รายงานวิจัยฉบับสมบูรณ์

โครงการ “การหาสารต้านอนุมูลอิสระในชาชงสมุนไพร 3 ชนิด โดยใช้วิธีเชื่อมต่อระบบการแยก การทดสอบฤทธิ์ และการหาสูตรโครงสร้าง”

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วันสิ้นสุดโครงการ 29 กันยายน 2551

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โครงการ"การหาสารต้านอนุมูลอิสระในชาชงสมุนไพร 3 ชนิด โดยใช้วิธีเชื่อมต่อระบบการแยก การทดสอบฤทธิ์และการหาสูตรโครงสร้าง"

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Executive summary

An HPLC was coupled on-line with colorimetric detection of antioxidant activity in order to separate antioxidants, determine their activities and obtain structural information in one run. The sample was chromatographed by HPLC and then the eluate was splited into two flows. The major part flowed to mass spectrometry (MS) interfaced with electrospray ionization (ESI) while the minor part mixed with the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The resulting decrease in visible light absorption correlated to the antioxidant contents. Furthermore, the interested antioxidant peak will be identified using the additional of MS/MS technique. This hyphenated technique was applied to investigate antioxidant compounds in the water extract of herbal tea; *Houttuynia cordata*, *Orthosiphon grandiflorus* and *Gynostemma pentaphyllum* (Thunb.) Makino**.**

Based on their mass spectra and fragmentation pattern; the antioxidant compounds of *Houttuynia cordata* tea were identified as quinic acid derivative, caffeic acid derivatives, procyanidin B, neo-chlorogenic acid, catechin, chlorogenic acid, crypto-chlorogenic acid and quercetin hexoside. Now that, *Orthosiphon grandiflorus***;** nine antioxidant compounds were identified, as danshensu, caftaric acid, rosmarinic acid, sagerinic acid, salvianolic acid B and four caffeic acid derivatives. Whereas in *Gynostemma pentaphyllum* (Thunb.) Makino, the antioxidant compounds were identified as, caffeoyl hexoside, maleic acid, caffeoylquinic acid, *p*-coumaroylhexose and kaempferol rutinoside. Composing of such strong free radical scavengers, these compounds in the herbal tea could be partly responsible for the ability to reduce oxidative stress.

Moreover the mass spectra and fragmentation pattern of the antioxidant compounds found in these three herbal teas were set as the data for structure elucidation of other plants.

Abstract

Characterisation of antioxidative activities of three herbals water extract using on-line liquid chromatographic- mass spectrometric techniques and DPPH based assay

Abstract

HPLC coupled on-line to both ESI-MS and a DPPH antioxidant assay is used for rapid screening of antioxidant compounds in plant extracts. Three herbal teas name *Houttuynia cordata, Orthosiphon grandiflorus* and *Gynostemma pentaphyllum* (Thunb.) Makino were characterized for the antioxidant compounds. *Houttuynia cordata* Thunb. (Saururaceae) has been used traditionally as immune stimulants and anticancer agent. Based on their mass spectra; the antioxidant compounds were identified as quinic acid derivative, procyanidin B, neo-chlorogenic acid, catechin, chlorogenic acid, crypto-chlorogenic acid and quercetin hexoside. *Orthosiphon grandiflorus* (Lamiaceae) is used for treating the kidney ailments and also is claimed to have antioxidant, anti-allergenic, anti-hypertensive and anti-inflammatory properties The structural elucidations of the active compounds was achieved by negative ionization LC-ESI-MS/MS. Based on their mass spectra related to antioxidant activity trace; nine compounds were identified, as danshensu, caftaric acid, rosmarinic acid, sagerinic acid, salvianolic acid B and four caffeic acid derivatives. *Gynostemma pentaphyllum* (Thunb.) Makino (Cucurbitaceae) is known as Punchakan in Thailand and has been used in traditional medicine as treating hepatitis, hyper-lipoproteinemia, cardiovascular disease and cancer. The chemical constituents of this plant are similarity to ginseng root, so that this plant has attracted much interest as a potential new medicinal plant. Based on their mass spectra; the antioxidant compounds were identified as, caffeoyl hexoside, maleic acid, *p*-coumaric acid derivative, *p*-coumaroylhexose, sinapic acid derivatives and kaempferol rutinoside. An on-line LC-ESI-MS coupled to DPPH assay for the simultaneous analyse of phenolic antioxidant compound in a single run resulting in significantly reducing of time and the amount of sample. Generally, this technique proved to be very powerful for the rapid characterization of antioxidant compounds in plant extracts.

Keywords:*Houttuynia cordata, Orthosiphon grandiflorus, Gynostemma pentaphyllum,* tea, LC-ESI-MS

บทคัดย่อ

การหาสารต้านอนุมูลอิสระในชาชงสมุนไพร 3 ชนิด โดยใช้วิธีเชื่อมต่อระบบการแยก การทดสอบฤทธิ์และการหาสูตรโครงสร้าง

บทคัดย่อ

การแยกสารโดยใช้เทคนิคโครมาโทกราฟีของเหลวสมรรถะสูงต่อเชื่อมกับการตรวจวัดชนิดของสารโดยวัดมวลโมเลกุล และทดสอบฤทธิ์ในการต้านอนุมูลอิสระโดยใช้สารดีพีพีเอชแบบต่อเนื่องในการทำครั้งเดียว เทคนิคการเชื่อมต่อระบบการแยกการทดสอบฤทธิ์และการหาสูตรโครงสร้างนี้ได้นำมาใช้ในการหาสูตรโครงสร้างของสารที่มีฤทธิ์ต้านอนุมูลอิสระในชาสมุนไพร 3 ชนิด ได้แก่ พลูคาว หญ้าหนวดแมว และ ปัญจขันธ์ พลูคาวเป็นพืชในตระกูล เซอลรูราซี ใช้ในการเพิ่มภูมิต้านทาน และต้านมะเร็ง จากข้อมูลการวิเคราะห์มวลโมเลกุลพบว่าสารที่มีฤทธิ์ต้านอนุมูลอิสระได้แก่ อนุพันธ์ของกรดควินิก สารโปรไซยานิดิน บี, นีโอโครโรจีนิก, คาทีชิน, โครโรจีนิก, คริบโตโครโรจีนิก และ เครอซีตินเฮกโซไซด์ หญ้าหนวดแมวเป็นพืชในตระกูล ลาเมียซี ใช้รักษาโรคไตและมีฤทธิ์ในการต้านอนุมูลอิสระ ต้านการแพ้ ต้านความดันสูง และต้านการอักเสบ การหาสูตรโครงสร้างของสารที่มีฤทธิ์ต้านอนุมูลอิสระทำโดยให้สารเกิดการไอออไนเซชั่นในแบบเนกาทีฟ พบสาร9 ชนิดได้แก่ แดนเชนสุ, กรดแคฟทาริก, กรดโรสมารินิก กรดเสกรินิก กรดซอลเวียโนลิก บี และอนุพันธ์ของกรดคาฟีอิก 4 ชนิด ปัญจขันธ์ เป็นพืชในตระกูลคูเคอบิทาซี ใช้ในการรักษาตับอักเสบ ภาวะลิโพโปรตีนในเลือดสูง โรคหัวใจ และโรคมะเร็ง สารสำคัญที่พบในพืชชนิดนี้จะคล้ายกับที่พบในโสม ทำให้พืชชนิดได้รับความสนใจในการนำมาใช้รักษาโรค สำหรับสารต้านอนุมูลอิสระที่พบได้แก่สารคาฟีโออิล เฮกโซไซด์, กรดมาลีอิก อนุพันธุ์ของกรดคูมาอิก คูมาโรอิล เฮกโซส อนุพันธุ์ของกรดไซเนพิก และแคมฟีออล รูติโนไซด์ เทคนิคการใช้วิธีเชื่อมต่อระบบการแยก การทดสอบฤทธิ์และการหาสูตรโครงสร้าง ในการวิเคราะห์ครั้งเดียว ทำให้สามารถลดเวลาในการวิเคราะห์และใช้ตัวอย่างปริมาณน้อย เทคนิคนี้มีประสิทธิภาพสูงในการหาสูตรโครงสร้างของสารต้านอนุมูลอิสระในสารสกัดจากพืช

คำสำคัญ พลูคาว หญ้าหนวดแมว ปัญจขันธ์ ชา LC-ESI-MS

Contents

CHAPTER 1

Introduction

There is thus currently a new interest in medicinal plant as a possible source of new leading compounds. A crude plant extract is a very complex mixture containing sometimes hundred or thousands different metabolites. It is essential to have access to methods, which lead to the rapid isolation and identification of bioactive natural products. The isolation and structure elucidation of unknown substances in biological materials such as plant extracts. The traditional way is to prepare extracts, test their biological activity then isolate the compounds by liquid chromatography, purify and perform off-line structure elucidation by a nuclear magnetic resonance (NMR) and a mass spectrometry (MS). The process has been lengthy and takes time month to years. By following only a bioactive guide fractionation procedure, there is a risk of unnecessary isolation of known plant constituents. Hyphenated techniques such as LC-MS or LC-PDA are used at the earliest state of separation. This analysis is valuable to detect compounds with interesting structures and to target their isolation, in order to perform an efficient investigation for antioxidant in plant extract. The developed system will be coupled with various detectors such as a photodiode array detector and a mass spectrometer. The information obtained from this method such as UV spectrum and molecular weight will be used for structural identification of the interested compounds. The achievement of structural information on the constituent of an extract at the earliest stage of separation is a strategic element for guiding an efficient and selective isolation procedure (Wolfender et al., 1998).

Antioxidants are dietary elements which give protection against many physiological and pathological processes caused by free radical reaction. Antioxidants have been used to prevent cancer, aging, and cardiovascular diseases (Jadhav et al.,1995). Natural antioxidants, such as flavonoids and other polyphenolic compounds, can be classified functionally into free radical scavengers, reducing agents, protectors against lipid peroxidation and quencher of reactive oxygen species. Natural antioxidants can represent an alternative to synthetic compounds such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) in food technology. In addition, they can serve as leads for the development of new drugs with the prospect of improving the treatment of several diseases.

Some high-performance liquid chromatography (HPLC) separation systems coupled on-line with antioxidant activity determination has been reported. These methods were aimed for rapid detection and separation of antioxidants in complex mixtures. Dapkevicius *et al.* (1999) used HPLC with on-line antioxidant activity detection through a post column reaction with luminol. The resulting chemiluminescence was measured. Koleva *et al.* (2000, 2001) developed the on-line detection of radical scavengers in HPLC eluates using the stable free radicals DPPH and ABTS. The combination of HPLC and a post column reaction with phosphomolybdenum reagent at acidic pH was developed by Cardenosa *et al.* (2002). The color complex was detected with an on-line absorbance detector. Yamaguchi *et al.* (1998) added DPPH to the sample and then injected it into HPLC system for the detection of the total radical scavenging activity in colored foods.

In recently, HPLC coupled with mass spectrometer was widely used to identify unknown compounds in biological sample. Even though there have been reports on this subject, only a small number of compounds were reported at one time in each individual study. Until now, the on-line-detection of some antioxidants by post-column reaction of eluates with free radicals, have been reported to be successfully applied to identify and quantify antioxidants in certain biological samples (Koleva et al., 2000; Nuengchamnong et al., 2005). The separation and activity determination provides information of an antioxidant compound. While, mass spectrometer provides information about the molecular weights and the molecular structures from its fragmentation data. This hyphenated technique gives a precise idea of plant constituents and has been widely and successfully used in the identification of phenolic compound in plant extracts without the need to isolate individual constituents.

In this study, the HPLC system will be coupled on-line with antioxidant activity detection unit consisting of a reactor and an UV-Visible detector. The developed techniques will be applied for rapid search of antioxidants from herbal teas. The HPLC system will also be coupled to mass spectrometer for the information of structure elucidation.

CHAPTER 2

Aim of the studies

This work presents a rapid method for separation and activity determination of antioxidant compounds in herbal teas sequentially. An HPLC separation system will be coupled with an FIA-detection system for on-line determination of antioxidant activity. The eluate from the column will be splited into two parts. The major part flow to MS system, while the minor part flow to the FIA-system and mixed with the free radical DPPH, and lead through a reaction coil. The resulting decrease in visible light absorption correlated to the antioxidant contents will be measured. The interested antioxidant peak will be identified using the additional LC-MS technique. The application of this hyphenated technique will be used for screening of antioxidants compounds in three herbal teas as water extract.

Aim of the studies

1. To screen the antioxidant compounds in *H. cordata, O. grandiflorus* and *G. pentaphyllum* herbal tea and to further identify those compounds using LC-MS coupled with DPPH assay
2. To make a MS fingerprint of the antioxidant compounds in these plants

CHAPTER 3

Rapid screening and identification of antioxidants in aqueous extracts of *Houttuynia cordata* using LC-ESI-MS coupled with DPPH assay

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Abstract

Houttuynia cordata Thunb. has been used traditionally as immune stimulant and anticancer agent. An aqueous extract of *H. cordata* tea showed high radical scavenging activity determined by off-line DPPH assays. Then, it was screened for its antioxidant components via an on-line DPPH radical scavenging technique coupled with a liquid chromatography-electrospray ionization mass spectrometer (LC-ESI-MS). Based on their mass spectra and fragmentation patterns; the antioxidant compounds were identified as quinic acid derivative, caffeic acid derivatives, procyanidin B, neo-chlorogenic acid, catechin, chlorogenic acid, crypto-chlorogenic acid and quercetin hexoside. LC-MS/MS in multiple reactions monitoring (MRM) mode was used to quantify these antioxidant compounds. Chlorogenic acid was found in the highest component in *H. cordata* tea.

Keywords: Houttuynia cordata; antioxidants; on-line; LC-ESI-MS; DPPH; tea

1. Introduction

Houttuynia cordata Thunb. (Saururaceae) is a pungent, heart-like leafed perennial herb native in Southeast Asia. It is called in Thai as Khao-tong or Plu-khao which means fishy smelling vegetable. Despite of its unpleasant smell, young leaves of *H. cordata* are popular vegetable in the Northern part of Thailand. It also has been used in traditional medicine for immune stimulization and as anticancer agent. *H. cordata* shows a variety of pharmacological activities such as anti-hypertension, and

seems to have anti-edema, detoxicant, anti-inflammatory, anti-pyretic, anti-purulent and diuretic activities (Probstle & Bauer, 1992 and Lu, Liang, Yi & Wu, 2006). In addition, additional activities of *H. cordata* against allergy, anaphylaxis, cancer and viral infection were reported (Chiang, Chang, Chen, Ng &Lin., 2003; Kwon, Kim, Shin, Seo, Yang & Ryu., 2003; Li, Chai, Lee, Han, Kim & Song, 2005). While, Hayashi, Kamiya & Hayashial. (1995) reported that it showed direct inhibitory activity against herpes simplex virus type I, influenza virus and human immunodeficiency virus type I (HIV-1) without showing cytotoxicity to the host. Moreover, Chiang and co-workers (2003) reported that *H. cordata* had selective activity against herpes simplex HSV-2. In the anti-viral aspect, *H. cordata* exhibited significant inhibitory activity on the severe acute respiratory syndrome (SARS) (Lau et al., 2008). *H. cordata* also acted as an antioxidant and antimutagenic agents (Chen, Liu, Chen, Chao & Chang, 2003). In general, the chemical components of *H. cordata* comprise of six major types, namely: volatile oils, flavonoids, alkaloids, fatty acids, sterols and polyphenolic acids (Bansiddhi et al., 2003; Toda, 2005; Meng, Dong, Zhou, Jiang, Leung & Zhao, 2007; Ch, Wen & Cheng, 2007). In the beginning, most of the studies mainly focused on the chemistry of the essential oils which have been considered responsible for the claimed clinical efficacy (Hayashi, Kamiya & Hayashi, 1995). Recently, Meng and co-workers (2005) developed an HPLC-DAD-MS method for analysis of *H. cordata* and characterized its major active chemical constituents as chlorogenic acid, quercetin-3-*O*-β-D-galactopyranosyl-7-*O*-β-D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyra-nosyl-7-*O*-β-D-glucopyranoside, rutin, hyperin, isoquercitrin, quercitrin, afzelin, quercetin, piperolactam A, and aristolactam B.

Antioxidants are usually found as complex mixtures in plant extracts. Some HPLC methods on-line coupled with the detection of antioxidative activity using post-column reaction of eluates with free radicals have been reported that they can be successfully applied for the identification and quantification of antioxidants in biological samples (Niederländer, van Beek, Bartasiute & Koleva, 2008; Nuengchamnong & Ingkaninan, 2009). Beside these, a free radical spiking technique also showed good detection efficacy (Shui, Leong & Wong, 2005). Our preliminary screening of four herbal teas indicated that *H. cordata* showed high antioxidant activity. However, so far there have been only few studies on the phenolic

constituents responsible for the antioxidant activity of *H. cordata*. The main objective of this study was therefore to screen the antioxidants in *H. cordata* and to further identify and quantified those compounds using LC-MS coupled with DPPH assay and LC-MS/MS.

2. Materials and methods

2.1. Materials and Reagents

The following herbal teas were examined: 1) *Orthosiphon grandiflorus* Bolding, 2) *Houttuynia cordata* Thunb, 3) *Morus alba* L. and 4) *Vernonia cinerea* L. The samples were obtained from Bangkratum hospital, Phitsanulok, Thailand.

The standard compounds, catechin, kaempferol were purchased from Wako (Pure Chemical Industries Ltd., Japan). Chlorogenic acid, quercetin dihydrate, ascorbic acid (vitamin C), quinic acid, butylated hydroxyanisole (BHA) and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was purchased from Fluka Chemie AG.(Buchs, Switzerland). Procyanidin B2 was purchased from Indofine (Somerville, NJ,USA). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Water was purified using Elga USF system (Bucks, England).

2.2. Sample preparation

Each sample (dry leaves) 250 mg was extracted with 2x10 ml 70% (v/v) methanol by sonication for 15 min then filtered through Whatman No.1 filter paper (Kent, England). The filtrate was adjusted to a volume of 25 ml. The extracts were directly test for antioxidant activity.

For structure elucidation and quantification, *H. cordata* dry leaves (2 g, 1 sachet) were infused in boiling water (100 ml) for 10 min, filtered through Whatman No.1. The filtrates was readjust to 100 ml and further filtered through a 0.2 μm Nylon syringe filter (Chrom Tech, Inc.MN, USA) and then directly injected into the LC-ESI-MS coupled with DPPH assay system.

2.3. Off-line DPPH assay

The potential free radical scavenging activity of plant extracts was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Different concentrations (10-10,000 μg/ml in 70% (v/v) methanol) of test samples were prepared. The reaction mixtures consisting of 75 μl of test samples and 150 μl of 0.2 mM DPPH in methanol were mixed in 96 well plates and incubated for 30 min. The absorbance (OD) was measured at 515 nm. The radical scavenging activity was obtained from the following equation: radical scavenging activity (%) = [(OD control-OD sample)/(OD control)]x 100. The free radical scavenging activity of plant extracts was express as EC_{50}, which was defined as the concentration in μg/ml of extract required to scavenge the formation of DPPH radicals by 50%. Percent radical scavenging activity was plotted against concentration (μg/ml), and the equation for the line was used to obtain the EC_{50} value. Measurement was performed in triplicate. Trolox, Ascorbic acid and BHA were used as positive controls.

2.4. LC-ESI-MS coupled with DPPH assay

The HPLC was coupled on-line to MS (line A) and a continuous flow DPPH assay (line B) as described in the previous study (Nuengchamnong & Ingkaninan, 2009). The set up system was shown in Figure 1. **Line A**; Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) is coupled to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with electrospray ionization interface. The chromatographic separation was achieved by a phenomenex Gemini column (5 μm, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, operated at 25 °C. The mobile phase consisted of solvent A (1 ml formic acid in 1 L of deionized water) and solvent B (methanol). The elution program started from 90:10 solvent A: solvent B for 4 min, then changed to 80:20 solvent A: solvent B in 6 min, and linearly increased to 10:90 solvent A: solvent B in 30 min and the ratio of solvent A: solvent B was constant at 10:90 for 5 min then changed to 90:10 solvent A: solvent B in 5 min and was kept constant at 90:10 solvent A: solvent B for 5 min for reconditioning of the column. Mass spectra were recorded within 55 min. The injection volume was 5 μl.

The flow rate was set at 600 µl/min. The Analyst 1.3.2 software was used for data acquisition and processing. The full scan mass spectra from *m/z* 100-1000 amu were acquired both in positive and negative ion modes. The optimum conditions of the interface were as follows: ESI-positive; ion spray voltage of 4500 V, curtain gas of 69 Kpa (10 psi), ion source gas 1 of 450 Kpa (65 psi), ion source gas 2 of 380 Kpa (55 psi). The interface temperature was set at 400°C. The entrance and declustering potential were 10V and 80V, respectively. ESI-negative; the condition was similar to ESI positive except the voltage was set in negative mode. **Line B** represents the continuous flow system for antioxidant activity detection. It consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter of 180 µm and a total reaction coil volume of 100 µL. The flow of 0.1mM DPPH was set to 200 µl/min and induced induction was detected as a negative peak at 515 nm using the UV-VIS detector (SPD 20AV, Shimadzu, Kyoto, Japan) The LC solution software was used for data acquisition and processing. The polarity of the signal output was reversed in order to obtain positive signals. The system was operated at 25 °C. Line A coupled to line B with a 24 cm length of 0.17 mm i.d. PEEK tubing by a Y-connector. The eluent flow was split in a ratio of 8:2 between the MS ion source and DPPH line. For the characterization of antioxidant peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between-5 to-50V and collision cell exit potential (CXP) of -6 V, DP in the range of -20 to -110 V. In positive mode, the ions were induced with CAD of 48 Kpa (7 psi). CE, CXP and DP were set in the range of 5-25, 3-13 and 20-100 V respectively. Quinic acid and kaempferol at concentration 100 µg/ml were added to the sample to synchronise an antioxidant peak in line B with the MS peak in line A.

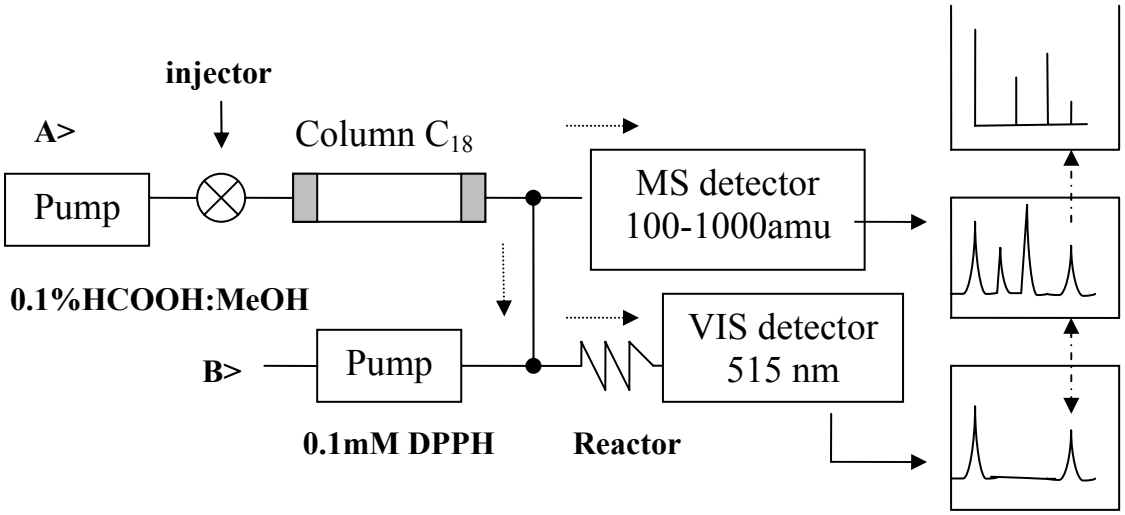


Fig.1. Scheme of the LC-ESI-MS coupled with DPPH assay. The arrows indicate flow direction.

2.5. Peak identification

Peak identification was performed by comparison of the retention time, mass spectra and fragmentation patterns with reference compounds and published data.

2.6. Quantitative analysis

For quantitative analysis of the antioxidants in *H. cordata* herbal tea, the same column was used and the gradient system was changed to shorten analysis time. The system was employed from solvent A: B (90:10) to (10:90) in 10 min, followed by isocratic elution with solvent A:B (10:90) for 5 min and linear gradient elution from solvent A:B (10:90) to (90:10) for 2 min and equilibration with solvent A:B (90:10) in 3 min before injecting a new sample. The flow rate was set at 600 µl /min. The injection volume was 5 µl. The MS parameters were operated in negative multiple reactions monitoring (MRM) scan mode. Optimal operating parameter of ESI-MS with maximum signal intensity of molecular ions and fragment ions were obtained by direct infusion of the standard solution at concentration 10 µg/ml in methanol, using a Harvard syringe pump (Syringe Pump 11 plus, Harvard apparatus Inc., Holliston, USA) at a flow rate of 5 µl/ml. The optimum conditions of the interface were as follows: ion spray voltage of -4500 V, curtain gas of 138 Kpa (20 psi), ion source gas

1 of 380 Kpa (55 psi), ion source gas 2 of 310Kpa (45 psi). The interface temperature was set at 400°C. The entrance potential was -10 V. The dwell time per transition was set at 100 msec. The individual optimized parameters for each compound are provided in Table 1. Chlorogenic acid, catechin and quercetin in various concentrations were used for preparation of calibration curves. The curves were generated by linear regression based on the peak area. Analyses were performed in triplicate. Other standards were not available; hence neo-chlorogenic acid and crypto-chlorogenic acid were quantified as chlorogenic acid equivalents and also procyanidin B and quercetin hexoside were quantified as catechin and quercetin equivalents, respectively. The limit of detection (LOD) was determined as the analyte signal having a peak area equal to three times of that of noise (S/N=3) using Analyst software.

Table 1 Mass parameters in negative MRM scan mode for quantitative analysis

Compound	Parent ion (m/z)	Product ion (m/z)	DP(V)	CE(V)	CXP(V)
chlorogenic acid	353.2	190.8	-120	-22	-30
catechin	288.9	245	-150	-22	-12
	288.9	125.1	-150	-30	-20
quercetin	300.9	151.0	-150	-30	-22
	300.9	178.9	-150	-30	-26

3. Results and discussions

3.1. DPPH assay for determination of the antioxidant activity of herbal teas

The antioxidant activities of four herbal teas were measured using DPPH assay. This assay is based on a measurement of the scavenging ability of antioxidants towards a stable radical, DPPH. The free radical DPPH, which shows absorption at 515 nm, is reduced to the corresponding hydrazine when it reacts with hydrogen donors and this can be detected as a negative signal (Brand-William, Cuvelier & Bersec,1995). The results of the screening are listed in Table 2. The results showed that methanolic extracts of *O. grandiflorus* and *H. cordata* had the highest activity

among all the teas tested. Further study was to identify the substances responsible for the antioxidant effect of *H. cordata* tea due to the popular used as health tea.

Table 2 DPPH free radical scavenging activities of methanol extracts of herbal tea

Family	**Species**	**Part used and their effects**	**EC_{50} (μg/ml)**
Lamiaceae	*O. grandiflorus*	Aerial part; for bladder stones.	217.22+12.56
Saururaceae	*H. cordata*	Aerial part: immunity stimulant, anticancer	341.50+17.17
Moraceae	*M. alba*	Leaves; antipyretic, diaphoretic, tonic, antitussive, diuretic, depurative	874.86+83.41
Asteraceae	*V. cinerea*	Leaves; poultice; for allergy, against conjuctivitis and cancer.	886.91+53.15
Standard compound			
BHA			4.48+2.13
Ascorbic acid (VitaminC)			6.71+0.17
Trolox			7.95+1.34

3.2. LC-ESI-MS coupled with DPPH assay for the rapid identification of antioxidants

An aqueous extract of *H. cordata* was subjected to the on-line LC-ESI-MS coupled with DPPH assay. The DPPH based antioxidant activity profile (Fig. 2a) exhibited that at least ten compounds showed antioxidant activity peak. The deprotonated molecular ion peaks of standard quinic acid and kaempferol were found at 5.7 and 39.3 min, respectively, from which the delay time between the read out from the MS detector and the corresponding peak from the antioxidant activity was calculated as 0.6 min. Therefore, the estimated retention time (t_R) values of the DPPH trace were minus 0.6 min. Negative ionization gave a better result than positive ionization (Fig. 2b). So, only negative ionization results are presented. The negative ions of the major active compounds are listed in Table 3 and the identification of these

compounds is proposed. The aqueous extract was used in this study as it will provide the same form of antioxidants as present in the tea.

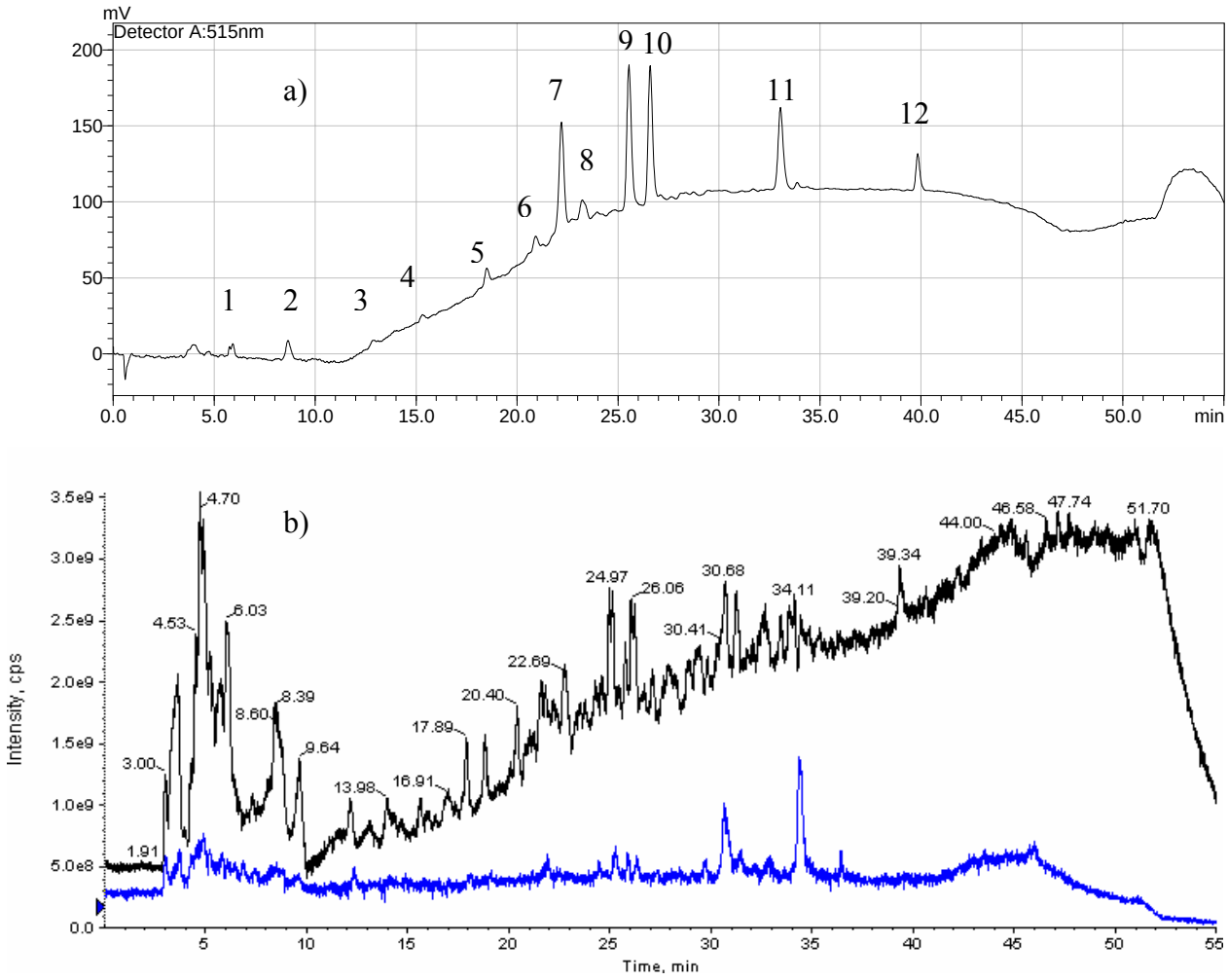


Fig. 2. Analysts of H. cordata aqueous extract using LC-ESI-MS coupled with DPPH assay a) The chromatogram from the antioxidant activity assay detection at 515 nm. b) The total ion current (TIC) output from the ESI-MS in negative mode (upper line) and positive mode (lower line). Conditions are described in the text. For peak assignments, see Table3. Peak 1 and 12 are quinic acid and kaempferol added to the extract for delay time measurement.

Peak 1 (t_R = 5.8 min) was a standard quinic acid at concentration 100 µg/ml as a delay time marker for two detectors. Peak 2 has a mass spectra and fragmentation

pattern nearly identical as standard quinic acid but difference in retention time (t_R 9.3 min). So that peak 2 was tentative identified as quinic acid derivative.

Peak 3-5 (t_R =12.3,14.7 and 17.9 min) with *m/z* 371.2 and fragmentation at *m/z* 197.3 [caffeic acid-H+18]$^-$, *m/z* 191.0 [quinic acid-H]$^-$, *m/z* 179.0 [caffeic acid –H]$^-$ and *m/z* 173.3 [quinic acid-H-H_2O]$^-$.These *m/z* are the characteristic of caffeic acid. Therefore these three compounds were identified as caffeic acid derivatives.

Peak 6 (t_R =20.3 min) exhibited [M-H]$^-$ base ion at *m/z* 577.3. It gave secondary fragment at *m/z* 289.0[M-288.3]$^-$ due to the cleavage of the inter-flavanoid C-C linkages with losses of 288.3 amu and *m/z* of 425.0 [M-152]$^-$ evolved from retro Diels-Alder fragmentation of the heterocyclic ring. The *m/z* of 407.2 [M-152-18]$^-$ resulted from water elimination of *m/z* 425. After comparison with standard procyanidin B2 (t_R 23.13 min, the fragmentation pattern is nearly identical but this compound came out before procyanidin B2 and also catechin (t_R 23.04 min). So that this compound might be procyanidin B. However, this compound cannot be indicate the stereochemistry by this MS technique.

Peak 8 (t_R =23.04 min) with *m/z* 289.0 [M-H]$^-$ and fragmentation at *m/z* 245.0 and 125.1(cleavage of the C ring) was identified as catechin by the comparison of the authentic standard.

Peak 9 (t_R =24.8 min) with *m/z* 353.6 and fragmentation at *m/z* 191.0 [quinic acid-H]$^-$, *m/z* 179.0 [caffeic acid –H]$^-$ and *m/z* 173.3 [quinic acid-H-H_2O]$^-$. By comparing its mass spectra and t_R with standard compound, this compound was assigned to be chlorogenic acid (5-*O*-caffeoyl quinic acid). Compounds 7 and 10 were most likely naturally occurring isomers of chlorogenic acid. According to their elution order, neo-chlorogenic acid (3-*O*-caffeoyl quinic acid) was eluted prior to crypto-chlorogenic acid (4-*O*-caffeoyl quinic acid) (Carini, Facino, Aldini, Calloni & Colombo, 1998). Therefore, peaks 7 and 10 were assigned as neo-chlorogenic acid and crypto-chlorogenic acid, respectively.

Peak 11 (t_R = 32.5 min) with a molecular ion at *m/z* 463.3 and fragment ions at *m/z* 300.0 and *m/z* 301.0 [M-162]$^-$ (loss of a hexose unit) was tentatively identified as quercetin hexoside. Quercetin aglycone eluted at 37.2 min

Peak 12 (t_R = 39.3 min) with *m/z* 285 was kaempferol standard at concentration 100 μg/ml was used as delay time marker for the two detectors.

The general structures of the antioxidant compounds identified in *H. cordata* are shown in Figure 4.

Table3 Identification of antioxidant compounds in water extracts of H. cordata by using their LC-ESI-MS-DPPH assay; data in negative ionization

Peak no.	**t_R (min)**	**ESI-MS (m/z)**		**Tentative ID**
		MS	MS/MS	
1	5.8	190.9	127.3	Quinic acid*
2	9.3	190.9	127.3	Quinic acid derivative
3	12.3	371.2	197.3,191.2,179.2,173.0	Caffeic acid derivative
4	14.7	371.2	197.3,191.2,179.2,173.0	Caffeic acid derivative
5	17.9	371.2	197.3,191.2,179.2,173.0	Caffeic acid derivative
6	20.3	577.0	425.0, 408.0, 306.2, 288.9	Procyanidin B**
7	22.2	353.6	191.0, 179.0, 173.3	Neo-chlorogenic acid
8	22.7	289.2	175.1, 158.7	Catechin**
9	24.8	353.6	191.0, 179.0, 173.3	Chlorogenic acid**
10	26.5	353.6	191.0, 179.0, 173.3	Crypto-chlorogenic acid
11	32.5	463.3	301.3, 300.0	Quercetin hexoside
12	39.8	285.0		Kaempferol*

* Standard compound added, ** compare with standard compound

3.3 Quantitative analysis of antioxidants using LC-MS/MS

Antioxidant compounds in *H. cordata* can be quantitatively analyzed using LC-MS/MS in MRM mode. In order to high throughput analysis, the elution profile was changed and the analysis was finished in 20 min. In our studies, chlorogenic acid, catechin and quercetin at various concentrations were used for the preparation of calibration curves. The regression equation of chlorogenic acid at concentrations of 5-100 μg/ml was y = 4.37e+005x-2.3e+006; with r value = 0.9985. The regression equation of catechin at concentrations of 1-100 μg/ml was y= 2.36e+004x+7.86e+003;

with r value = 0.9991. The regression equation of quercetin at concentrations of 1-100 μg/ml was y= 9.26e+004x+6.6e+004; with r value 0.9977. The limits of detection (LOD) with signal to noise ratio of three were determined. LOD of chlorogenic acid, catechin and quercetin was 2.5, 2.5 and 5.0 ng per injection respectively. Neo-chlorogenic acid and crypto-chlorogenic acid were quantified by using a linear regression line of chlorogenic acid. Procyanidin B and quercetin hexoside were also quantified as catechin and quercetin. The extracts mass in MRM quantification of the standard compounds and compound equivalent in the *H. cordata* tea aqueous extracts were shown in Figure 3.The amounts of antioxidant compounds found in *H. cordata* tea were shown in Table 4. The quinic acid derivative and caffeic acid derivatives were not quantified due to contain in a small amount.

The major antioxidative components in this plant were found to be chlorogenic acid and its derivatives. Several reports showed that chlorogenic acid exhibited antivirus, anticancer, anti-inflammation activities, and anxiolytic-like effects (Jin et al. 2005, Nakamura et al. 1997; Jiang, Satoh, Watanabe, Kusama & Sakagami, 2001; Bouayed,Rammal, Dicko, Younos, & Soulimani, 2007). Some of these compounds such as chlorogenic acid and quercetin glycoside have already been reported in *H. cordata* (Meng, Leung, Jiang, Dong, Zhao & Xu, 2005). However, this is the first time that the presence of catechin, and procyanidin B type in the aqueous tea extract are reported. The results suggest that these compounds could be partly responsible for antioxidant activity of *H. cordata* tea.

Table 4 Content of phenolic antioxidant found in *H. cordata* aqueous extract

Compound	**Content (μg/g)**
Chlorogenic acid	2813.5+12.5
Neo- Chlorogenic acid	916.5+10.5
Crypto- Chlorogenic acid	603.5+2.9
Catechin	28.0+0.8
Procyanidin B	52.4+5.21
Procyanidin B	189.5+0.55
Quercetin hexoside	725.0+13.2

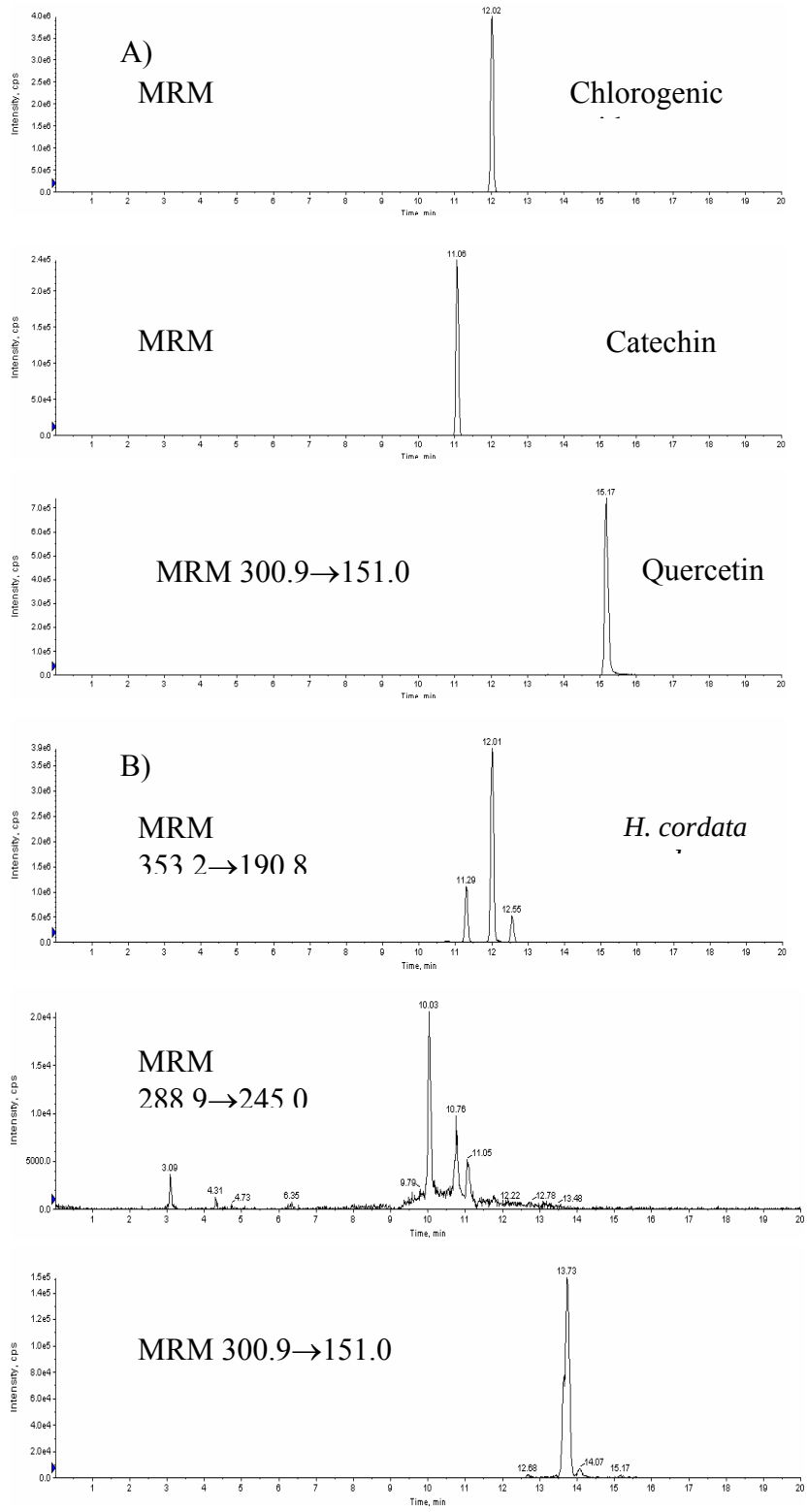
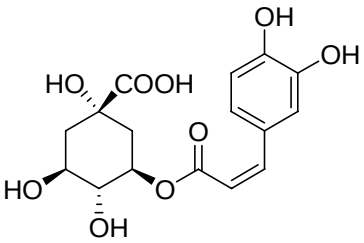
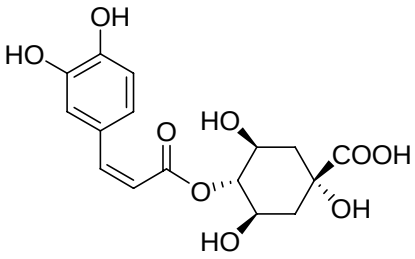


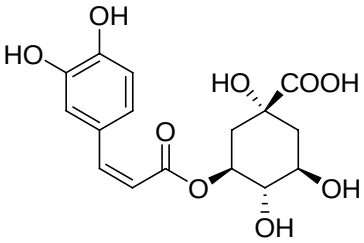
Fig. 3. MRM extract mass for quantitation of A) standard compounds; chlorogenic acid, catechin and quercetin. B) Compounds that equivalent to standard found in *H. cordata* tea.



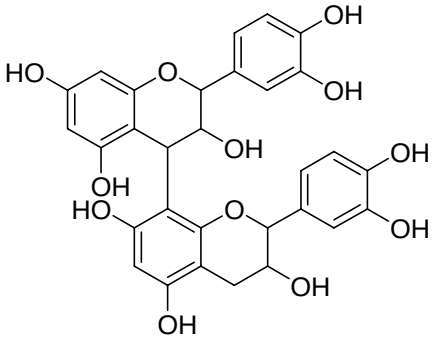
5-*O*-caffeoylquinic acid
(chlorogenic acid)



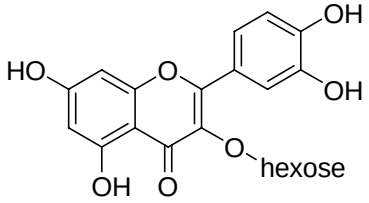
4-*O*-caffeoylquinic acid
(crypto-chlorogenic acid)



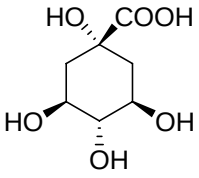
3-*O*-caffeoylquinic acid
(neo-chlorogenic acid)



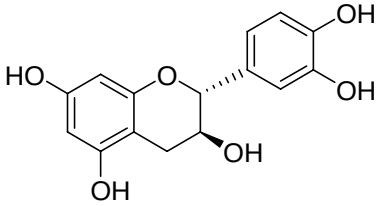
procyanidin B



quercetin hexoside



quinic acid



catechin

Fig.4. Structure of antioxidant compounds identified in the *H. cordata* tea.

4. Conclusions

The preparative isolation of the active compounds of herbal extracts in adequate quantities for off-line spectral and biological analysis is a laborious and time consuming. In this study, the use of on-line rapid screening of the antioxidants in herbal tea and their subsequent identification are reported. The quantitation in MRM mode is advantage in case of derivative compounds can be determined. Antioxidants in the aqueous extract of *H. cordata* mainly consisting of chlorogenic acids and its derivatives, catechin and procyanidin B were characterized using on-line LC-MS coupled with DPPH assay for the first time.

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CHAPTER 4

Identification and characterization of phenolic antioxidants in water extract of *Orthosiphon grandiflorus* tea by LC-ESI-MS/MS coupled to DPPH assay

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Abstract

Aqueous extract from *Orthosiphon grandiflorus* tea was on-line screened for its antioxidant components based on its capacity to scavenge free DPPH (1,1-diphenyl-2-picrylhydrazyl) radical after the separation on LC gradient condition. The structural elucidation of the active compounds was achieved by negative ionization LC-ESI-MS/MS. Based on their mass spectra and fragmentation patterns related to antioxidant activity trace; nine compounds showing strong DPPH scavenging were identified to be danshensu, caftaric acid, rosmarinic acid, sagerinic acid, salvianolic acid B and four caffeic acid derivatives. In addition, the quantitation of antioxidant compounds was performed using LC-MS/MS in multiple reactions monitoring (MRM) mode. Rosmarinic acid was found as a major component that responsible for the antioxidant activity of this plant.

Keywords: *Orthosiphon grandiflorus*, caffeic acid derivatives, antioxidant, on-line; LC-MS/MS, DPPH, tea

1. Introduction

Orthosiphon grandiflorus Bolding [syn.: *O. aristatus* Blume, *O. stamineus* Benth, *O. spicatus* Thunb, *Ocimum aristatum* BI.; Lamiaceae] is used extensively as a traditional folk medicine in Southeast Asia for the treatment of a wide range of diseases. It is used for treating the ailments of the kidney (Arafat et al., 2008), and also claimed to have antioxidant, anti-allergenic, anti-hypertensive and anti-inflammatory properties (Akowuah et al., 2005; Masuda et al., 1992). It is reportedly

effective for anti-fungal and anti-bacterial purposes (Hossain et al., 2008; Chen et al., 1989). People in some areas of Japan consume it as a healthy tea for body detoxication. The flower of *O. grandiflorus* (Cat's whisker grass tea) is white and bluish with filaments resembling a cat's whiskers. It, also known as Java tea, *Misai Kucing* (Malaysia), *Kumis Kucing* or *Remujung* (Indonesia), *Rau meo* (Vietnam), *Se-Cho* or *Myit-shwe* (Myanmar) *Nego no hige* (Japan) and *Yaa Nuat Maeo* (Thailand).

Studies on *O. grandiflorus* revealed the presence of various classes of compounds. The major components of *O. grandiflorus* leaves are the polyphenols such as polymethoxylated flavonoids; sinesetine, eupatorine and the caffeic acid derivatives; rosmarinic acid and 2,3 dicaffeoyltartaric acid (Sumaryono et al., 1991; Olah et al., 2003). In addition, several diterpenes and oxygenated diterpenes were isolated and reported (Stampoulis et al.,1999; Tezuka et al.,2000; Awale et al., 2003; Nguyen et al., 2004).

. Recently, HPLC coupled with mass spectrometer has been widely used to identify unknown compounds in complex mixtures. The on-line-detections of some antioxidants by post-column reaction of eluates with free radicals have been reported to be successfully applied to identify and quantify antioxidants in certain biological samples (Niederländer et al., 2008; Nuengchamnong & Ingkaninan, 2009). The separation and activity determination provides information of an antioxidant compound while mass spectrometer provides information about the molecular weight and the molecular structure from its fragmentation data. This hyphenated technique gives a precise idea of plant constituents and has been widely and successfully used in the identification of phenolic compounds in plant extracts without the need to isolate individual constituents.

From preliminary screening, *O. grandiflorus* aqueous extract showed excellent free radical scavenging activity using DPPH assay. It is, therefore, interesting to identify the principle components responsible for their antioxidant activities. To achieve this goal, on-line LC-ESI-MS/MS coupled to DPPH assay was used to identify and quantify the antioxidant components in *O. grandiflorus* extract.

2. Materials and methods

2.1 Chemical and reagents

Standard caffeic acid, rosmarinic acid and free radical 1,1 diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemie (Steinheim,

Germany). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Formic acid (analytical grade) was purchased for Merck (Darmstadt, Germany). Water was purified using Elga USF system (Bucks, England).

2.2 Plant material

The tea sample was supplied from the Bangkratum hospital, Phitsanulok, Thailand. Each tea bag contains dried leaves of *O. grandiflorus* (2 g). The indication was used to promote diuresis and dissolve stone with size less than 0.5 cm.

2.3 Sample preparation

O. grandiflorus tea (5 g) was extracted with boiling water of 150 ml for 5 min. After cooling at around room temperature (25°C), the tea were filtered by filter paper (Whatman no.1, Kent, England). The filtrate was then further filtered through a 0.2 μm Nylon syringe filter (Chrom Tech Inc., MN, USA) prior to injection into the HPLC-MS-DPPH assay system.

2.4 HPLC coupled on-line to ESI-MS and DPPH assay

The system was mentioned in the previous work (Nuengchamnong & Ingkaninan, 2009). The delay time between MS trace and visible trace was ca 0.6 min.

The HPLC was coupled on-line to MS (line A) and a continuous flow DPPH assay (line B) as shown in Figure 1. **Line A**; Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) was coupled to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with electrospray ionization interface. The chromatographic separation was achieved with a phenomenex Gemini column (5 μm, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, operated at 25 °C. The mobile phase consisted of solvent A (1 ml formic acid in 1 L of deionized water) and solvent B (methanol). The elution program started from 90:10 solvent A:solvent B for 4 min, then change to 80:20 solvent A:solvent B in 6 min, linearly increase to 10:90 solvent A:solvent B in 30 min and the ratio of solvent A:solvent B was constant at 10:90 for 5 min then changed to 90:10 solvent A:solvent B in 5 min and kept constant at 90:10 solvent A:solvent B for 5 min for reconditioning of the column. A mass

spectra was recorded within 55 min. The injection volume was 5 µl. The flow rate was set at 600 µl /min. The Analyst 1.3.2 software was used for data acquisition and processing. The full scan mass spectra from *m/z* 100-1000 amu were acquired in negative ion modes. The optimum conditions of the interface were as follows: ESI-negative; ion spray voltage of -4500 V, curtain gas (N_2) of 69 Kpa (10 psi), ion source gas 1 (air, for nebulizing) of 450 Kpa (65 psi), ion source gas 2 (air, for drying solvent) of 380 Kpa (55 psi). The interface temperature was set at 400°C. The entrance potential (EP) and declustering potential (DP) were -10V and -80V, respectively. **Line B** represents the continuous flow system for antioxidant activity detection. It consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter of 180µm and a total reaction coil volume of 100 µL. The flow of 0.1mM DPPH was set to 200 µl/min and induced bleaching was detected as a negative peak at 515 nm using the UV-VIS detector (SPD 20AV, Shimadzu, Kyoto, Japan), and the LC solution software was used for data acquisition and processing. The polarity of the signal output was reversed in order to obtain positive signals. The system was operated at 25 °C. Line A couple to line B with a 24 cm length of 0.17 mm i.d. PEEK tubing by a Y-connector. The eluent flow was split between the MS ion source and DPPH line in a ratio of 8:2. For the characterization of antioxidant peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between-5 to-50V and collision cell exit potential (CXP) of -6 V, DP in the range of -20 to -110 V.

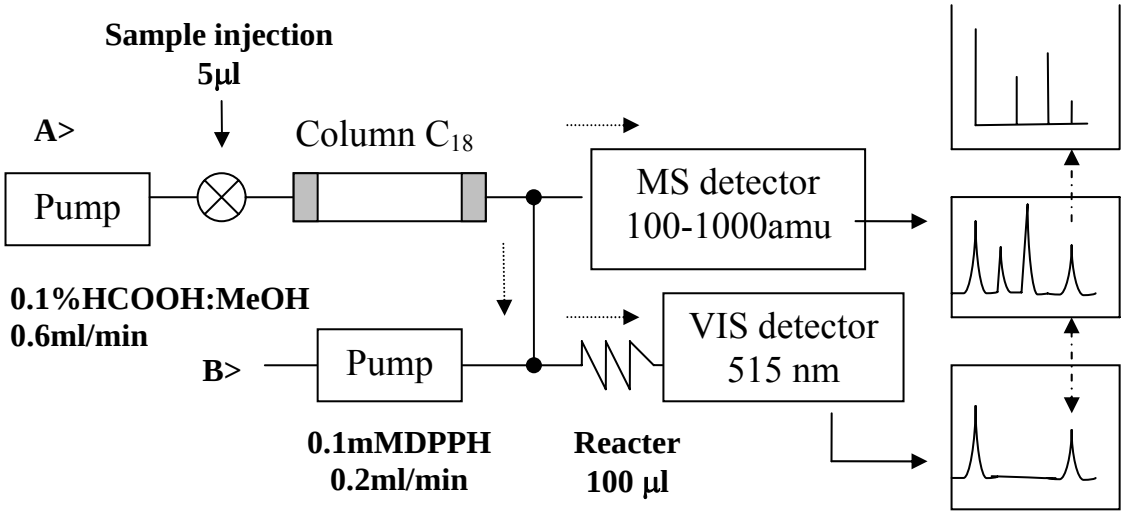


Figure 1. Scheme of the HPLC on-line coupled to the ESI-MS and DPPH assay. The arrows indicate flow directions.

2.5 Peak identification

Peak identification was performed by comparison of the retention time and mass spectra with standard compounds and literatures data.

2.6 Quantitative analysis

For quantitative analysis of antioxidants in *O. grandiflorus* herbal tea, the same HPLC condition was used. The MS parameters were operated in negative multiple reactions monitoring (MRM) mode using precursor ion $[M-H]^-$ and their product ions. Rosmarinic acid and caffeic acid at various concentrations were used for preparation of calibration curves. The curves were generated by linear regression based on peak area**.** The analyses were performed in triplicate. The other compounds were quantitates as rosmarinic acid or caffeic acid equivalent. The condition for detection of caffeic acid and its derivatives was at *m/z* 178.9→ 134.9 amu with DP, CE and CXP at -70,-23 and -20 V, respectively. For detection of rosmarinic acid, two conditions were used. The first one was set at *m/z* 358.8→ 160.9 amu with DP, CE and CXP at -60,-26 and-8 V. The second one was set at *m/z* 358.8→197.0 amu with DP,CE and CXP at -60,-27and-13 V respectively. The IS was set at -4500V and dwell time was set at 100 msec for both compounds.

3. Results and discussion

3.1. LC-ESI-MS coupled with DPPH assay for the rapid identification of antioxidants

In this study, we used a readily available free radical scavenging assay coupled with HPLC-MS/MS as a tool to directly screen and characterize antioxidants in herbal tea. The DPPH assay is based on a measurement of scavenging ability of antioxidants towards a stable radical, DPPH. The free radical DPPH, which shows absorption at 515 nm, is reduced to the corresponding hydrazine when it reacts with hydrogen donors and this can be detected as a negative signal (Nuengchamnong & Ingkaninan, 2009). The DPPH based antioxidant activity profile (Fig. 2a) shows that at least nine compounds had antioxidant activity. Mass spectral data obtained in negative ionization mode of major active compounds are listed in Table 1 and the identification of these compounds is proposed. Negative ionization gave a better result than positive so that only negative ionization was presented.

In the negative ionization, the *m/z* data show that all of the compounds had ion at *m/z* 197, 179 and 359 indicated that these compounds were caffeic acid derivatives.

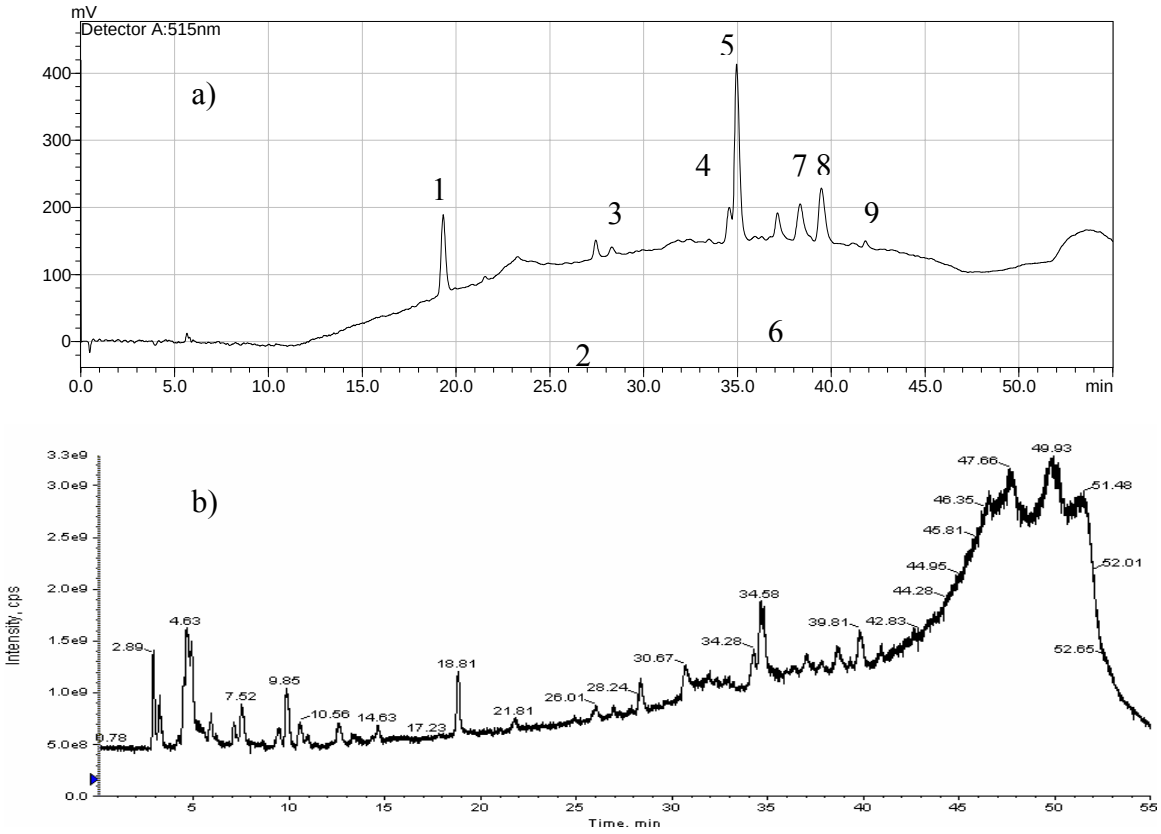


Figure 2. HPLC separation of *O.grandiflorus* with simultaneous antioxidant activity assay and MS detection a) the chromatogram from the antioxidant activity assay detection at 515 nm b) The total ion current (TIC) output from the ESI-MS in negative mode. For peak assignments, see Table1. Conditions are described in the text.

Peak 1 (t_R=19.3 min) showed *m/z* at 197.2 $[M-H]^-$ and 395.3$[2M-H]^-$. The fragment ions at *m/z* 134.9$[M-18-44]^-$ and 179.0 $[M-18]^-$ were also observed. This compound can be identified as Danshensu, the hydrated form of caffeic acid (Liu et al., 2007).

Peak 3 (t_R=28.3 min) showed a molecular ion at *m*/*z* 311 (MW 312). The fragments of a tartaric acid (base peak, *m*/*z* 149) and caffeic acid (*m*/*z* 179) were observed. In addition, there was low signal produced by the caffeic acid decarboxylation at *m*/*z* 135. The compound was thus identified as caftaric acid corresponding to the report of Llorach and co worker, 2008. In particular, this

compound is *cis*-isomer because of the absence of *m/z* 623 which will be seen in *trans*-isomer corresponding to adduct formation of two individual molecules of caftaric acid (Schutz et al., 2005).

Table1 Identification of antioxidant compounds in water extracts of *O. grandiflorus* by using their LC-ESI-MS-DPPH assay data in negative ionization. t_R was the retention time at the antioxidant detector.

Peak no.	t_R (min)	ESI-MS (*m/z*)		Tentative ID
		MS	MS/MS	
1	19.3	197.2	134.9, 179.0,395.3	Danshensu
2	27.4	317.1	134.9, 167.0, 179.0, 197.1,	Caffeic acid derivative
3	28.3	311.4	135.0, 149.2, 179.2, 223.0	Caftaric acid
4	34.6	792.1	359.0, 584.6, 630.2	Caffeic acid derivative
5	35.0	359.0	134.9, 160.9, 179.2, 197.0	Rosmarinic acid [a]
6	37.2	497.9	179.2, 196.9, 336.1, 359.6	Caffeic acid derivative
7	38.3	719.6	359.4, 521.5, 538.9	Sagerinic acid
8	39.5	717.7	359.6, 520.0	Salvianolic acid B
9	41.8	343.1	161.0, 179.2, 181.1, 197.1, 297.8, 313.2	Caffeic acid derivative

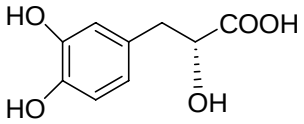
[a] compare with standard compound

Peak 5 (t_R=35.0 min) showed a molecular ion at *m/z* 359.0 (MW 360). The fragmentation at *m/z* 179 showed the presence of caffeic acid moiety. The *m/z* at 197 indicated the 2-hydroxy derivative of hydrocaffeic acid. The dehydrated ion fragments of these compounds at *m/z* 161 correspond well with that of rosmarinic acid. The information mass spectra as well as the comparison with the standard compound led to an unambiguous identification of peak 5 to that of rosmarinic acid.

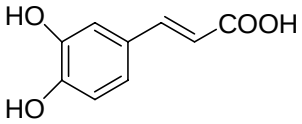
Peak 7 (t_R=38.3 min) showed *m/z* of 719 corresponding to $[M-H]^-$ and a base peak at *m/z* 359 corresponding to$[2M-H]^-$ of a rosmarinic acid dimer where dimerization had occurred by a [2+2] union of the olefinic moieties (cyclobutane structure). This compound was proposed as Sagerinic acid (Lu & Foo, 1999).

Peak 8 (t_R=39.5 min) with $[M-H]^-$ at *m/z* 717 was a condensation product of two rosmarinic acid molecules via an oxidative cyclization leading to the formation of 1,2-dihydronaphthalene ring structure. This can be salvianolic acid E, B, L and isosalvianolic acid B which have the same molecular weight but different in elution time. By comparison with the data of Hu and co worker (2005), this compound was tentative identified as Salvianolic acid B or Lithospermic acid B (Watzke et al.,2006).

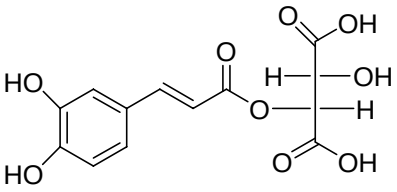
Peak 2 (t_R=27.4 min) showed $[M-H]^-$ at *m/z* 317.1. The fragmentation pattern at *m/z* 197.1, 179.0, 167.0 and 134.9 was identical to that of caffeic acid. The *m/z* at 179 indicated the loss of 138 amu can be assigned as danshensu combine with *p*-hydroxy benzoic acid and 120 amu loss of water similar to peak 6 (t_R=37.2 min) loss of 138 amu from rosmarinic acid so that these two compounds need more data to identify, in this experiment just say caffeic acid derivatives. In the same fashion, peak 4 (t_R=34.6 min) with *m/z* at 792.1$[M-H]^-$ and fragmentation at *m/z* 630.2 $[M-162]^-$, 584.6[M-162-45.5] and *m/z* 359.0 was defined as rosmarinic acid derivative. Peak 9 (t_R=41.8 min) with *m/z* 343.1 and fragmentation at *m/z* 161.0, 181.1, 197.1$[M-145.9]^-$, 179.2 might be danshensu rhamnoside or coumaoryl group. In general MS experiment, flavonoid glycosides will lose the bound sugar groups consecutively and produce the corresponding aglycone, the major sugar groups such as glycosyl, rhamnosyl or the coumaroyl group. A difficulty in the characterization of flavonoids is that coumaroyl group has the same mass as the rhamnosyl group. Both have mass of 146 amu. But coumaoryl conjugate retains on the column longer than rhamnosyl (Mullen et al.,2002). So, this compound was identified as caffeic acid derivative. The chemical structures of these antioxidants found in *O. grandiflorus* were showed in Fig.3.



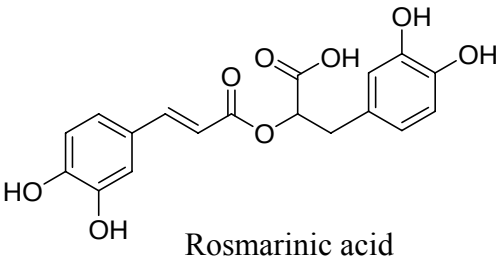
Danshensu



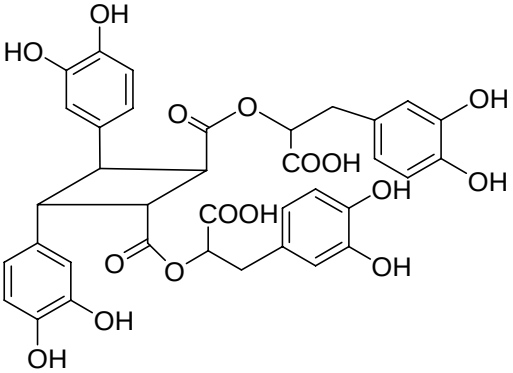
Caffeic acid



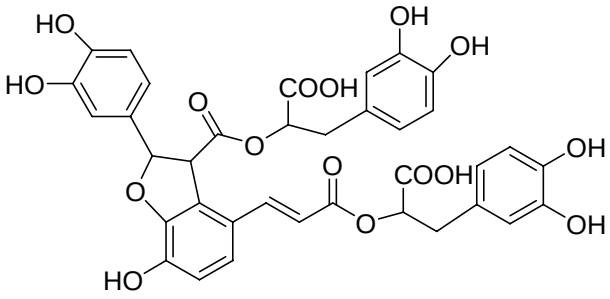
Caftaric acid



Rosmarinic acid



Sagerinic acid



Salvianolic B

Figure3. Chemical structures of antioxidants found in *O. grandiflorus*.

3.2. Quantitative analysis of antioxidants in O. grandiflorus using LC-MS/MS

Antioxidant compounds in *O. grandiflorus* can be quantitatively analyzed using LC-MS/MS in MRM mode. In our studies, caffeic acid and rosmarinic acid at various concentrations were used for the preparation of calibration curves. The regression equation of caffeic acid at concentrations of 0.1-10μg/ml was

y=2.84e+006x+6.69e+005; with r value of 0.9966. The regression equation of rosmarinic acid at concentrations of 0.1-10 μg/ml was y=2.93e+006x+3.43e+005; with r value of 0.9995. The limits of detection (LOD) with signal to noise ratio of three were determined. LOD of caffeic acid and rosmarinic acid were 0.25 ng and 0.05 ng on column respectively. The MRM quantification of these compounds in *O. grandiflorus* tea is shown in Table 2.

Table 2 Contents of phenolic antioxidants found in *O. grandiflorus* aqueous extract

Compound	**Content (μg/g)**
Danshensu	31.63 ± 2.69
Caffeic acid derivative	211.72 ± 16.54
Caftaric acid	239.55 ± 23.73
Caffeic acid derivative	8.54 ± 1.32
Rosmarinic acid	165.50 ± 9.28
Sagerinic acid	96.15 ± 1.48
Salvianolic acid B	165.70 ± 9.5

Caffeic acid derivatives exhibit various properties, such as antioxidative, antimutagenic, antiinflamatory, hepatoprotective and antimicrobial (Findley et al., 1985; Liam et al., 2006; Lu & Foo, 2001; Tewtrakul et al., 2003). In addition, rosmarinic acid shows antithrombotic, antiallergic, as well as antiviral activity against herpes simplex virus and human immunodeficiency virus (Findley et al., 1985; Lu & Foo, 2001; Parejo et al., 2004;Tewtrakul et al., 2003). The order antioxidant activity of caffeic acid derivatives is caffeic acid< danshensu< rosmarinic acid< salvianolic acids (Zhao et al., 2008; Kim & Lee, 2004; Lu & Foo, 2001; Liu et al., 2007). The level of caffeic acid derivatives, including rosmarinic acid, have been reported to as high as 94.6% in hot water extract (Sumaryono et al.,1991). Rosmarinic acid was found not only in *O. grandiflorus* but also in other species such as dittany, turkish oregano, sweet marjoram oregano, rosemary, thyme (Triantaphyllou et al., 2001; Justesen, 2000).

4. Conclusion

An on-line LC-ESI-MS coupled to DPPH assay for the simultaneous analyse of phenolic antioxidant compound in a single run resulting in significantly reducing of time and the amount of sample. The interference from complex matrices can be avoided using the specific MRM technique. Generally, this technique proved to be very powerful for the rapid characterization of antioxidant compounds in plant extracts. The results from the study indicate that *O. grandiflorus*, composes of such strong free radical scavengers thus it has potential for applications in food and health product industry.

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CHAPTER 5

On-line characterlization of phenolics antioxidants in water extract of *Gynostemma pentaphyllum* (Thunb.) Makino by LC-ESI-MS/MS coupled to DPPH assay

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Abstract

Gynostemma pentaphyllum (Thunb.) Makino belongs to the family Cucurbitaceae. In Thailand is known as Panchakhan. It has been used in traditional medicine for treating hepatitis, hyperlipoproteinemia, cardiovascular disease and cancer. The chemical constituents of this plant are close to that of ginseng root, so this plant has attracted interest as a potential food supplement. Since antioxidant activity in this plant has not been studies so far, in this study we have attempted for the first time to investigate the antioxidant component in this plant using on-line LC-MS/MS. The sample was extracted with boiling water, concentrated and direct injected to the system. LC-ESI-MS/MS coupled to DPPH assay system was used to separate screen for antioxidant activity and identify the antioxidant compound. Peak identification was performed by comparison of the retention time and mass spectra with reference compounds and published data. Based on their mass spectra; the antioxidant compounds were identified as caffeoyl glucoside, maleic acid, *p*-coumaric acid derivative, *p*-coumaroylhexose, sinapic acid derivative and kaempferol rutinoside. This on-line LC-ESI-MS/MS coupled with DPPH assay is very useful for rapid screening of antioxidant compounds.

Keywords: *Gynostemma pentaphyllum*, LC-ESI-MS/MS, DPPH assay, tea

1. Introduction

Gynostemma pentaphyllum (Thunb.) Makino belongs to the family Cucurbitaceae. It is a perennial creeping herb grows a wild in Southern China, Japan, India, Korea and Thailand. It is called Jiaogulan and Panchakhan in China and Thailand respectively. It has been used in traditional Chinese medicine (TCM) to treat bronchitis and asthma. In Thailand, this plant is found growing wild on highlands in the northern region where it is also cultivated for commercial purposes and is a well known herbal tea in the recent year. Phytochemical studies of this plant resulted in the finding of 130 dammarane-type glycosides which are closely related to gingseng saponins. They are called gypenosides (Razmovski et al.,2005). Because of this similarity to the expensive ginseng root, *G. pentaphyllum* has attracted much interest as a potential new botanical drug. The presence of functional components in *G. pentaphyllum* such as flavonoids, saponins, carotenoids, chlorophylls, and heteropolysaccharide are believed to be responsible for this beneficial effect (Huang et al., 2008, Lv et al., 2009). Wang & Luo, 2007 reported that a fraction of water soluble polysaccharide had the higher scavenging effects on superoxide radicals and inhibitory effects on self oxidation of 1,2,3-phentriol which comparable to vitamin C and so should have been explored as a novel potential antioxidant. The chemical constituent of this plant was reported as rutin quercetin and trace amount of chlorogenic acid (Tsai, et al., 2008). Pharmacological studies of *G. pentaphyllum* and/or the isolated saponins have shown a variety of interesting activities such as antitumor, cholesterol-lowering, immunopotentiating, antiulcer (Lewis and Hanson, 1991), antioxidant and others (Chen et al.,1996; Hou et. al.,1991; Li and Lau,1993; Norberg et al., 2004). The water extract of this plant was proven not to produce any significant chronic toxic effect (Attawish et al., 2004). For this reason, it is claimed that drinking tea made of *G. pentaphyllum* could regularly promote good health and lessen the severity of many disorders.

Antioxidants are usually found as complex mixtures in plant extracts. Some HPLC methods on-line coupled with the detection of antioxidative activity using post-column reaction of eluates with free radicals have been reported that they can be successfully applied for the identification and quantification of antioxidants in biological samples (Niederländer et al., 2008; Nuengchamnong & Ingkaninan, 2009). Beside these, a free radical spiking technique also showed good detection efficacy

(Shui, Leong & Wong, 2005). However, so far there have been only few studies on the phenolic constituents responsible for the antioxidant activity of *G. pentaphyllum* The main objective of this study was therefore to screen the antioxidants in *G. pentaphyllum* and to further identify those compounds using LC-ESI-MS/MS coupled to DPPH assay

2. Materials and methods

2.1. Chemical and reagents

Free radical 1,1 diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Formic acid (analytical grade) was purchased form Merck (Darmstadt, Germany). Water was purified using Elga USF system (Bucks, England).

2.2. Plant material

The tea sample was supplied from the Bangkatum hospital, Phitsanulok, Thailand.

2.3. Sample preparation

Five gram of *G. pentaphyllum* tea was extracted with boiling water (150 ml) for 5 min, filtered through Whatman No.1. The filtrate was concentrated by rotary evaporator and blown to dryness under a stream of nitrogen. The extract was reconstituted with 1 ml 70% methanol and filtered through a 0.2 μm Nylon syringe filter (Vertical Chromatography Co., Ltd.,Thailand) and then directly injected into the LC-ESI-MS/MS coupled to DPPH assay system.

2.4. LC-ESI-MS/MS coupled to DPPH assay for the rapid identification of antioxidants in water extract of G. pentaphyllum

The HPLC was coupled on-line to MS (line A) and a continuous flow DPPH assay (line B) as described in the previous study (Nuengchamnong & Ingkaninan, 2009) Figure 1. Line A; Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) is coupled to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with electrospray ionization interface. The chromatographic separation was achieved with a phenomenex Gemini column (5 μm, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, operated at 25 °C. The mobile phase consisted of solvent A (1 ml formic acid in 1 L of deionized water) and solvent B (methanol). The elution program increased from 90:10 solvent A: solvent B for 4 min, then changed to 80:20 solvent A: solvent B in 6 min, linearly increased to 10:90 solvent A: solvent B in 30 min and the ratio of solvent A: solvent B was constant at 10:90 for 5 min then changed to 90:10 solvent A: solvent B in 5 min and kept constant at 90:10 solvent A: solvent B for 5 min for reconditioning of the column. A mass spectra was recorded within 55 min. The injection volume was 5 μl. The flow rate was set at 600 μl /min. The Analyst 1.3.2 software was used for data acquisition and processing. The full scan mass spectra from *m/z* 100-1000 amu were acquired both in positive and negative ion modes. The optimum conditions of the interface were as follows: ESI-positive; ion spray voltage of 4500 V, curtain gas of 69 Kpa (10 psi), ion source gas 1 of 450 Kpa (65 psi), ion source gas 2 of 380 Kpa (55 psi). The interface temperature was set at 400°C. The entrance and declustering potential were 10V and 80V, respectively. ESI-negative; the condition was similar to ESI positive except the voltage was set in negative mode. **Line B** represents the continuous flow system for antioxidant activity detection. It consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter oϕ 180 μm and a total reaction coil volume of 100 μL. The flow of 0.1mM DPPH was set to 200 μl/min and induced bleaching was detected as a negative peak at 515 nm using the UV-VIS detector (SPD 20AV, Shimadzu, Kyoto, Japan) The LC solution software was used for data acquisition and processing. The polarity of the signal output was reversed in order to obtain positive signals. The system was operated at 25°C. Line A coupled to line B with a 24 cm length of 0.17 mm i.d. PEEK tubing by a Y-connector. The eluent flow was split in a ratio of 8:2 between the MS ion source and DPPH line. For the characterization of antioxidant

peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between-5 to-50V and collision cell exit potential (CXP) of -6 V, DP in the range of -20 to -110 V. In positive mode, the ions were induced with CAD of 48 Kpa (7 psi). CE, CXP and DP were set in the range of 5-25, 3-13 and 20-100 V respectively.

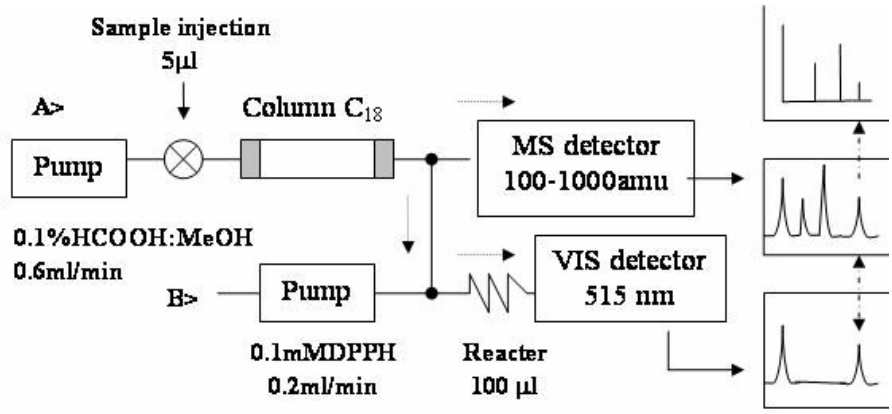


Figure1 Scheme of the LC-ESI-MS/MS coupled to DPPH assay system

2.5. Peak identification

Peak identification was performed by comparison of the retention time and mass spectra with reference compounds and published data.

3. Results and discussion

The phenolic antioxidants in *G. pentaphyllum* were monitored by mass spectrometry and radical scavenger detection. These analyses were carried out in the water extract to ensure the consumer intake. A typical chromatogram of the antioxidants trace and total ion detected in mass trace are shown in Fig. 2. The negative ionization give a good results than positive ionization so that in this work the identification of all compounds did in negative ionization mode. Table 1 gives the MS and MS/MS characteristics of the chromatographic antioxidant peak, along with their proposed structure. Up to 9 different phenolic compounds were detected in the aqueous extracts.

Compound 1, with $[M-H]^-$ at *m/z* 341.3 and fragments pattern at *m/z* 179.2 and 161.1 correspond to caffeoyl hexoside. Ion at *m/z* 179 and 161 are deprotonated caffeic acid and dehydrated caffeic acid, respectively (Määttä et al.,2003).

Compound 4 with $[M-H]^-$ at *m/z* 231.4 and MS/MS ion at *m/z* 115.2 and 116.3 , the dimeric form of maleic acid.

Mass spectra of compound 6 in negative ion mode showed the molecular ion $[M-H]^-$ at *m/z* 325.6. MS/MS base ion *m/z* 163.8 resulting from loss of 162 amu (glucose or galactose), and other fragment ion at *m/z* 145.7 suggests that compound 6 is a coumaric acid sugar derivative (Määttä et al.,2003) and compound 5 with $[M-H]^-$ at *m/z* 371.3 and the fragmentation pattern gave *m/z* at 209.0,190.8 and 163.0 similar to coumaric acid so that this compound is proposed as *p*-coumaric acid derivatives.

Compound 7 with $[M-H]^-$ at *m/z* 431.5 and MS/MS ion at 223.1 [sinapic acid-H]$^-$ and *m/z* 205.5 [sinapic acid-H-18]$^-$ was identified as sinapic acid derivative (Ferreres, et al., 2006).

Compound 9 had a deprotonated molecular ion at *m/z* 593.3 and fragments at *m/z* 447.2, 428.9, 284.3, 285.1, which suggests that it is a kaempferol rutinoside as reported by Kao, et al., 2008.

Compound 2, 3 and 8 could not be identified in this study based on the data available. The structures of the compounds found were shown in Fig. 3.

Antioxidant activity of *G. pentaphyllum* is likely to be small because of their relatively low concentration. Moreover, flavonols in *G. pentaphyllum* are glycosilated, which is known to significantly reduce their antioxidant potency. This data were relevant to the reported of Tsai (Tsai et al., 2008).

Table1 Identification of antioxidant compounds in water extract of *G.pentaphyllum* by LC-ESI-MS/MS coupled to DPPH assay

Peak No.	t_R	MS	MS/MS	Identification
1	4.8	341.3	179.2,161.1,143.0	Caffeoyl hexoside
2	6.0	525.7	262.9	Unidentified
3	6.8	439.4	304.4, 244.6	Unidentified
4	11.7	231.4	115.2,116.3,147.3,144.2	Maleic acid
5	21.5	371.3	209.0,190.8,163.0,119.2	*p*-Coumaric acid derivatives
6	23.4	325.6	145.7,163.8,119.2	*p*-Coumaroylhexose
7	25.6	431.5	223.1,179.0,205.5	Sinapic acid derivative
8	30.5	497.7	479.1,451.3	Unidentified
9	31.3	593.3	447.2, 428.9, 284.3,285.1	Kaempferol rutinoside

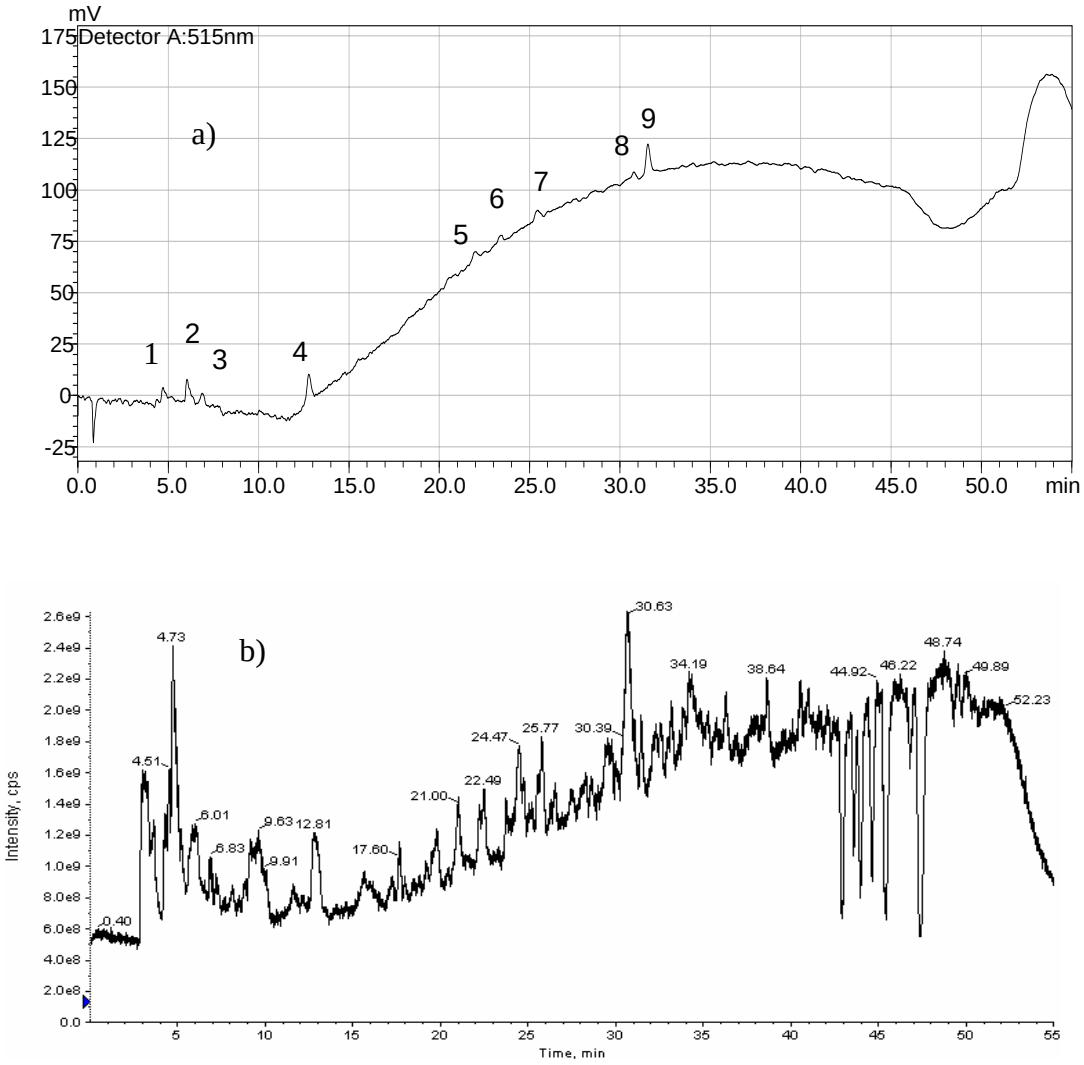
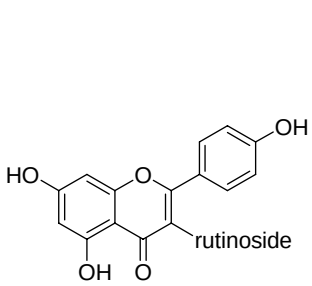
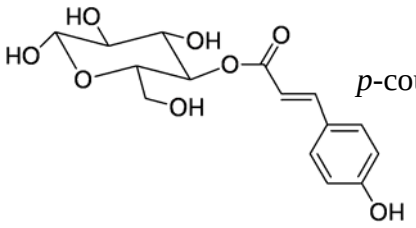


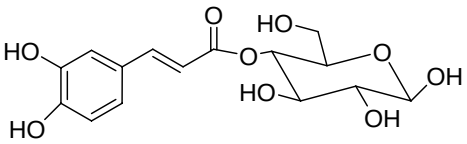
Figure 2 The analysts of *G. pentaphyllum* aqueous extract using LC-ESI-MS coupled with DPPH assay. a) the chromatogram from the antioxidant activity assay detection at 515 nm. b) The total ion current (TIC) output from the ESI-MS in negative mode. For peak assignments, see table1.



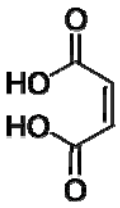
Kaempferol rutinoside



p-coumaroylhexose



Caffeoyl glucoside



Maleic acid

Figure 3 Structures of some antioxidant compounds identified in the *G. pentaphyllum*

4. Conclusions

G. pentaphyllum is a well known edible and medicinal plant in oriental countries. Most studies were focus on saponin compounds. However, this study showed the presence of phenolic compounds which also play an important role in antioxidant activities of this plant. In this study, the use of on-line LC-ESI-MS coupled with DPPH assay was proved to be efficient tool in rapid screening of antioxidants in complex herbal extracts.

Acknowledgements

The financial support of the project DBG-4980011, from the Thailand Research Fund (TRF) is gratefully acknowledged.

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CHAPTER 6
Mass spectra fingerprint

1. Antioxidant compounds in aqueous extract of *Houttuynia cordata*

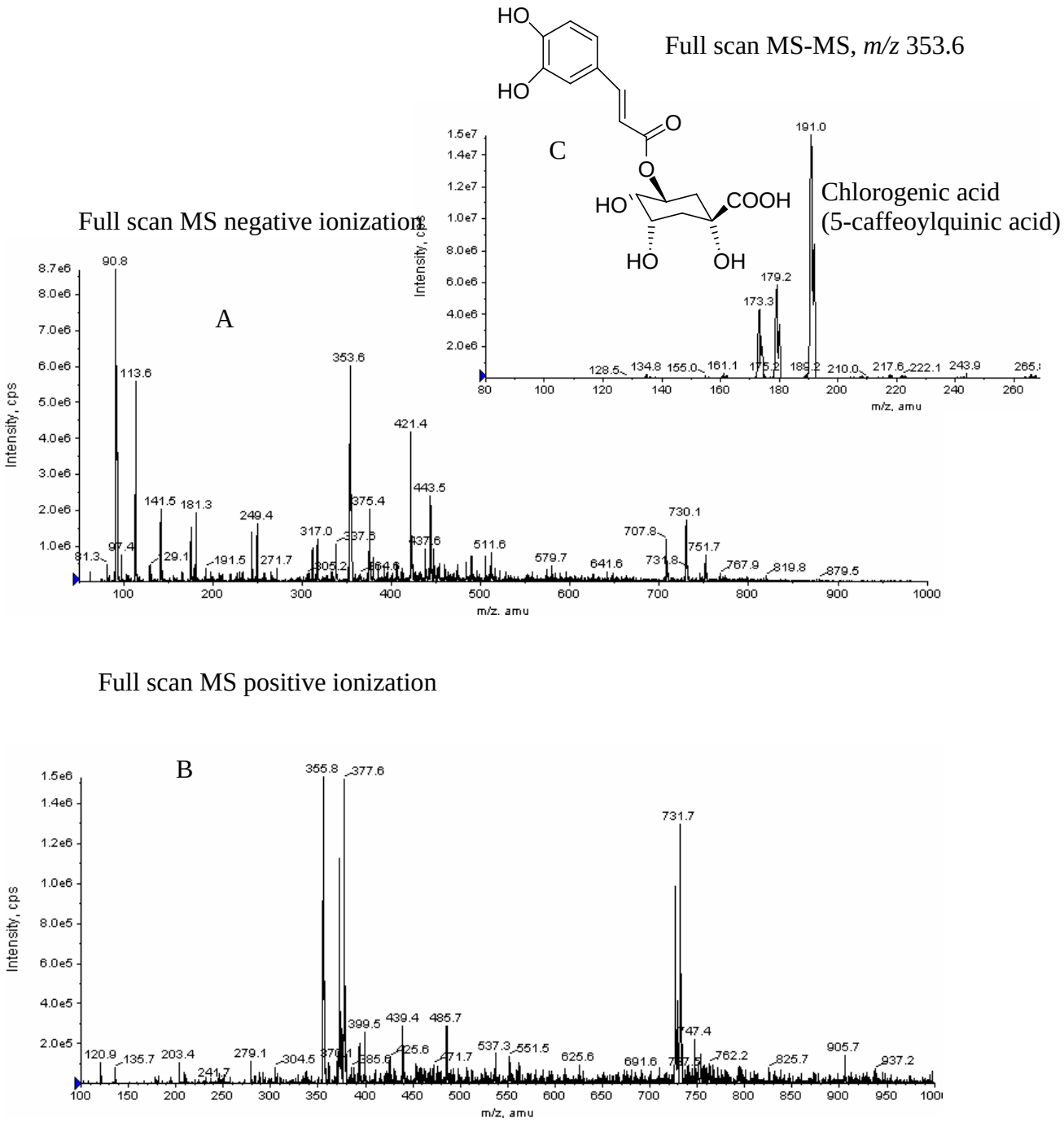

Figure 1. (A) full scan MS spectrum of the peak at 25.6 min in negative ionization, (B) full scan MS spectrum of the peak at 25.6 min in positive ionization, (C) fragmentation spectrum(MS/MS) of the *m/z* 353.6

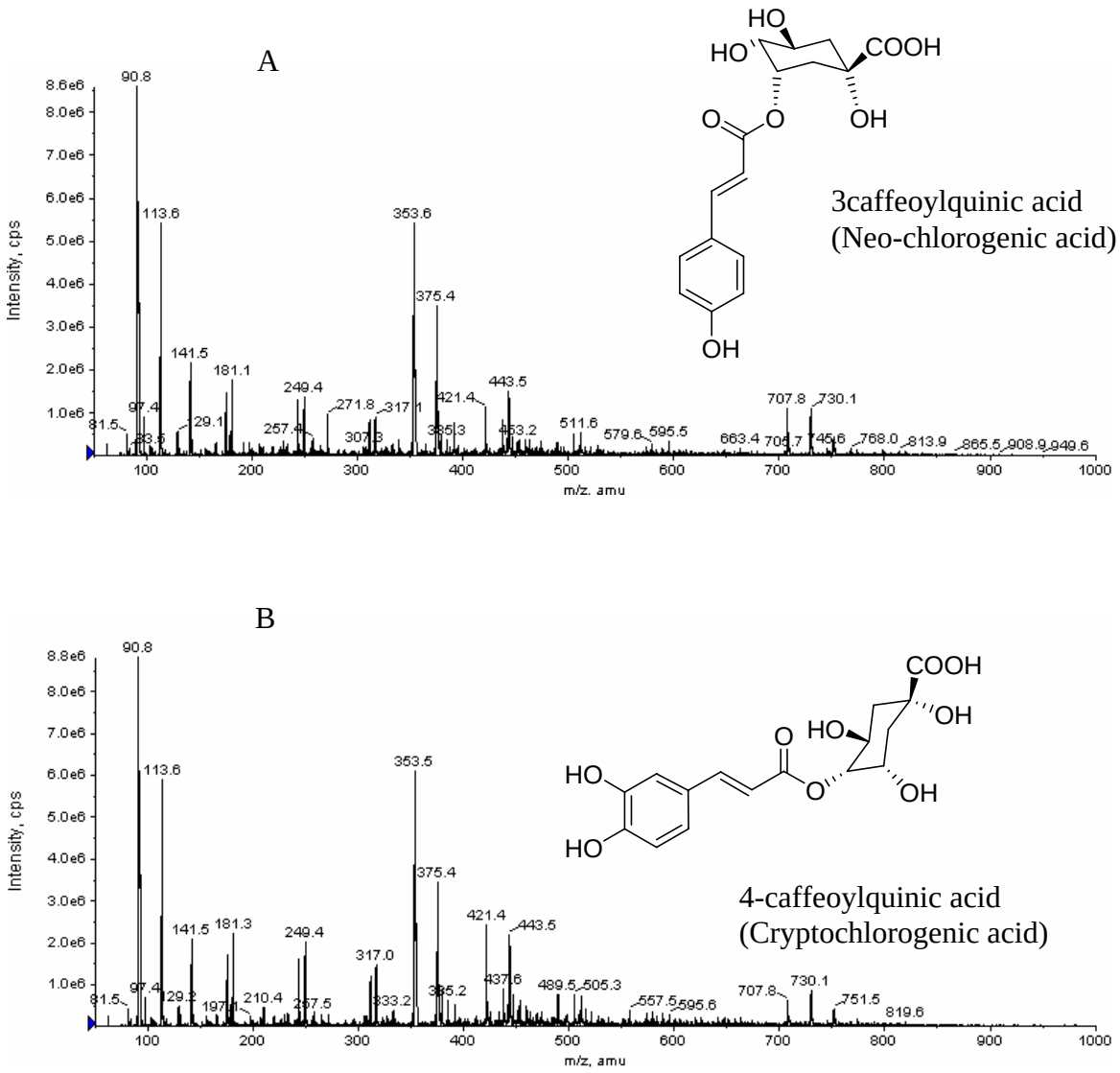

Figure 2 Full scan MS spectrum in negative ionization of the peak at t_R (A) 22.4 min, (B) 27.3 min

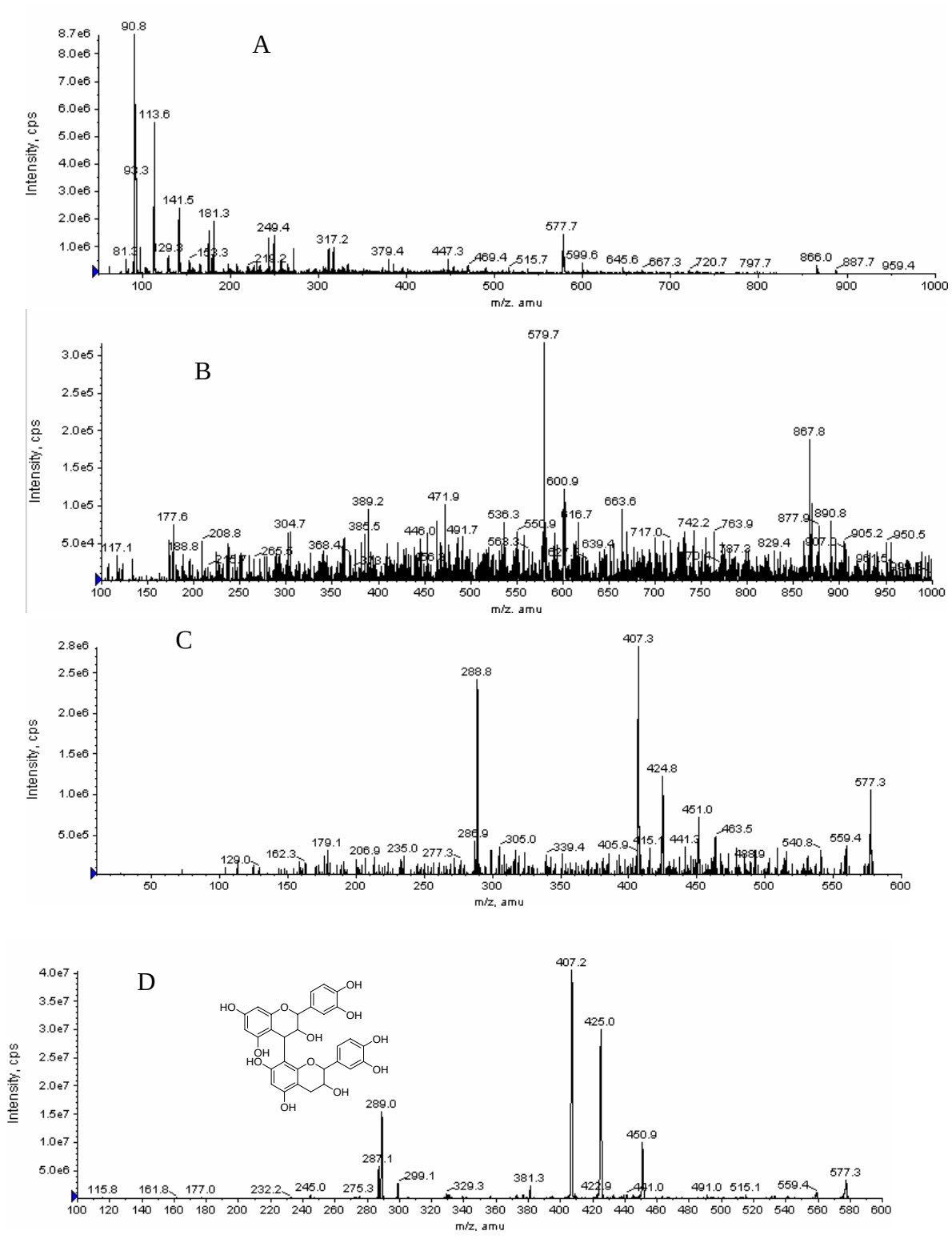

Figure 3. (A) full scan MS spectrum of the peak at 21.8 min in negative ionization, (B) full scan MS spectrum of the peak in positive ionization, (C) fragmentation spectrum(MS/MS) of the *m/z* 577.7 (D) fragmentation spectrum(MS/MS) of standard procyanidin B2

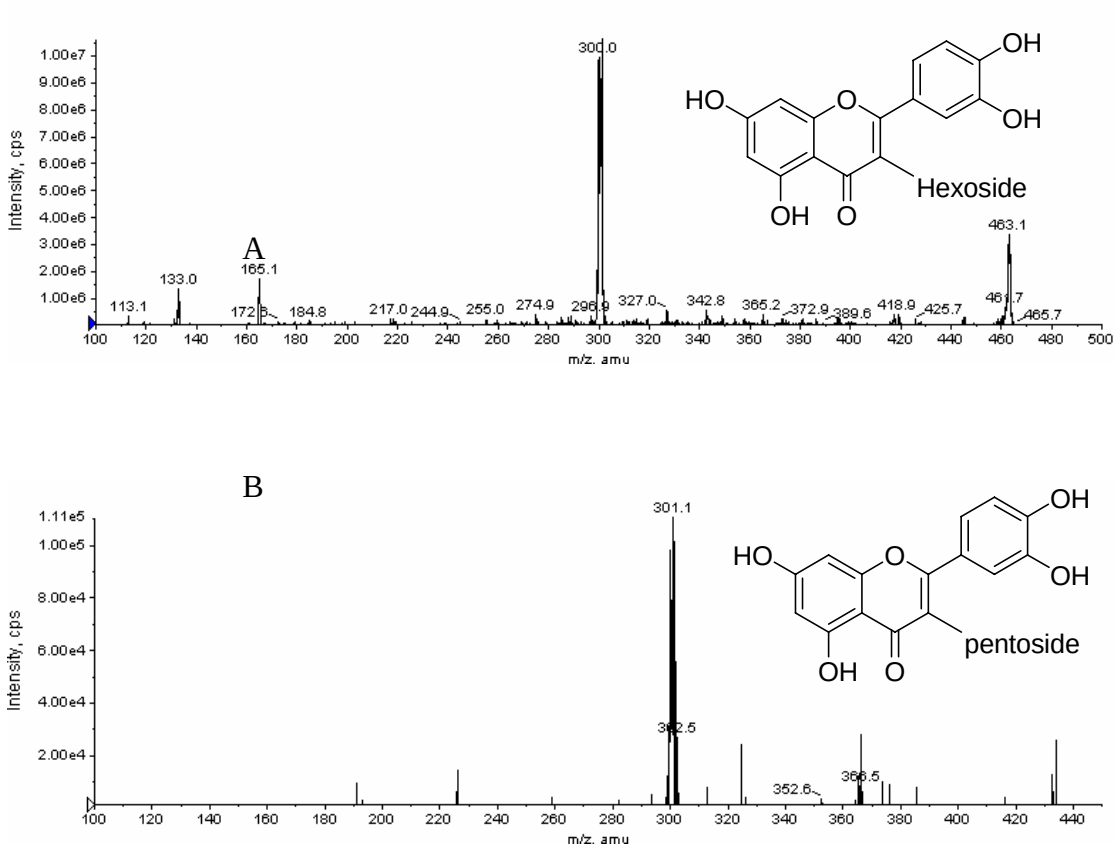

Figure 4. Fragmentation spectrum(MS/MS) in negative mode of the (A) *m/z* 463.3 peak at 33.4 min, (B) *m/z* 433.7 peak at 34.5min

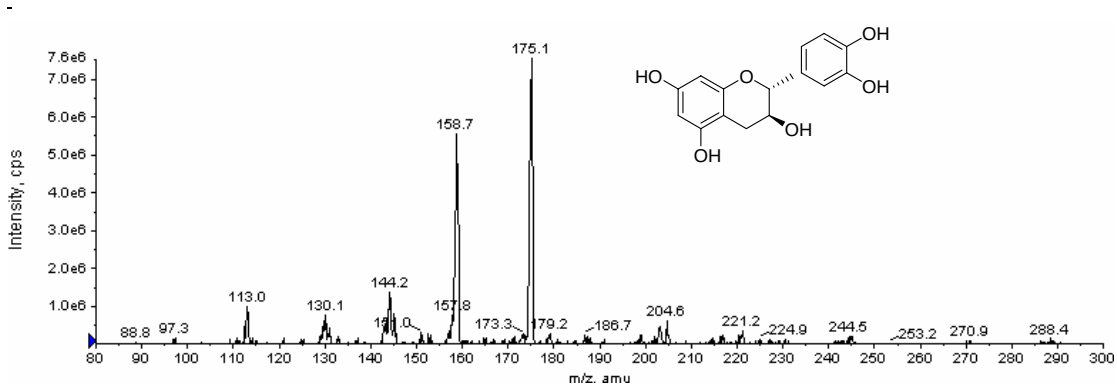

Figure 5 Fragmentation spectrum (MS/MS) in negative mode of the *m/z* 289.2 peak at 23.8 min

2.Antioxidant compounds in water extracted of *Orthosiphon grandiflorus* (ESI-negative ionization)

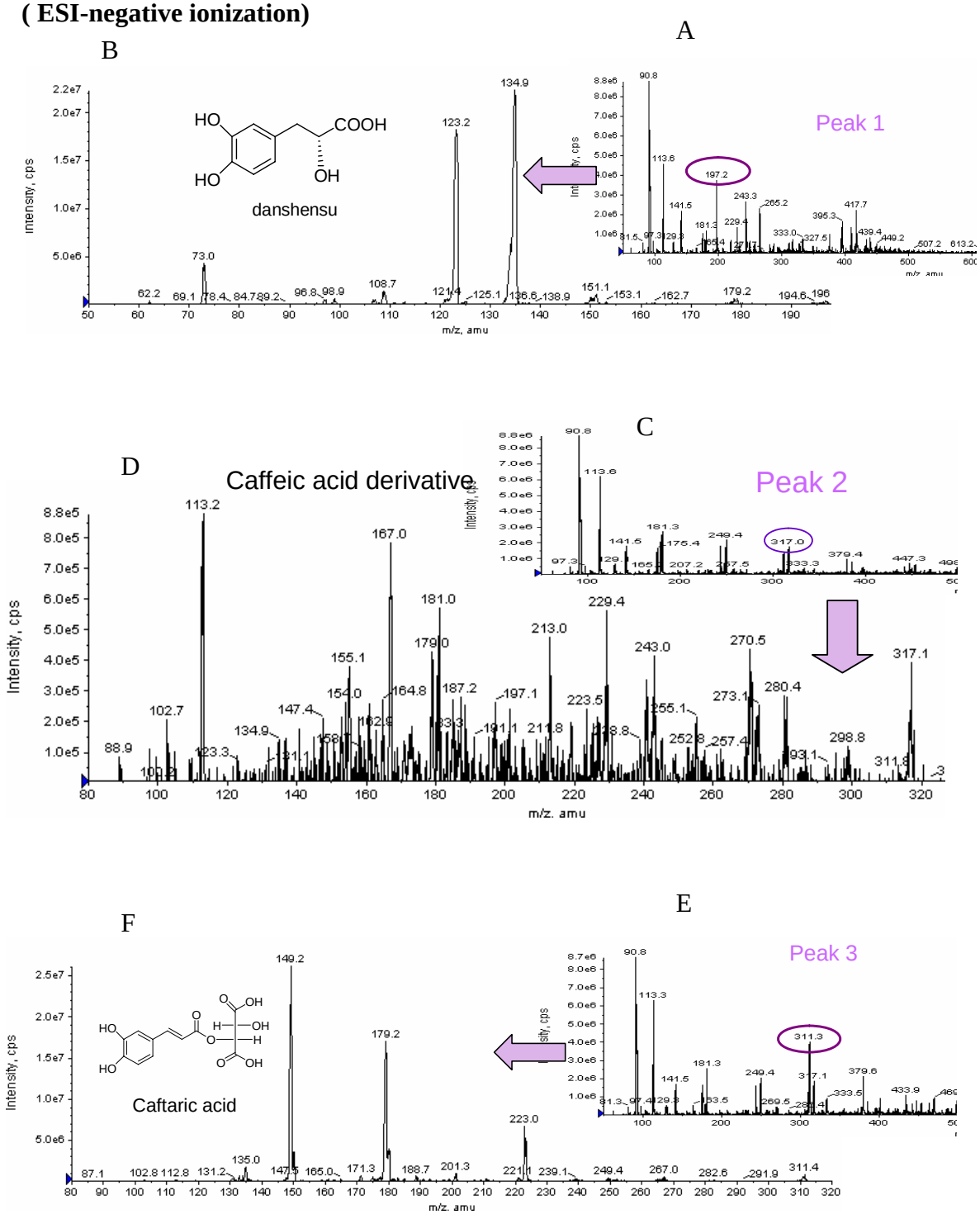

Figure 1. (A) full scan MS spectrum of the peak at 19.3 min (B) fragmentation spectrum(MS/MS) of the *m/z* 197.2 (C) full scan MS spectrum of the peak at 27.4 min (D) fragmentation spectrum(MS/MS) of the *m/z* 317.1 (E) full scan MS spectrum of the peak at 28.3 min (F) fragmentation spectrum(MS/MS) of the *m/z* 311.6

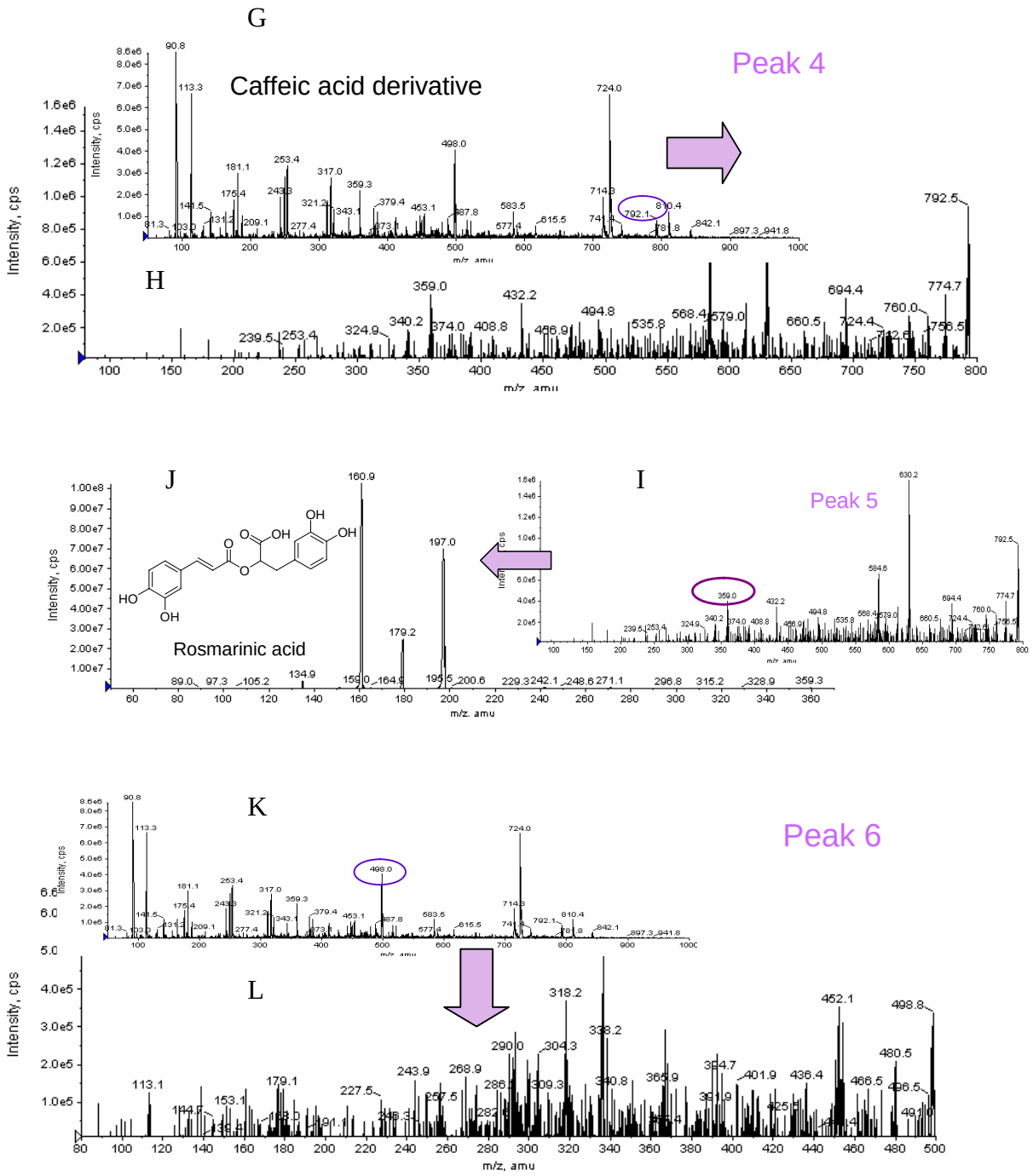

Figure 2 (G) full scan MS spectrum of the peak at 34.6 min (H) fragmentation spectrum(MS/MS) of the *m/z* 792.1 (I) full scan MS spectrum of the peak at 35.0 min (J) fragmentation spectrum(MS/MS) of the *m/z*359.0 (K) full scan MS spectrum of the peak at 37.2 min (L) fragmentation spectrum(MS/MS) of the *m/z* 498.8

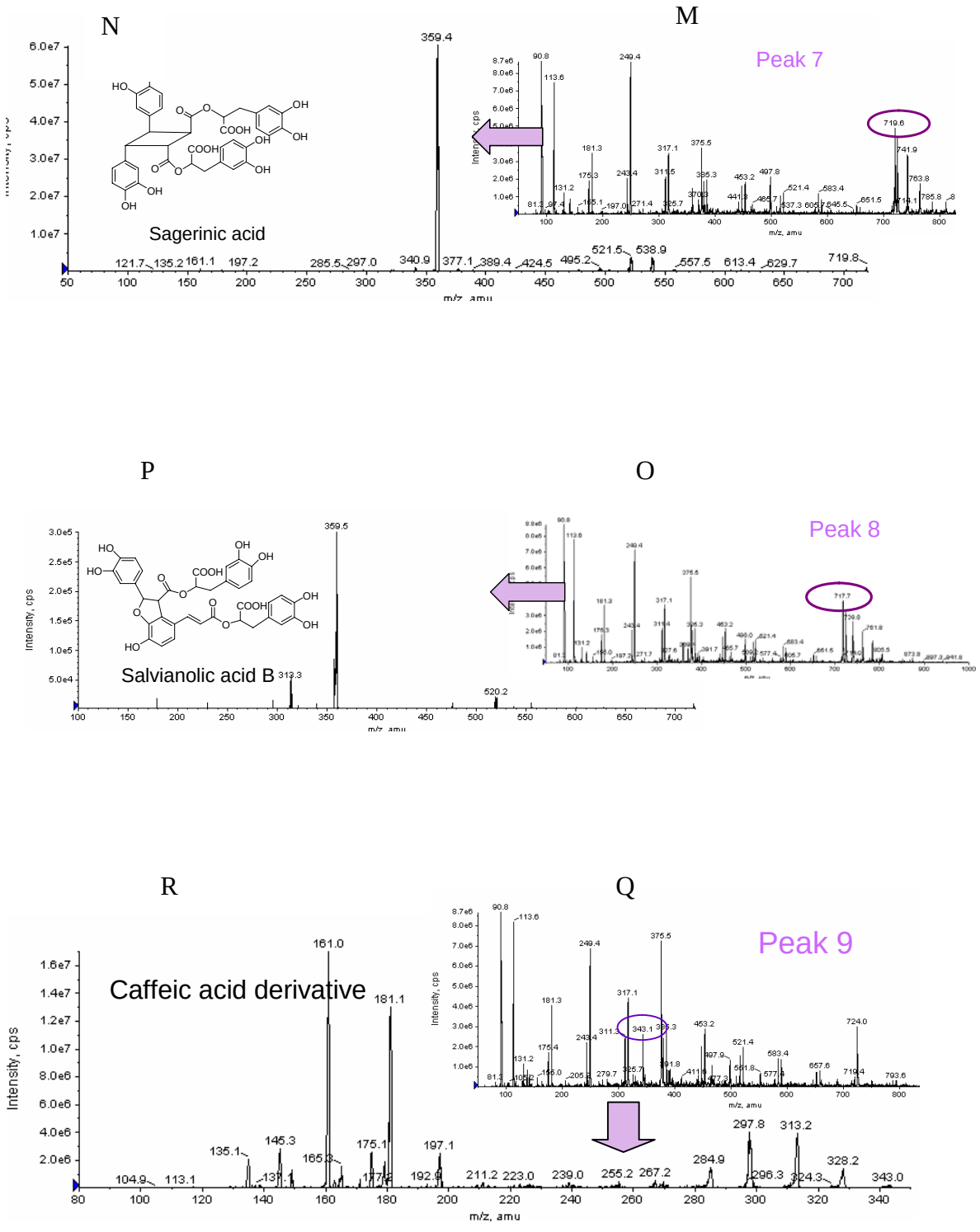

Figure 3. (M) full scan MS spectrum of the peak at 38.3 min (N) fragmentation spectrum(MS/MS) of the *m/z* 719.6 (O) full scan MS spectrum of the peak at 39.5 min (P) fragmentation spectrum(MS/MS) of the *m/z* 717.7 (Q) full scan MS spectrum of the peak at 41.8 min (R) fragmentation spectrum(MS/MS) of the *m/z* 343.1

3. Antioxidant compounds in water extracted of *Gynostemma pentaphyllum* (ESI negative ionization)

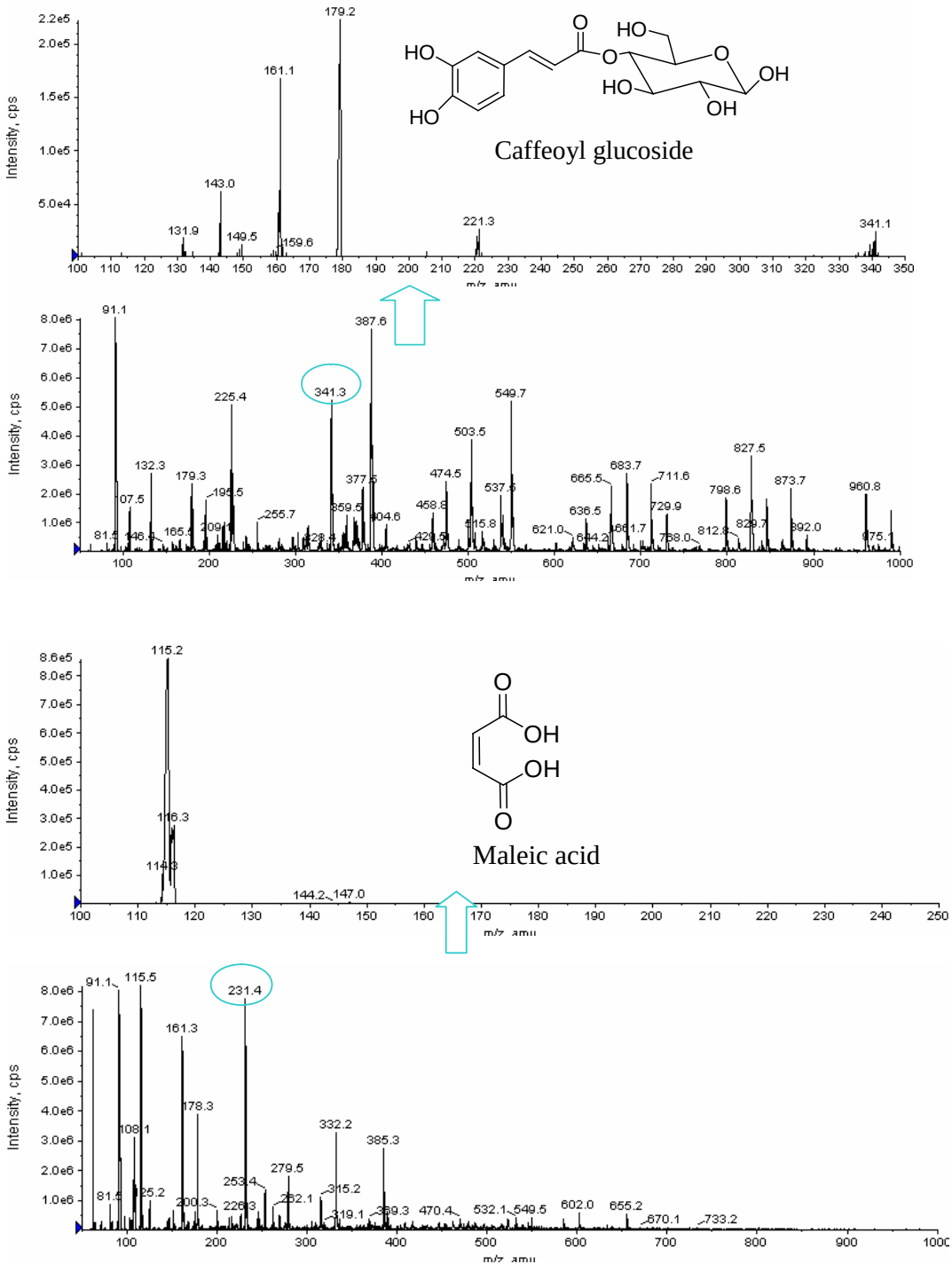

Figure 1. (A) full scan MS spectrum of the peak at 4.8 min (B) fragmentation spectrum(MS/MS) of the *m/z* 341.3 (C) full scan MS spectrum of the peak at 11.7 min (D) fragmentation spectrum(MS/MS) of the *m/z* 231.4

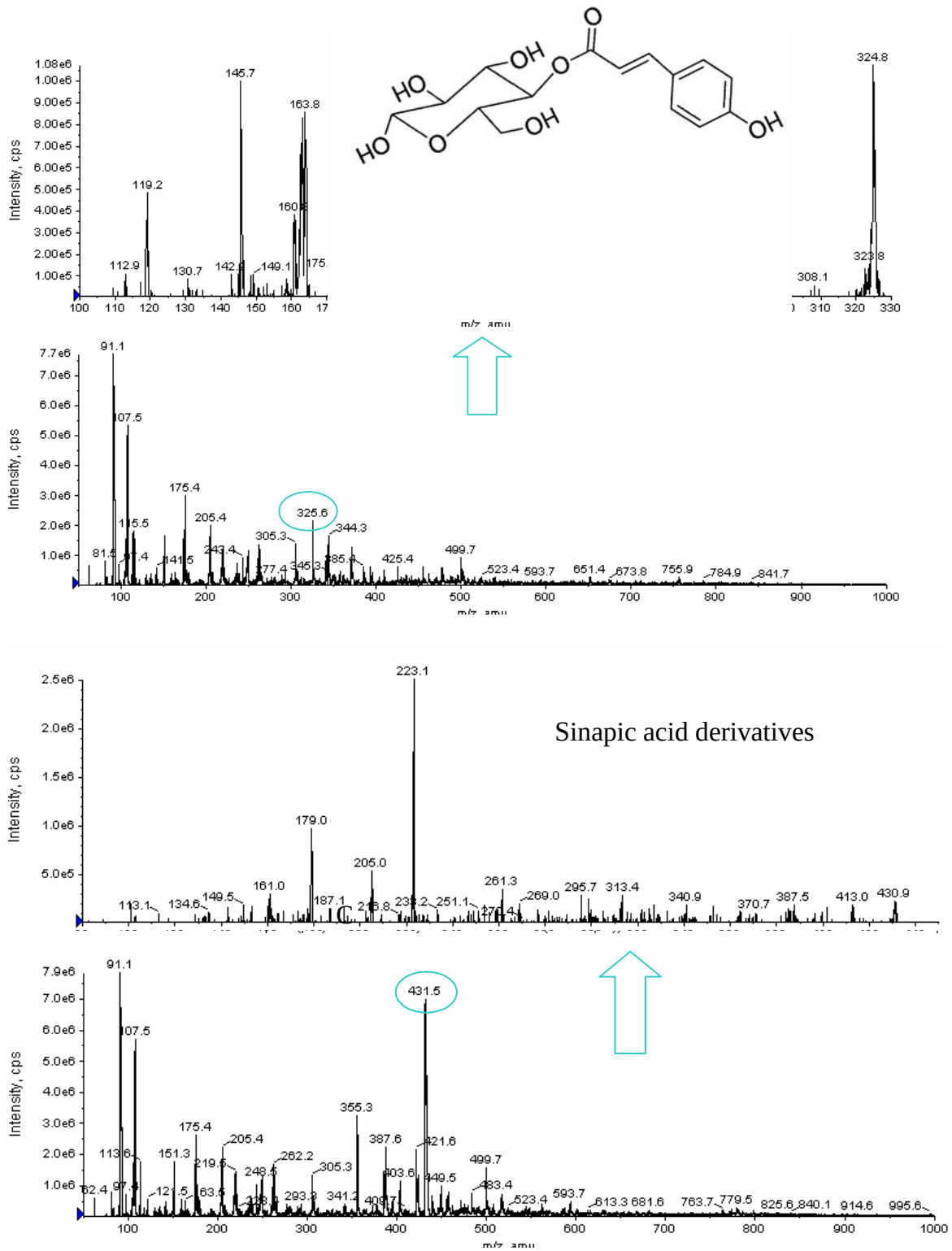

Figure 2. (M) full scan MS spectrum of the peak at 38.3 min (N) fragmentation spectrum(MS/MS) of the *m/z* 719.6 (O) full scan MS spectrum of the peak at 39.5 min (P) fragmentation spectrum(MS/MS) of the *m/z* 717.7 (Q) full scan MS spectrum of the peak at 41.8 min (R) fragmentation spectrum(MS/MS) of the *m/z* 343.1

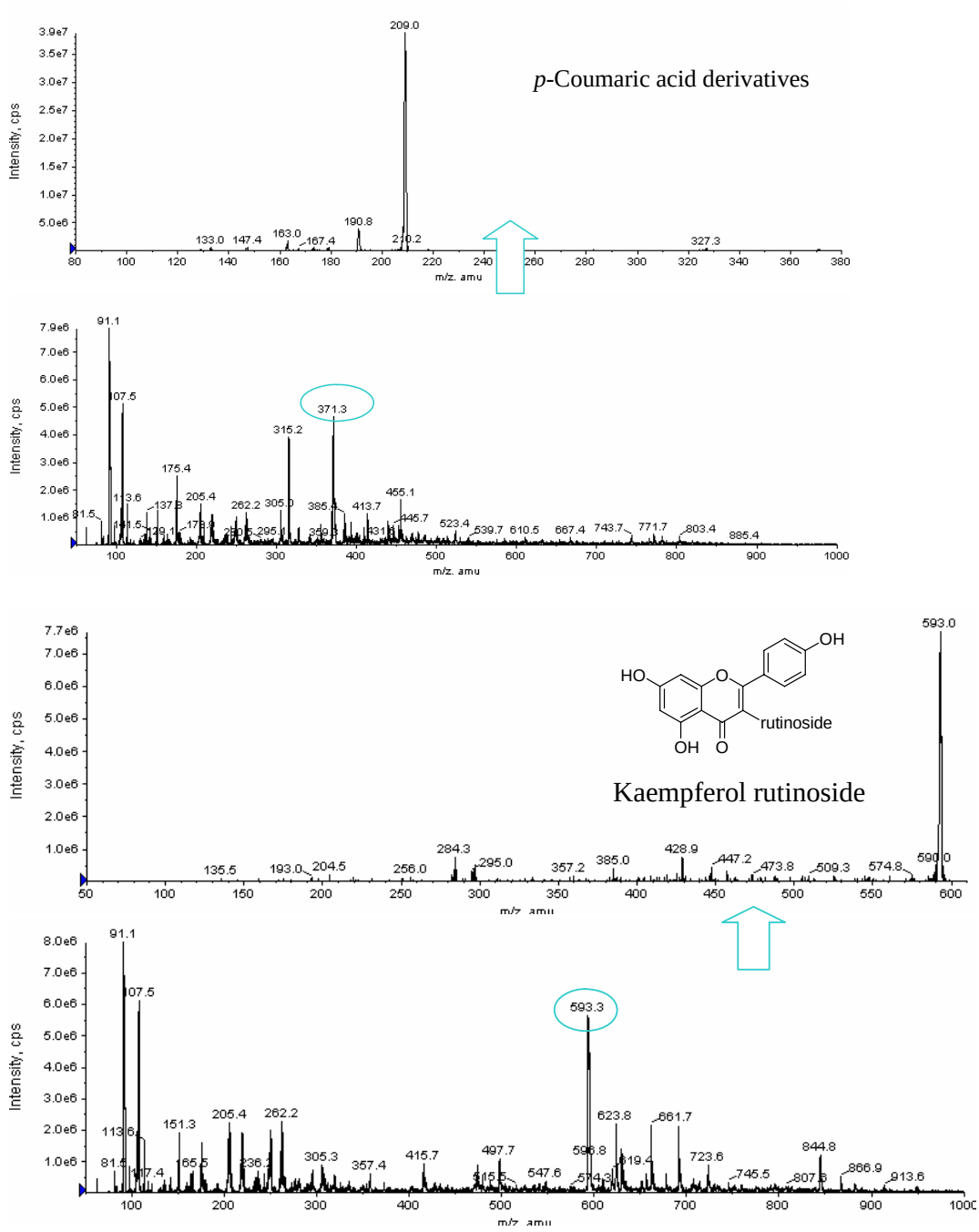

Figure 3. (M) full scan MS spectrum of the peak at 38.3 min (N) fragmentation spectrum(MS/MS) of the *m/z* 719.6 (O) full scan MS spectrum of the peak at 39.5 min (P) fragmentation spectrum(MS/MS) of the *m/z* 717.7 (Q) full scan MS spectrum of the peak at 41.8 min (R) fragmentation spectrum(MS/MS) of the *m/z* 343.1

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(chapter1)

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Publications and Presentations

List of publications

1. Rapid screening and identification of antioxidants in aqueous extracts of *Houttuynia cordata* using LC-ESI-MS coupled with DPPH assay
(Submitted for Food chemistry)

2. Identification and characterization of phenolic antioxidants in water extract of *Orthosiphon grandiflorus* tea by LC-ESI-MS/MS coupled to DPPH assay
(Manuscript is to be prepared)

Presentations

1. Rapid screening and identification of antioxidants in *Houttuynia cordata* herbal tea using LC-ESI-MS coupled with DPPH assay

การประชุมเสนอผลงานวิจัยทางเภสัชศาสตร์ ประจำปี2550 ครั้งที่24 วันที่12 ธันวาคม 2550 อาคาร 80 ปี คณะเภสัชศาสตร์จุฬาลงกรณ์มหาวิทยาลัย

2. Identification and characterization of phenolic antioxidants in water extract of *Orthosiphon grandiflorus* tea by LC-ESI-MS/MS coupled to DPPH assay

At 7th Joint Meeting of AFERP, ASP,GA,PSE & SIF ; Natural products with pharmaceutical, nutraceutical, cosmetic and agrochemical interest, Athenaeum Intercontinental, Athen, Greece 3-8 August 2551

การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่8; 16-18 ตุลาคม 2551 โรงแรมฮอลิเดย์อินน์ รีสอร์ท บีช ชะอำ จ.เพชรบุรี

3. On-line characterlization of phenolics antioxidants in water extract of *Gynostemma pentaphyllum* (Thunb.) Makino by LC-ESI-MS/MS coupled to DPPH assay

Outstanding Poster Presentation at the 2nd International conference on Natural Products for Health and Beauty at Naresuan University, Phayao, Thailand

RAPID SCREENING AND IDENTIFICATION OF ANTIOXDANTS IN WATER EXTRACTS OF *HOUTTUYNIA CORDATA* THUNB. USING LC-ESI-MS COUPLED WITH DPPH ASSAY

Nitra Nuengchamnong[1], Kamlai Krittasilp[2] and Kornkanok Ingkaninan[3]
[1]Regional Medical Sciences Center Phitsanulok, Department of Medical Sciences, Ministry of Public Health. 65000 Thailand.
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INTRODUCTION

Houttuynia cordata **Thunb. (Saururaceae) has been used in traditional medicine for the treatment of allergy, anaphylaxis, cancer and viral infection[1-4]. The herb is a perennial native to Southeast Asia. It has a thin stalk and hearth liked leaf. In Thailand, it is called "Kaew-tong or Plu-kaew". It is used not only as medicine but also as food.**

The components in *Houttuynia cordata* are reported as rutin, quercetin, quercitrin and hyperoside[5]. Volatile oils are also found in this plant. Due to the presence of many bioactive ingredients, it is necessary to find a rapid method for screening active compounds. In this work, the on-line HPLC separation, activity determination together with structure identification method of the antioxidant from this plant was demonstrated.

EXPERIMENT SECTION

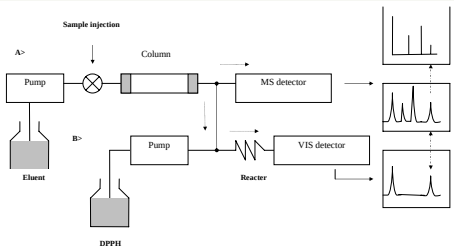


Figure1 Scheme of the HPLC on-line coupled to ESI-MS and DPPH assay. The arrows indicate flow directions.

Condition

Column: Gemini column (5 μm, 250 x 4.6 mm i.d.)

Mobile phase: MeOH:0.1% formic acid; gradient elution in 55 min.

Injection volume: 5μl;

Flow rate: A; 600 μl/min (LC)

B; 200 μl/min (0.1mM DPPH)

Split ratio 8:2 (MS:DPPH line); Reactor volume:100 μl

Detector: 515nm (DPPH line)

MS full scan 100-1000 amu ESI negative and positive ionization

Sample preparation

H. cordata **leaves (5 g) were extracted with boiling water (150 ml) for 5 min, and the filtrate was evaporated on a rotatory evaporator. The crude extracted was redissolved in 1 ml of 70% MeOH.**

Peak identification

Peak identification was performed by comparison of the retention time and mass spectra with reference compounds and published data.

ACKNOWLEDGEMENT

The financial support from the Thailand Research Fund (DBG-4980011) is acknowledged.

RESULTS AND DISCUSSION

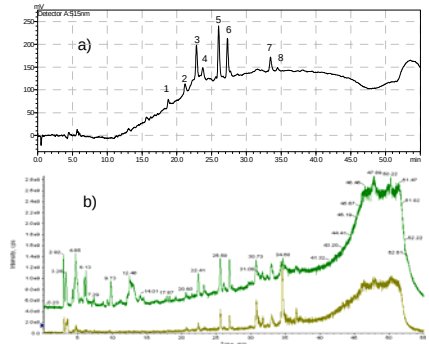
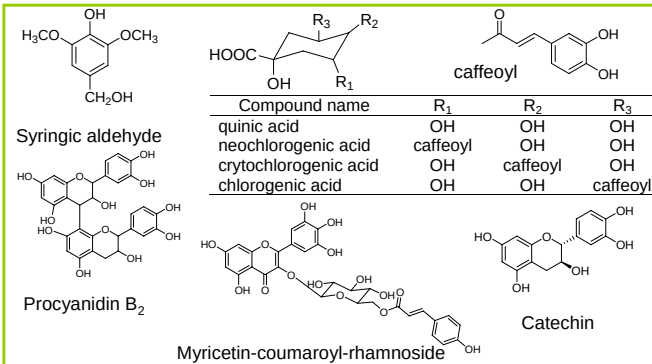


Figure 2 HPLC separation of a *H. cordata* water extract with simultaneous antioxidant activity assay and MS detection a) the chromatogram from the antioxidant activity assay detection at 515 nm. b) The total ion current (TIC) output from the ESI-MS in negative mode (green) and positive mode (brown). For peak assignments, see table1.

The DPPH based antioxidant activity profile (Fig. 2a) shows that at least eight compounds have antioxidant activity. The negative and positive ions of major active compounds are listed in table 1 and identification of these compound are proposed. Peak assignments were confirmed by mass spectrometric behaviors both in negative and positive ionization mode. Identification of compound 4 was based on the comparison of its retention time with the reference compound. By comparing the elution order of the caffeoyl quinic acid with published data[6], compound 3, 5, 6 might be neochlorogenic acid, chlorogenic acid and cryptochlorogenic. The structures of these compounds are shown in Figure 3. The intensity of peaks depend on the types and amounts of the compounds in the sample.

Table1 Identification of antioxidant compounds in water extracts of *Houttuynia cordata* by using their LC-MS-DPPH assay data

Peak no.	t_R (min)	ESI-MS (*m/z*) mode	ESI-MS (*m/z*) $[M-H]^-$, $[M+H]^+$	Tentative ID
1	18.7	-	181.3,165.6,151.0	Syringic aldehyde
2	21.8	-	577.7,289.0	Procyanidin B2
		+	579.9, 601.0,409.0,453.0	
3	22.4	-	353.6,707.8	Caffeoyl quinic acid
		+	355.8,377.6,731.7	
4	23.8	-	289.0	Catechin
5	25.6	-	353.6	Caffeoyl quinic acid
6	27.3	-	353.6	Caffeoyl quinic acid
7	33.4	-	317.0,463.3,609.5	Myricetin-coumaroyl-rhamnoside
8	34.5	+	340.6,680.0,702.0	Not identified



Compound name	R_1	R_2	R_3
quinic acid	OH	OH	OH
neochlorogenic acid	caffeoyl	OH	OH
crytochlorogenic acid	OH	caffeoyl	OH
chlorogenic acid	OH	OH	caffeoyl

Figure 3 Structures of antioxidant compounds identified in the *Houttuynia cordata* Thunb.

Conclusion

HPLC with a DPPH assay linked to a triple quadrupole mass spectrometer was proved to be extremely helpful for peak assignment and further characterization of antioxidants. This is a clear example of how a hyphenated technique can accelerate the procedure for identification of active compounds in plants.

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Identification and characterization of phenolic antioxidants in water extract of *Orthosiphon grandiflorus* tea by LC-ESI-MS/MS coupled to DPPH assay

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INTRODUCTION

Orthosiphon grandiflorus Bolding [syn.: *O. aristatus* Blume, *O. stamineus* Benth., *O. spicatus* Thumb, *Ocimum aristatum* Bl.; [Lamiaceae] is a popular traditional folk medicine extensively used in Southeast Asia and some parts of tropical Australia. It is used for treating the ailments of the kidney, It is also claimed to have anti-allergenic, anti-hypertensive and anti-inflammatory properties. Orthosiphon is sometimes used to treat gout, diabetes, hypertension and rheumatism. It is reportedly effective for anti-fungal and anti-bacterial purposes. People in some areas of Japan consumed orthosiphon tea to facilitate body detoxication.

In preliminary screening, *O. grandiflorus* water extract was an excellent free radical scavenger. However, it is still not clear which compounds are responsible for this scavenging ability. The on-line LC-ESI-MS/MS coupled to DPPH assay was applied for the systematic characterization and identification of antioxidants in *O. grandiflorus* water extract.

EXPERIMENT SECTION

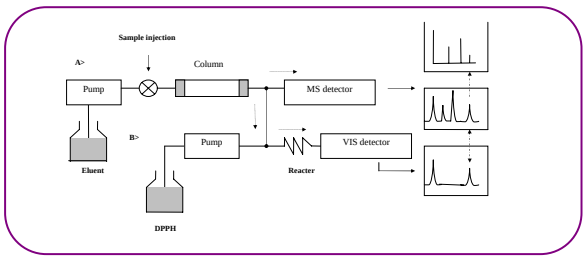

Figure1 Scheme of the on-line LC-ESI-MS/MS coupled to DPPH assay. The arrows indicate flow directions.

Condition

Column: Gemini column (5 μm, 250 x 4.6 mm i.d.)

Mobile phase: MeOH:0.1% formic acid; gradient elution in 55 min.

Injection volume: 5μl;

Flow rate: A; 600 μl/min (LC)

B; 200 μl/min (0.1mM DPPH)

Split ratio 8:2 (MS:DPPH line); Reactor volume:100 μl

Detector: 515nm (DPPH line)

MS full scan 100-1000 amu ESI negative

Sample preparation

H. cordata leaves (5 g) were extracted with boiling water (150 ml) for 5 min, and the filtrate was filter through 0.2 μm Nylon syringe and then directly injected to the system.

Peak identification

Peak identification was performed by comparison of the retention time and mass spectra with reference compounds and published data.

ACKNOWLEDGEMENT

The financial support from the Thailand Research Fund (DBG-4980011) is acknowledged.

RESULTS AND DISCUSSION

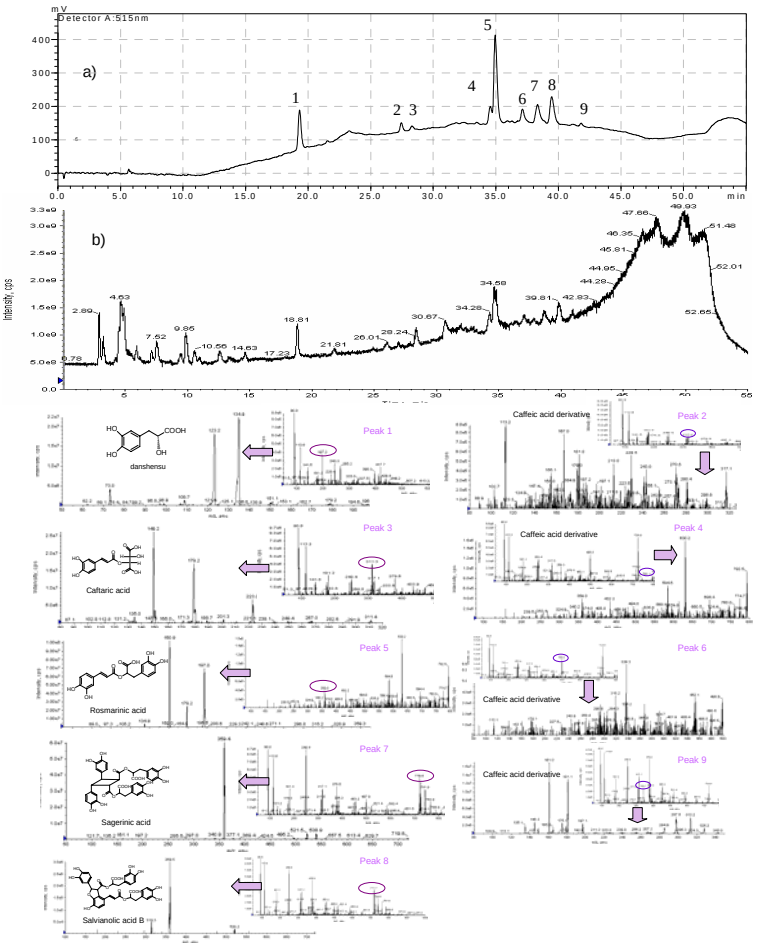

Figure 2. HPLC separation of *O.grandiflorus* with simultaneous antioxidant activity assay and MS detection a) the chromatogram from the antioxidant activity assay detection at 515 nm b) The total ion current (TIC) output from the ESI-MS in negative mode. MS and MS/MS spectra corresponding to peaks 1-9 of active compounds are shown.

In the negative ionization, the *m/z* data show that all of the compounds had ion at *m/z* 197, 179 and 359 indicated that these compounds are phenolic acids of caffeic acid derivatives. Based on their mass spectra related to antioxidant activity trace; nine compounds were identified as danshensu, caftaric acid, rosmarinic acid, sagerinic acid, salvianolic acid B and four caffeic acid derivatives. Rosmarinic acid has been identified in extracts of the following species; Dittany, Tuekish, Oregano, sweet Marjoram, Rosemary and Thyme.

Conclusion

HPLC with a DPPH assay linked to a triple quadrupole mass spectrometer was proved to be extremely helpful for peak assignment and further characterization of antioxidants. This is a clear example of how a hyphenated technique can accelerate the procedure for identification of active compounds in plants.

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On-line characterlization of phenolics antioxidants in water extract of *Gynostemma pentaphyllum* (Thunb.) Makino by LC-ESI-MS/MS coupled to DPPH assay

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[2]Bangkatum Hospital, Phitsanulok, Ministry of Public Health, 65000,Thailand
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INTRODUCTION

Gynostemma pentaphyllum **Makino (Cucurbitaceae) is known as Jiaogulan in china and as Panchakhan in Thailand and as Amachazuru in Japan. It has been clinically used for depressing cholesterol level, regulating blood pressure, strengthening the immune system, treating chronic bronchitis and gastritis and reducing inflammation. Phytochemical studies of this plant have identified about 130 dammarane-type glycosides (called gypenosides) closely related to the ginseng saponins. In a search for addition constituents of the antioxidant activity in this plant, we attempted to use a on-line LC-MS/MS coupling to DPPH assay for identification of the phenolic antioxidant compounds in *G. pentaphyllum***

EXPERIMENT SECTION

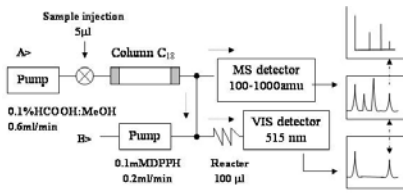


Figure1 Scheme of the HPLC on-line coupled to ESI-MS and DPPH assay.

Condition

Column: Gemini column (5 mm, 250 x 4.6 mm i.d.)
Mobile phase: MeOH:0.1% formic acid; gradient elution in 55 min.
Injection volume: 5ml;
Flow rate: A; 600 ml/min (LC)
B; 200 ml/min (0.1mM DPPH)
Split ratio 8:2 (MS:DPPH line); Reactor volume:100 ml
Detector: 515nm (DPPH line)
MS full scan 100-1000 amu ESI negative ionization

Sample preparation

G. pentaphyllum **leaves (5 g) were extracted with boiling water (150 ml) for 5 min, concentrate and the filtrate was direct injected to the on-line system.**

Peak identification

Peak identification was performed by comparison of the retention time and mass spectra with reference compounds and published data.

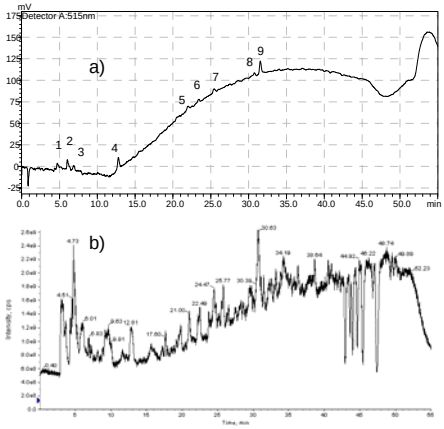
SUMMARY AND CONCLUSION

HPLC with a DPPH assay linked to a triple quadrupole mass spectrometer was proved to be extremely helpful for peak assignment and further characterization of antioxidants. Based on their mass spectra; the antioxidant compounds were identified as, Caffeoyl glucoside, Maleic acid, *p*-coumaric acid derivatives, *p*-coumaroylhexose, sinapic acid derivatives and Kaempferol rutinoside. This is a clear example of how a hyphenated technique can accelerate the procedure for identification of active compounds in plants.

ACKNOWLEDGEMENT

The financial support from the Thailand Research Fund (DBG-4980011) is acknowledged.

RESULTS



The DPPH based antioxidant activity profile (Fig. 2 a) shows that at least nine compounds have antioxidant activity. The negative ionization of major active compounds are listed in table 1 and identification of these compounds are proposed. Peak assignments were confirmed by fragmentation (MS/MS). The structures of these compounds are shown in Figure 3. The intensity of peaks depend on the types and amounts of the compounds in the sample.

Figure 2 HPLC separation of *G. pentaphyllum*, a water extract with simultaneous antioxidant activity assay and MS detection a) the chromatogram from the antioxidant activity assay detection at 515 nm. b) The total ion current (TIC) output from the ESI-MS in negative mode. For peak assignments, see table1.

Table1 Identification of antioxidant compounds in water extract of *G.pentaphyllum* by LC-ESI-MS/MS coupled to DPPH assay

Peak No.	t_R	MS	MS/MS	Identification
1	4.8	341.3	179.2,161.1,143.0	Caffeoyl glucoside
2	6.0	525.7	262.9	Unknown
3	6.8	439.4	304.4, 244.6	Unknown
4	11.7	231.4	115.2,116.3,147.3,144.2	Maleic acid
5	21.5	371.3	209.0,190.8,163.0,119.2	*p*-Coumaric acid derivatives
6	23.4	325.6	145.7,163.8,119.2	*p*-Coumaroylhexose
7	25.6	431.5	223.1,179.0,205.5	Sinapic acid derivatives
8	30.5	497.7	479.1,451.3	Unknown
9	31.3	593.3	447.2, 428.9, 284.3,285.1	Kaempferol rutinoside

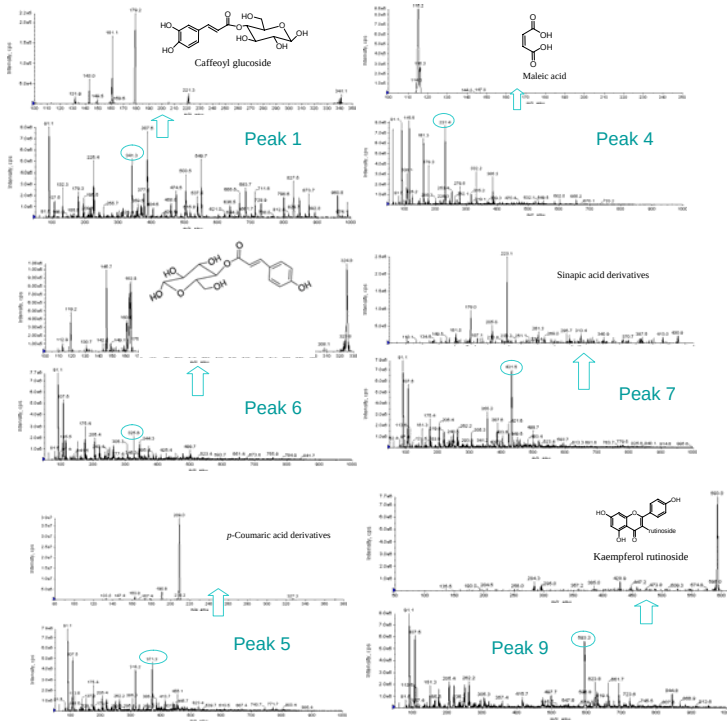


Figure 3 Structures of antioxidant compounds identified in the *G. pentaphyllum*

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