two stages. First, diluted recombinant human MMP-13 (rh-MMP-13, 10 µg/ml) with dilution buffer B was activated by adding 5 µl of activator 1 (APMA) at 35°C for 60 minutes. Second, appropriate amounts of test samples that diluted by solution B and reaction buffer to the wells were added to adjust the final volume to 160 µl. The reaction was initiated by

adding 100 μ l substrate solution to each well. The collagenase reaction was stopped by adding 10 μ l of the stop solution to each well after incubating at room temperature for approximately 30 minutes. The reaction fluorescence intensity was determined at λ_{em} =450 nm and λ_{ex} =360 nm with LS- PLATE manager 2001 (Wako, Osaka, Japan). The MMP-13 activity was determined by comparing with a standard response curve using buffer instead of inhibitor in similar conditions. The inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. All treatments were carried out in 3 replications.

3.2.5. DPPH radical scavenging assay

Free radical scavenging activity of extracts was determined using the DPPH method as described previously in **Chapter 2** with following different: The measurement was performed using a Shimadzu UV-Vis spectrophotometer (mini 1240).

3.2.6. PMS-NADH system superoxide radical scavenging assay

The superoxide radical scavenging ability of extracts was assayed following the method as described previously in **Chapter 2**.

3.2.7. Determination of reducing power assay

The reducing power was performed according to the method of Singh (2007). Various concentrations of samples (25-200 μ g/ml) in 200 μ l of methanol were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium ferricyanide (1% w/v). The mixture was incubated at 50 $^{\circ}$ C for 20 min. After terminating the reaction by adding trichloroacetic acid (10% w/v), the mixture was centrifuged at 1000 \times g for 10 min. The

supernatant of solution 0.5 ml was mixed with 0.5 ml distilled water and 0.1 ml FeCl₃ (0.1% w/v). After 5 min later, the absorbance was measured at 700 nm in a microplate reader (Benchmark plus microplate 170-6930j1, BIO-RAD Company).

3.2.8. Ferrous metal ions chelating assay

The ferrous ion-chelating potential of the extracts was investigated according to the method of Dinis *et al.* (1994). Briefly, the extracts samples and EDTA (1000 µg/ml) in 0.8 ml was added to a solution of 2 mM FeCl₂ (0.02 ml). The reaction was initiated by the addition of 5mM ferrozine (0.2 ml). The mixture was shaken vigorously and keep at room temperature for 10 minutes. Absorbance of the solution was then measured spectrophotometrically at 562 nm against a blank. The ferrous ion-chelating capability of extracts was calculated using the following equation:

$$\text{Metal chelating effect (\%)} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}}] \times 100\%$$

3.2.9. Amount of total phenolic compounds

The amount of total phenolics in the methanol extracts was measured using the Folin-Ciocalteu reagent method (Djeridane *et al.*, 2006). The methanol solution of each extract (0.2 ml, 500 µg/ml) was added in a test tube. An amount of 0.5 ml of each distilled water and the Folin-Ciocalteu reagent were added in the tubes. After 1 min later, 0.8 ml of sodium carbonate solution (7.5%) was added and the mixture was allowed to stand for 30 min with intermittent shaking. Absorbance was measured at 760 nm using a Shimadzu UV-Vis spectrophotometer (Mini 1240). The total phenolic content was expressed as gallic acid equivalents (GAE) in mg per g extract.

3.2.10. LC/MS spectrometer

LC/MS spectra were obtained using a Sciex API 2000 LC-MS/MS System as described previously in **Chapter 2**.

3.3. Results and discussion

3.3.1. MMP-13 inhibitory activity

The MMP-13 inhibitory activities of the 13 plants were shown in **Table 2**. Except for *Z. officinale*, *C. roseus* and *F. microcarpa*, the tested plants showed certain inhibitory effect against MMP-13, however their IC₅₀ values varied among plant species. The highest inhibitory activity was obtained with the extract of *C. longa* (IC₅₀, 27.8 µg/ml), followed by those of *O. basilicum* and *C. aromatica* (IC₅₀, 81.7 and 85.8 µg/ml, respectively). However, no plants extracts could have greater MMP-13 inhibitory ability than the positive control CBC (a selective synthetic MMP-13 inhibitor, IC₅₀, 0.15 µM). In general, the MMP-13 inhibitory activities of these plants were proportional to applied dose. The methanolic extracts of *C. longa*, *O. basilicum* and *C. aromatica* exhibited maximum MMP-13 inhibitory properties comparing to the other plants; they were selected for further chemical analysis.

Table 2. MMP-13 inhibitory, DPPH radical scavenging activities and total phenolic content by 13 medicinal plants extracts in Okinawa

Samples	MMP-13 inhibitory activity IC ₅₀ (µg/ml)	DPPH radical scavenging activity EC ₅₀ (µg/ml)	Total phenolic content as gallic acid equivalents (GAE mg/g extract)
<i>P. orientale</i>	172.1 ± 2.0	152.4 ± 2.4	32.5 ± 2.0
<i>A. zerumbet</i>	222.8 ± 4.5	24.5 ± 1.5	82.7 ± 2.3
<i>Z. officinale</i>	NI	39.5 ± 1.1	104.7 ± 1.5
<i>C. longa.</i>	27.8 ± 2.9	36.6 ± 1.4	141.7 ± 1.5
<i>C. aromatica</i>	85.8 ± 0.6	86.4 ± 2.0	84.5 ± 0.3
<i>C. roseus</i>	NI	141.8 ± 3.1	28.0 ± 0.6
<i>O. aristatus</i>	120.0 ± 4.5	57.7 ± 2.6	53.3 ± 0.3
<i>E. applanata</i>	154.2 ± 14.1	47.3 ± 3.0	83.3 ± 1.7
<i>H. cordata</i>	139.0 ± 20.4	66.7 ± 2.7	53.0 ± 1.0
<i>P. asiatica</i>	174.5 ± 24.0	88.5 ± 1.5	26.7 ± 0.3
<i>S. sebeana</i>	288.1 ± 18.2	17.2 ± 1.1	116.7 ± 5.8
<i>F. microarpa</i>	NI	15.7 ± 0.8	123.3 ± 4.3
<i>O. basilicum</i>	81.7 ± 2.2	36.2 ± 1.4	88.3 ± 1.5
Curcumin (µM)	3.6 ± 1.1	23.1 ± 1.1	ND
Rosmarinic acid (µM)	2.9 ± 0.8	11.7 ± 0.6	ND
CBC (µM)	0.15 ± 0.04	ND	ND
Trolox (µM)	ND	17.6 ± 1.2	ND

NI: no inhibitory activity; ND: not determined; the values represent means ± S. E. (n=3).

3.3.2. Antioxidant activity

The methanol extracts prepared from 13 medicinal plants assayed by the 4 different antioxidant methods including DPPH free radicals scavenging, reducing power, PMS-NADH system superoxide radical scavenging and metal chelating ability (**Table 2** and **Figs. 1, 2** and **3**).

As shown in Table 2, three extracts (*F. microcarpa*, *S. sebeana* and *A. zerumbet*) exhibit EC₅₀ values below 30 µg/ml, indicating high potential as DPPH free radical

scavenger. By another two assays (reducing power and PMS-NADH system superoxide radical scavenging, **Figs. 1 and 2**), these three extracts also showed higher antioxidant activity than other ten extracts. However PMS-NADH assay could not be performed with some plant extracts due to a strong precipitate formed in the reactions. It should be noted that the extract of *F. microcarpa* showed highest antioxidant in DPPH and reducing power assay, and exhibited high activity even in PMS-NADH superoxide radical scavenging assay. As showed in **Fig. 3**, extracts of *C. longa*, *C. aromatica*, *H. cordata* and *F. microcarpa* exhibited higher Fe²⁺ ion chelating ability than other extracts. Antioxidant activity of extracts of *C. longa*, *C. aromatica*, *A. zerumbet* and *O. basilicum* has been reported by previous researchers. They found that some phenolic compounds, such as curcumin, rosmarinic acid and quercetin were responsible for this activity (Ramsemak *et al.*, 2000; Jayasinghe *et al.*, 2003). To our knowledge no previous studies on any antioxidant assay or phenolic content had been reported for the extracts of *F. microcarpa* and *S. sebeana*. Isolation and identification of some antioxidant compounds from these two extracts will be our future works.

3.3.3. Total phenolic content

As one of the most important antioxidant plant components, phenolic compounds were widely investigated in many medicinal plant and vegetables (Djeridane *et al.* 2006). The amounts of total phenolic compounds in the methanol extracts of 13 medicinal plants were shown in **Table 2**. The amount of total phenolics varied in different plants and ranged from 26.7 to 141.7 mg GAE /g of methanol extract. The highest total phenolic levels have been detected in *C. longa*, *F. microcarpa* and *S. sebeana* (141.7, 123.3 and 116.7 mg/g extract, respectively) and the lowest in *P. asiatica*, *C. roseus* and

P. orientale (26.7, 28.0 and 32.5 mg/g extract, respectively). It has been noted that the extract of *C. longa* showed highest inhibitory activity against MMP-13, while the extracts of *F. microcarpa* and *S. sebeana* showed highest antioxidant activity in DPPH radical scavenging, PMS-NADH superoxide radical scavenging and reducing power ability assays. We can deduce that the strong antioxidant of the extracts may be attributed to its high level of phenolic compounds. These data were in accordance with previous research (Gorinstein *et al.*, 2003; Maisuthisakul *et al.*, 2007), which have shown that high total polyphenols content increases antioxidant activity and there was a linear correlation between phenolic content and antioxidant activity.

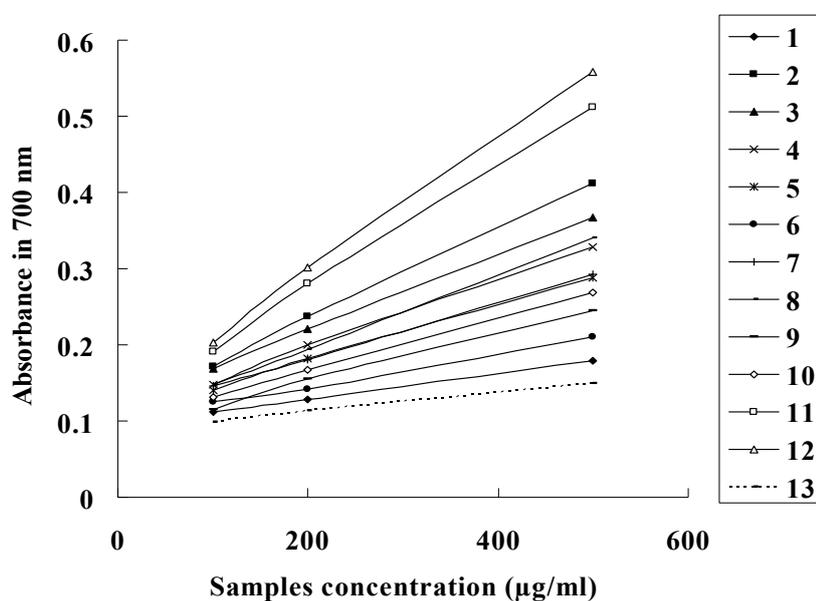


Fig. 1. Evaluation of antioxidant activity of methanol extracts of 13 medicinal plants using the reducing power method; 1: *P. orientale*, 2: *A. zerumbet*, 3: *Z. officinale*, 4: *C. longa*, 5: *C. aromatica*, 6: *C. roseus*, 7: *O. aristatus*, 8: *E. applanata*, 9: *H. cordata*, 10: *P. asiatica*, 11: *S. sebeana*, 12: *F. microcarpa*, 13: *O. basilicum*.

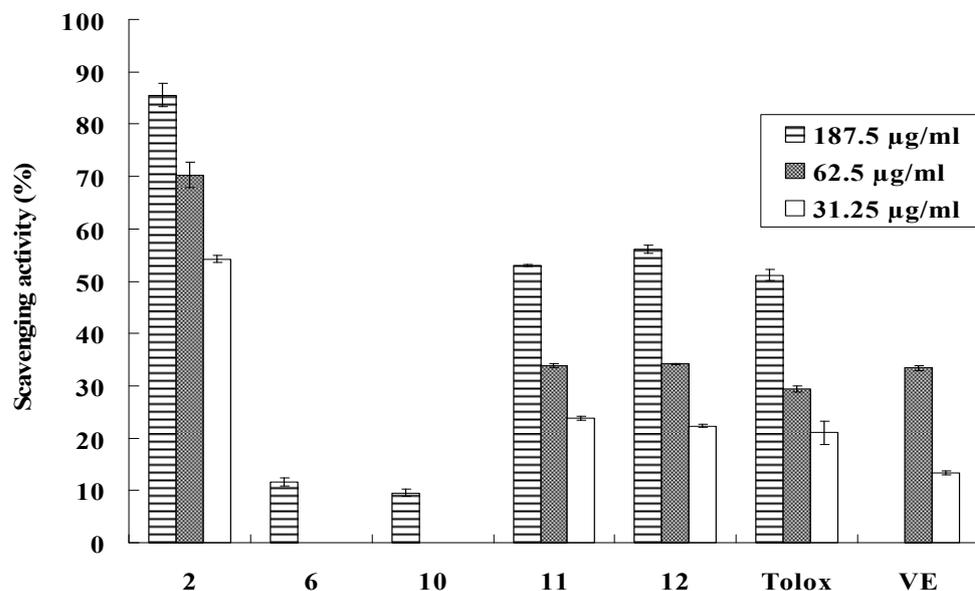


Fig. 2. Antioxidant activity evaluation of methanol extracts of 13 medicinal plants using the superoxide radical scavenging activity method; the values represent means \pm S. E. (n=3). 2: *A. zerumbet*, 6: *C. roseus*, 10: *P. asiatica*, 11: *S. sebeana*, 12: *F. microarpa*.

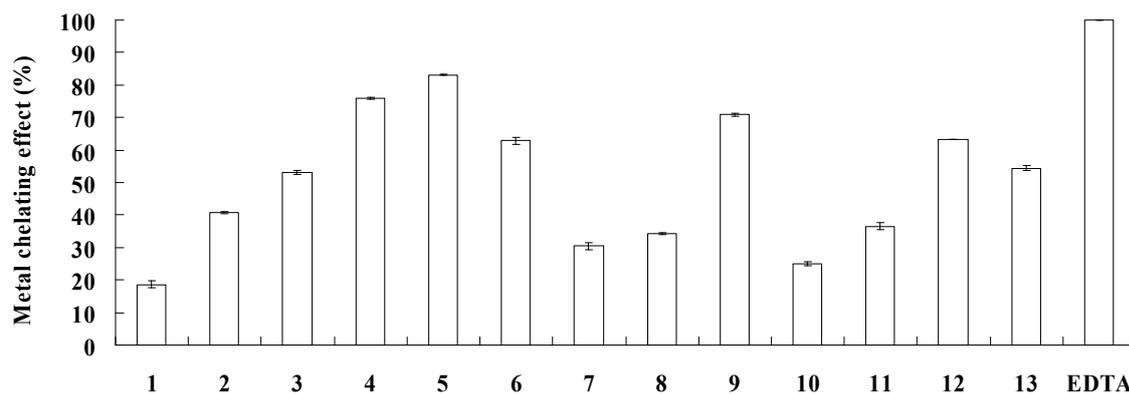


Fig. 3. Antioxidant activity of methanol extracts of 13 medicinal plants using the metal chelating effect method; the values represent means \pm S. E. (n=3); the number 1-13 were described in the legend of Fig. 1.

3.3.4. Chemical composition and biological activity

Because *C. longa*, *O. basilicum* and *C. aromatica* showed greater MMP-13 inhibitory properties than other plant species, they were therefore analyzed by LC-MS to determine potential chemicals involved in the biological activities as mentioned above. By comparing the retention time, MS spectra and UV with those of standards, and from literature (Bais *et al.*, 2002; Jayaprakasha *et al.*, 2005), curcumin were identified in *C. longa* and *C. aromatica*, and rosmarinic acid was detected in *O. basilicum* (**Fig. 4**). However, curcumin and rosmarinic acid can not be detected in other ten species plants by LC-MS. The contents of curcumin and rosmarinic acid were also quantified by LC-MS. As showed in **Table 3**, curcumin was the major compounds as they exist in rather high quantities in *C. longa* (58.6 mg/g extract) and *C. aromatica* (28.7 mg/g extract), as rosmarinic acid was also the main compound in *O. basilicum* (47.3 mg/g extract).

Table 3. The contents of curcumin in *C. longa* and *C. aromatica* and rosmarinic acid in *O. basilicum*

Plants	Compounds content (mg/g methanol extract)	
	Curcumin	Rosmarinic acid
<i>C. longa</i>	58.6 ± 1.1	ND
<i>C. aromatica</i>	28.7 ± 0.8	ND
<i>O. basilicum</i>	ND	47.3 ± 1.0

The values represent means ± S. E. (n=3); ND: not detected.

The MMP-13 inhibitory activity *in vitro* of curcumin and rosmarinic acid were examined and shown in **Table 2**. Both two compounds exhibited high inhibitory effect (IC₅₀, 3.6 and 2.9 μM, respectively). Obviously, rosmarinic acid exerted higher

inhibitory activity than that of curcumin, while two compounds exhibited dose-dependent activity. However, the inhibitory strength against the MMP-13 of the two natural compounds was lesser than that of the positive control CBC.

The rhizomes of the *C. longa* and *C. aromatica* have been long used as indigenous medicines for the treatment of a variety of inflammatory conditions and other diseases. Chemical composition analysis research showed that the main component present in the rhizome of *C. longa* includes three pyrazole analogues of curcuminoids (curcumin, monodemethoxycurcumin and bisdemethoxycurcumin) (Ramsewak *et al.*, 2000, Jayaprakasha *et al.*, 2005). Hong *et al.* (2006) also reported the content of curcumin in *C. longa* and *C. aromatica* (3.6% and 0.3%, respectively). Curcumin was an active constituent of *C. longa* and *C. aromatica*, which were used in clinical Chinese medicine as aromatic stomachic and choleric (Ching *et al.* 2001). Curcumin and related species have a wide array of pharmacological and biological activities. Ramsewak *et al.* (2000) and Selvam *et al.* (2005) found that *C. longa* extract and curcumin exhibited significant COX-1 and COX-2 inhibitory activity *in vitro*. Cytotoxicity, antioxidant, anti-inflammatory and anti-cancer activities of curcumin have been assessed in various assays (Jayaprakasha *et al.*, 2005; Maheshwari *et al.*, 2006; Johnson *et al.*, 2007). Except curcumin, other compounds in extract of *C. longa* might be also responsible for its MMP-13 inhibitory and antioxidant activities. However, in this study, the two substances monodemethoxycurcumin and bisdemethoxycurcumin were not quantified and examined for their biological activities as they could neither been purchased nor successfully isolated in our laboratory. In addition, *O. basilicum* is also an important medicinal plant and culinary herb and is marketed worldwide (Loughrin *et al.*, 2001). The extract of *O. basilicum* leaves showed inhibitory activity against HIV-1 reverse

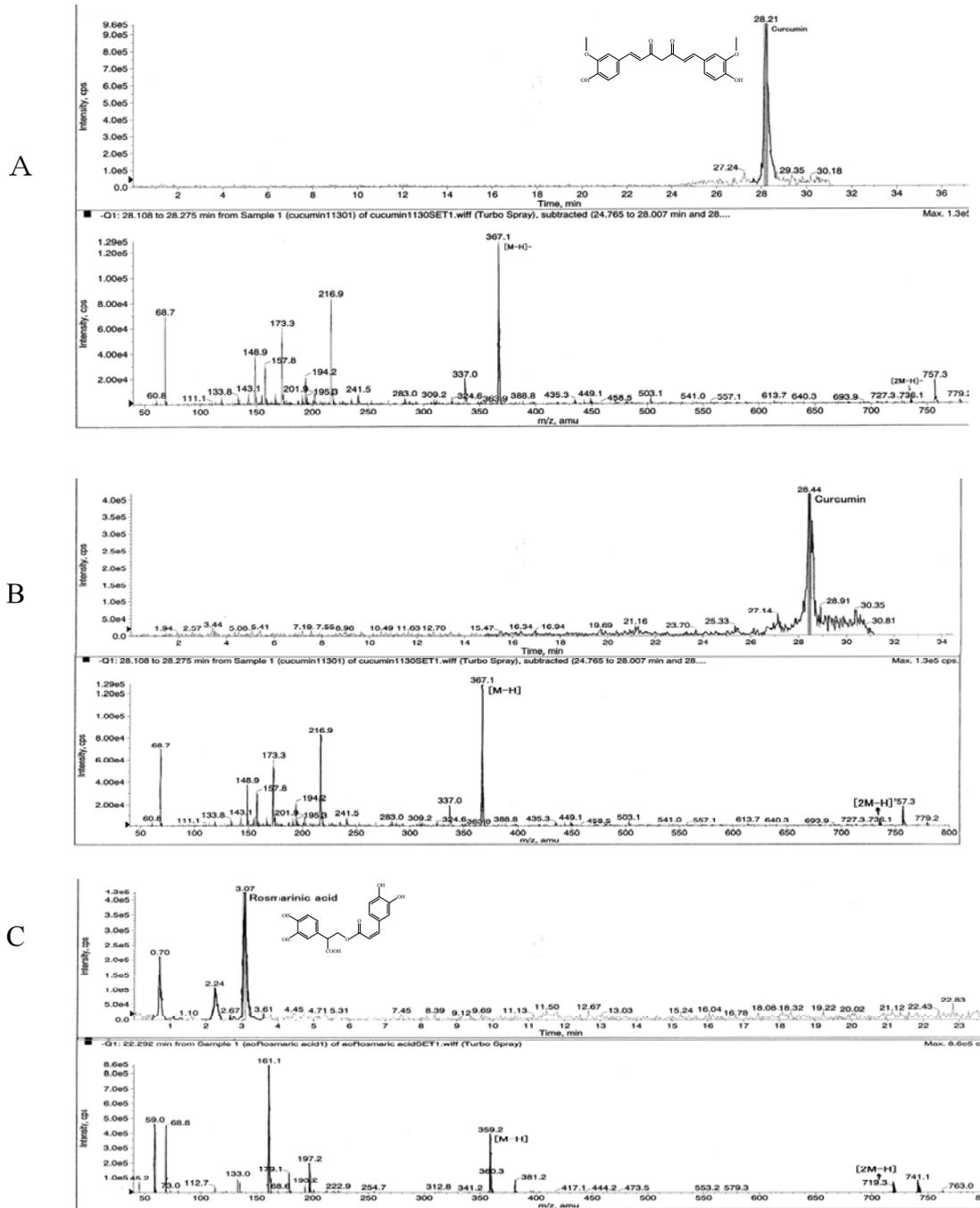


Fig. 4. The LC-MS chromatograph of curcumin in methanol extracts of *C. longa*. (A), *C. aromatica* (B) and rosmarinic acid in methanol extracts of *O. basilicum* (C).

transcriptase (Yamasaki *et al.*, 1998). Rosmarinic acid was one of the most abundant caffeic acid esters present in *O. basilicum* (Jayasinghe *et al.*, 2003), and this compound have been reported to have antioxidant, anti-HIV, and anti-inflammatory or cyclooxygenase and lipoxygenases inhibitory activities (Kelm *et al.*, 2000; Petersen *et al.*, 2003). These evidences indicated that curcumin and rosmarinic acid have been shown to possess wide range of pharmacological activities.

3.4. Conclusion

Among the 13 Okinawa medicinal plants, the extracts of *C. longa*, *O. basilicum* and *C. aromatica* showed high inhibitory effect against MMP-13, and remarkable DPPH radical scavenging, reducing power, and metal chelating abilities. In addition, *C. longa* has the highest phenolic content among the screened plants. Curcumin and rosmarinic acid showed promising MMP-13 inhibitory activity and may be responsible for the strong MMP-13 inhibitory and antioxidant activities of the related plants. *F. microcarpa* and *S. sebeana* exhibited strong antioxidant activities in all applied assays; it might also be attributed to their high level of phenolic compounds. The results shown here may provide some scientific evidence for the use of several medicinal plants from Okinawa, for treating tumours, inflammatory diseases and arthritis.

CHAPTER 4

Evaluation of antioxidant and antibacterial activities of

Extract from *Ficus microcarpa* L. fil.

Abstract

Antioxidant and antibacterial activities were evaluated by the extracts of *F. microcarpa*, which is one of the traditional medicinal plant and food additives in Okinawa, Japan. Results showed that methanol extracts of bark, fruits and leaves of *F. microcarpa* exhibited excellent antioxidant activities and also possessed antibacterial activity against tested Gram-positive and Gram-negative bacteria. Ethyl acetate fraction of bark extract (BE) exerted strong antioxidant and antibacterial effects and contained high amount of total phenolics (436 GAE mg/g extract). EC₅₀ values of BE were 4.83, 1.62 and 63.2 µg/ml in DPPH, ABTS^{•+}, superoxide radicals scavenging methods, respectively. Inhibition zones of BE against *Bacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Achromobacter polymorph* were 18.0, 15.5, 16.5, 16.0 and 8.0 mm, respectively. Twelve phenolic compounds were identified in BE fraction by GC-MS and HPLC analyses. The strong antioxidant and antibacterial activities of *F. microcarpa* bark extract may be attributed to its high level of phenolic compounds.

Keywords: *Ficus microcarpa*; Antioxidant activity; antibacterial activity; phenolic compounds; flavonoid

4.1. Introduction

Ficus microcarpa L. fil. (Chinese banyan tree, Moraceae) is a popular ornamental tree grown widely in many tropical regions of the world. It is native from Ceylon to India, Southern China, Ryukyu Islands, Australia, and New Caledonia (Wagner and Herbst, 1999). Its dried leaves, aerial roots and bark have been used as folk herbs for perspiration, alleviating fever and relieving pain in Okinawa Islands (Yosikawa, 1983, Tawata and Oota, 1985). Okinawa Soba (Okinawa Noodle) is a famous Okinawa traditional food which is made by kneading wheat with the lye of wood ash. The quality of the noodle depends on the used type of wood ash. In Okinawa, the *F. microcarpa* was one of the main kinds of trees used for the lye for preparing Okinawa Soba (Nakama, 2003). Previous research showed that the charcoal ash from *F. microcarpa* had the best quality for the Okinawa Soba (Yokuta, 1982). Many triterpenoids (lupenyl acetate, friedelin, glutinol, epifriedelinol, β -amyrin acetate, and β -amyrin), phenolic compounds (protocatechuic acid, marmesin, trans-catechin, 4, 5-dihydroblumenol) and isoflavones have been isolated from aerial roots, bark, leaves and fruits of *F. microcarpa* and some compounds showed piscicidal, germination inhibitory and antifungal activities (Higa *et al.* 1987; Higa *et al.* 1996; Kuo and Li, 1997; Kuo and Chiang, 1999, 2000). Furthermore, oleanonic acid, acetylbetulonic acid, betulonic acid, acetylursolic acid, ursolic acid, 3-oxofriedelan-28-oic acid and 3B-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 (Chiang *et al.* 2005). However, the antioxidant and antibacterial activities of this plant have not previously been reported.

In recent years, much research has focused on antioxidant activity of phenolic

compounds in traditional medicinal plants, and a positive correlation was observed between the high phenolic content and the strong antioxidant activity (Cai *et al.* 2004, Djeridane *et al.* 2006). Phenolic compounds can play an important role in preventing body cells from injuries by hydrogen peroxide, preventing cells and the organs of man from damage of unsaturated fatty acids by lipid peroxides and absorbing and neutralizing free radicals (Sroka and Cisowski, 2002,). Many of these phytochemicals possess significant antioxidant capacities that have been associated with lower occurrence and mortality rates of several human diseases.

In this study, the antioxidant and antibacterial activities, total phenolics, total flavonoids of methanol extracts of bark, leaves and fruits of *F. microcarpa* were investigated. As the ethyl acetate fraction of bark extract (BE) showed the strongest activity, it was therefore selected and analyzed by GC-MS and HPLC.

4.2. Materials and Methods

4.2.1. Plant material

Bark, fruits (tender green fruits) and leaves of *F. microcarpa* were collected from the campus of University of the Ryukyus, Okinawa, Japan.

4.2.2. Solvents and reagents

Potassium persulfate, β -carotene, 2,2-azinobis (3-ethylbenzothiazoline-6-fulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ampicillin, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Nacalai Tesque, Japan, and Sigma, Germany. Gallic acid, rutin, Folin-Ciocalteu reagent, sodium

carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, catechol, *p*-vinylguaiacol, syringol, *p*-propylphenol, vanillin, syringaldehyde and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

4.2.3. Preparation of the methanol extract

The dried (60°C, 48 h) and finely ground samples of bark, leaves and fruits (6 g each) were separately extracted with 35 ml of 100% methanol for 12 h at room temperature with shaking. After filtration, the plant materials were extracted twice in the same conditions. The methanol extracts obtained from each sample were collected, filtered, dried under vacuum and then re-dissolved in methanol and stored under refrigeration for further analysis. The extraction yields of the methanol extracts of *F. microcarpa* are shown in **Table 1**.

Table 1. Extraction yields of bark, leaves and fruits of *F. microcarpa* extracts obtained under methanol soaking system

Samples	Raw material (g)	Extract powder (g)	Extraction yield (%)
Bark	6.00	0.93	15.5
Leaves	6.00	0.84	14.0
Fruits	6.00	0.71	11.8

4.2.4. Purified extract

Sixty-five grams of the dried powdered bark were extracted with methanol (3 times with a total of 800 ml of methanol) for 24 h at room temperature. The methanol extracts were collected, filtered and dried under vacuum to yield 7.1 g. The dried methanol

extract was suspended in 10% aqueous methanol and was successively extracted with n-hexane and ethyl acetate. The obtained extracts, in addition to the aqueous solution remaining after extraction, were filtered and concentrated under vacuum to receive 1.6, 0.5 and 4.9 g of hexane (BH), ethyl acetate (BE) and aqueous (BW) fractions, respectively. Each extract was dissolved in methanol and refrigerated for further experiments.

4.2.5. DPPH radical scavenging assay

Free radical scavenging activity of extracts was determined using the DPPH method as described previously in **Chapter 3**.

4.2.6. Total antioxidant activity (ABTS assay)

The total antioxidant activity values of *F. microcarpa* extracts were measured by the improved ABTS^{•+} method as described by Baltrusaityte *et al.* (2007) with minor modification. In this test, ABTS^{•+} radical cation was generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate (K₂S₂O₈) after incubation at room temperature in the dark condition. Before used, the ABTS^{•+} solution was diluted with 80% ethanol to get an absorbance of 0.700 ± 0.050 at 734 nm, the spectrophotometer was preliminary blanked with 80% ethanol. ABTS^{•+} solution (3.9 ml, absorbance of 0.700 ± 0.050) was added to 0.1 ml of the tested samples 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:

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

4.2.7. PMS-NADH system superoxide-radical scavenging assay

The superoxide scavenging ability of extracts was assayed as described previously in **Chapter 2**.

4.2.8. β -carotene/linoleic acid system antioxidant assay

Antioxidant activity was performed according to the method described by Elzaawely, Xuan and Tawata (2005) with minor modifications. An aliquot of 1.0 mg β -Carotene was dissolved in 10 ml CHCl_3 . Linoleic acid 40 mg and 400 mg Tween-40 were mixed with 1 ml of the CHCl_3 solution. The CHCl_3 was evaporated under vacuum at 45°C for 4 min, then 100 ml oxygenated water was added, and the mixture was vigorously shaken. The β -Carotene –linoleic acid emulsion (330 μl) was distributed in a 96-well microplate. Methanolic solutions (14 μl , 125 $\mu\text{g}/\text{ml}$) of the sample extracts and positive control Trolox were added into the plate. An equal amount of methanol was used as control. Absorbance was read at 492 nm after incubation for every 15 min until 180 min at 45°C using a microplate reader (Benchmark plus microplate 170-6930j1, BIO-RAD company). Tests were carried out in triplicate. The antioxidant activity of the extracts and Trolox were evaluated in terms of the bleaching of β -Carotene using the following formula:

$$\text{Antioxidant activity (\%)} = [1 - (\text{OD}_0 - \text{OD}_{180} / \text{OD}_0^* - \text{OD}_{180}^*)] \times 100$$

Where OD_0 and OD_0^* are the absorbance values measured at zero incubation time for the test samples and control, respectively, and OD_{180} and OD_{180}^* are the corresponding absorbance values measured after incubation for 180 min.

4.2.9. Amount of total phenolic compounds

Total phenolics in the methanol extracts of bark, leaves and fruits of *F. microcarpa* and sub-fractions of bark extract was measured using the Folin-Ciocalteu reagent method as described previously in **Chapter 3**.

4.2.10. Total flavonoids content

The amount of total flavonoids in the extracts was measured spectrophotometrically according to the method reported by Djeridane *et al.* (2006). This method based on the formation of a complex flavonoid-aluminium, having the absorbance maximum at 430 nm. Rutin was used to make a calibration curve. One ml of methanolic extract (500 $\mu\text{g/ml}$) was mixed with 1 ml of 2% AlCl_3 methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with a Shimadzu UV-Vis spectrophotometer (Mini 1240). The flavonoids content was expressed as rutin equivalents in mg per g extract.

4.2.11. Antibacterial activity

Antibacterial activities of extracts from *F. microcarpa* and positive control ampicillin were tested against *Bacillus subtilis*, *Bacillus cereus*, *Bacillus brevis*, *Achromobacter polymorph*, *Mycobacterium avium*, and *Escherichia coli* by using a disc diffusion method introduced by Jenny *et al.* (2003) with minor modifications. All bacterial strains have been kindly provided by Laboratory of Microbiology, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan. The 24 h test bacterial broth culture (1×10^6 organisms/ml) with 0.5 ml was poured evenly over the surface of 9 cm

diameter Petri dishes containing nutrient agar media 15 ml. The plates were placed at 37°C for approximately 30 min until bacterial overlay have been dried on the surface. Four filter paper discs (6 mm diameter, Whatman) were placed on the surface of one Petri dish. Forty μ l of test samples (10 mg/ml for samples and 0.1 mg/ml for ampicillin) that were dispersed in distilled water then sterilized by filtration through a 0.2 μ m membrane filter and gently injected on the disk. The plates were subsequently incubated at the 37°C for 24 h. Zones of inhibition were calculated by measuring diameter in mm (including disk). Experiments were performed in duplicate and the inhibition zones were compared with those of ampicillin and sterile distilled water.

4.2.12. GC-MS analysis

The BE fraction was analyzed by GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan) equipped with 30-m \times 0.25 mm DB-5MS column (Agilent Technologies, J&W Scientific Products, Folsom, CA). The carrier gas was helium. The temperature program was set as follow: 100°C hold for 5 min, raised at 4°C /min to 280°C, and hold for 5 min. The injector and detector temperatures were set at 250°C and 280°C, respectively. The ion source and interface temperatures were set at 200°C and 250°C, respectively. The mass range was scanned from 50 to 900 amu. The control of the GC-MS system and the data peak processing were controlled by means of Shimadzu's GC-MS solution software, version 2.4. Compound identification was verified based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST147. LIB. database of the GC-MS system (Shimadzu).

4.2.13. HPLC analysis

Phenolic compounds in BE were measured at 280 nm by a Shimadzu HPLC

(SCL-10 A vp, Shimadzu Co., Japan). Separation was carried out on a Synergi 4u Hydro-RP 80A column (150 mm × 4.60 mm, Phenomenex Company, USA). A gradient elution was performed with solvent 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 μ l. Identification of the phenolic compounds was carried out by comparing their retention times to those of standards.

4.2.14. Statistical analysis

The statistical analyses were performed by a one-way ANOVA and the Student's *t*-test. The results were expressed as the mean \pm SE. to show variations in the various experimental. Differences are considered significant when $P < 0.05$.

4.3. Results

4.3.1. Antioxidant activities

The methanol extracts prepared from bark, fruits and leaves of *F. microcarpa* exhibited strong antioxidant activity assayed by the 4 different methods including DPPH and ABTS^{•+} free radicals scavenging, PMS-NADH system superoxide radical scavenging and β -carotene-linoleic acid system (Table 2 and Fig. 1). The methanol extract of bark showed stronger antioxidant activity than those of leaves and fruits in ABTS^{•+}, PMS-NADH and β -carotene/linoleic acid system, however no significant difference was found between bark and fruits in DPPH assay.

Table 2. Antioxidant effect (EC₅₀) of *F. microcarpa* extracts in DPPH radical scavenging, ABTS⁺ radical scavenging and PMS-NADH superoxide radical scavenging assays

extracts	EC ₅₀ (µg/ml)		
	DPPH assay	ABTS ⁺ assay	PMS-NADH assay
Bark	7.9 ± 0.1d	4.0 ± 0.0d	97.5 ± 2.8d
Fruits	7.3 ± 0.0d	9.2 ± 0.1c	179 ± 2.8c
Leaves	21.4 ± 0.1b	10.2 ± 0.0b	223 ± 2.2b
BH	379 ± 13.0a	265 ± 10.1a	>1000a
BE	4.8 ± 0.0e	1.6 ± 0.0e	63.2 ± 1.3f
BW	11.6 ± 0.1c	4.0 ± 0.0d	83.9 ± 2.8e
Trolox	4.4 ± 0.0e	0.5 ± 0.0f	181 ± 5.8c

Concentration of samples in assays was expressed as final concentration; Superscript letters with different letters in the same column indicate significant difference (P<0.05). Each value in the table was expressed as mean ± SE (n=3). (BH, BE and BW were hexane, ethyl acetate and water fractions of bark extract of *F. microcarpa*, respectively).

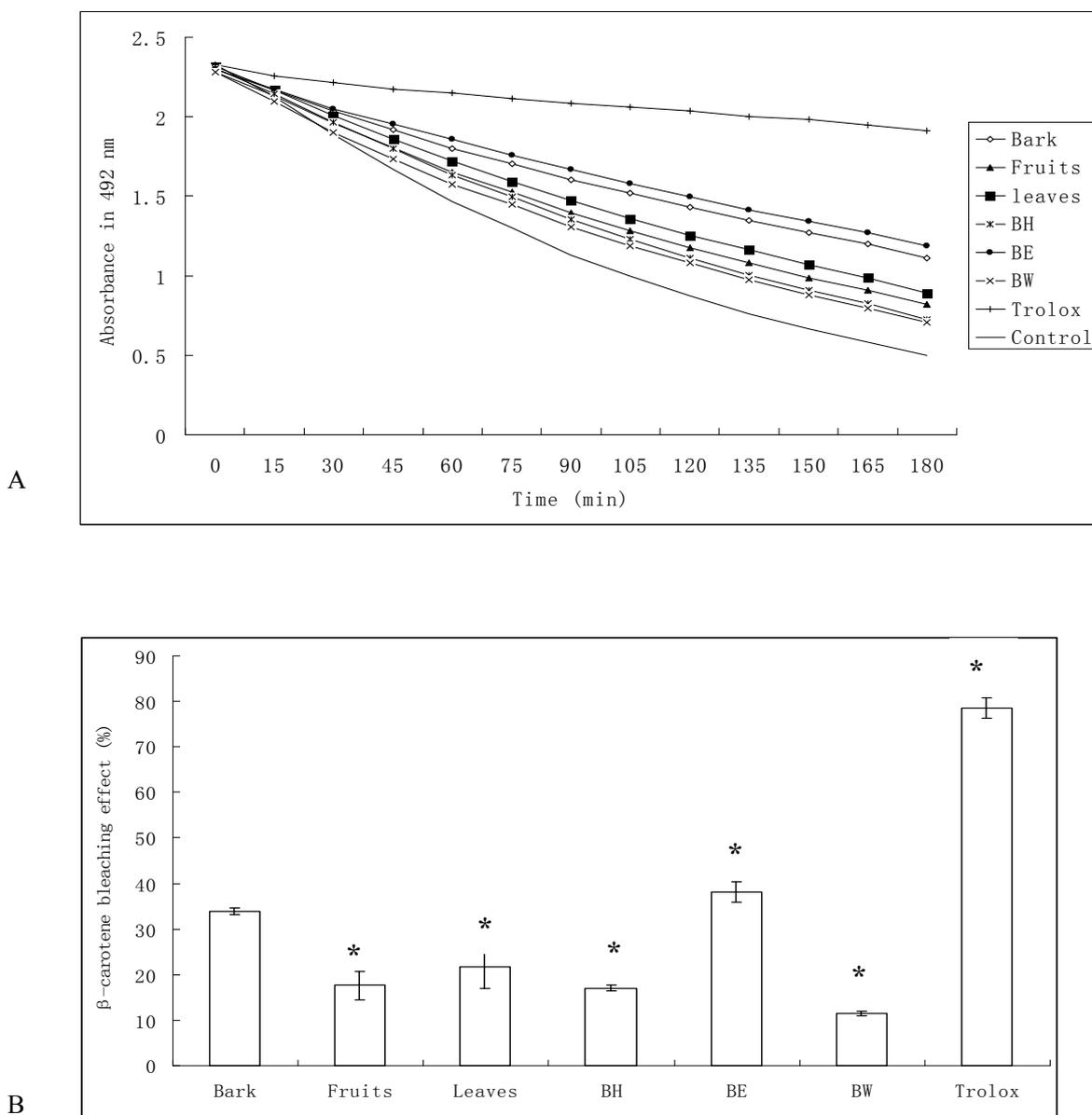


Fig. 1. Antioxidant effect of the methanol extracts of bark, leaves and fruits and sub-fractions of bark extract of *F. microcarpa* on β -carotene linoleate model system assays; (A) kinetics of β -carotene bleaching effect by samples as measured by decrease of absorption at 492 nm during 180 min; (B) β -carotene bleaching effect by samples after incubation 180 min at 45 °C; The values represent means \pm S. E. (n=3); * p <0.05 vs. methanol extract of bark (BH, BE and BW were hexane, ethyl acetate and water fraction of bark extract respectively).

As bark extract possessed higher antioxidant activity than other parts, it was further separated into fraction BH, BE and BW. Among these three fractions, fraction BE was the most effective in scavenging DPPH and ABTS^{•+} free radicals, scavenging superoxide radicals generated in PMS-NADH system. Its EC₅₀ values in these 3 assays were 4.83, 1.62 and 63.2 µg/ml, respectively. Trolox was used as positive control and its corresponding EC₅₀ values were 4.39, 0.54 and 264 µg/ml, respectively (**Table 2**).

As showed in **Table 3**, catechol exhibited a very strong radical scavenging activity (EC₅₀: 1.28 and 114 µg/ml for DPPH and PMS-NADH assays, respectively), which was higher than that displayed by Trolox. Likewise, *p*-vinylguaiacol and syringol effectively scavenged DPPH free radicals (EC₅₀: 8.76 and 5.44 µg/ml, respectively). On the contrary, vanillin did not show any antiradical activity.

Table 3. Antioxidant effect (EC₅₀) of standard phenolic compounds in DPPH radical scavenging, and PMS-NADH superoxide radical scavenging assays

Samples	EC ₅₀ (µg/ml)	
	DPPH assay	PMS-NADH assay
Catechol	1.3 ± 0.0	114 ± 7.9
Vanillin	>1000	425 ± 23.3
Syringaldehyde	74.1 ± 0.7	274 ± 26.9
<i>p</i> -Propylphenol	320 ± 3.0	>1000
<i>p</i> -Vinylguaiacol	8.8 ± 0.0	>1000
Syringol	5.4 ± 0.0	>1000

Each value in the table was expressed as mean ± SE (n=3).

4.3.2. Total phenolic compounds and flavonoids content

As one of the most important antioxidant plant components, phenolic compounds are widely investigated in many medicinal plant and vegetables (Yizhong et al. 2004, Djeridane et al. 2006). The amounts of total phenolic compounds and total flavonoids in the methanol extracts of bark, fruits and leaves as well as BH, BE and BW fractions prepared from the bark of *F. microcarpa* are shown in **Table 4**. The bark contained a significantly higher amount of total phenolics than fruits and leaves (237, 179 and 127 mg GAE/g extract, respectively). Furthermore, total phenolics were accounted in greater amounts in BE than BW and BH fractions prepared from the bark and were 436, 194 and 41.7 mg GAE/g extract, respectively.

Table 4. Total phenolic and flavonoid content in *F. microcarpa* extracts

Samples	Total phenolic content as gallic acid equivalents (GAE mg/g extract)	Total flavonoid content as rutin equivalents (mg/g extract)
Bark	237 ± 0.6b	6.3 ± 0.03c
Fruits	179 ± 6.8d	6.1 ± 0.03c
Leaves	128 ± 1.2e	9.4 ± 0.6a
BH	41.7 ± 1.0f	1.5 ± 0.1e
BE	436 ± 2.1a	8.0 ± 0.1 b
BW	194 ± 0.8c	2.0 ± 0.1d

Each value in the table is represented as mean ± SE (n=3); superscript letters with different letters in the same column indicate significant difference (P<0.05); BH, BE and BW are hexane, ethyl acetate and water fraction of *F. microcarpa* bark extract respectively.

4.3.3. Relationship between antioxidant activity and total phenolic content

The antioxidant activity and total phenolic content of *F. microcarpa* extracts were positively correlated (**Fig. 2 A, B, C and D**). Values of the correlation coefficient (R^2) were 0.919, 0.836, 0.813 and 0.534 for ABTS, DPPH, PMS-NADH, β -carotene bleaching methods, respectively. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *F. microcarpa* extracts.

4.3.4. Antibacterial activity

As summarized in **Table 5**, methanol extracts of bark, fruits and leaves showed antibacterial activity against both Gram-positive bacteria (*B. brevis*, *B. cereus*, and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *A. polymorph*). The bark extract possessed significantly higher antibacterial activity than fruits and leaf extracts. However, no antibacterial activity was observed from methanol extracts of bark, fruits and leaves against *M. avium*, BE fraction that was separated from the bark was significantly active against all bacterial strains including *M. avium*.

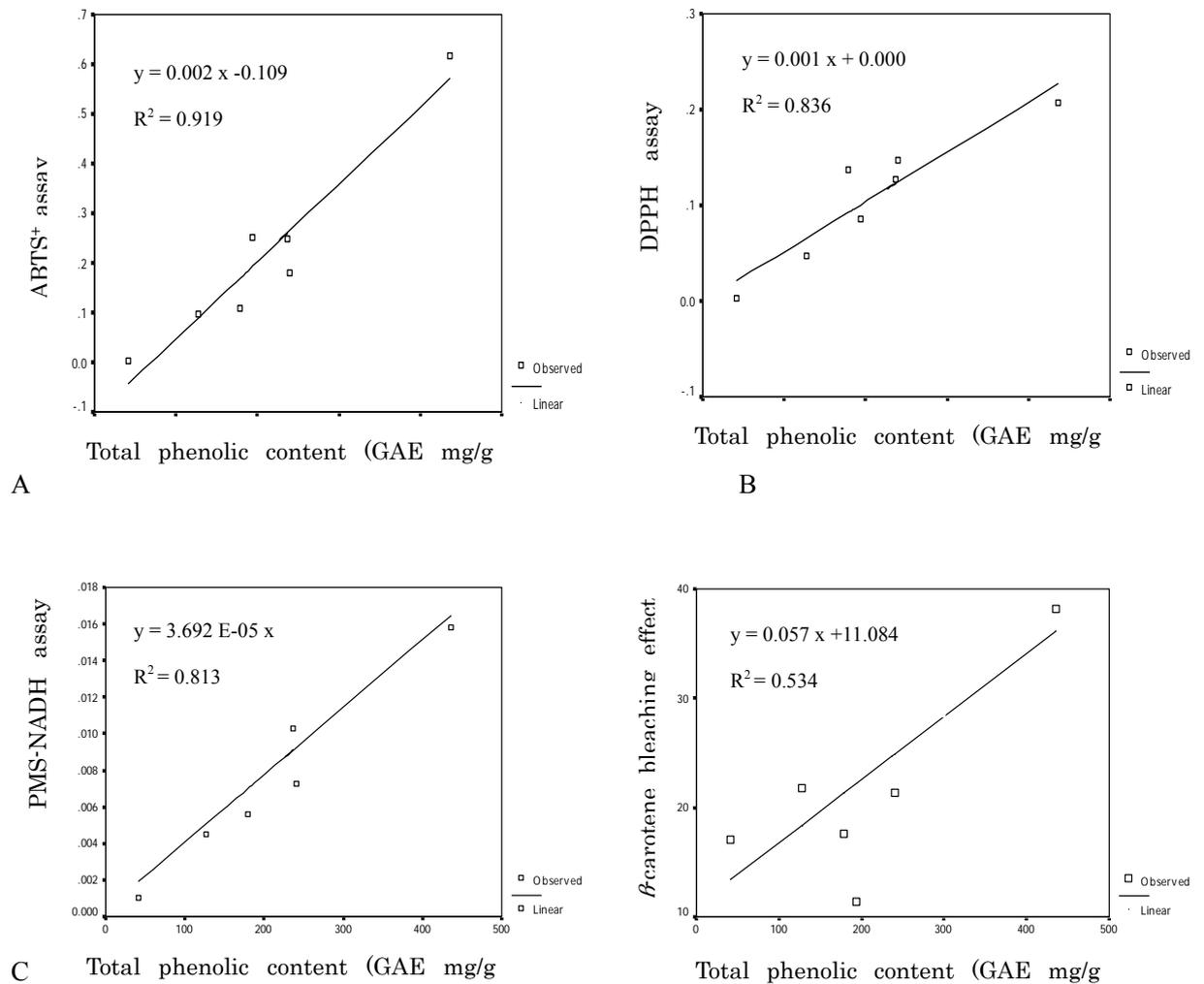


Fig. 2. Correlation between total phenolic content as gallic acid equivalents (GAE mg/g extract) of *F. microcarpa* extracts and their antioxidant capacity as determined by ABTS⁺ assay (A), DPPH assay (B), PMS-NADH assay (C) and β -carotene bleaching effect (D).

Table 5 Antibacterial activity of different extracts^a of *F. microcarpa* and ampicillin^b using disc diffusion method (inhibition zones, mm)

Bacteria	Bark	Fruits	Leaves	BE	Ampicillin
<i>Bacillus brevis</i>	17.5 ± 2.5b	11.3 ± 0.0c	11.9 ± 1.4c	18.2 ± 2.4b	34.5 ± 0.5a
<i>Bacillus cereus</i>	14.7 ± 1.2a	13.8 ± 1.0a	10.9 ± 0.1b	15.5 ± 2.5a	—
<i>Bacillus subtilis</i>	15.3 ± 1.3b	12.9 ± 0.1c	12.0 ± 0.5c	16.8 ± 1.8b	30.0 ± 0.0a
<i>Escherichia coli</i>	17.8 ± 0.8b	11.2 ± 0.4c	7.5 ± 0.0d	16.0 ± 0.5b	27.0 ± 0.5a
<i>Mycobacterium avium</i>	—	—	—	7.3 ± 0.8b	34.5 ± 0.5a
<i>Achromobacter polymorph</i>	8.0 ± 1.0b	—	—	7.3 ± 0.8b	21.0 ± 2.5a

Each value in the table is represented as mean ± SE (n=2); Superscript letters with different letters in the same low indicate significant difference (P<0.05); ^a 400 µg/disc of each extract (dispersed in water) used to load the sterile disc; ^b 1 µg/disc of ampicillin (dissolved in water) was used to load the sterile disc; “—” indicated no inhibition zone.

4.3.5. Analysis of ethyl acetate fractions by GC-MS and HPLC

By activity-guided fractionation, ethyl acetate fraction (BE) was separated from the bark. Since this fraction (BE) contained the highest phenolic content and showed the strongest antioxidant and antibacterial activities, it was therefore analyzed by GC-MS and HPLC to determine its chemical composition that may contribute to this activity. The GC-MS analysis showed that BE contained a variety of phenolic compounds (Fig. 3). By comparing the MS spectral data with those of standards and MS library, twelve phenolic compounds were identified (Table 6). Of which, the contents of seven

phenolic compounds including protocatechuic acid, catechol, *p*-vinylguaiacol, syringol, *p*-propylphenol, vanillin and syringaldehyde were quantified in BE fraction by HPLC (Table 7).

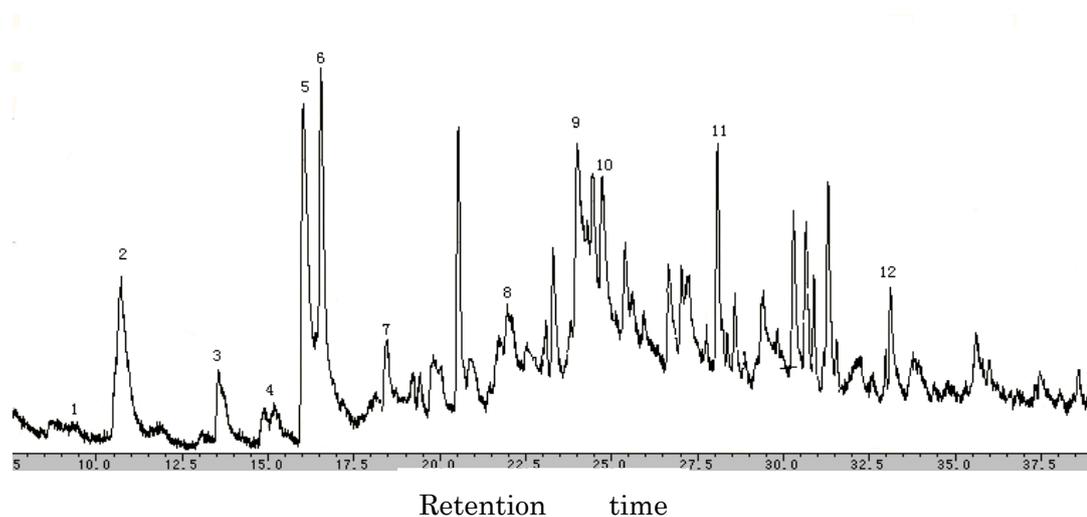


Fig. 3. GC-MS chromatogram of BE from *F. microcarpa*. 1. Catechol; 2. Coumaran; 3. *p*-Vinylguaiacol; 4. Syringol; 5. *p*-Propylphenol; 6. Vanillin; 7. *p*-Propylguaiacol; 8. Iovanillic acid; 9. 4-n-Propylresorcinol; 10. Syringaldehyde; 11. Protocatechuic acid; 12. Oleanolic acid.

Table 6. Chemical composition of phenolic compounds detected by GC-MS in BE fraction

No	Compound	Retention time (min)	Molecular weight	Major peaks
1	Catechol	10.42	110	110, 92, 81, 64
2	Coumaran	10.7	120	120, 91, 63, 39
3	<i>p</i> -Vinylguaiacol	13.6	150	150, 135, 107, 77
4	Syringol	14.9	154	154, 139, 111, 93
5	<i>p</i> -Propylphenol	16.0	136	136, 107, 77, 39
6	Vanillin	16.5	152	152, 123, 109, 81
7	<i>p</i> -Propylguaiacol	18.5	166	166, 137, 122, 94
8	Isovanillic acid	22.0	168	168, 153, 125, 97
9	4- <i>n</i> -Propylresorcinol	24.0	152	152, 123, 77
10	Syringaldehyde	24.7	182	182, 167, 152, 123
11	Protocatechuic acid	28.1	154	154, 137, 81, 63
12	Oleanolic acid	33.1	456	456, 423, 410, 203

Table 7. Phenolic compounds content of BE fraction of *F. microcarpa* by HPLC

Compound	Content mg/g extract
Protocatechuic Acid	6.6 ± 0.2
Catechol	11.1 ± 0.00
Vanillin	4.27 ± 0.02
Syringaldehyde	8.96 ± 0.29
Syringol	173 ± 1.12
<i>p</i> -Vinylguaiacol	4.40 ± 0.07
<i>p</i> -Propylphenol	10.5 ± 0.78

Each value in the table is represented as mean ± SE (n=3).

4.4. Discussion

The results in this study revealed that the methanol extracts from bark, fruits and leaves of *F. microcarpa* showed strong antioxidant and antibacterial effects and contained high amounts of phenolic compounds. These findings may provide a pharmacological explanation for some of its uses in folk medicine in Ryukyu Islands. It has been reported that free radical scavenging and antioxidant activity of many medicinal plants are responsible for their therapeutic effect against cancer, tissue inflammatory, cardiovascular disease (Cai *et al.* 2004). Thus, the antipyretic and analgesic action of *F. microcarpa* may be associated with its antioxidant activity. Previous research suggested that other species of the genus *Ficus* (*Moraceae*) such as *F. racemosa* and *F. carica* possessed various biological activities including antioxidant

effects. Racemosic acid isolated from *F. racemosa* showed potent inhibitory activity against COX-1 and 5-LOX *in vitro* and demonstrated strong antioxidant activity (Li et al. 2004). The methanol extract of *F. racemosa* showed a significant antitussive activity of 56.9 % inhibition at a dose of 200 mg/kg (Bhaskara et al. 2003). Leaf extracts of *F. carica* exhibited antioxidant capacity and had a high phenolic content (Konyalioglu et al. 2005).

The methods employed in this study are simple and provided reproducible results showing antioxidant properties of *F. microcarpa* extracts. It was important to examine the correlation between the content of the main antioxidant compounds (total phenolics) and the antioxidant capacity of the studied extracts. The correlation of the total phenolic content and the antioxidant capacity as determined by tree used tests was positively high. These data were in accordance with previous research (Gorinstein et al. 2003; Maisuthisakul et al. 2007), which have shown that high total polyphenols content increases antioxidant activity and there was a linear correlation between phenolic content and antioxidant activity. Six phenolic compounds were identified in BE fraction and showed high DPPH radical scavenging capacity except for vanillin. These results were in agreement with previous results documented by Bortolomeazzi et al. (2007). Phenolic compounds have been extensively investigated since the past 30 years. They have one or more aromatic rings bearing hydroxyl groups that are potentially able to act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Rice-Evans et al. 1996; Mattei et al. 1998). However, compounds other than phenolics in the ethyl acetate fraction may also involve in the antioxidant properties of *F. microcarpa*. Rodri'guez et al. (2007) found many phenolic compounds in wines possessing high antibacterial effect. Other research found that guarana seed methanol

extract had high antibacterial activity, when this methanol extract also possessed high phenolics content (Majhenič *et al.* 2007). These are in agreement with our study. Previous research carried out on *F. microcarpa* reported the presence of several triterpenoids such as oleanolic acid, rusolic acid, α -hydroxyursolic acid, protocatechuic acid and maslinic acid in the fruits, aerial roots and bark (Higa *et al.* 1987; Higa *et al.* 1996; Kuo and Chaiang, 1999, 2000). It has been shown that ursolic acid, oleanoic acid and other triterpenoids were efficient protectors against lipid peroxidation (Adhikari *et al.* 2006; Assimopoulou *et al.* 2005).

The fruit of this plant are small and are eaten by numerous fruit eating birds and possible other animals such as bats, rodents and parrots. Torymid wasp, one kind of insect also attacks the fruits of *F. microcarpa*, which was first found in 1976 (Starr *et al.* 2003). These evidences indicated that extracts from *F. microcarpa* could be used as a potential antioxidant and antibacterial resource.

As far as we know, this is the first study concerning the antioxidant capacity, antibacterial activity and total phenolic content of *F. microcarpa*. Since this plant is distributed in a large quantity in many tropical regions of the world and its bark extract showed a significant antioxidant and antibacterial activities, our work suggest that the bark of *F. microcarpa* may be utilized as effective and safe antioxidant and antibacterial source. However, searching for further bioactive compounds which are responsible for the biological activities of *F. microcarpa* is needed.

CHAPTER 5

**Biological activity and composition of extract from aerial root
of *Ficus microcarpa* L. fil.**

Abstract

Total phenolic content, antioxidant and antibacterial activities of *Ficus microcarpa* aerial roots were determined on methanol extract and its sub-fractions (hexane, ethyl acetate, n-butanol and water). The ethyl acetate fraction possessed the highest amount of phenolic compounds evaluated by Folin-Ciocalteu reagent method. It also showed the highest antioxidant activity when tested the following methods: DPPH, ABTS^{•+} radical-scavenging, superoxide radical scavenging, reducing power and β -carotene linoleic acid bleaching. On the other hand, high phenolics content extracts (ethyl acetate, methanol and n-butanol) showed significantly higher inhibitory activity than low phenolics content extracts (water and hexane) against five tested Gram positive and Gram negative bacteria. Protocatechuic acid, catechol, *p*-vinylguaiacol, syringol, *p*-propylphenol, vanillin, *p*-propylguaiacol, isovanillic acid, 4-n-Propylresorcinol, syringaldehyde and oleanolic acid contained in ethyl acetate fraction were identified by GC-MS and HPLC based on their cochromatography with standard compounds. Protocatechuic acid, catechol and syringol exhibited nearly same antioxidant activity as did by positive control Trolox using the mentioned assays. Results here showed that the ethyl acetate fraction has strong antioxidant and antibacterial activities, which are correlated with its high level of phenolic compounds.

Key words: *Ficus microcarpa* aerial root; phenolics; antioxidant; antibacteria activity

5.1. Introduction

Medicinal plants derived phytochemical preparations with dual functionalities in preventing lipid oxidation and microbial spoilage have tremendous potential for extending shelf-life of food products with minimal use of synthetic preservatives. Food-grade phosphates, lactates and flavouring agents have long been known to possess dual functions in preserving foods (Raccach, 1984). Some natural phenolic antioxidants, such as caffeic acid, *p*-coumaric acid, chlorogenic acid and ferulic acid exhibited antimicrobial activity (Garrote *et al.*, 2004). Many researchers have investigated the dual functions of plants extracts that can preserve food quality and safe to the consumers. The essential oil and methanol extract of endemic *Zataria multiflora* Boiss showed excellent antioxidant and antibacterial activity (Sharififar *et al.*, 2007). Wong *et al.*, (2006) have evaluated the antioxidant and antibacterial activity of methanol and water extracts from parsley and cilantro, two kinds of culinary herbs and confirmed that the phenolic compounds extracted from these plants were responsible for their dual functional activities in preventing lipid oxidation and microbial spoilage.

In our screening program looking for potential biological activities of plants from Okinawa islands, it was found that *F. microcarpa* showed high antioxidant activity in our preliminary work.

The aims of this study were to evaluate the total phenolic content, antioxidant and antibacterial activities of aerial root extracts of *F. microcarpa*, and to examine the relationship between total phenolic content, antioxidant and antibacterial activities. The fraction that exhibited the highest activities was further analyzed and identified by GC-MS and HPLC.

5.2. Materials and Methods

5.2.1. Plant material

Aerial roots of *F. microcarpa* were collected from the campus of University of the Ryukyus, Okinawa, Japan.

5.2.2. Solvents and reagents

Potassium persulfate, β -carotene, 2,2-azinobis (3-ethylbenzothiazoline-6-fulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ampicillin, potassium ferricyanide, trichloroacetic acid, FeCl_3 and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Sigma Aldrich, Germany. Gallic acid, rutin, Folin-Ciocalteu reagent, sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, protocatechuic acid, catechol, syringol, oleanolic acid and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

5.2.3. Sample extraction and separation

Five hundred gram of the dried and powdered aerial root was extracted with methanol (3 times with a total of 3000 ml of methanol) at room temperature for 36 h. The methanol extracts were collected, filtered and dried under vacuum to yield 54.6 g. The dried methanol extract was suspended in 10% aqueous methanol and was successively extracted with hexane, ethyl acetate and n-butanol. The obtained extracts, in addition to the aqueous solution remained after the extraction, were filtered and dried under vacuum to give 5.8, 3.5, 9.1 and 35.1 g of hexane, ethyl acetate, n-butanol and

water fractions, respectively. Each extract was dissolved in methanol and refrigerated for further experiments.

5.2.4. DPPH radical scavenging assay

Free radical scavenging activity of extracts and standard compounds was determined using the DPPH method (Saha *et al.*, 2004; Boskou *et al.*, 2006) as described previously in Chapter 3.

5.2.5. Total antioxidant activity (ABTS assay)

The total antioxidant activity values of aerial root extracts and standard compounds were measured by the improved ABTS^{•+} method as described by Baltrusaityte *et al.* (2007) with minor modification. The method was previously introduced in **Chapter 4**.

5.2.6. PMS-NADH system superoxide-radical scavenging assay

The superoxide scavenging ability of extracts and standard compounds was assayed following the method of Lau *et al.* (2002) as described previously in **Chapter 2**.

5.2.7. β -carotene/linoleic acid system antioxidant assay

The assay was performed according to the method described by Elzaawely *et al.* (2005) which was previously introduced in **Chapter 4**.

5.2.8. Determination of reducing power assay

The reducing power of aerial root extracts and standard compounds was performed according to the method of Oyaizu (1986) as previously described in **Chapter 3**.

5.2.9. Amount of total phenolic compounds

The amount of total phenolics in the aerial root methanol extract and sub-fractions was measured using the Folin-Ciocalteu reagent method as described in **Chapter 3**.

5.2.10. Antibacterial activity

Antibacterial activities of extracts from *F. microcarpa* and ampicillin were tested against *Bacillus subtilis*, *Bacillus cereus*, *Bacillus brevis*, *Achromobacter polymorph*, *Mycobacterium avium*, and *Escherichia coli* by using a disc diffusion method as described in **Chapter 4**.

5.2.11. GC-MS analysis

The sample was analyzed by GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan) equipped with 30-m×0.25 mm DB-5 column (Agilent Technologies, J&W Scientific Products, Folsom, CA). The conditions of machine were previously described in Chapter 4. Compound identification was verified based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST147. LIB. database of the GC-MS system (Shimadzu).

5.2.12. HPLC analysis

Compounds in *F. microcarpa* extracts were analyzed with a sensitivity of 0.1 µg between the wavelengths 220 to 280 nm by a Shimadzu HPLC chromatography with UV-vis spectra (SCL-10 A vp, Shimadzu Co., Japan). Separation was carried out on a Synergi 4u Hydro-RP 80A column (150 mm × 4.60 mm, Phenomenex Company, USA).

A gradient elution was performed with solvent 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 μ l. Identification of the phenolic compounds was carried out by comparing their retention times and ultraviolet spectra with those of standards.

Stock solutions of the extracts and authentic compounds were prepared in methanol to final concentrations of 3 and 0.5 mg/ml, respectively. The concentration range used for calibration of the authentic compounds was 5-100 μ g/ml. The all samples were injected in duplicate.

5.2.13. *Statistical analysis*

The statistical analyses were performed by one-way ANOVA and the Student's *t*-test. The results were expressed as the mean \pm SE. (n=3) to show variations in the various experimental. Differences are considered significant when $P < 0.05$.

5.3. Results and discussion

5.3.1. *Total phenolic content in extracts*

Phenolic compounds in plants comprise a comparatively large class of secondary metabolites with varies potential bioactivities, such as antioxidant, antibacterial and anti-inflammatory (Yizhong *et al.*, 2004; Djeridane *et al.*, 2006).

Table 1. Total phenolic content and antioxidant activity of the methanol extract and sub-fractions from aerial root of *F. microcarpa* and some standard compounds

Samples	EC ₅₀ (µg/ml)			Total phenolic content (GAE mg/g extract)
	DPPH assay	ABTS ⁺ assay	PMS-NADH assay	
Methanol	6.8 ± 0.1d	5.6 ± 0.1c	137.5 ± 6.4d	240.3 ± 4.3c
Hexane	173.0 ± 5.8a	75.1 ± 0.8a	>1000a	45.2 ± 1.3e
Ethyl acetate	6.0 ± 0.0d	1.8 ± 0.0e	89.7 ± 2.6f	391.9 ± 2.5a
N-butanol	11.2 ± 0.1c	3.0 ± 0.0d	115.2 ± 3.2e	286.9 ± 5.1b
Water	36.5 ± 0.2b	12.4 ± 0.3b	211.1 ± 10.7b	92.0 ± 2.5d
Trolox	4.4 ± 0.0e	0.5 ± 0.0f	180.7 ± 5.8c	
Protocatechuic acid	6.2 ± 0.1	ND	43.0 ± 0.4	
Catechol	1.3 ± 0.0	ND	113.7 ± 7.9	
Syringol	5.4 ± 0.0	ND	>1000	
Oleanolic acid	>1000	ND	>1000	

Each value in the table was expressed as mean ± SE (n=3). Superscript letters with different letters in the same column indicate significant difference (P<0.05). Concentration of samples in assays was expressed as final concentration. “ND” indicated not determined.

The amounts of total phenolic compounds in the methanol extract and sub-fractions prepared from the aerial root of *F. microcarpa* were shown in **Table 1**. The total phenolic content of methanol extract was 240.3 GAE mg/g extract. The highest total phenolic content was found in ethyl acetate fraction (391.9 GAE mg/g extract) and followed by n-butanol fraction (286.9 GAE mg/g). In water and hexane fractions, phenolic compounds were present in lesser extent. The order of total phenolic content in

methanol extract and sub-fractions was established as follow: ethyl acetate > n-butanol > methanol > water > hexane (p<0.05).

5.3.2. Analysis of ethyl acetate fraction by GC-MS and HPLC

Since ethyl acetate fraction contained the highest phenolic content and showed the strongest antioxidant and antibacterial activities, it was further analyzed by GC-MS and HPLC to determine its chemical composition that may contribute to these activities. The GC-MS analysis showed that ethyl acetate fraction contained a variety of phenolic compounds (**Fig. 1**). Protocatechuic acid, catechol, *p*-vinylguaiacol, syringol, *p*-propylphenol, vanillin, *p*-propylguaiacol, isovanillic acid, 4-n-propylresorcinol, syringaldehyde and oleanolic acid were identified (**Table 2**). The structures of these compounds were established by cochromatography with standards, MS library and spectroscopic evidences. To confirm the GC-MS analysis result, ethyl acetate fraction was analyzed again by HPLC, the chromatography coupled with detector responses at 220 and 280 nm overlaid was presented in **Fig 2**. Protocatechuic acid, catechol, syringol and oleanolic acid were identified by comparing their retention times and UV spectra with authentic standards analyzed under identical conditions. Quantitative data was also calculated from their respective calibration curves. These four authentic compounds were purchased from Sigma-Aldrich. The other seven compounds could not be quantified and examined for their activities as neither these authentic compounds could be purchased from any chemical company nor successfully isolated in our laboratory. As showed in **Table 3**, the major component present in the fraction was identified as syringol (93.5 mg/g). The content of catechol was 28.2 mg/g. The other two compounds, oleanolic acid and protocatechuic acid, showed 13.4 and 6.6 mg/g, which have been

reported to be isolated from methanol extract of *F. microcarpa* by other researchers (Higa *et al.*, 1987; Kuo & Chiang, 1999).

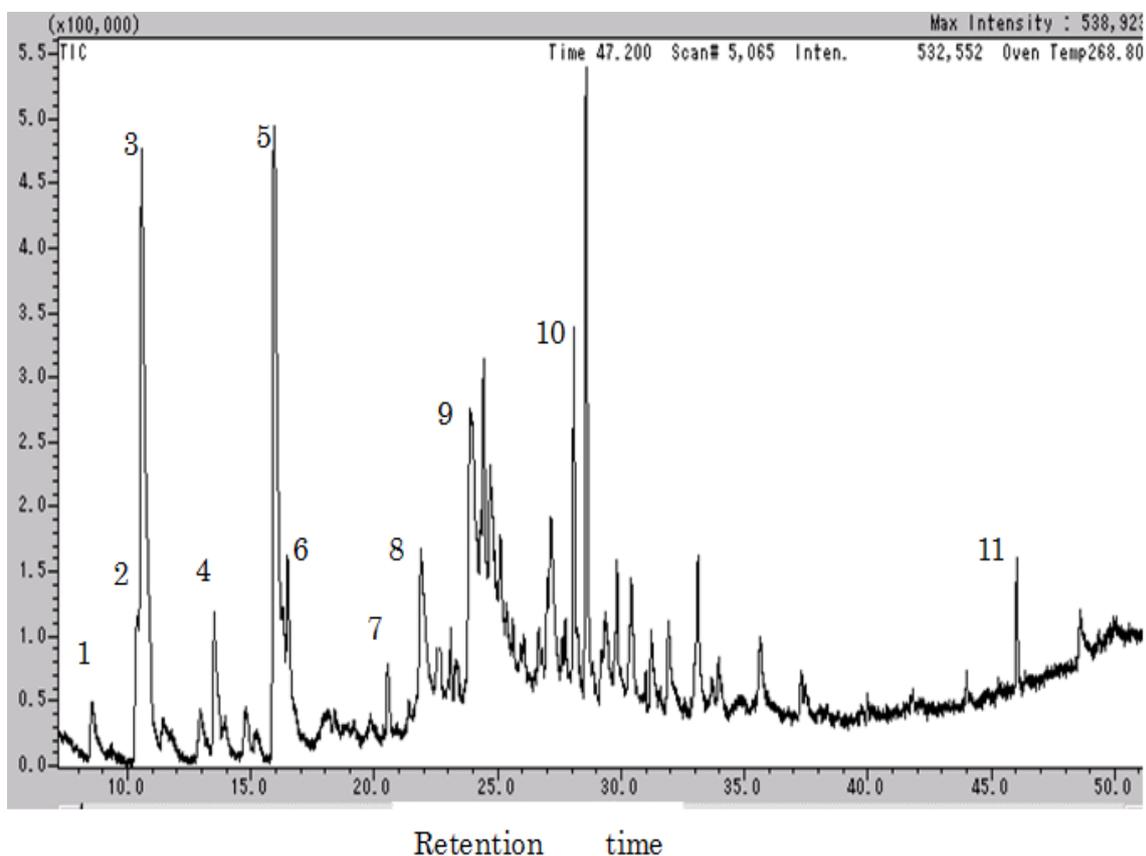


Fig. 1. GC-MS chromatogram of ethyl acetate fraction from *F. microcarpa*.

1. Protocatechuic acid, 2. Catechol, 3. *p*-Vinylguaiacol, 4. Syringol, 5. *p*-Propylphenol, 6. Vanillin, 7. *p*-Propylguaiacol, 8. Isovanillic acid, 9. 4-*n*-Propylresorcinol, 10. Syringaldehyde, 11. Oleanolic acid.

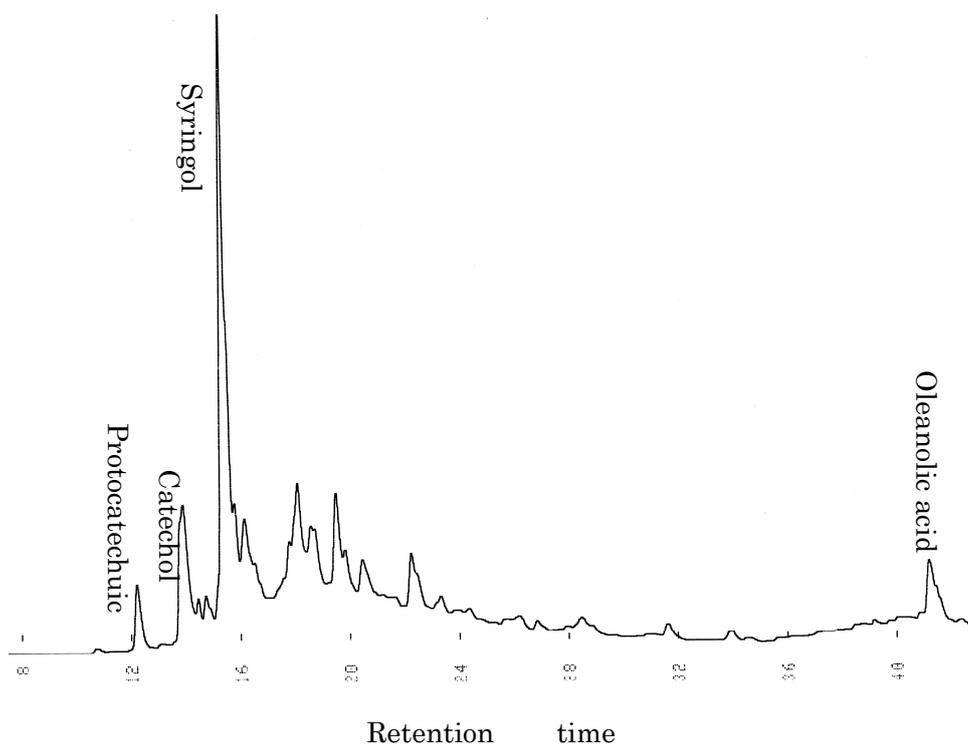


Fig. 2 HPLC chromatogram of ethyl acetate fraction from *F. microcarpa*.

Table 2. Chemical composition in ethyl acetate fraction of *F. microcarpa* detected by GC-MS

No	Compounds	Retention time (min)	Molecular weight	Major peaks
1	Protocatechuic acid	8.657	154	154, 137, 109, 81
2	Catechol	10.420	110	110, 92, 81, 64
3	<i>p</i> -Vinylguaiacol	10.868	150	150, 135, 107, 77
4	Syringol	13.902	154	154, 139, 111, 93
5	<i>p</i> -Propylphenol	16.039	136	136, 107, 77, 39
6	Vanillin	16.538	152	152, 123, 109, 81
7	<i>p</i> -Propylguaiacol	20.464	166	166, 137, 122, 94
8	Isovanillic acid	21.859	168	168, 153, 125, 97
9	4- <i>n</i> -Propylresorcinol	24.324	152	152, 123, 77
10	Syringaldehyde	28.242	182	182, 167, 152, 123
11	Oleanolic acid	46.105	456	392, 248, 203, 189

Table 3 Compounds retention time (RT), UV_{max} and content (mg/g extract) in ethyl acetate fraction by HPLC chromatography with UV-vis spectra

<i>Compounds</i>	<i>RT (min)</i>	<i>UV_{max} (nm)</i>	<i>Content mg/g extract</i>
Oleanolic acid	42.514	208	13.4 ± 0.4
Protocatechuic acid	12.524	294, 258, 217, 212	6.6 ± 0.2
Catechol	14.621	220, 201	28.2 ± 0.0
Syringol	15.857	207, 269	93.5 ± 1.1

Each value in the table is represented as mean \pm SE (n=3).

5.3.3. Antioxidant activities

The antioxidant activity of plant extract is often attributed to its redox effects, which function as a reducing agent, or acting as a hydrogen donor, singlet oxygen quencher and hydrogen peroxide or lipid peroxide scavenger (Sroka & Cisowski, 2003). Many researches have suggested that phenolic compounds are major components responsible for antioxidant activity in plants (Gorinstein *et al.*, 2003; Maisuthisakul *et al.*, 2007).

5.3.3.1. Reducing power of extracts

Antioxidant activity has been proposed to be related to its reducing power. Therefore, to assess the electron donating power of methanol extract and sub-fractions of aerial root of *F. microcarpa*, their ability to reduce iron (III) to iron (II) was investigated. The reducing ability of the extracts in different concentrations was given in **Fig. 3 A**. The order of reducing power has been established as follow: ethyl acetate > n-butanol > methanol > water > hexane. This order of reducing power of the extracts was the same order as total phenolics contents in these extracts. Except hexane, other extracts were capable of reducing iron (III) ions and did so in a linear concentration-dependent manner. As showed in **Fig. 3 B**, protocatechuic acid, catechol and syringol all exhibited excellent reducing power while oleanolic acid did not show any activity even at high concentration (200 μ g/ml). The results suggested that the plant extracts have the ability to donate electrons, thus they can scavenge for radicals. However, the reducing ability from these extracts was weaker than that of positive control, Trolox.

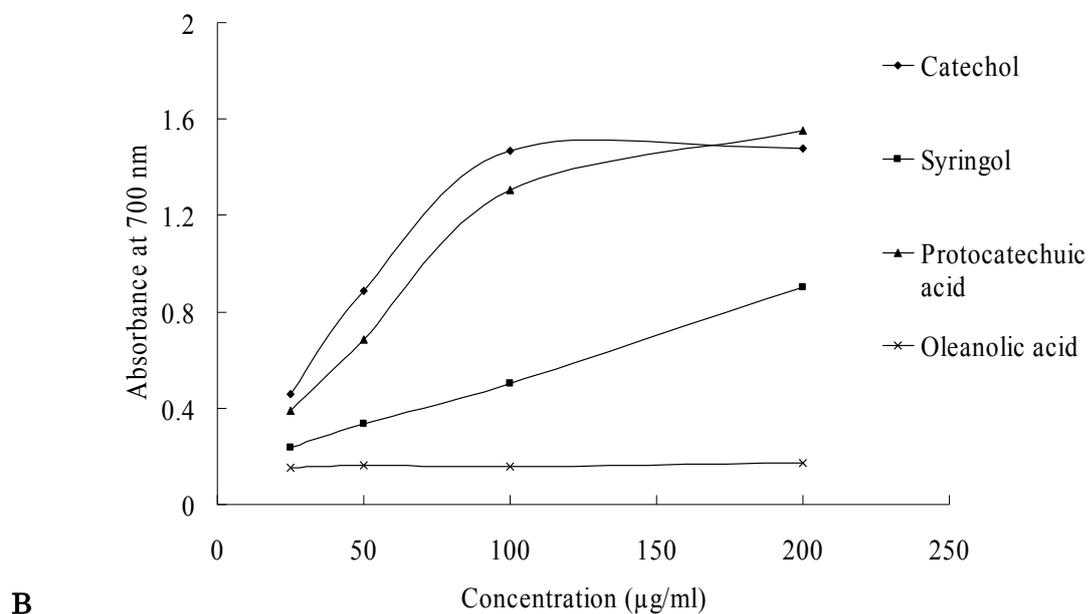
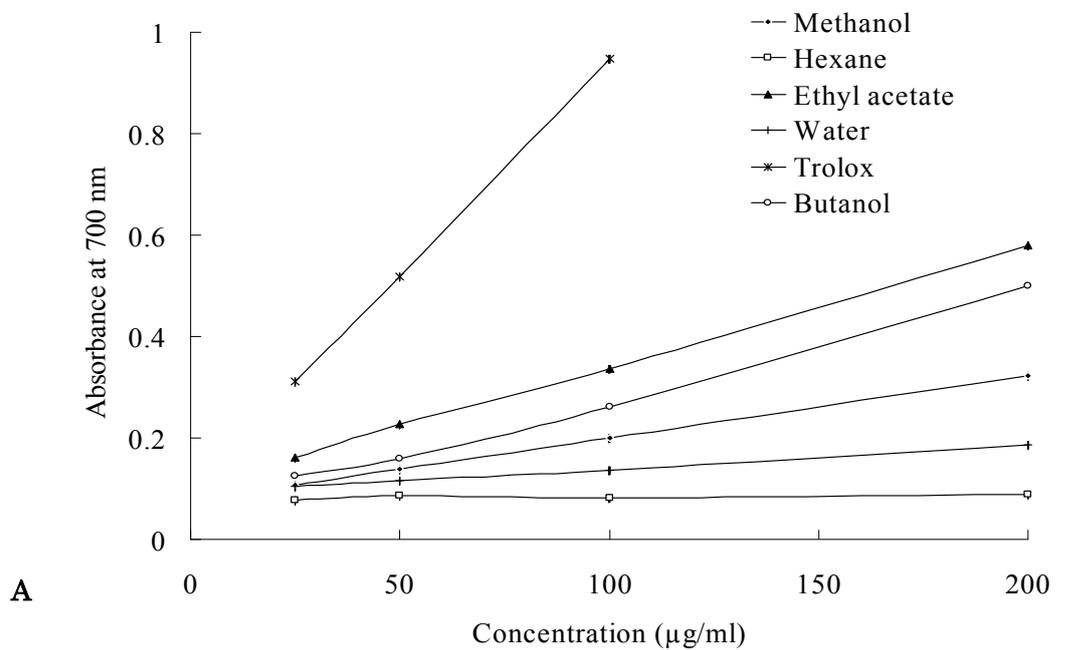


Fig. 3. Reducing power of the methanol extract and sub-fractions from aerial root of *F. microcarpa* (A) and reducing power of some standard compounds (B). The values represent means \pm S. E. (n=3). Concentration of samples in assays was expressed as final concentration

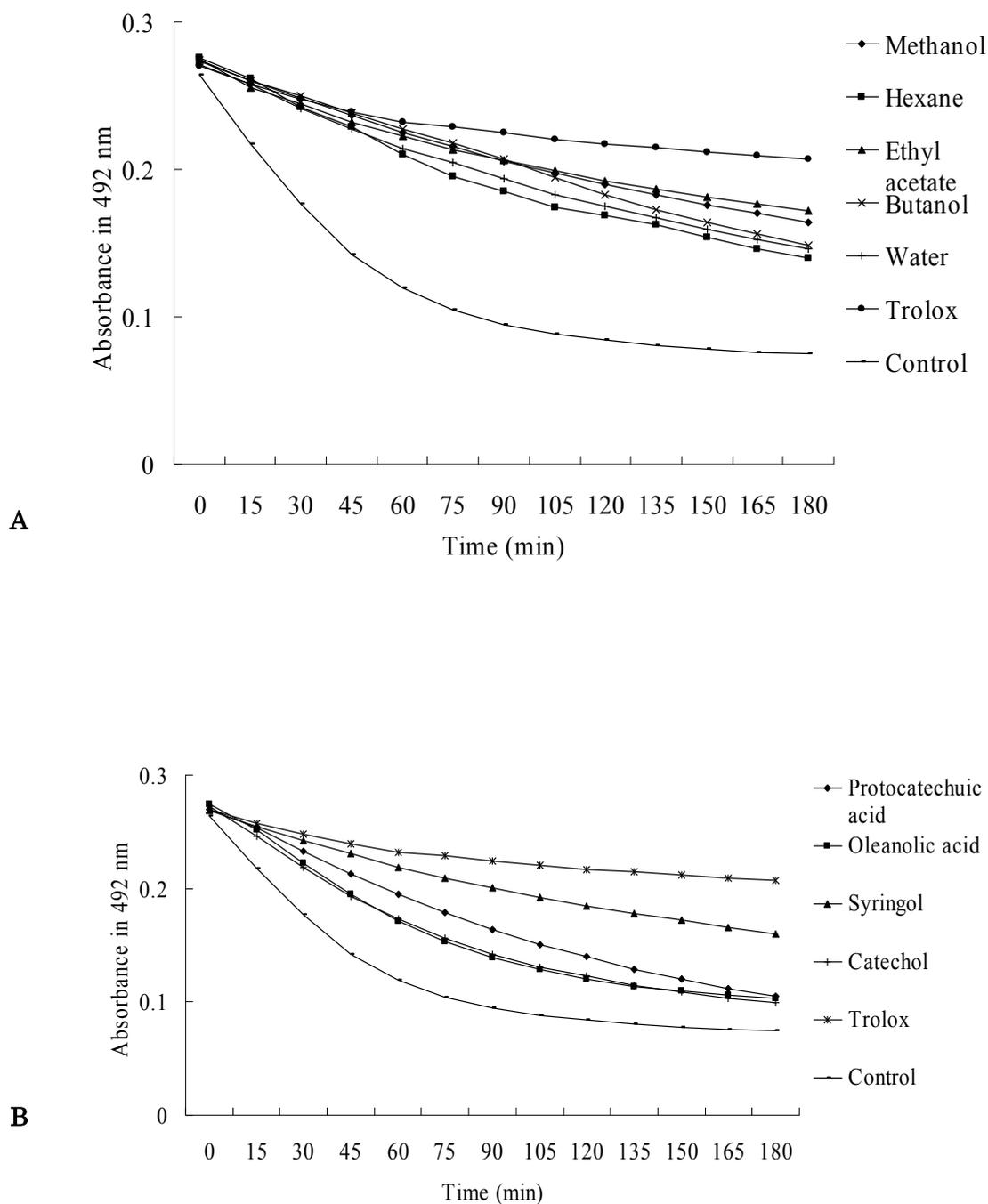


Fig. 4 Antioxidant effect of the methanol extract and sub-fractions from aerial root of *F. microcarpa* (A) and some standard compounds (B) on β -carotene linoleate model system assays

5.3.3.2. β -carotene/linoleic acid system antioxidant assay

β -carotene/linoleic acid system bleaching assay was considered to be a good model for membrane-based lipid peroxidation (Ferreria *at al.*, 2006). Plant extract that inhibits β -carotene bleaching can be described as a free radical scavenger and primary antioxidant. In order to simulate oxidation of membrane lipid oxidation, β -carotene/linoleic acid system was selected to evaluate the antioxidant effect of the extracts of *F. microcarpa* and authentic compounds. As showed in **Fig. 4 A**, all the extracts of *F. microcarpa* showed inhibition on β -carotene oxidation in different levels comparing with control (only methanol). The ethyl acetate fraction and methanol extract demonstrated high ability to inhibit the bleaching of β -carotene by scavenging linoleate-derived free radicals, while n-butanol, water and hexane fractions showed weaker ability than the former. The four authentic compounds also showed different levels of inhibition on β -carotene oxidation (**Fig. 4 B**). This result suggested that the antioxidant effect of the extracts of *F. microcarpa* could be related to their phenolic compounds.

5.3.3.3. DPPH free radical scavenging

One of the mechanism by which antioxidant bring about their action is scavenging free radicals. The assay of scavenging DPPH free radicals offers a convenient and accurate method for determining the oxidizable groups of natural or synthetic antioxidants. This free radical serves both as an oxidizing substrate and as reaction indicator molecule. Therefore it is necessary to determine the free radical scavenging ability of the *F. microcarpa* extracts. The DPPH radical scavenging activity (EC₅₀) of *F. microcarpa* extracts, standard compounds and Trolox was shown in **Table 3**. Based on

the result, the order of the activity was as follow: ethyl acetate (6.0 $\mu\text{g/ml}$) > methanol (6.8 $\mu\text{g/ml}$) > n-butanol (11.2 $\mu\text{g/ml}$) > water (36.5 $\mu\text{g/ml}$) > hexane (173.0 $\mu\text{g/ml}$) (lower EC_{50} values means higher activity). Comparing total phenolic content with the DPPH radical scavenging ability of all extracts, the correlation coefficient was high ($R^2 = 0.836$). Protocatechuic acid, catechol, and syringol showed high DPPH radical scavenging activity (EC_{50} : 6.2, 1.3 and 5.4 $\mu\text{g/ml}$, respectively). These results suggested that the strong DPPH radical scavenging ability of ethyl acetate fraction was closely related to the high levels of phenolic compounds and due to the scavenging of the radical by hydrogen donation.

Based on the above analysis result, we can deduce that extract from aerial root of *F. microcarpa* was a potential antioxidant in preservation of foodstuffs, drug products and cosmetics, in which free radical mediated chain reactions that resulted in lipid oxidation and subsequent deterioration of these products. The extracts may also prove to have therapeutic potential as free radicals were believed to be involved in the pathogenic cascade of events in many diseases.

5.3.3.4. *ABTS*^{•+} free radical scavenging

Even similar with the mechanism of DPPH free radical scavenging assay, *ABTS*^{•+} model is another method that is commonly used to evaluate antioxidant activity of plant extract. This assay overcomes the limitations of the former method (DPPH model) such as solubility, and spectral interference. The *ABTS*^{•+} model is more versatile as both the polar and non-polar samples can be assessed for their scavenging activity and the spectral interference is minimized as the absorption maximum used is 760 nm (a wavelength not normally encountered by natural products). Additionally, this method is

more sensitive than DPPH model to test radical scavenging ability of sample. The ABTS⁺ radical scavenging ability (EC₅₀) of the extracts was presented in **Table 3**. It can be seen that the hierarchy of EC₅₀ values was as ethyl acetate (1.8 µg/ml) < n-butanol (3.0 µg/ml) < methanol (5.6 µg/ml) < water (12.4 µg/ml) < hexane (75.1 µg/ml). Since lower EC₅₀ value means higher antioxidant activity, the activity order of these extracts was reversed. Comparing with previously presented total phenolics content of the extracts, it can be seen that the correlation between ABTS⁺ radical scavenging ability and total phenolics content of all extracts was positively high (R² = 0.936). The result suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *F. microcarpa* extracts. This was also in accordance with previous researches (Gorinstein *et al.*, 2003; Maisuthisakul *et al.*, 2007), which have shown that high total polyphenols content increases antioxidant activity and there was a linear correlation between phenolic content and antioxidant activity.

It has been reported that free radical scavenging and antioxidant activity of many medicinal plants are responsible for their therapeutic effect against cancer, tissue inflammatory and cardiovascular disease (Cai *et al.*, 2004). Thus, the antipyretic and analgesic action of *F. microcarpa* may be associated with its antioxidant activity.

5.3.3.5. PMS-NADH superoxide anion radical scavenging

The superoxide radicals (O₂^{·-}) are the most common ROS formed *in vivo*. These radicals can easily initiate the peroxidation of membrane lipids leading to the accumulation of lipid peroxides and damaging a wide range of essential molecules (Liu & Ng, 2000). Therefore, the ability of scavenging O₂^{·-} is one of the important methods to evaluate the antioxidant activity of *F. microcarpa* extracts and authentic compounds.

As showed in **Table 3**, most extracts exhibited certain $O_2^{\cdot -}$ scavenging activity in a concentration dependent manner. However, hexane fraction, syringol and oleanolic acid, have a poor scavenging activity and their EC_{50} could not be determined. The EC_{50} value of ethyl acetate fraction (89.7 $\mu\text{g/ml}$) was the lowest in all extracts, even was significant ($p < 0.05$) lower than that of Trolox (180.7 $\mu\text{g/ml}$). Two standard phenolic compounds, protocatechuic acid and catechol exhibited very excellent $O_2^{\cdot -}$ scavenging activity (values of EC_{50} were 43.0 and 113.7 $\mu\text{g/ml}$, respectively). The result showed that ethyl acetate fraction possessed superoxide radical scavenging activity, which can be of potential health interest as it may be effective in reducing the level of $O_2^{\cdot -}$. Many diseases are believed to be derived from high level of $O_2^{\cdot -}$ in body (Valéry *et al.*, 2007).

5.3.4. Antibacterial activity

Extracts from many medicinal plants have antibacterial activities, noted against foodborne Gram-positive and Gram-negative bacteria, as well as yeasts and fungi. As summarized in **Table 4**, aerial root extracts of *F. microcarpa* at the amount of 200 $\mu\text{g/disc}$ showed certain antibacterial activity against both Gram-positive bacteria (*B. brevis*, *B. cereus*, and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *A. polymorph*) compared with control (only DMSO). Ethyl acetate, n-butanol and methanol extracts possessed significantly ($p < 0.05$) higher activity against these five bacteria than hexane and water extracts. All extracts did not show any inhibition against *M. avium*. Except for its inhibition on *B. cereus*, ampicillin exhibited much higher inhibitory activity against the rest five bacteria than *F. microcarpa* extracts do.

5.4. Dissussion

Some researches reported that herbs which had antimicrobial activity due to the presence of phenolic compounds contain a polar isopropyl functional group (Frag *et al.*, 1989). In the present study, high phenolics content extracts (ethyl acetate, methanol and n-butanol) showed significantly ($p < 0.05$) higher inhibitory activity than low phenolics content extracts (water and hexane) against five tested bacteria. Certainly, the chemical composition of *F. microcarpa* extract might influence the antibacterial effects. The high content of catechol, syringol and the other phenolic compounds identified in the ethyl acetate fraction might contribute for its antimicrobial properties. Catechol was found to have antibacterial effects on *pseudomonas putida*, *P. pyocyanea* and *corynebacterium xerosis* at 5-10 mM concentration (Kocacaliskan *et al.*, 2006). Rodri'guez Vaquero *et al.* (2007) found that many phenolic compounds in wines possess high antibacterial effect. Guarana seed methanol extract has high antibacterial activity, and the methanol extract possessed high phenolics content (Majhenič, Škerget & Knez, 2007). Baydar *et al.* (2006) reported that tested fifteen species of bacteria were inhibited by three different grape seed extracts, and these extracts all showed high content of total phenolic compounds. Rauha *et al* (2000) have tested antimicrobial effects by 29 Finnish plant extracts and 13 phenolic substances and confirmed that flavone, quercetin naringenin and some extracts were effective in inhibiting the growth of the organisms. These evidences are in accordance to our present study. In fact, the antibacterial activity of phenolics is well known. Some mechanisms of antimicrobial ability by phenolics were as follows: phenolics can denature proteins of bacteria, being generally classified as surface-active agents (Sousa *et al.*, 2006); phenolics react with cellular membrane components, which impairs both function and integrity of cells (Raccach, 1984); and the

reducing ability of phenolics can influence the oxidation reduction potential (E_h) of bacterial growth in which the produced potential negative or positive E_h cause growth inhibition against some bacteria (Jay, 1996).

Table 4 Antibacterial activity of the methanol extract and sub-fractions from aerial root of *F. microcarpa* and ampicillin^b using disc diffusion method (inhibition zones, mm)

Bacteria	Methanol	Hexane	Ethyl acetate	n-butanol	Water	Ampicillin	DMSO
<i>Bacillus brevis</i>	12.8 ± 0.5b	11.9 ± 0.3c	13.4 ± 0.7b	13.0 ± 0.3b	10.0 ± 0.2d	34.3 ± 0.7a	8.5 ± 0.2e
<i>Bacillus cereus</i>	13.1 ± 0.2b	11.4 ± 0.5c	14.5 ± 0.4a	14.0 ± 0.4a	10.5 ± 0.3d	6.5 ± 0.3e	—
<i>Bacillus subtilis</i>	11.6 ± 0.9b	10.5 ± 0.2c	12.5 ± 0.5b	12.0 ± 0.1b	10.5 ± 0.4c	30.0 ± 0.0a	6.8 ± 0.5d
<i>Escherichia coli</i>	12.7 ± 0.4c	11.4 ± 0.2c	14.5 ± 0.4b	14.5 ± 0.2b	11.5 ± 0.2c	27.0 ± 0.3a	—
<i>Achromobactor polymorph</i>	11.0 ± 0.8bc	11.3 ± 0.3bc	12.0 ± 0.1b	9.0 ± 0.1c	—	34.5 ± 0.3a	6.5 ± 0.0d
<i>Mycobacterium avium</i>	—	—	—	—	—	21.0 ± 1.4	—

^a 200 µg/disc of each extract (dissolved in DMSO) and ^b 1 µg/disc of ampicillin (dissolved in water) used to load on sterile disc; Each value in the table is represented as mean ± SE (n=3); Superscript letters with different letters in the same row indicate significant difference (P<0.05); “—” indicated inhibition zone was not >6 mm (diameter of disc).

5.5. Conclusion

As far as we know, this was the first study concerning the antioxidant capacity, antibacterial activity and total phenolic content of aerial root extract of *F. microcarpa*.

The results revealed that the methanol extracts and sub-fractions showed strong antioxidant and antibacterial effects and contained high amounts of phenolic compounds. The compositional analysis showed that most compounds in ethyl acetate fraction identified as phenolics and some have proven to possess antioxidant activity in our study. Therefore, it is possible that they are contributing to the antioxidant effects of the extracts. On the other hand, our study also showed that the phenolic compounds extracted from *F. microcarpa* are responsible, in part, for antibacterial activity. These findings may provide a pharmacological explanation for some of its uses in folk medicine in Ryukyu Islands.

The fruits of this plant are small and are eaten by numerous fruits eating birds and possible other animals such as bats, rodents and parrots. This evidence indicated that extract from *F. microcarpa* could be used as a potential antioxidant and antibacterial resource. However, further studies are needed to isolate more pure compounds from *F. microcarpa* extracts that are responsible for antioxidant or antibacterial activity.

CHAPTER 6

**Isolation and identification of antioxidant and antiallergic
compounds from *Ficus Microcarpa* L. fil. bark**

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. 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 ethyl acetate fraction exhibited the strongest activity, it was selected for further purification by repeated Sephadex LH-20 column 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, ¹H and ¹³C-NMR. Except protocatechuic acid and catechin, other five compounds were first reported from this plant. All isolated compounds showed strong antioxidant activity when tested by all applying methods. Catechin, epicatechin, procyanidin B1 and procyanidin B3 exhibited excellent inhibitory activity against hyaluronidase. The results indicated that the extract of *F. microcarpa* bark may be utilized as potential antioxidant and hyaluronidase inhibitor in functional food industry.

Keywords: *Ficus microcarpa*; hyaluronidase inhibitory; anti-allergic; antioxidant; phenolics

6.1. 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 pain in local place (Tawata and Oota, 1985). Chiang *et al.* (2005) reported that oleanonic acid, acetylbetulonic acid, betulonic acid, acetylursolic acid, ursolic acid, 3-oxofriedelan-28-oic acid and 3B-acetoxy-25-hydroxy-11-oxo-12, 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.

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 GC-MS and HPLC (Ao *et al.* 2008). However, many other bioactive phenolic compounds that cannot be identified by GC-MS were still unclear in *F. microcarpa* extract. In the present work, inhibitory activity on hyaluronidase of *F. microcarpa* extract was investigated. By bioassay-guided chromatographic fractionation, seven phenolic compounds were isolated and these compounds also were evaluated for hyaluronidase inhibitory and antioxidant activity.

6.2. Materials and Methods

6.2.1. Chemicals

Hyaluronidase (type IV-S: from bovine testes), hyaluronic acid sodium salt, disodium cromoglycate (DSCG), potassium persulfate, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS),

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Sigma Chemical Co. (SEIKO CO.,LTD. Japan). Protocatechuic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

6.2.2. Plant material

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

6.2.3. 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 its 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 remained 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 ethyl acetate and butanol fractions showed the higher hyaluronidase inhibitory and antioxidant activities than other two fractions, they have been subjected to further purification procedures (**Fig. 1**).

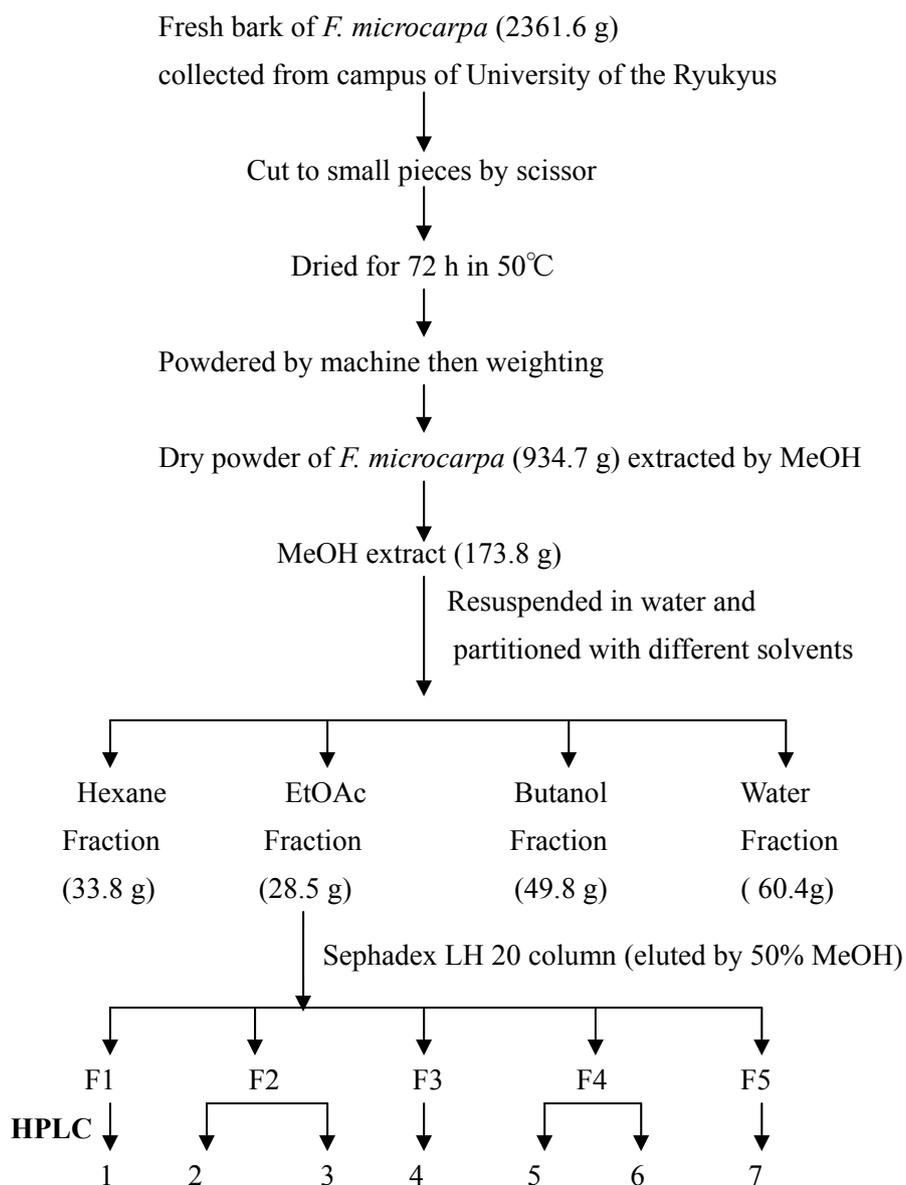


Fig. 1. Separation scheme of bioactive compounds from *F. microcarpa*

6.2.4. Isolation procedures

Although butanol fraction showed high hyaluronidase inhibitory activity, its chemical composition could not be investigated. By MALDI-TOF MS and LC-MS analysis (data not shown), it was confirmed that butanol fraction contained rich-polymeric proanthocyanidin compounds (condensed tannins). To determine 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 NMR of higher oligomers makes the spectra difficult to interpret.

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 μ m PVDF filter media, Whatman). One hundred fifty ml of fraction was divided 3 times applied to a 140 cm \times 3 cm i.d. glass column packed with Sephadex LH-20 (Lipophilic Sephadex, bead size: 25-100 μ , 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 fraction of 15 ml / tube was collected continuously with a SF-2120 fraction collector (Advantec Toyo Kalsha, Ltd. Japan). The all collected fractions were analyzed by HPLC with a Synergi 4u Hydro-RP 80A column (150 mm \times 4.60 mm, Phenomenex Company, USA) at 280 nm for checking the polyphenols content. A gradient elution was performed with solvent 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 μ l. The fractions with similar retention time peaks were pooled and all tubes were divided into five fractions (F1-F5) (**Fig. 2**). This procedure was repeated two times to obtain sufficient amounts with yields

varying from 125 mg to 300 mg. The fractions F1-F5 were further purified and by preparative HPLC with Shim-Pack PRC-ODS column (20×250 mm, SHIMADZU company), under the buffer of methanol : acetonitrile : 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.

6.2.5. Instrumentation

Melting points were measured on a BüCHI 535 (FLAWIL/SCHWEIZ Co., Switzerland). The ^1H (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 TMS and the coupling constants are given in Hz. 2D NMR experiments (^1H , ^{13}C - COSY, HMQC, HMBC) were obtained using standard pulse sequences. ESI-MS spectra were obtained using a Sciex API 2000 LC-MS/MS System (Model Sciex API 2000, Applied Biosystems, Langen, Germany).

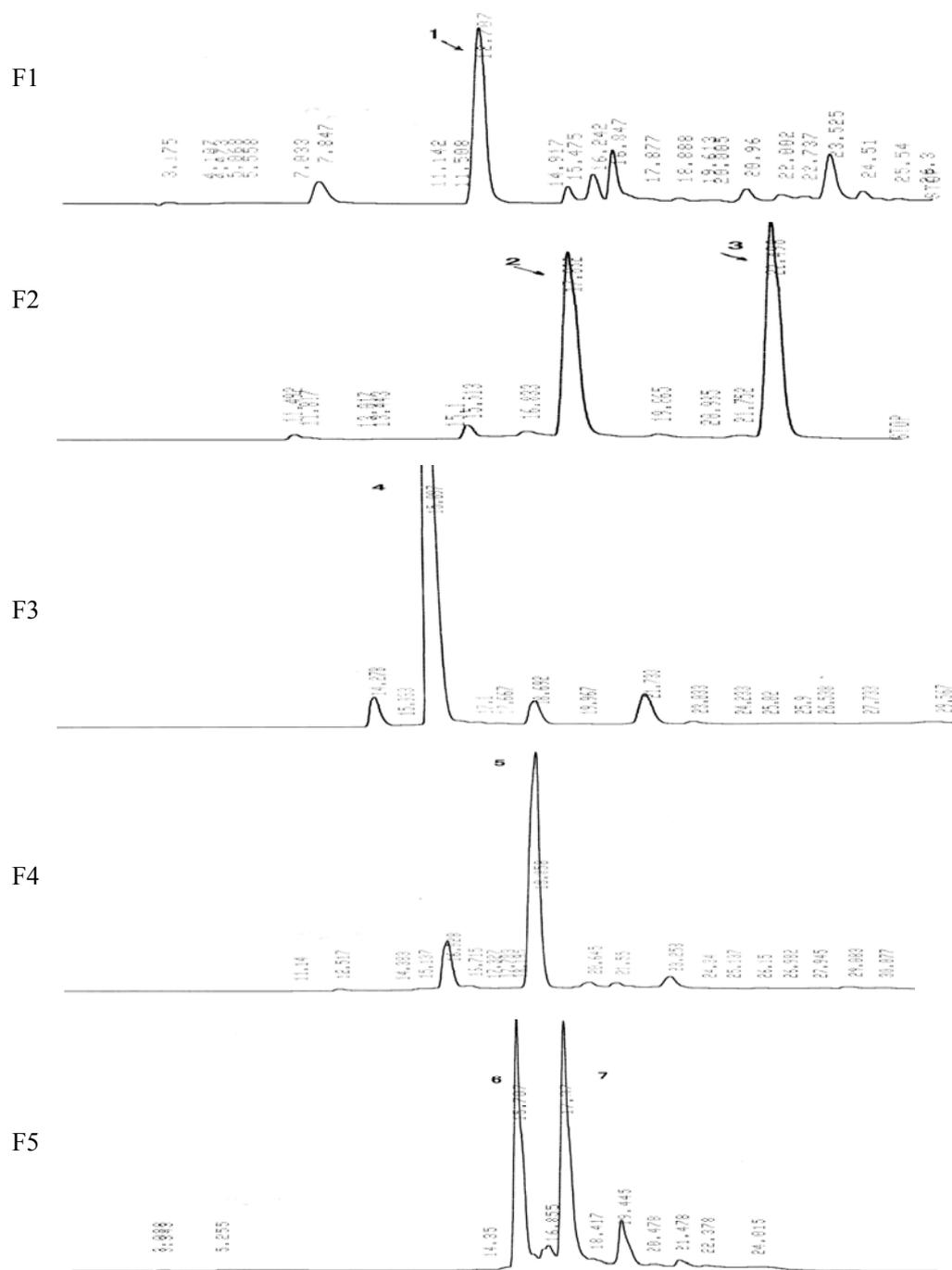


Fig. 2. HPLC chromatograms as monitored by UV absorption at 280 nm for the fractions F1 to F5 in 50% methanol, as obtained by Sephadex LH-20 column chromatography of the ethyl acetate extract of *F. microcarpa* bark. The peaks numbered with arabic numerals in the order of increasing retention time correspond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

6.2.6. Estimation of hyaluronidase inhibitory activity

Hyaluronidase inhibitory activity of samples was determined spectrophotometrically according to the methods introduced by Ito et al. (1998) and Lee and Kim (1999) with minor modification. Fifty μl of bovine hyaluronidase (4000 units/ml) dissolved in 0.1 M acetate buffer (pH 4.0) was mixed with 100 μl of a different concentration of samples dissolved in acetate buffer except hexane fraction (first dissolved in small amount of DMSO, then dispersed in acetate buffer). After incubated at 37°C for 20 min, 100 μl of 1.25 mM calcium chloride was added in reaction mixture for activating hyaluronidase, then again incubated at 37°C for 20 min. Reaction was initiated by adding 0.25 ml hyaluronic acid sodium salt (0.8 mg/ml dissolved in acetate buffer). After incubated 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 in reaction mixture, then the mixture was boiled in water bath 3 min. After cooled on ice, 3 ml of *p*-DAB reagent (10 g of *p*-DAB dissolved in 12.5 ml of 10 N HCl and 87.5 ml acetic acid. Before used, the reagent was 10 times diluted with acetic acid) was added to the reaction mixture, and incubated 37°C for 20 min again. After incubation, measurement was conducted at the absorbance of 585 nm against a blank (without hyaluronidase and test samples). The percent inhibition was calculated as follows: Inhibition (%) = $[A-(B-C)]/A \times 100\%$

A: absorbance of control (only acetate buffer, without test sample), B: absorbance involving test samples, C: absorbance of test samples alone, without hyaluronidase.

6.2.7. DPPH Radical scavenging assay

DPPH free radical scavenging activity of extracts and isolated compounds was determined using the DPPH method as described previously in **Chapter 3**.

6.2.8. Total antioxidant activity (ABTS assay)

ABTS⁺ free radical scavenging activity of extracts and purified compounds was determined using the total antioxidant assay method as described previously in **Chapter 4**.

6.2.9. Superoxide-radical scavenging activity by PMS-NADH system

The superoxide scavenging ability of extracts and compounds was assayed as described previously in **Chapter 3**.

6.3. Results

6.3.1. Identification of the isolated compounds

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

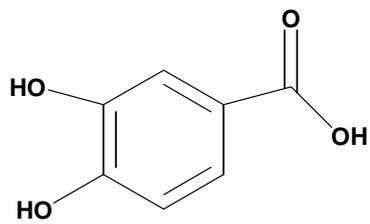
Compound 2 (28 mg, $t_R = 10.8$ min) was purified from F2, a white powder, MP: 204-206°C, ESI-MS: negative ion, m/z: 353.4, [M-H]⁻; 375.3, [M+Na]⁻, ¹H and ¹³C-NMR spectral data analysis confirmed **2** was chlorogenic acid (De Almeida et al., 1998).

Compound 3 (18 mg, $t_R = 15.2$ min) was purified from F2, 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. Spectral of ¹H and ¹³C-NMR of **compound 3** were similar with that of **compound 2**,

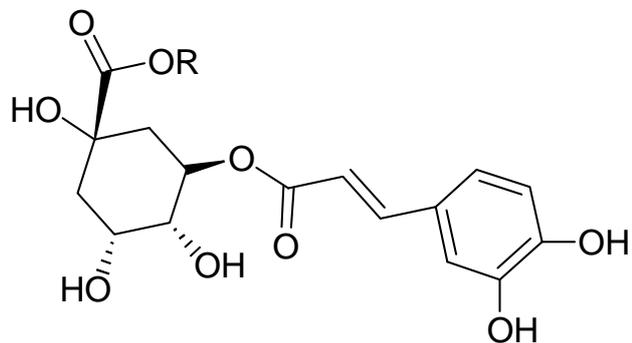
but comprising typical methoxy signal (δ_{H} 3.70 and δ_{C} 53.0, OCH₃). The positions of methoxy and carbonyl ester substitution were determined by HMBC. Compound **3** was identified as methyl chlorogenate (Deyama et al., 1987; Kweon et al., 2001).

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 same the spectra of MS-MS, except different retention time. By analysis of ¹H, ¹³C-NMR, COSY, HMQC and HMBC, **compounds 4** and **5** were identified as catechin and epicatechin, respectively (Bae et al., 1994; Khallouki et al., 2007).

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, respectively. Because the two compounds have similar retention time, purified processes were repeated 3 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 spectra of MS-MS. Compounds **6** and **7** were hydrolyzed as the following method: treatment 0.5 mg compound with 0.1 N hydrochloric acid in ethanol (2 ml) at 60°C for 15 min. Result of analyzing the hydrolyzates of compounds 6 and 7 by HPLC with a ODSpak column (150 mm × 4.60 mm, Shodex, Co. Japan) showed that catechin and epicatechin were detected in hydrolyzate of compound 6, while only catechin was detected in hydrolyzate of compound **7** (**Fig. 4**). By analysis of ¹H, ¹³C-NMR again, compounds **6** and **7** were identified as procyanidin B1 (Thompson et al., 1972; Shoji et al., 2003) and procyanidin B3 (Thompson et al., 1972; Bruyne et al., 1996; Rohr et al., 2000), respectively.

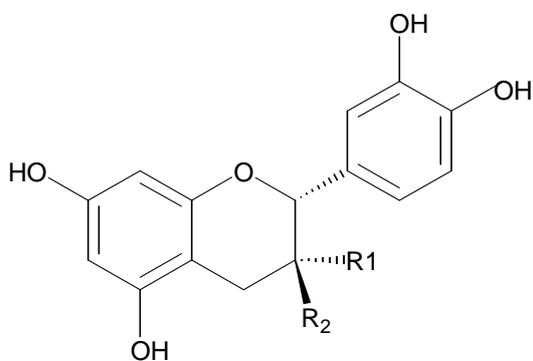


1. Protocatechuic acid



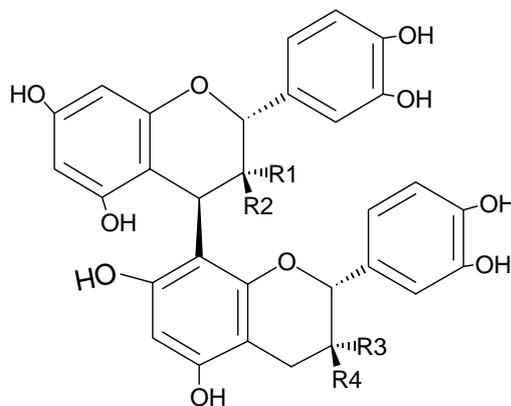
2. Chlorogenic acid: R=H

3. Methyl chlorogenate: R=CH₃



4. (+)-Catechin: R1=H, R2=OH

5. (-)-Epicatechin: R1=OH, R2=H



6. Procyanidin B1: R1=OH, R2=H, R3=H, R4=OH

7. Procyanidin B3: R1=H, R2=OH, R3=H, R4=OH

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

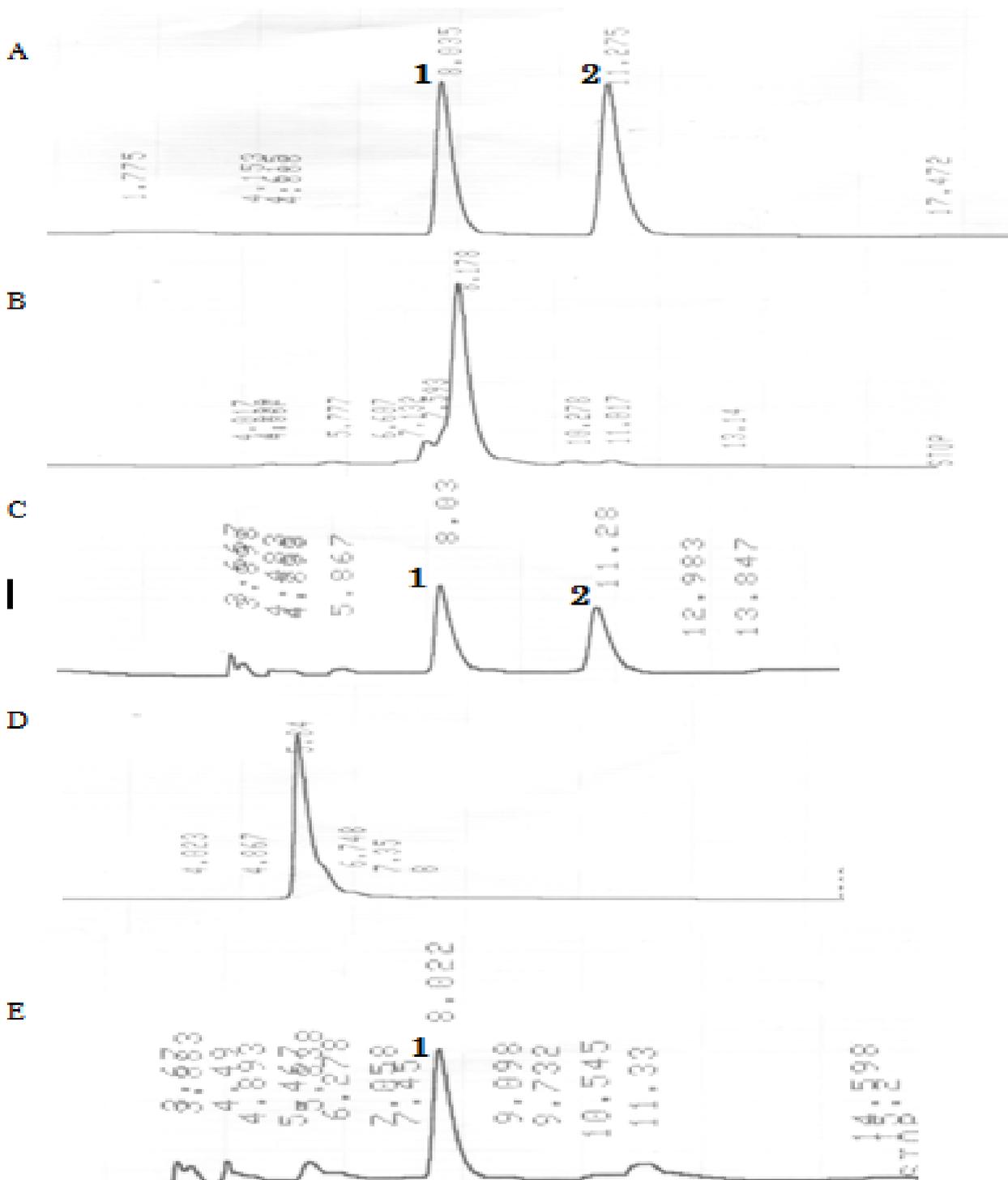


Fig. 4. HPLC chromatograms as monitored by UV absorption at 280 nm (ODSpak column (150 mm × 4.60 mm, Shodex, Co. Japan) for catechins, epicatechin, compounds 6, 7 and their Hydrolyzate; A: standard of catechin and epicatechin (200 $\mu\text{g/ml}$); B: Compound 6; C: Hydrolyzate of 6; D: Compound 7; E: Hydrolyzate of 7; 1, catechin; 2, epicatechin.

6.3.2. Hyaluronidase inhibitory activity by the extracts

Hyaluronidase inhibitory activity of methanol extract of *F. microcarpa* bark and its fractions is illustrated in **Fig. 5**. Methanol extract exhibited inhibitory activity (3.4, 17.3, and 39.4%) against hyaluronidase at the concentration of 100, 200 and 400 $\mu\text{g/ml}$, respectively. Hexane, ethyl acetate, *n*-butanol and water fractions were prepared from methanol crude extract of *F. microcarpa* bark. Of which, ethyl acetate and *n*-butanol fractions exhibited the stronger hyaluronidase inhibitory activity (83.7 and 81.4 %, respectively) at 400 $\mu\text{g/ml}$. From our previous study, methanol extract of *F. microcarpa* bark as well as its fractions ethyl acetate and *n*-butanol showed strong antioxidant activity assayed by different methods (Ao *et al.*, 2008). Since ethyl acetate and *n*-butanol fractions had remarked antioxidant and hyaluronidase inhibitory activities, they subjected to further purification.

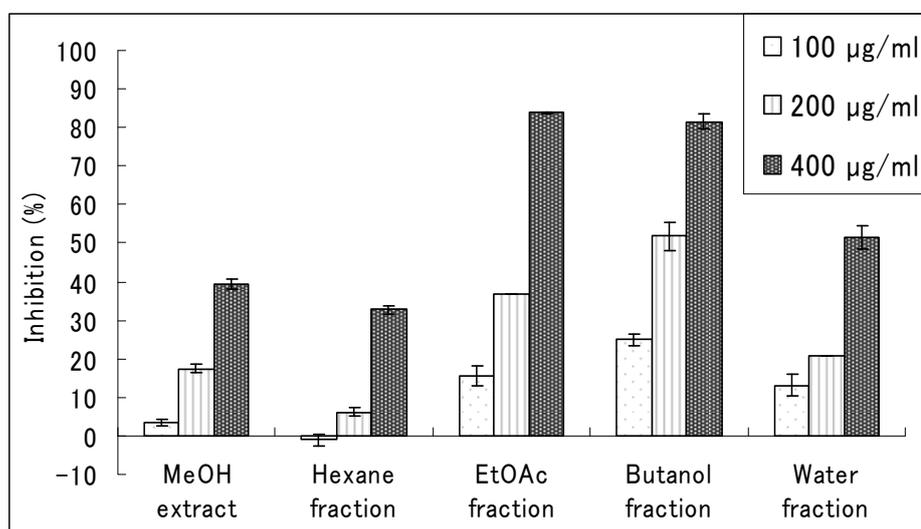


Fig. 5. Hyaluronidase inhibitory activity by methanol extract and fractions of *F. microcarpa*, each value in the figure was expressed as mean \pm SD (n=3), concentration of samples in assays was expressed as final concentration.

6.3.3. Hyaluronidase inhibitory activity by isolated compounds

Seven phenolic compounds were isolated from ethyl acetate fraction by sephadex LH-20 and preparative HPLC. The isolated compounds and antiallergic drug DSCG (positive control) were evaluated for hyaluronidase inhibitory activity (**Fig. 6**). In assay, protocatechuic acid, methyl chlorogenate were only prepared one concentration (500 μM), other compounds prepared three different concentrations. Most compounds effectively inhibited the hyaluronidase activity with increasing concentration. Protocatechuic acid, 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 compared with DSCG (51.1%), catechin, epicatechin, procyanidin B1 and procyanidin B3 exhibited comparable inhibitory activity 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%).

6.3.4. 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).

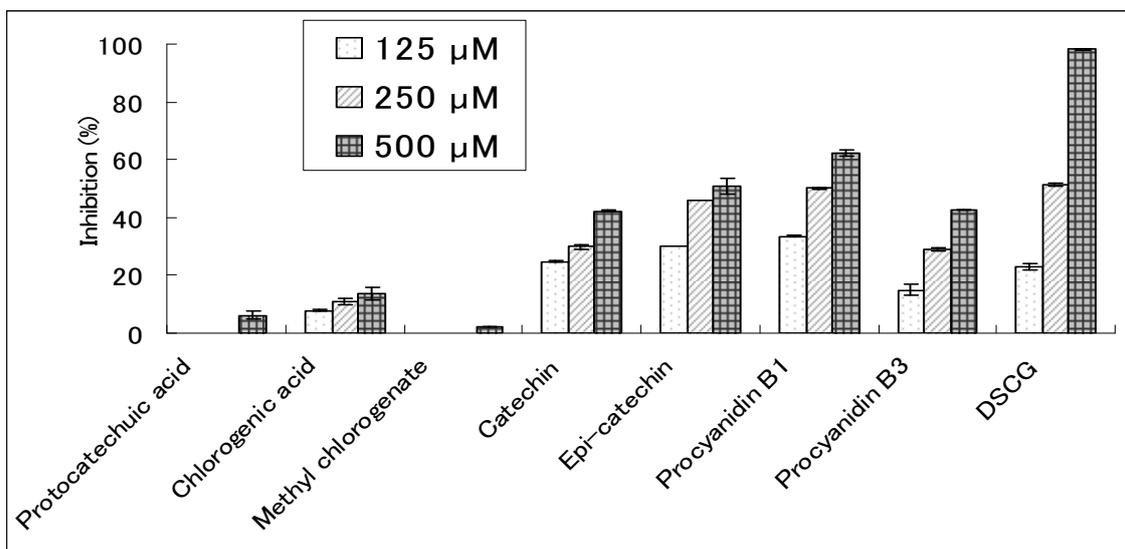


Fig. 6. Hyaluronidase inhibitory activity by the purified compounds of *F. microcarpa*, each value in the figure was expressed as mean \pm SD (n=3), concentration of samples in assays was expressed as final concentration.

Table 1. Antioxidant Activity of Isolated Compounds from *F. microcarpa*

Compounds	Antioxidant activity (EC ₅₀ , μ M)		
	DPPH ^a	PMS-NADH ^b	ABTS ⁺ ^c
Protocatechuic acid	40.3 \pm 1.4	279.2 \pm 2.8	19.6 \pm 1.3
Chlorogenic acid	61.6 \pm 0.9	65.8 \pm 2.0	28.2 \pm 1.0
Methyl chlorogenate	31.9 \pm 1.1	54.3 \pm 1.4	16.0 \pm 0.4
Catechin	11.4 \pm 0.5	67.4 \pm 1.8	4.1 \pm 0.5
Epi-catechin	11.3 \pm 1.0	71.0 \pm 1.4	2.9 \pm 0.4
Procyanidin B1	12.4 \pm 0.7	79.4 \pm 0.9	–
Procyanidin B3	14.0 \pm 0.4	80.5 \pm 1.7	–
Trolox	17.6 \pm 0.7	722.0 \pm 3.4	2.0 \pm 0.3

^aDPPH Radical Scavenging activity. ^b Superoxide free radical scavenging activity. ^c ABTS⁺ free radical scavenging activity. “–” Not determined. Each value in the table was expressed as mean \pm SD (n=3). Concentration of samples in assays was expressed as final concentration.

6.4. Discussion

The purpose of this research was to isolate and identify anti-allergic and antioxidant compounds from the bark of *F. microcarpa*, which is a well-known as a medicinal herb for perspiration, alleviating fever and relieving pain in Okinawa Islands (Tawata and Oota, 1985). 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 and preparative HPLC. Compounds 1 and 4 were previously isolated from *F. microcarpa* by Kou and Li (Kuo and Li, 1997), however, as far as we know, compounds 2, 3, 5, 6 and 7 were reported for the first time from this plant. Komatsu *et al.* (2003) reported that contents of catechin and epicatechin as the major components in the green tea were about 4 mg and 10 mg/5 g powdered green tea, 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) than tea catechins. Chlorogenic acid was recorded in 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 are widely recognized to have antioxidant activity (Jung *et al.*, 1999; Landrault *et al.*, 2001). Our study revealed that these compounds are responsible for the antioxidant properties of *F. microcarpa*. Furthermore, the antiallergic activity of chlorogenic acid, catechin, epicatechin, procyanidin B1 and procyanidin B3 has been under intensive investigations (Maffei *et al.*, 1994; Ito *et al.*, 1998; Kanda *et al.*, 1998). In our study, at the concentration of 500 μ M, 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%). Contrarily, at the concentration of 125 μ M, 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 DSCG did at lower sample concentration.

Table 2. Compounds Content in *F. microcarpa* Bark and Ethyl Acetate Fraction

	Compounds content (mg/g DW)	
	Bark ^a	Ethyl acetate fraction ^b
Catechin	6.7 ± 0.6	220 ± 1.9
Epi-catechin	5.0 ± 0.9	60 ± 1.5
Chlorogenic acid	6.7 ± 0.4	101 ± 1.2
Methyl chlorogenate	–	–
Protocatechuic acid	–	–
Procyanidin B1	–	–
Procyanidin B3	–	–

^a compounds content in dried powder of *F. microcarpa* bark; ^b compounds content in dried ethyl acetate fraction; “– “ not measuring. Each value in the table was expressed as mean ± SD (n=3).

The result indicated that isolated compounds 2, 4, 5, 6 and 7 both showed high antioxidant activity and hyaluronidase inhibitory activity. Many Researches also reported that some antioxidants showed high anti-allergic activity (Ito *et al.*, 1998; Sala *et al.*, 2003). 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 which, 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 of using *F. microcarpa* in the folk medicine in Ryukyu Islands. Further investigation is needed to separate individual proanthocyanidin molecules from the butanol fraction of *F. microcarpa*.

CHAPTER 7

General conclusion

Three traditional Chinese medicines: *Sophora subprostrata*, *Hemsleya amabilis*, *Hedyotis diffusa* and thirteen Okinawan medicinal plants are commonly used as herbal medicines for anticancer and arthritis, analgesic, antipyretic and anti-inflammatory purposes. However, the mechanisms of disease treatments and active components of most of these plants have not been clearly elucidated up to now. Inhibitory ability against cyclooxygenase (COX), collagenase 3 (MMP-13) and hyaluronidase were used to evaluate the anti-tumor and anti-inflammatory activities of plant extract or compounds. The antioxidant activity is among the first links between chemical reactions and biological activity. In present study, (1) *S. subprostrata*, *H. amabilis* and *H. diffusa* have been investigated for cyclooxygenase inhibitory and antioxidant activities. The thirteen Okinawan medicinal plants were screened for MMP-13 inhibitory and antioxidant activities. (2) Some major components were purified or identified from the plants that showed higher biological activities. (3) The isolated compounds were tested for bioactivities and structures were elucidated.

Dried powders of the three Chinese herbs were separately extracted with methanol. Solvent was removed under vacuum, and the dried methanol extracts were re-dissolved in methanol for measuring: COX inhibitory activities by peroxidase and product assays; antioxidant activities by DPPH and PMS-NADH system assays. Since methanol extract of *S. subprostrata* showed the highest activity in COX inhibition and antioxidant experiments by applied assays in the three herbs, it was selected for large-scale extraction and isolation of bioactive compounds. The two alkaloids matrine and oxymatrine were isolated from chloroform fraction of the aerial portion of *S. subprostrata* by activity-guided chromatographic fractionation and the structures were analyzed by LC-MS, LC-MS/MS, ¹H-NMR, and ¹³C-NMR. The yield of matrine and

oxymatrine in *S. subprostrata* were 0.46% and 3.93% per dry weight, respectively. The results indicated that matrine and oxymatrine had high inhibitory activity against the COX (matrine, IC₅₀: 7.8 and 47 µg/ml for COX-1 and COX-2, respectively; oxymatrine, IC₅₀: 52.5 and 102.2 µg/ml for COX-1 and COX-2, respectively). Matrine and oxymatrine also showed high antioxidative activities by superoxide radicals scavenging (EC₅₀: 7.5 and 6.2 µg/ml, respectively).

The methanol extracts of thirteen Okinawan medicinal plants were screened for MMP-13 inhibitory and antioxidant activities. MMP-13 inhibitory activity was measured by a MMP-13 inhibitor assay kit, and antioxidant activity was performed by DPPH radical scavenging, superoxide radical scavenging, reducing power and metal chelating ability methods. Among the tested plants, *Curcuma longa*, *Ocimum basilicum* and *Curcuma aromatica* showed high inhibitory effect on MMP-13, with IC₅₀ values of 27.8, 81.7 and 85.8 µg/ml, respectively. The chemical compositions of these three extracts were analyzed by LC-MS. Curcumin was identified and quantified in extracts of *C. longa* and *C. aromatica* (58.6 and 28.7 mg/g extract, respectively). Rosmarinic acid was identified in extract of *O. basilicum* (47.3 mg/g extract). Both curcumin and rosmarinic acid exhibited high MMP-13 inhibitory activity (IC₅₀: 3.6 and 2.9 µM, respectively). On the other hand, the extracts of *Ficus microcarpa* and *Smilax sebeana* exerted stronger antioxidant activities than the other extracts of plants in all applied methods. These two plants and *C. longa* possessed high phenolic content when analyzed using Folin-Ciocalteu's reagent. *F. microcarpa* is one of the traditional medicinal plants and food additives (for Okinawan Soba) in Okinawa and a few papers reported its antioxidant activity up to now. As this plant exhibited excellent antioxidant activity by screening, therefore it was selected for further research. Results showed that methanol

extracts of bark, aerial roots of *F. microcarpa* exhibited higher antioxidant activities and also possessed better antibacterial activity against tested Gram-positive and Gram-negative bacteria than that of fruits and leaves. By further separation, ethyl acetate fraction of bark extract (BE) and aerial root (RE) exerted much stronger antioxidant and antibacterial effects than other fractions and contained high amounts of total phenolics (436, 391 GAE mg/g extract). EC₅₀ values of BE and RE were 4.83 and 6.0, 1.62 and 1.8, 63.2 and 89.7 µg/ml in DPPH, ABTS^{•+}, superoxide radicals scavenging methods, respectively. Inhibition zones of BE and RE against *Bacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Achromobacter polymorph* were 18.0 and 13.4, 15.5 and 14.5, 16.5 and 12.5, 16.0 and 14.5, 8.0 and 12.0 mm, respectively. Twelve and eleven phenolic compounds were identified in BE and RE fraction by GC-MS and HPLC analyses, respectively. Methanol extract of *F. microcarpa* bark also showed potential inhibitory activity against hyaluronidase. It was fractionated into hexane, ethyl acetate, butanol and water fractions. As ethyl acetate fraction exhibited the strongest activity, it was selected for further purification by repeated Sephadex LH-20 column and preparative HPLC. Seven compounds were isolated and identified as protocatechuic acid, chlorogenic acid, methyl chlorogenate, catechin, epicatechin, procyanidin B1 and procyanidin B3 by analyses of ESI/MS, UV, ¹H and ¹³C-NMR. Chlorogenic acid, methyl chlorogenate, epicatechin, procyanidin B1 and procyanidin B3 were reported for the first time in *F. microcarpa*. All isolated compounds showed strong antioxidant activity when tested by DPPH, ABTS⁺ and superoxide radical scavenging methods. Catechin, epicatechin, procyanidin B1 and procyanidin B3 exhibited excellent inhibitory activity against hyaluronidase.

This study provided some scientific supports for using the herb as anticancer,

anti-arthritis, analgesic, antipyretic and anti-inflammatory in traditional folk medicine in China and Okinawa. The findings suggested that extract from *F. microcarpa* might be useful for antioxidant and antibacterial materials in food industry.

APPENDIX

**Charts of antibacterial effects of the extracts and NMR, mass
and UV spectroscopy analysis of compounds isolated from *F.*
*microcarpa***

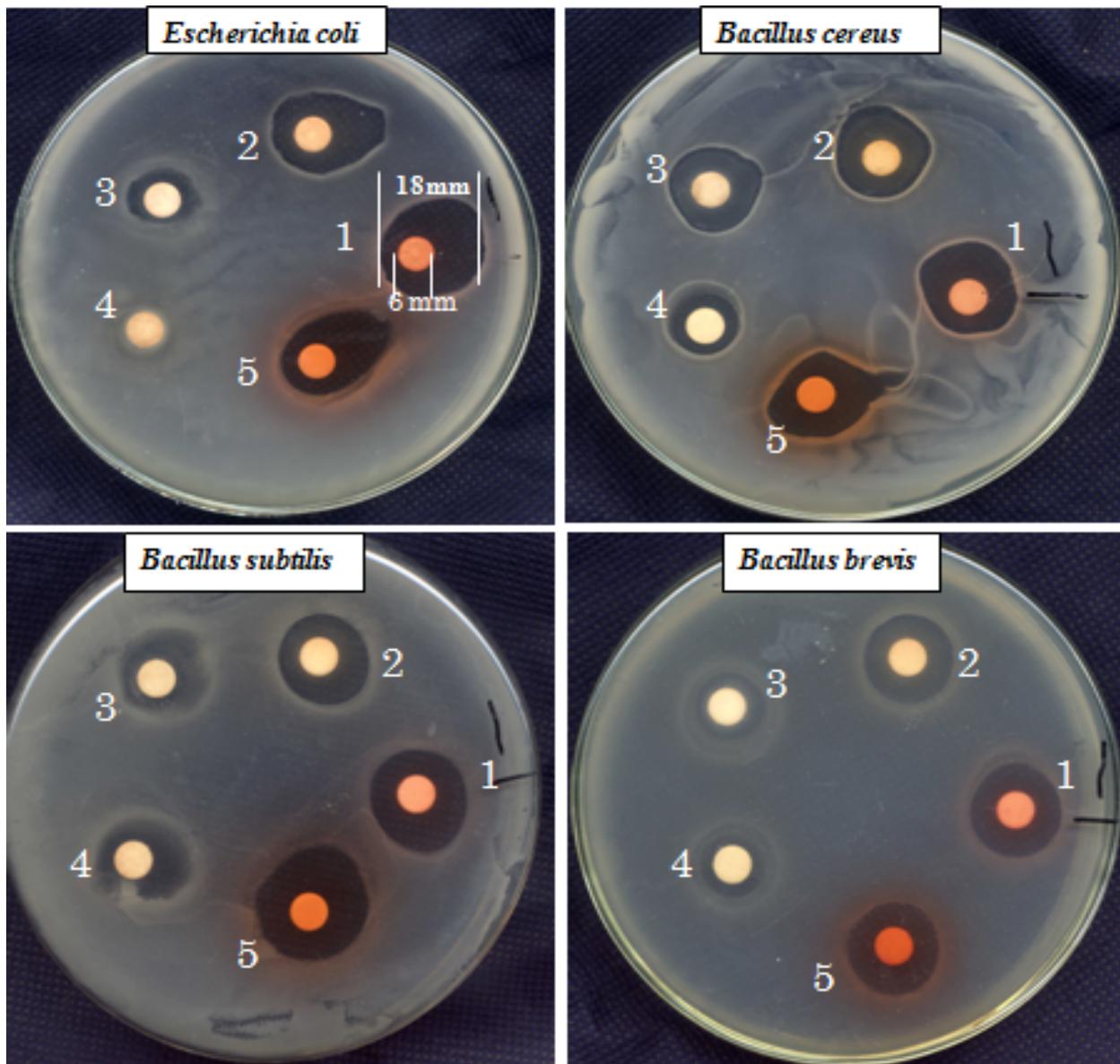


Chart 1. Antibacterial activity of *F. microcarpa* by disc diffusion method (inhibition zones, cm); 300 µg/disc of each extract (dissolved in water) was used to load on a sterile disc; The Arabic number in each figure means: 1. Bark extract, 2. Aerial root extract, 3. Fruit extract, 4. Leaves extract, 5. Ethyl acetate fraction of bark extract.

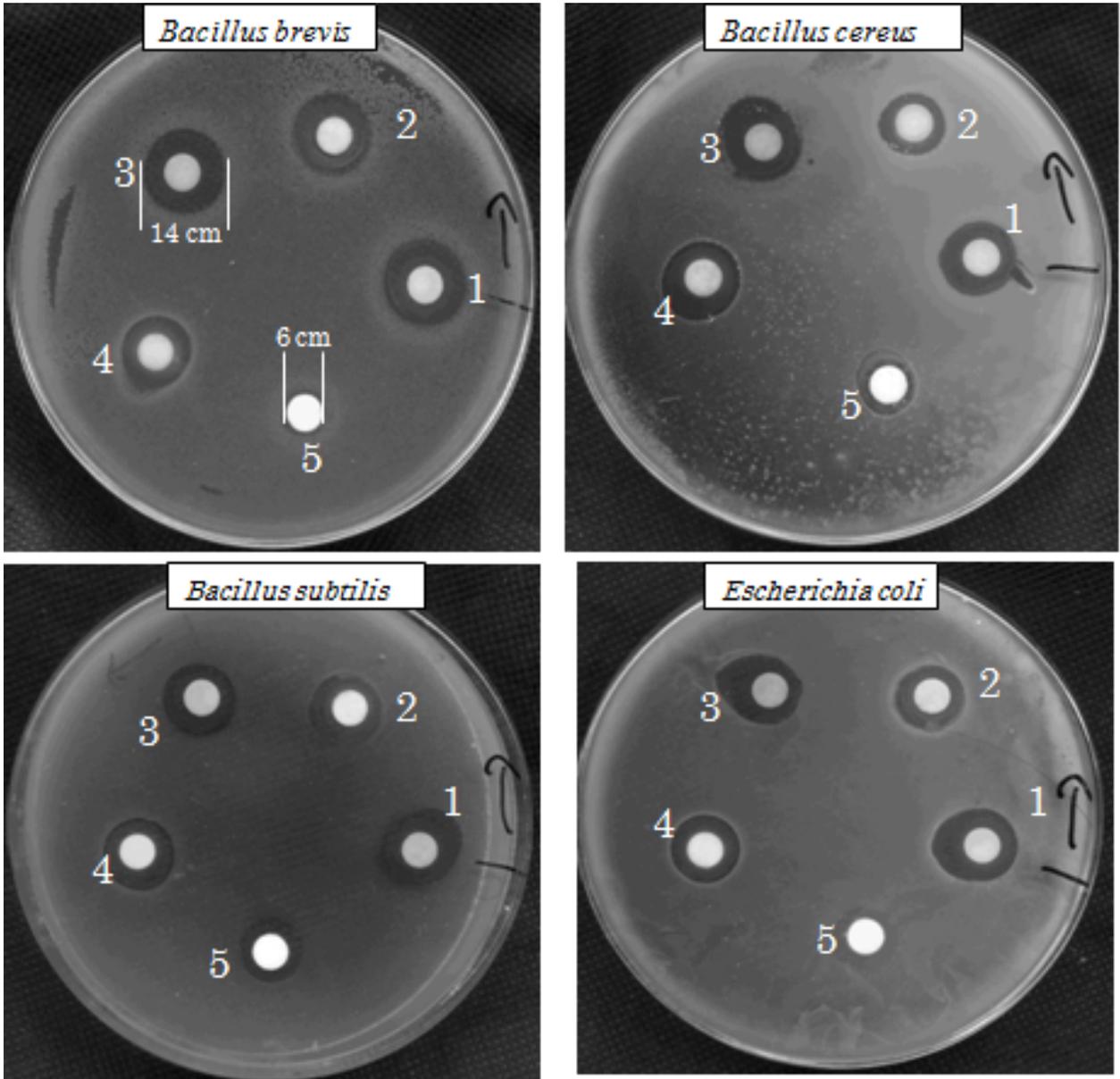


Chart 2. Antibacterial activity of aerial root extracts of *F. microcarpa* by disc diffusion method (inhibition zones, cm); 200 µg/disc of each extract (dissolved in DMSO) was used to load on a sterile disc; The Arabic number in each figure means: 1. Methanol extract, 2. Hexane fraction, 3. Ethyl acetate fraction, 4. Water fraction, 5. DMSO (negative control).

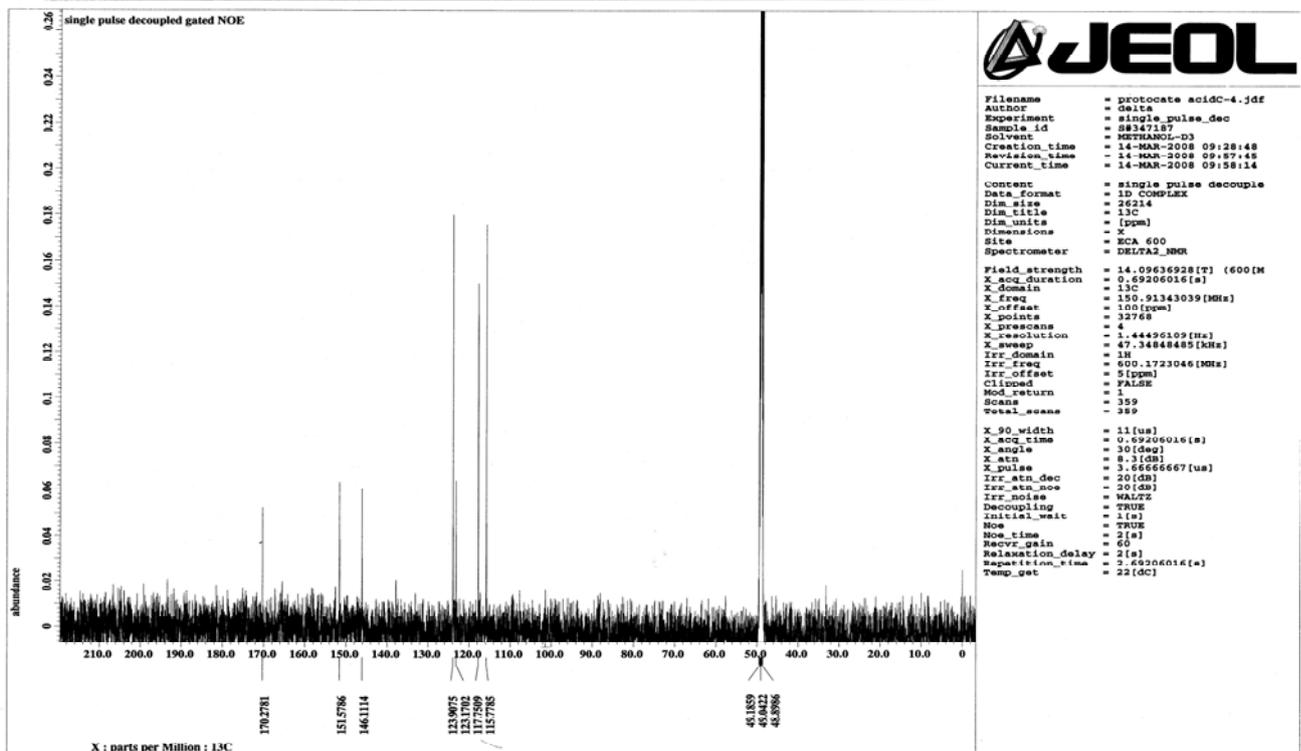
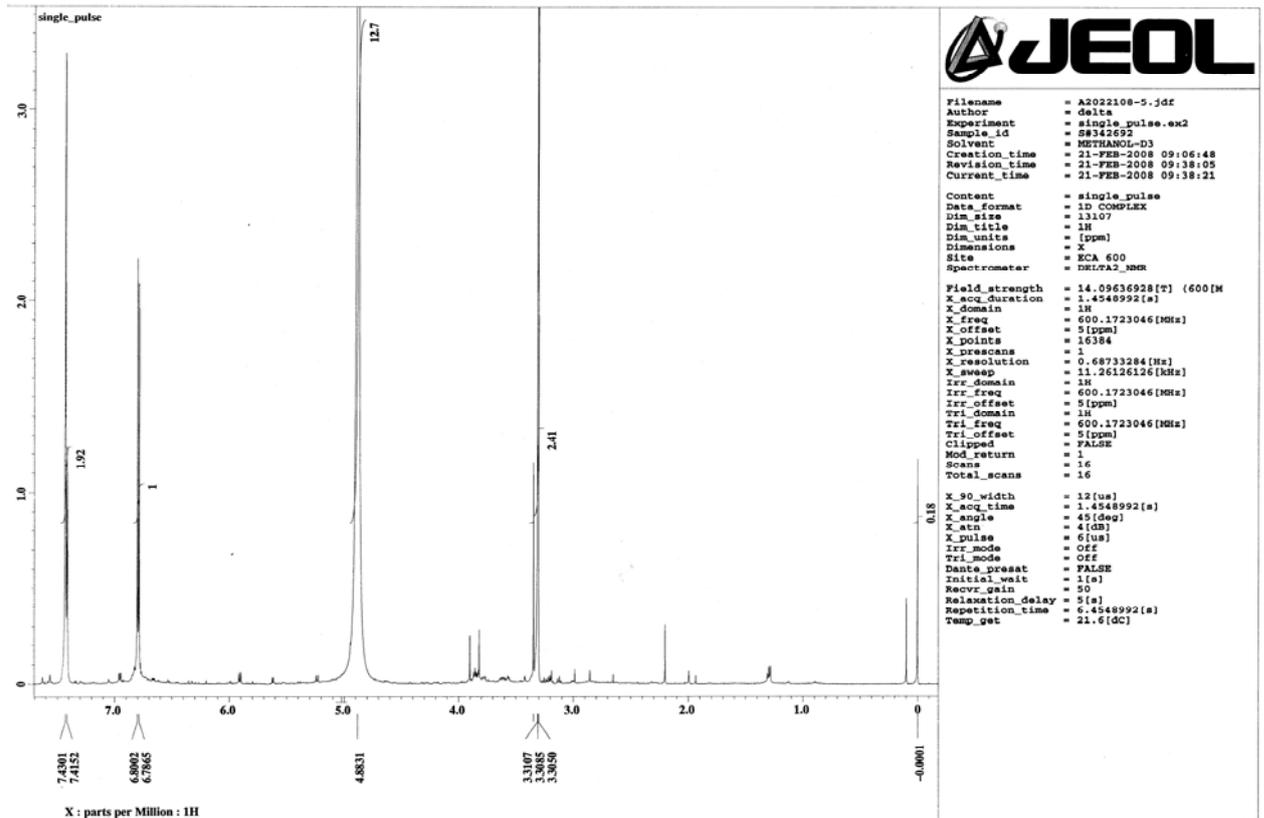


Chart 3-1. ^1H and ^{13}C -NMR of isolated protocatechuic acid from *F. microcarpa*.

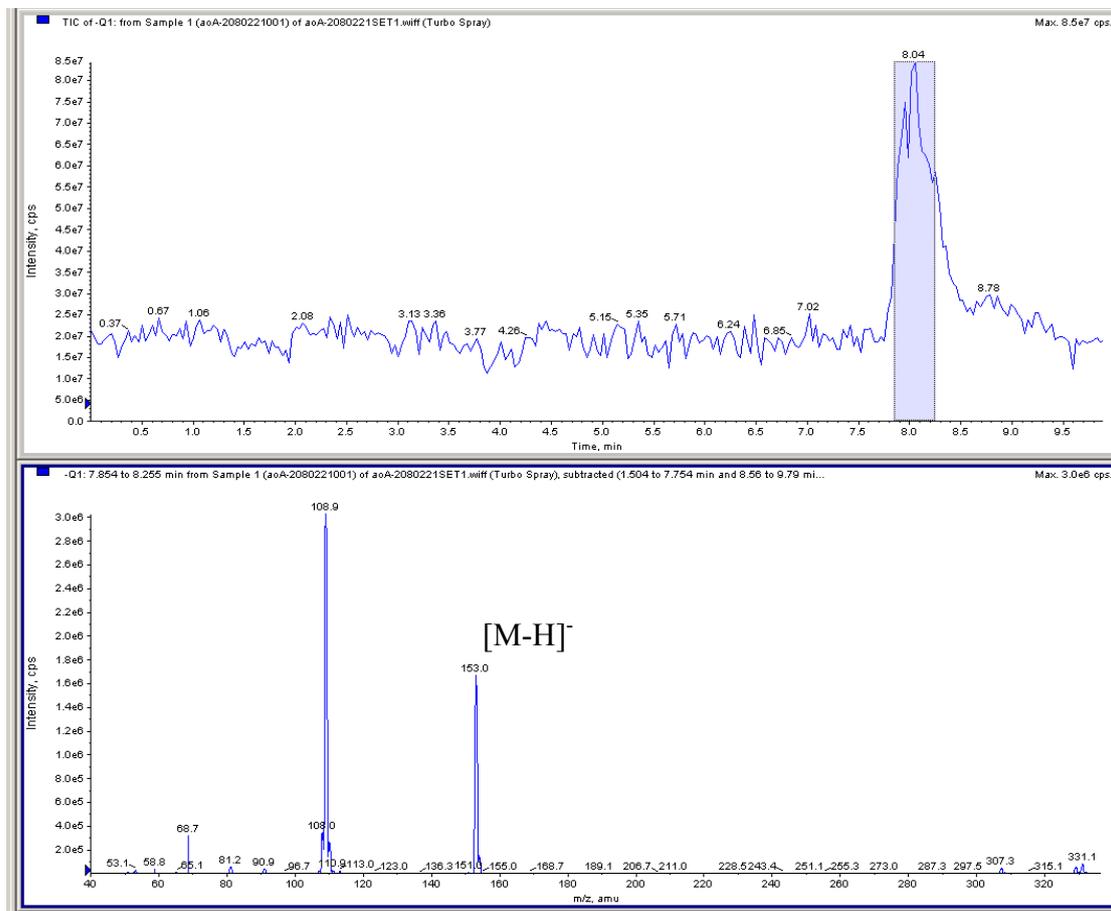


Chart 3-2. LC-MS of isolated protocatechuic acid from *F. microcarpa*.

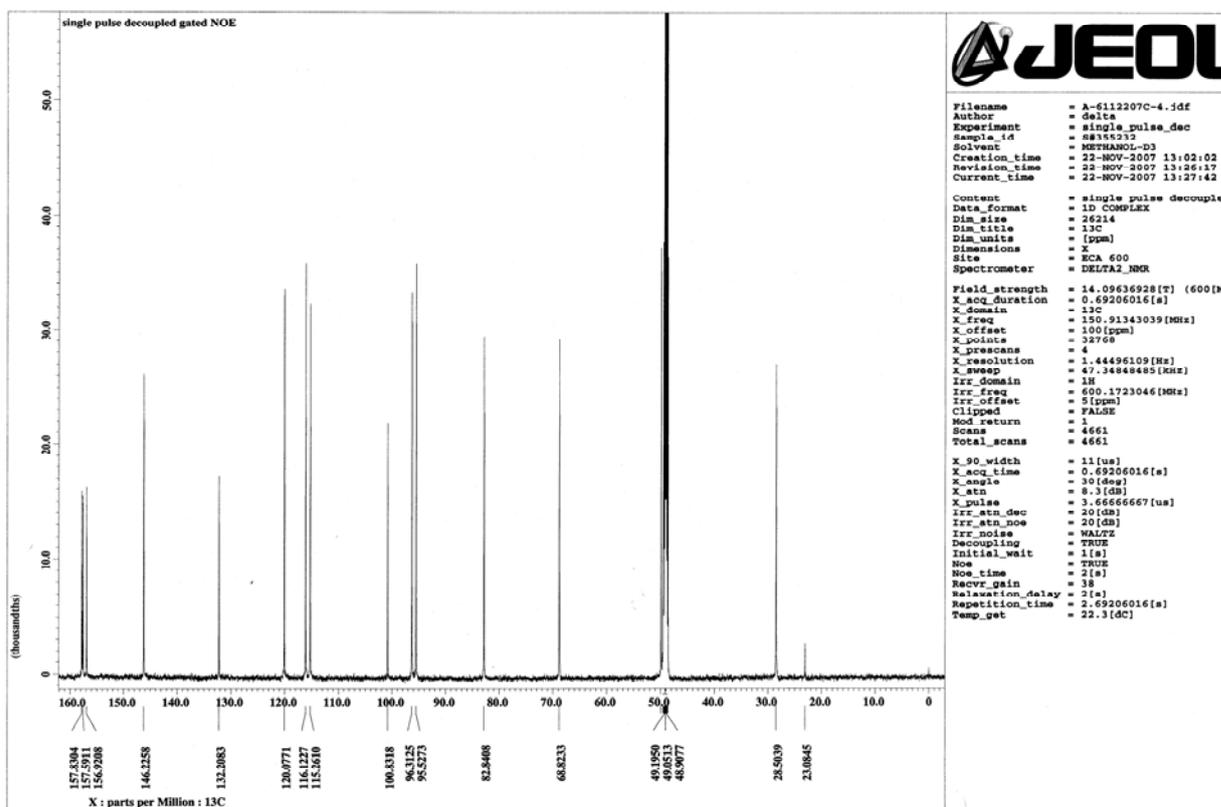
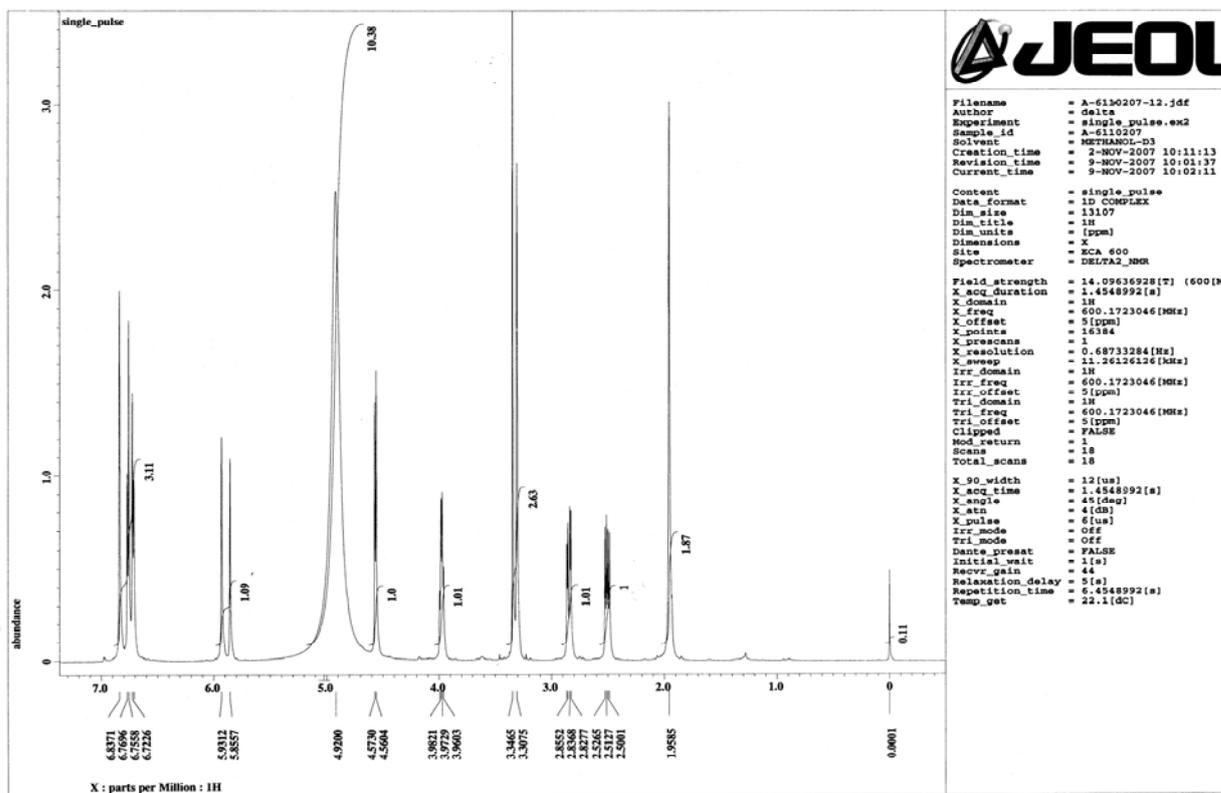


Chart 4-1. ^1H and ^{13}C -NMR of isolated catechin from *F. microcarpa*.

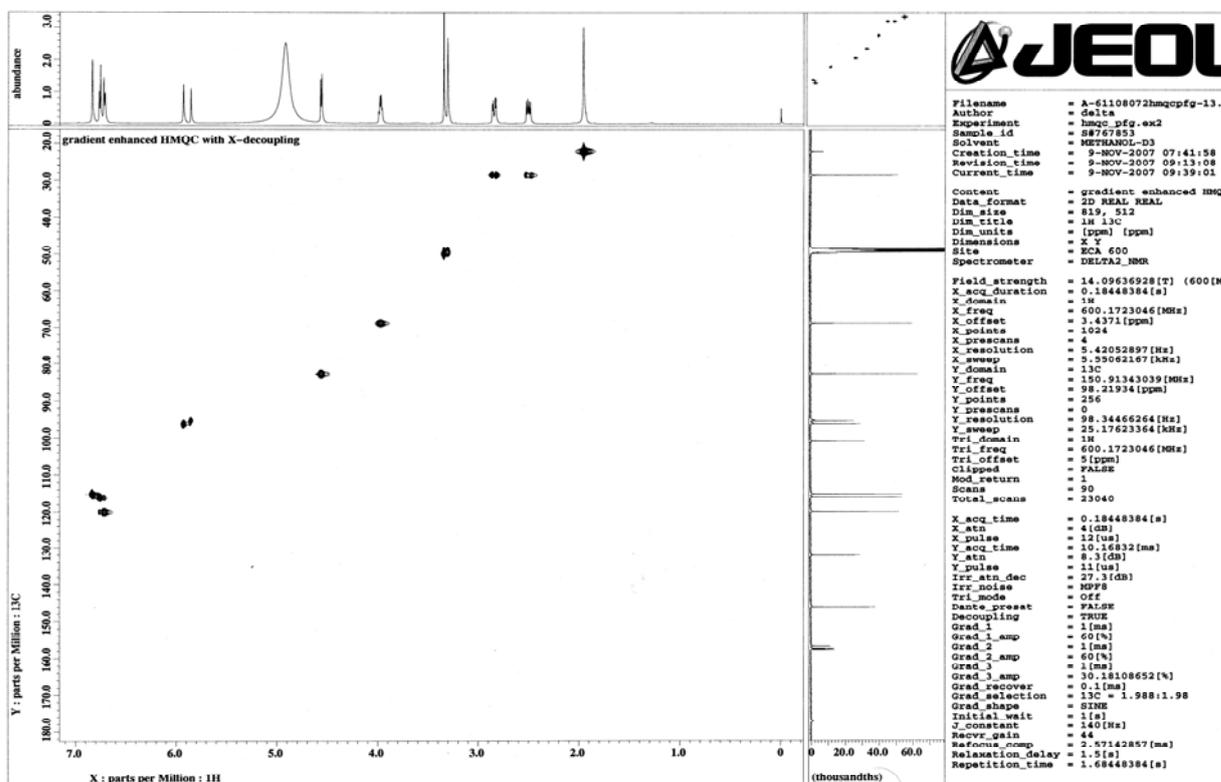
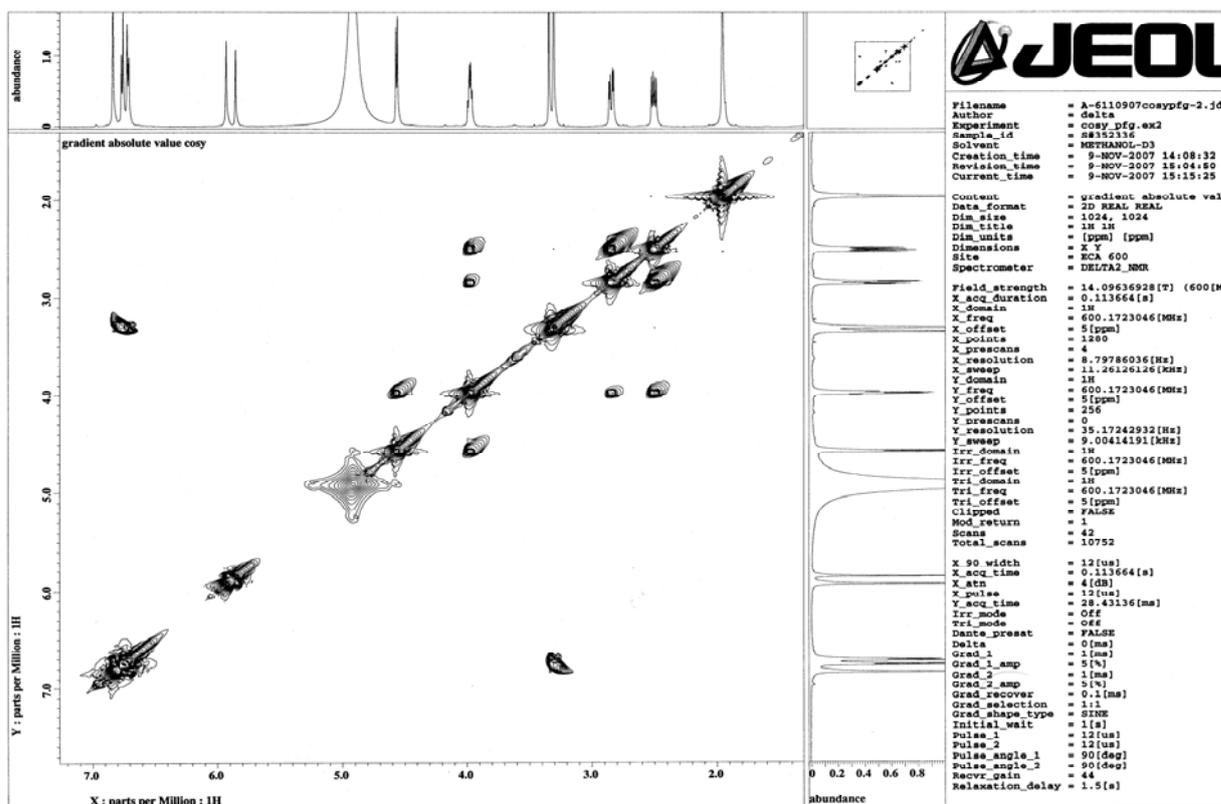


Chart 4-2. COSY and HMQC of isolated catechin from *F. microcarpa*.

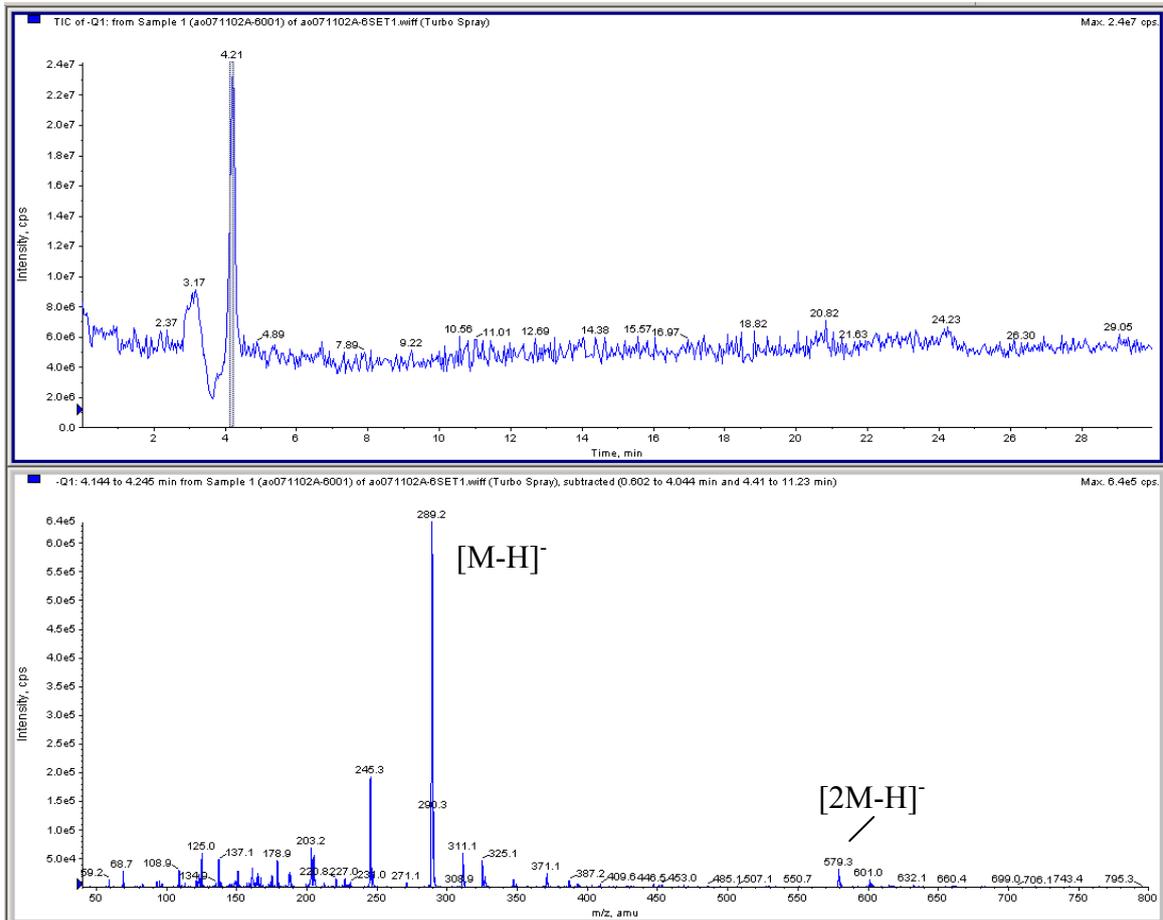


Chart 4-3. LC-MS of isolated catechin from *F. microcarpa*.

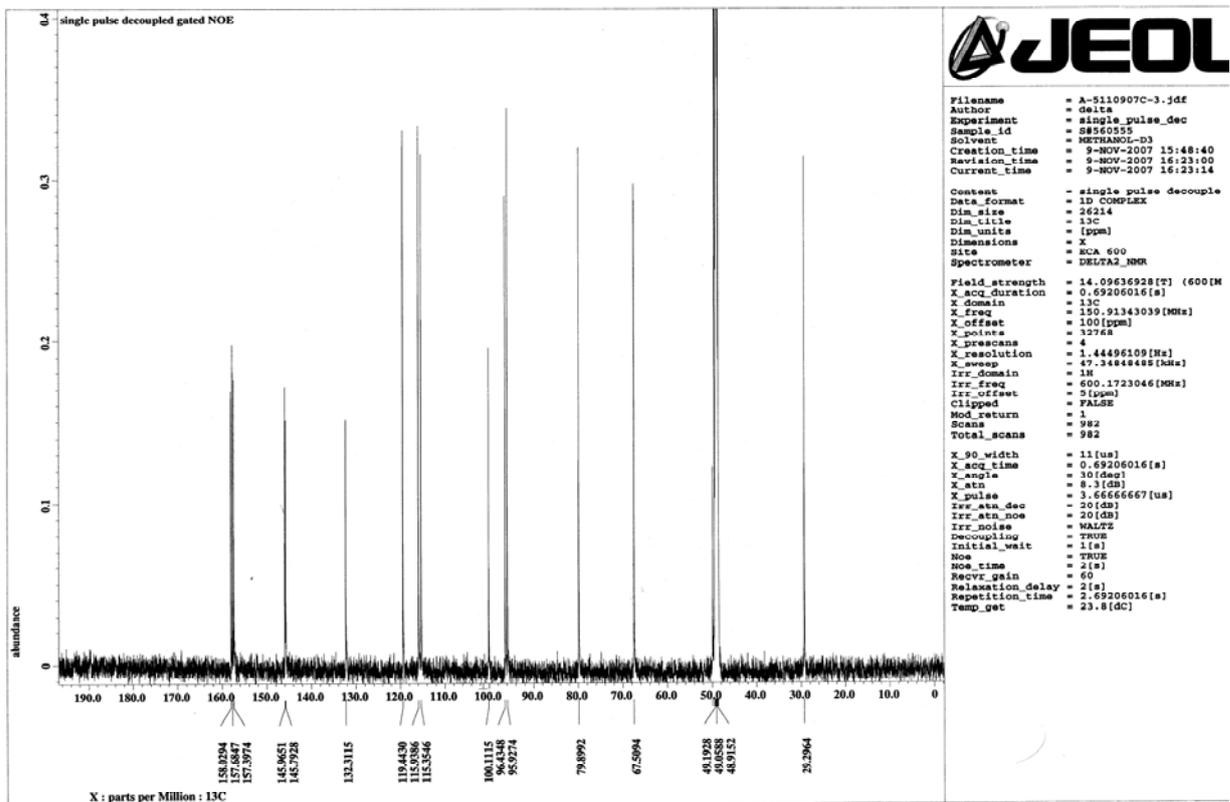
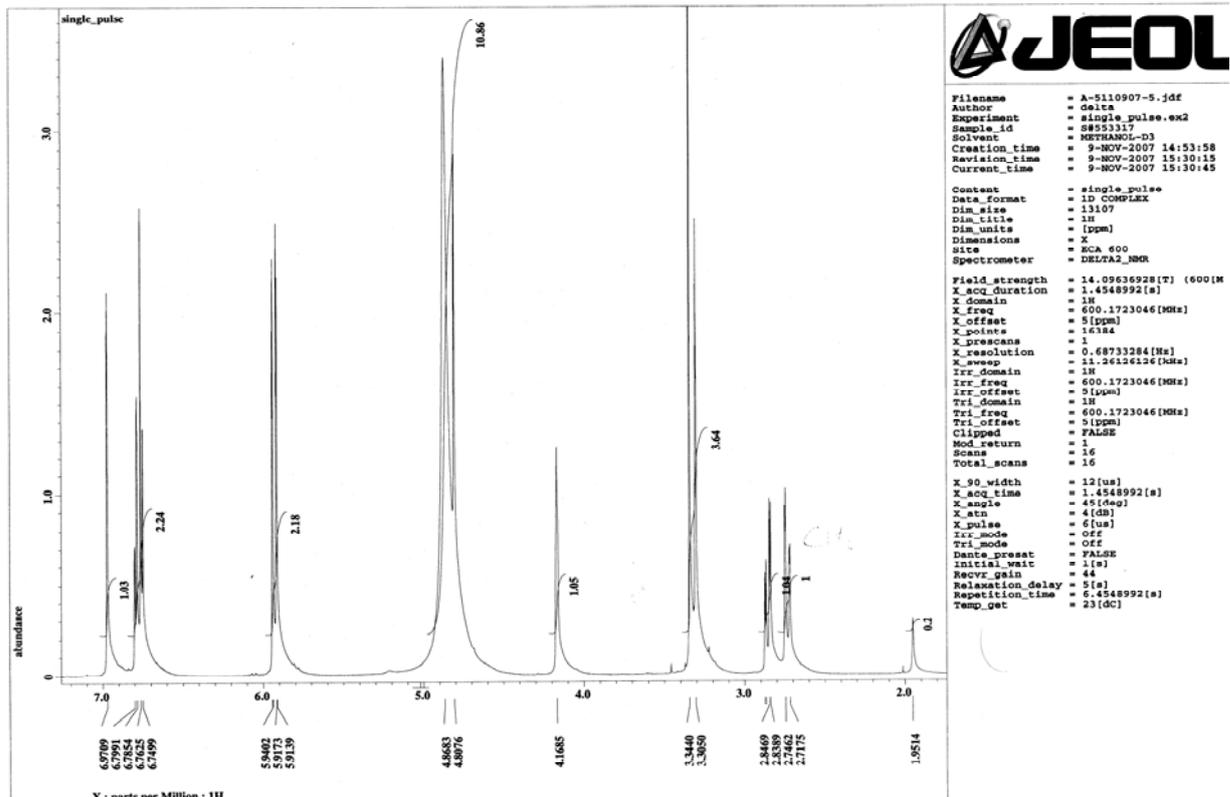
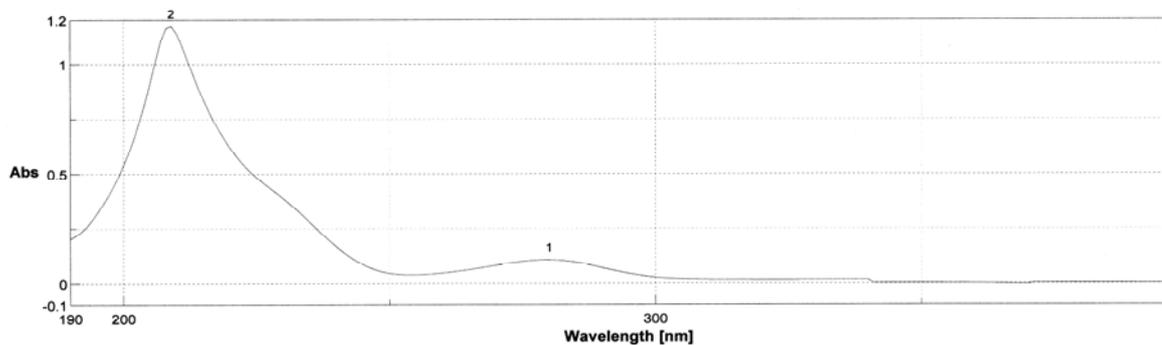


Chart 5-1. ^1H and ^{13}C -NMR of isolated epicatechin from *F. microcarpa*.



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 近赤外バンド幅 4.0 nm
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 測定範囲 400 - 190 nm
 データ取込間隔 1nm
 走査速度 1000nm/min
 試料番号 18
 繰り返し回数 1
 試料名 お客様各位
 測定者
 コメント

ファイル名 Memory#3

A-5

V-570-5

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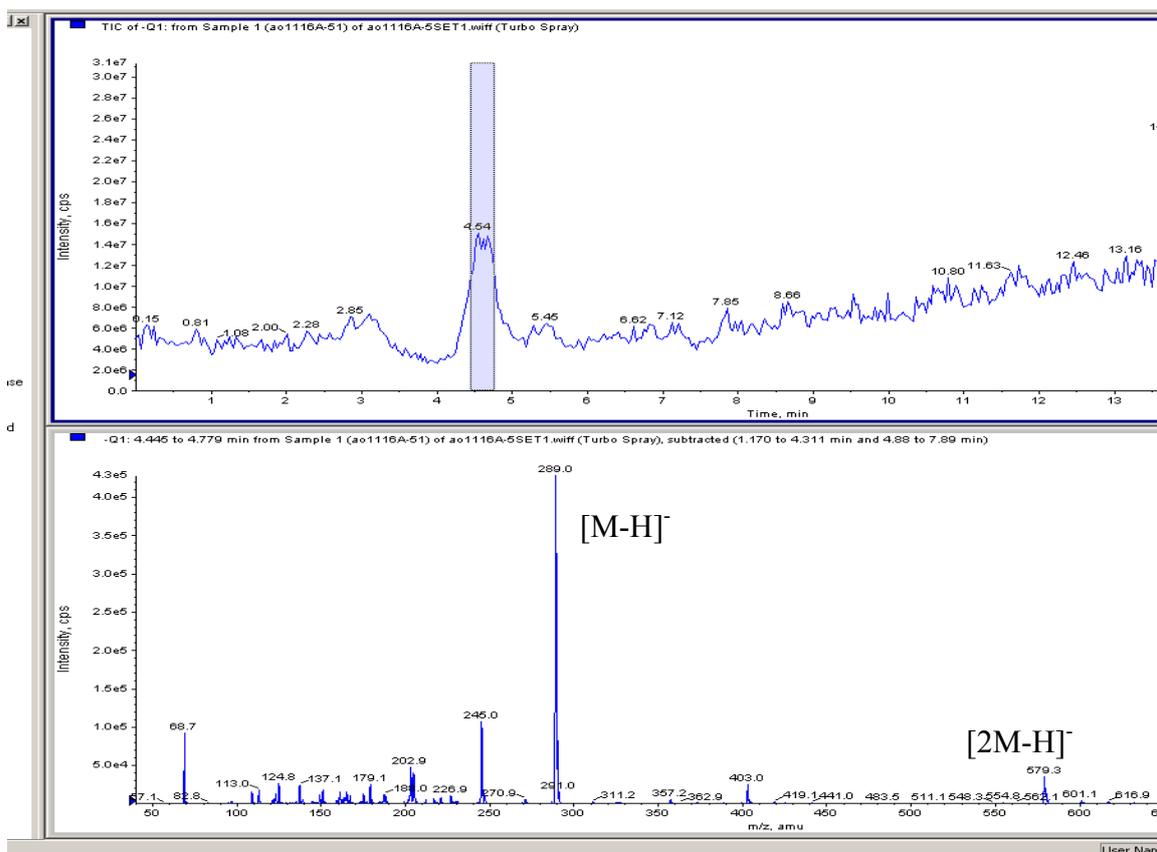


Chart 5-2. UV and LC-MS of isolated epicatechin from *F. microcarpa*.

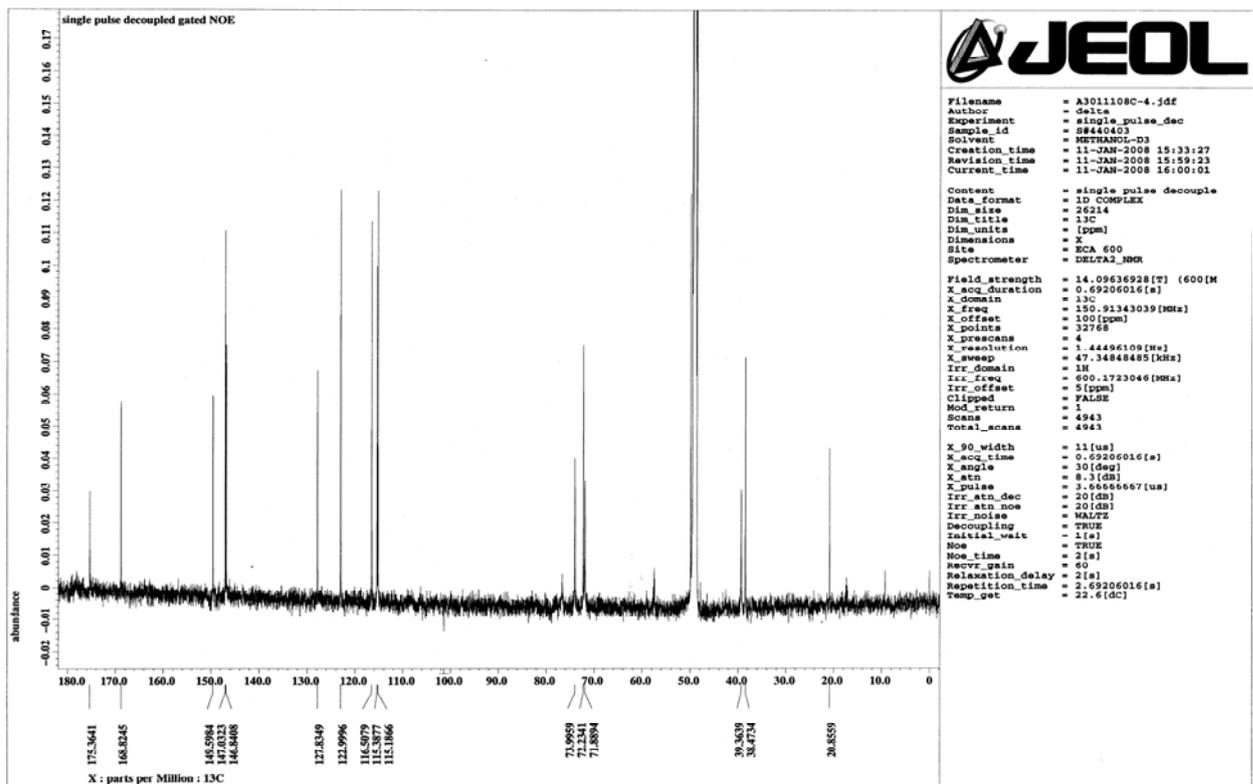
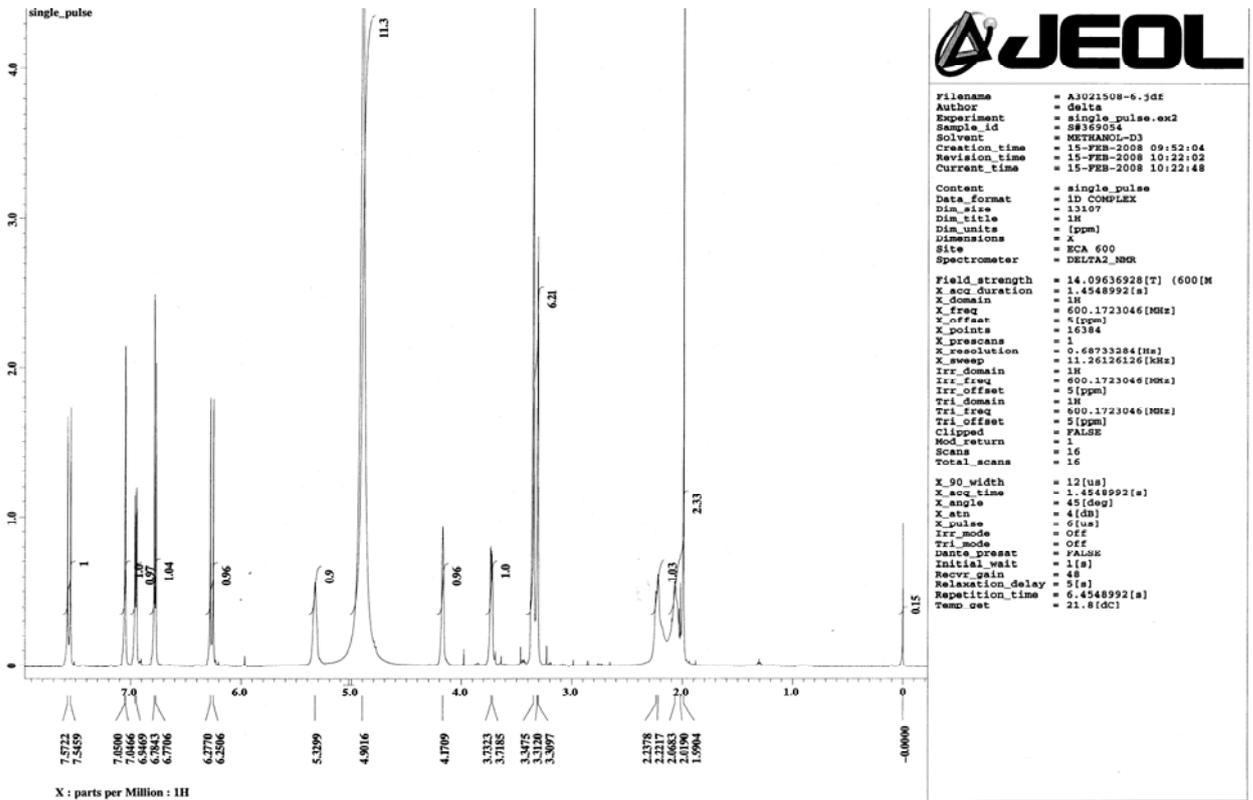
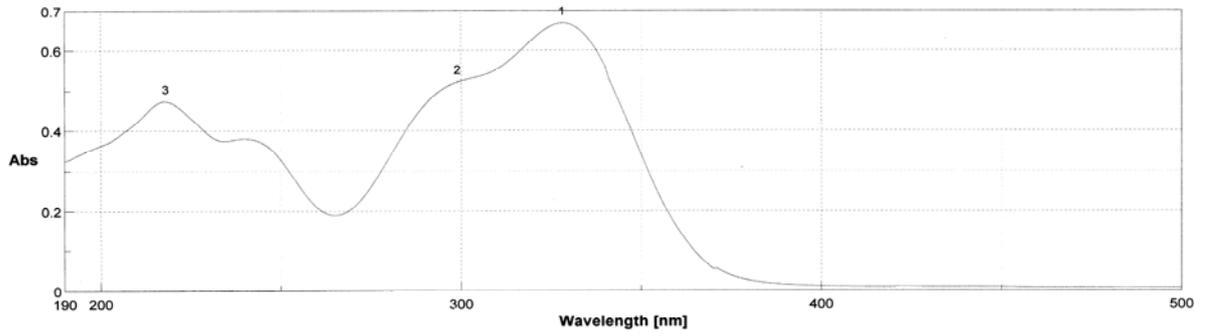


Chart 6-1. ^1H and ^{13}C -NMR of isolated chlorogenic acid from *F. microcarpa*.



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 近赤外バンド幅 4.0 nm
 レスポンス Medium
 測定範囲 500 - 190 nm
 データ取込間隔 1nm
 走査速度 1000nm/min
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 繰り返し回数 1
 試料名 お客様各位
 測定者
 コメント

ファイル名 A-3 Memory#1

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			3	218	0.47418

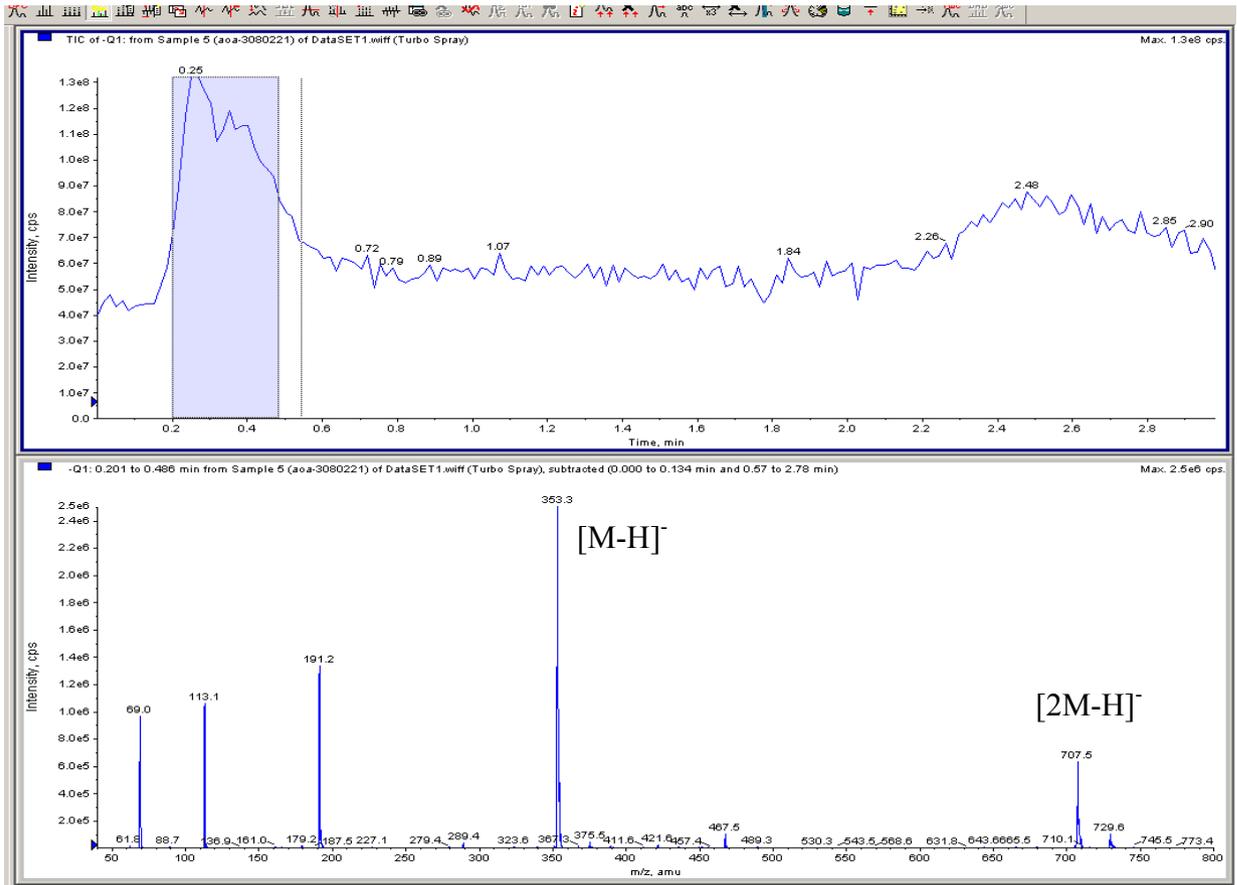


Chart 6-2. UV and LC-MS of isolated chlorogenic acid from *F. microcarpa*.

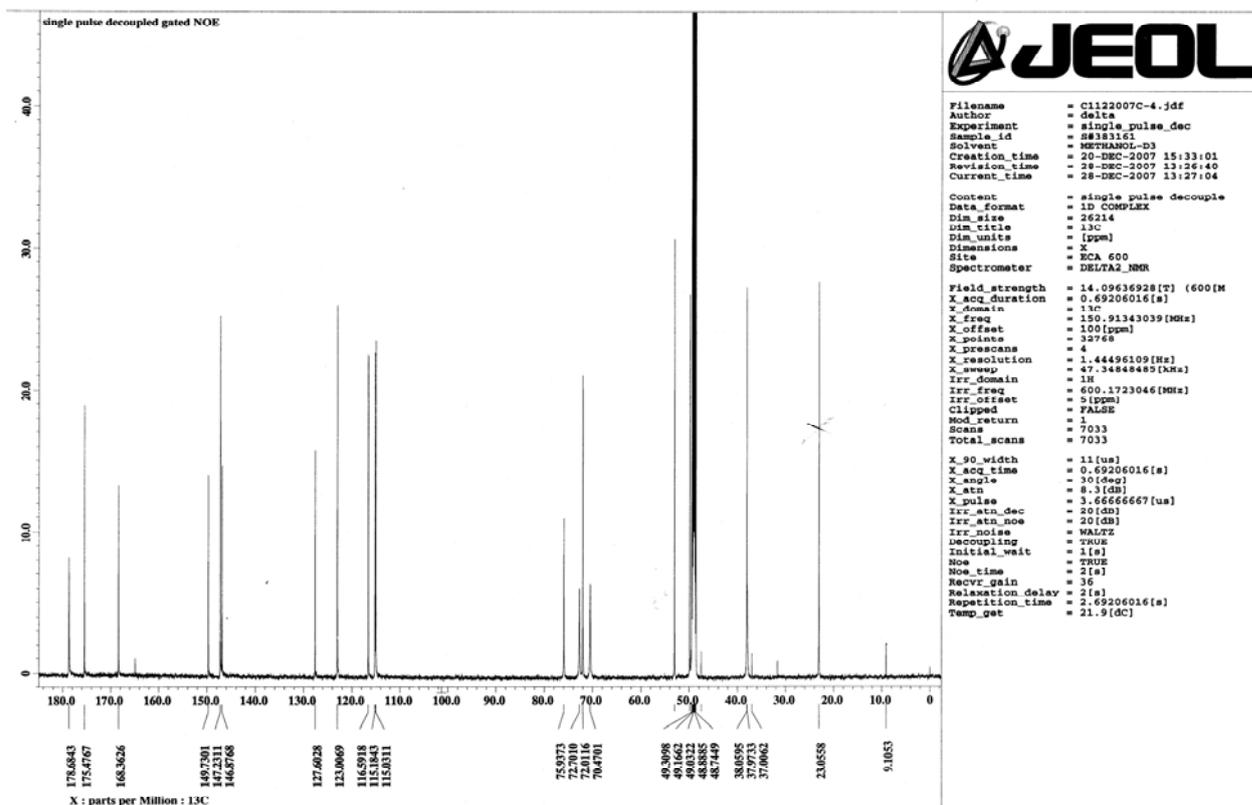
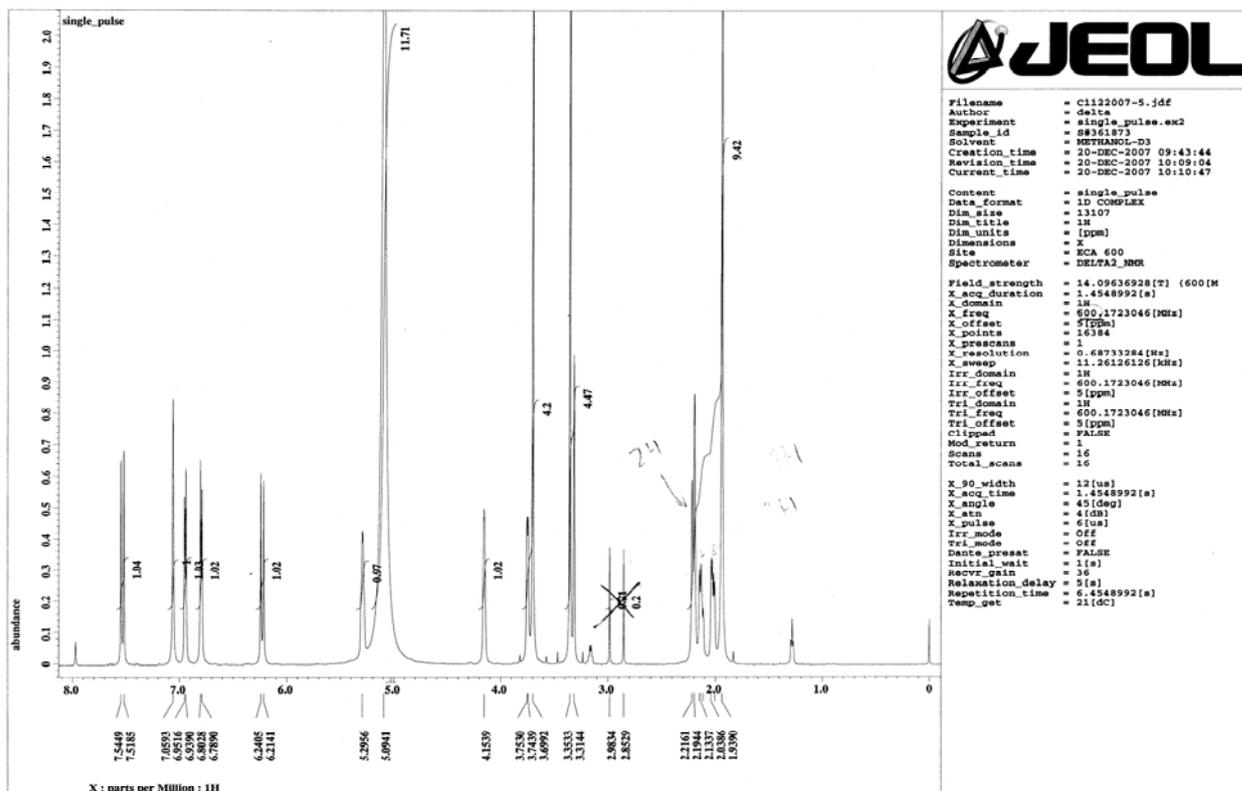


Chart 7-1. ^1H and ^{13}C -NMR of isolated methyl chlorogenate from *F. microcarpa*.

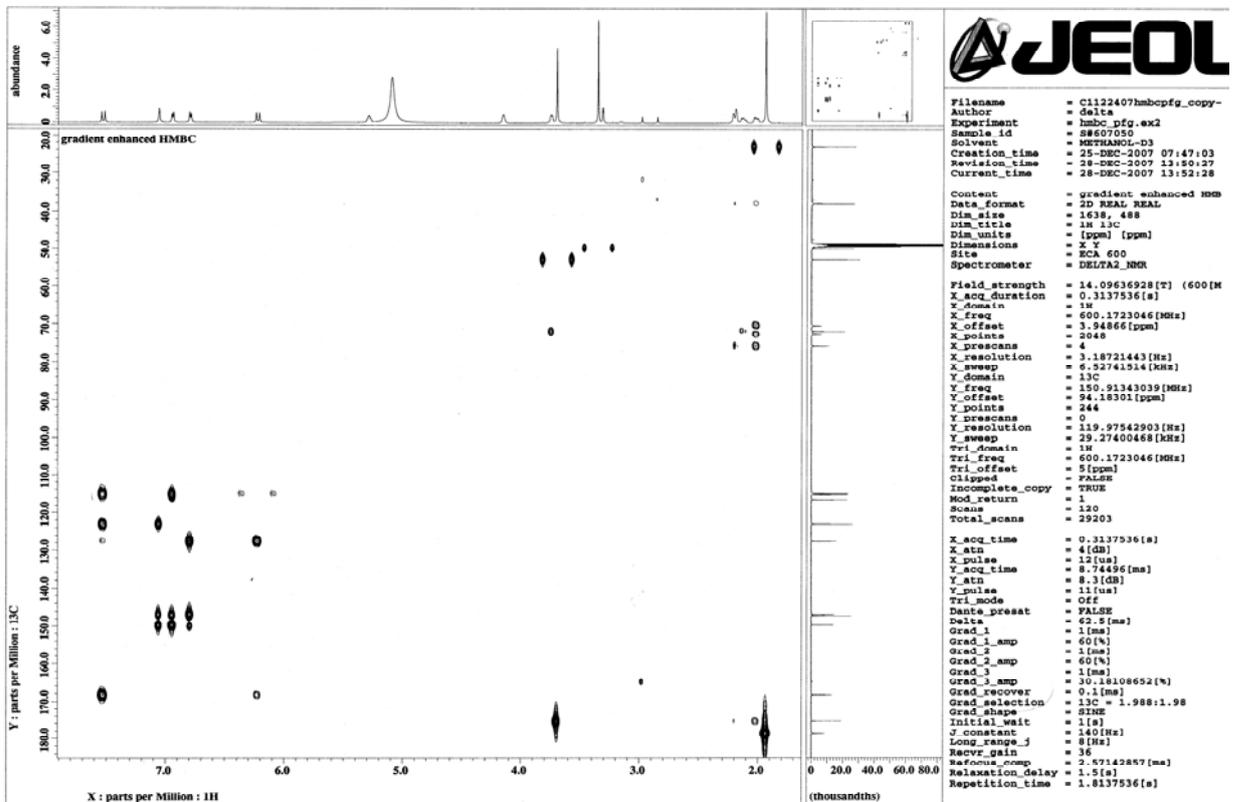
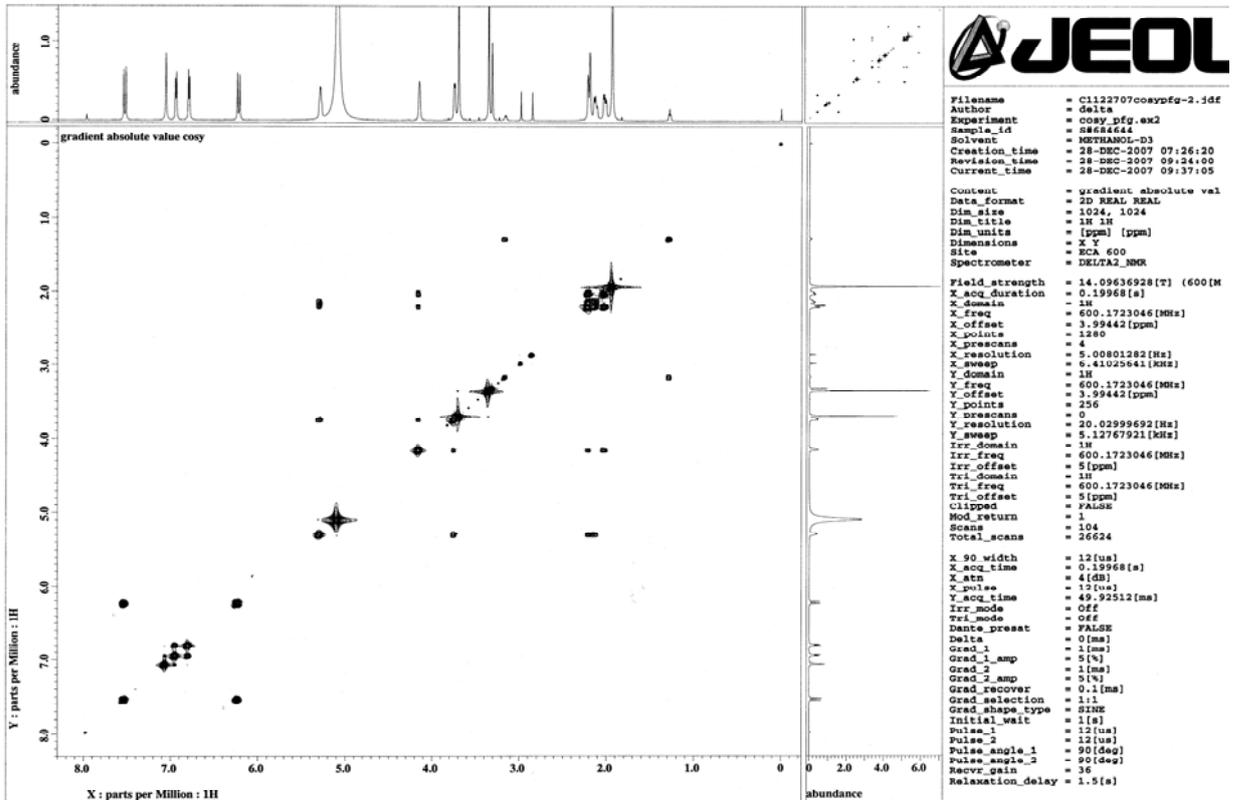
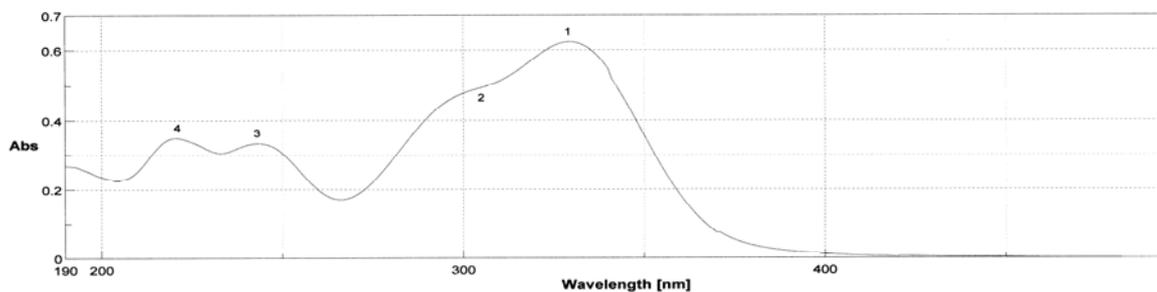
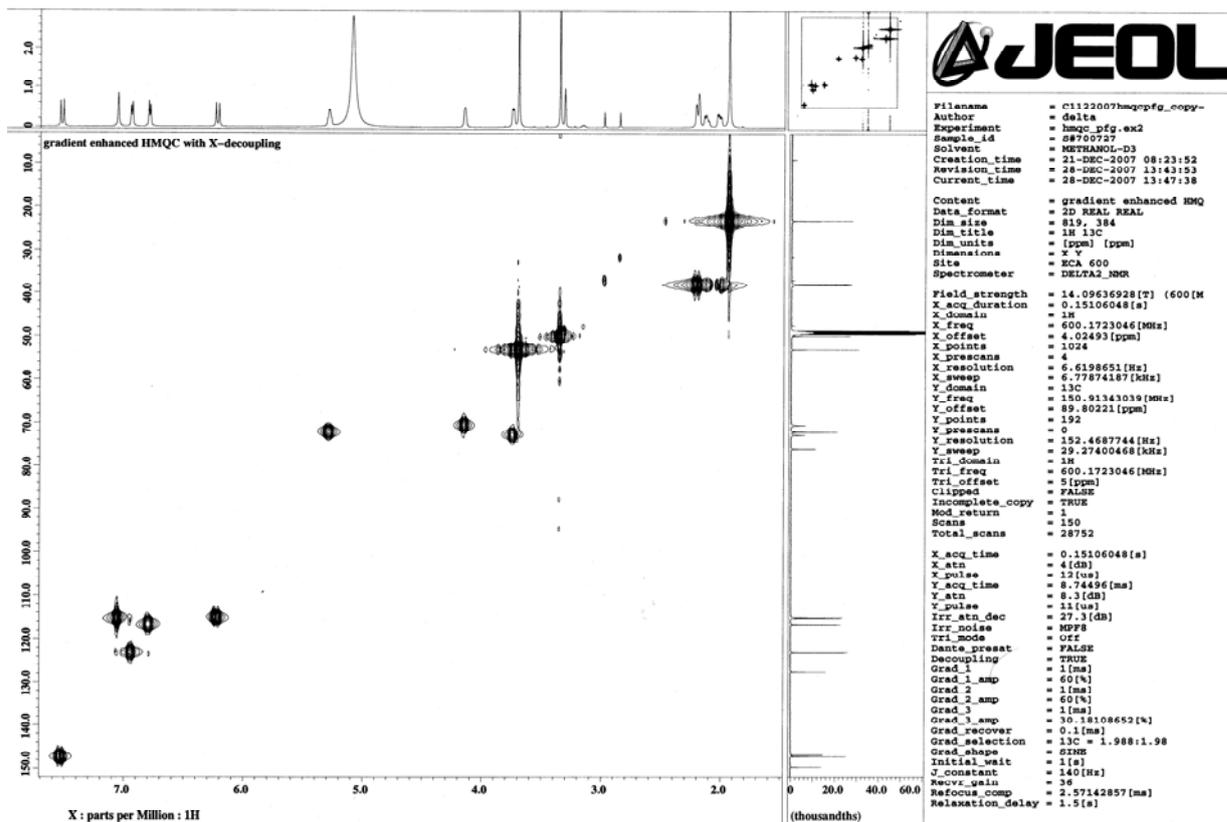


Chart 7-2. COSY and HMBC of isolated methyl chlorogenate from *F. microcarpa*.



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近赤外バンド幅 4.0 nm
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データ取込間隔 1nm
走査速度 1000nm/min
試料番号 20
繰返し回数 1

試料名 お客様各位
測定者 コシノ

ファイル名 C-1 Memory#2

No.	nm	Abs	No.	nm	Abs	No.	nm	Abs	No.	nm	Abs
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Chart 7-3. HMQC and UV of isolated methyl chlorogenate from *F. microcarpa*.

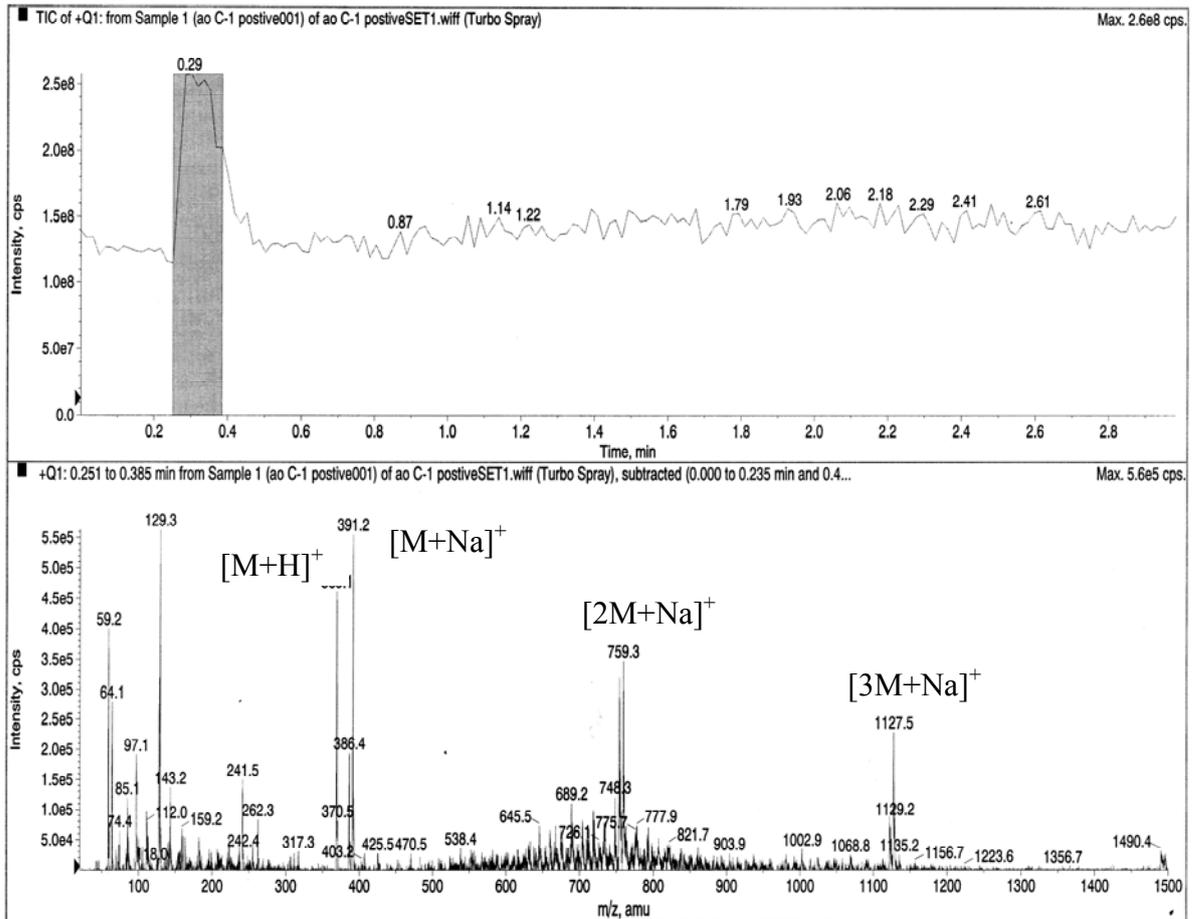
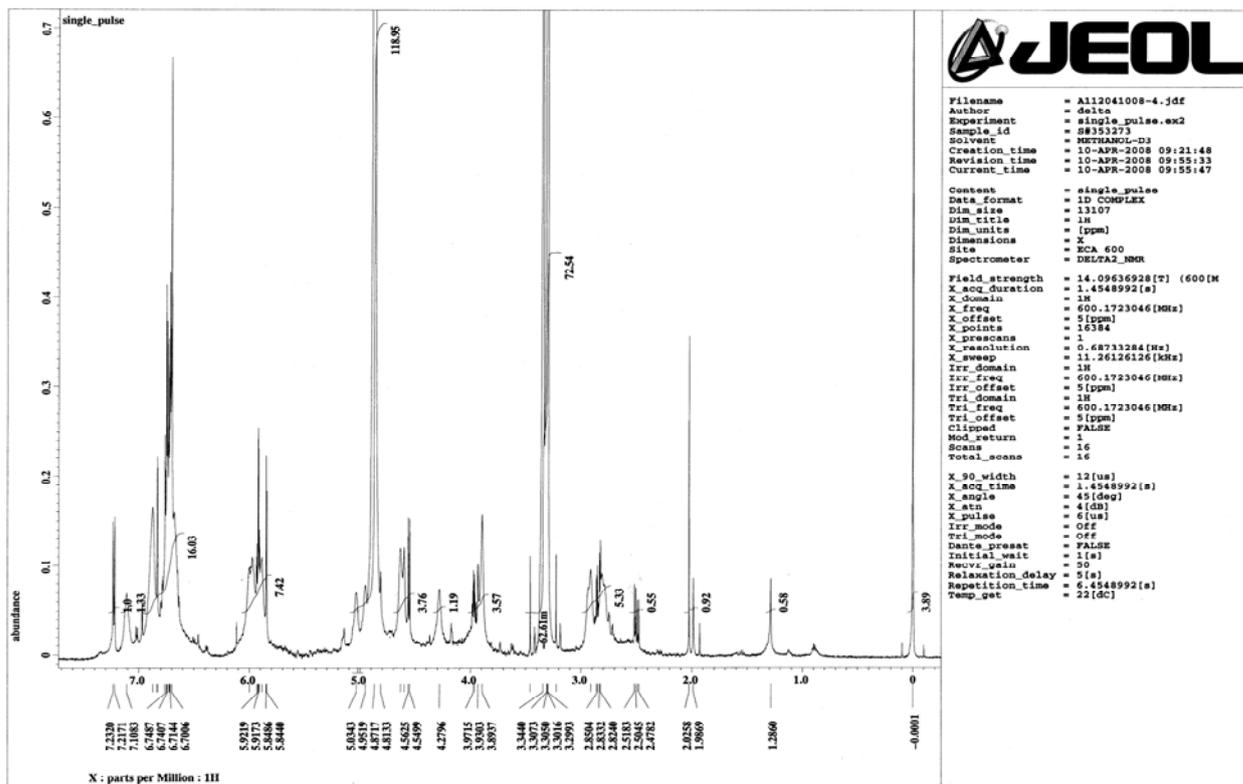


Chart 7-4. LC-MS of isolated methyl chlorogenate from *F. microcarpa*.



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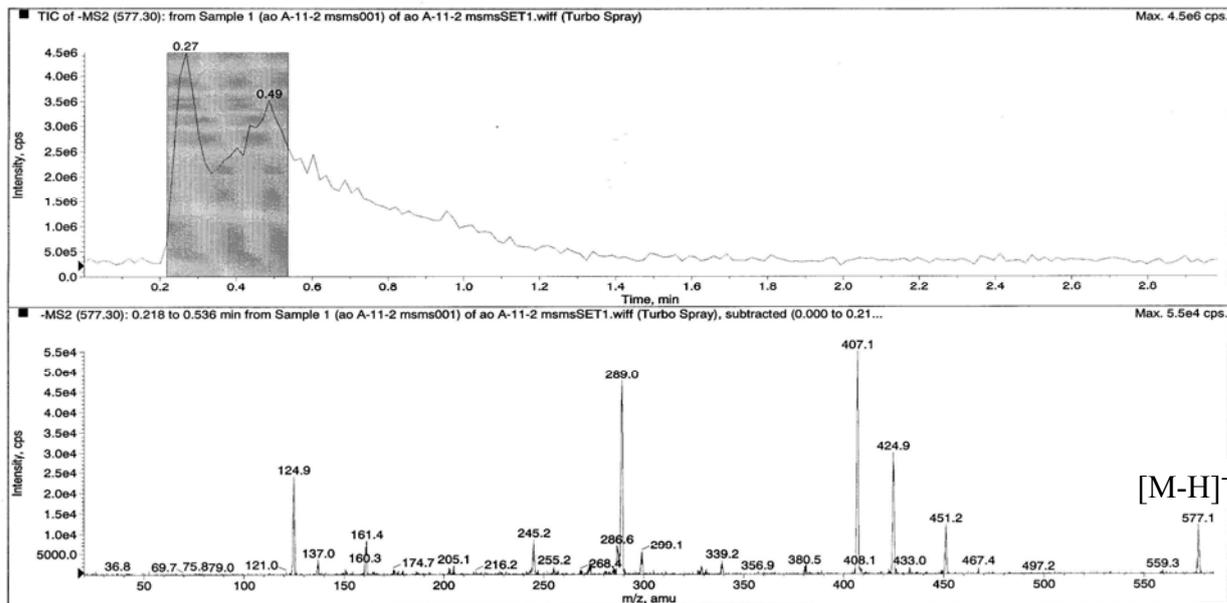


Chart 8. ¹H-NMR and LC-MS/MS of isolated procyanidin B1 from *F. microcarpa*.

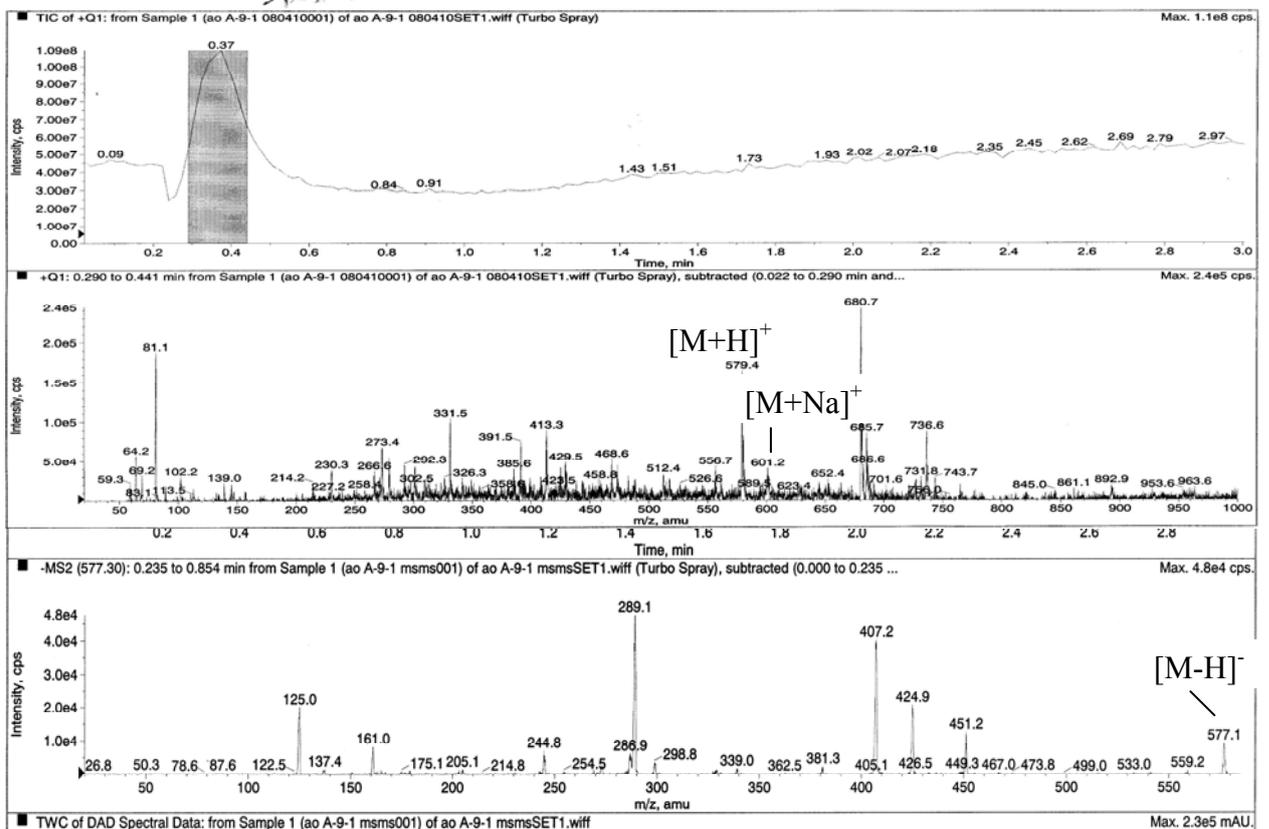
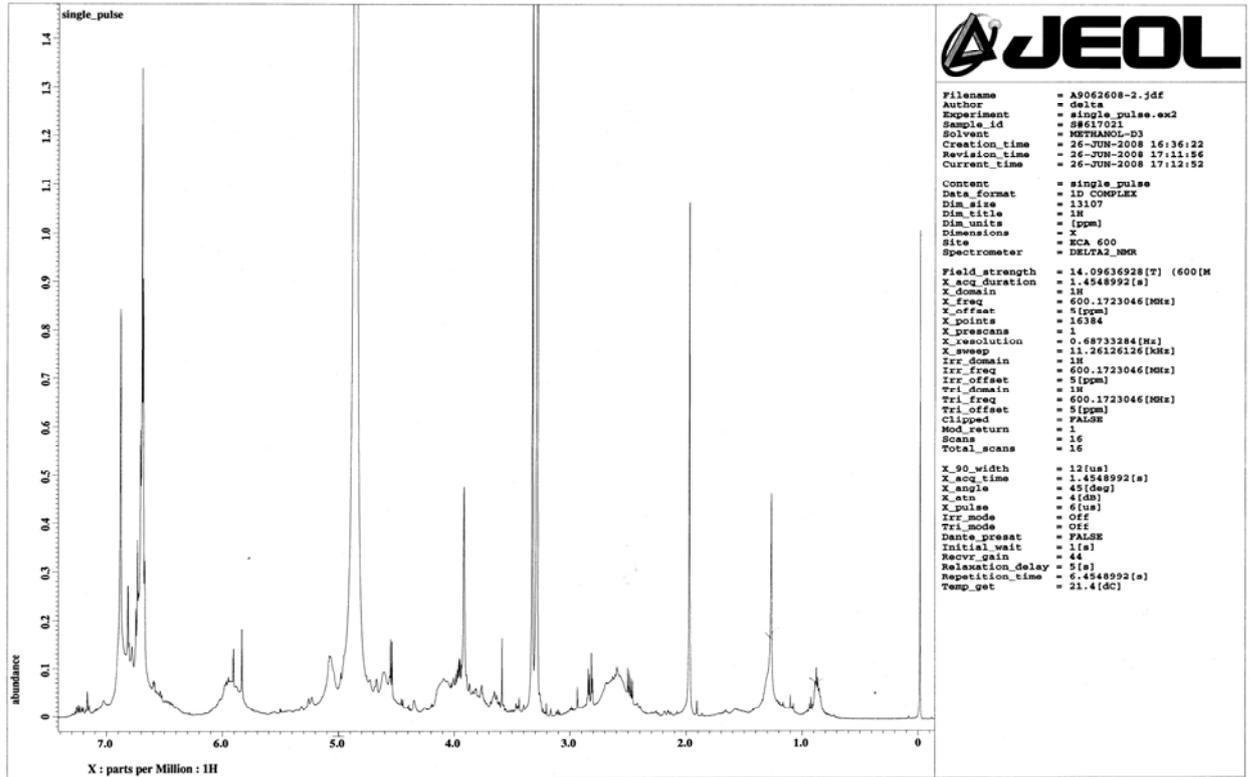


Chart 9. ¹H-NMR and LC-MS/MS of isolated procyanidin B3 from *F. microcarpa*.

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薬用植物由来の抗酸化、抗菌及び抗炎症活性物質に関する研究
Studies on antioxidant, antibacterial and anti-inflammatory active
compounds from medicinal plants

研究で用いた 3 種類の中国産漢方薬と 13 種類の沖縄県産薬草については抗炎症、抗腫瘍、抗関節炎等の活性を有することが報告されている。しかし、それら薬草の活性成分や何故活性を示すかについてはまだ解明されていない。Cyclooxygenase (COX), Collagenase 3 (MMP-13) 及び Hyaluronidase の阻害活性は薬草抽出物や化合物が有する抗炎症作用及び抗癌作用の解明のためによく使用される。本研究では、それぞれの薬草の抗酸化、抗菌及び炎症に関連する酵素の阻害活性を評価し、活性が高い試料から有効成分を精製し、同定することを目的としている。

3 種類の漢方薬について COX 阻害活性と抗酸化活性を測定した結果、*Sophora subprostrata* が最も高い活性を示した。*S. subprostrata* の抽出物から活性成分として Matrine と Oxymatrine をカラムクロマトグラフィーおよび HPLC で単離精製した。Matrine の IC_{50} 値は COX-1 に対して 7.8 $\mu\text{g/ml}$ 、COX-2 に対して 47 $\mu\text{g/ml}$ を示した。Oxymatrine の IC_{50} 値は COX-1 に対しては 52.5 $\mu\text{g/ml}$ 、COX-2 に対しては 102.2 $\mu\text{g/ml}$ を示した。本研究は Matrine と Oxymatrine が COX の阻害活性を示す最初のレポートとなり、*S. subprostrata* の民間抗炎症薬剤として利用されている事実を科学的に解明した。

13 種類の沖縄産薬草について MMP-13 阻害活性と抗酸化活性を測定した結果、秋ウコン、春ウコンおよびバジルのメタノール抽出物には強い MMP-13 阻害活性を有する成分があることが確認された。これらの薬草の抽出物中に Curcumin と Rosmarinic acid が含まれることを LC-MS で同定及び定量した。Curcumin と Rosmarinic acid は強い MMP-13 阻害活性を示し、それぞれ IC_{50} が 3.6 および 2.9 μM の活性を示した。また、ガジュマルの抽出物は抗酸化活性が最も高いことを明らかにした。これら粗抽出物はヘキサン、酢酸エチル、ブタノールを用いて分離し、酢酸エチル画分は他の画分よりすべて抗酸化、抗菌活性及びポリフェノール含有量が高いことがわかった。GC-MS と HPLC を用いて、酢酸エチル画分中に 12 種類のフェノール物質を同定した。さらに、樹皮の抽出物は Hyaluronidase 阻害活性を示し、前に述べた方法で画分すると、酢酸エチル画分は最も強い Hyaluronidase 阻害活性を示した。Sephadex LH-20 コラムクロマトグラフィー及び分取 HPLC を用いて、酢酸エチル画分から Protocatechuic acid、Chlorogenic acid、Methyl chlorogenate、Catechin、Epicatechin、Procyanidin B1 及び Procyanidin B3 を単離精製し、ESI-MS、 $^1\text{H-NMR}$ 、および $^{13}\text{C-NMR}$ で分子構造を解析した。Protocatechuic acid と Catechin を除く他の

成分は本研究で初めてガジュマル成分中に存在することを報告した。精製された化合物は全て強い抗酸化活性を示し、Catechin、Epicatechin、Procyanidin B1 及び Procyanidin B3 は高いHyaluronidase 阻害活性を示すことを明らかにした。

これらの結果から、本研究は沖縄県産の薬草中には強い生物活性を示す植物成分があるという新たな情報を与えるものである。また、ガジュマルからの抽出物には強い抗酸化、抗炎症および抗菌物質を示す有効かつ安全な資源として機能性食品および製薬業界で将来利用可能であることを示唆している。