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Microwave-Assisted Extraction and Simultaneous Characterization of Medicinal Compounds from *Coscinium fenestratum*

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requirements for a doctoral degree of engineering

Microwave-Assisted Extraction and Simultaneous Characterization of Medicinal Compounds from *Coscinium fenestratum*

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ABSTRACT

The identification of the components of herbal medicines is of great importance in controlling their quality and gaining a better understanding of their pharmacological effects. In addition, it is necessary to investigate alternative extraction methods because some conventional extraction methods could cause an adverse impact on the environment. The methods for simultaneous characterization of medicinal compounds using liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS) and for effective extraction of protoberberine alkaloids using microwave-assisted extraction (MAE) from *Coscinium fenestratum* were successfully developed. A total of 32 compounds, including 2 benzylisoquinoline alkaloids, 3 aporphine alkaloids, 12 quaternary protoberberine alkaloids, 10 8-oxoprotoberberine alkaloids, 3 tetrahydroprotoberberine alkaloids, and a steroid compound were simultaneously separated and characterized by matching the empirical molecular formulae with those published in literature and the multi-stage mass spectrometry (MS^n) data obtained using structural information from IT, accurate mass measurement obtained from TOF MS, and HPLC separation. A total of 20 compounds, including 4 novel natural products were identified or tentatively identified for the first time from *C. fenestratum*. MAE of protoberberine alkaloids were studied in both an open (at atmospheric pressure) and closed vessel (under a pressurized condition). The extraction yields obtained by using MAE under optimum conditions for 15 min (60% EtOH, material/solvent ratio 1 g:40 mL, microwave output power 300 W) in an open vessel at a boiling point of 83°C and in a closed vessel at 160°C were higher than those obtained using Soxhlet extraction for 8 h and extraction with 0.2% sulfuric acid for 24 h. Extraction using water as a solvent at 160°C for 15 min yielded the same level of berberine amount as that obtained using 0.2% sulfuric acid for 24 h. This high extraction yield could be due to the alcohol-like characteristics of water at high temperature. Berberine degradation was observed at a temperature above 160°C. An additive such as EtOH or NaCl could reduce berberine degradation. MAE is more effective than conventional extraction methods with regard to the extraction time and extraction yield for extracting protoberberine alkaloids from *C. fenestratum*.

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ABBREVIATIONS

ASEAN	=	Association of Southeast Asian Nations
BER	=	Berberine
C. fenestratum	=	*Coscinium fenestratum*
C. janonica	=	*Coptis japonic*a
CAM	=	Complementary and alternative medicine
CDL	=	Curved Desolvation Line
CID	=	Collision induced dissociation
ESI	=	Electro spray ionization
FTA	=	Free trade agreement
GC	=	Gas chromatography
GNI	=	Gross national income
HM	=	Herbal medicine
HPLC	=	High performance liquid chromatography
IT	=	Ion trap
JAT	=	Jatrrorhizine
LC	=	Liquid chromatography
LC/IT TOF MS	=	Liquid chromatography hybrid ion trap time-of-flight mass spectrometry
MAE	=	Microwave-assisted extraction
MS	=	Mass spectrometry
MS^n	=	Multi-stage mass spectrometry
NMR	=	Nuclear magnetic resonance
ODS	=	Octa Decyl Silyl ($C_{18}H_{37}Si$)
P. amurense	=	*Phellodendron amurense*
PAL	=	Palmatine
PDA	=	Photo diode array
PSE	=	Pressurized solvent extraction
SFE	=	Supercritical fluid extraction
SIM	=	Selected ion monitoring
TIC	=	Total ion chromatogram
TLC	=	Thin-layer chromatography
TM	=	Traditional medicine
TOF	=	Time-of-Flight
UV	=	Ultra violet ray
WHO	=	World Health Organization

CHAPTER 1

INTRODUCTION

1.1. General background

Traditional medicine (TM) has played an important role in the health care and medication of people in many regions of the world from the ancient history. The World Health Organization (WHO) estimated that 65–80% of the world population used TM, and complementary and alternative medicine (CAM) as the primary form of health care[1-3].

The use of TM remains widespread in developing countries, while the used of CAM is increasing rapidly in developed countries[1, 4]. Approximately 80% of population in Africa use TM to help meet their health care. In Asia and Latin America, populations continue to use TM as a result of historical circumstances and cultural beliefs. In China, TM accounts for around 40% of all health care delivered. The broad use of TM in developing countries is attributable to its accessibility, affordability and cultural acceptability. In Europe, North America and other industrialized regions, CAM are becoming increasingly popular. For example, the percentage of the population which has used CAM at least once is 48% in Australia, 70% in Canada, 42% in USA, and 75% in France. The popular use of CAM in many developed countries is may be due to the concern about the adverse effects of allopathic medicine. For many patients with heart disease, cancer, diabetes and mental disorders, CAM appears to offer gentler means of managing such diseases than does allopathic medicine[5].

Herbal treatments are the most popular form of TM, and are highly lucrative in the international marketplace. The global market sale of herbal medicine reached US$ 19 billion in 2006 and estimated to surpass US$ 26 billion in 2011 with the growth rate of 10–15% per year[6, 7]. This trend offers a great opportunity for developing countries those

have potential in the development of their herbal medicines as an important industry. The development of herbal product including medicinal plants and spices for export can help to increase income among farmers, reduce poverty, stimulate entrepreneurship, and create a favorable business environment to integrate into the global market place[8].

One of the countries that has this potential is Lao P.D.R, a landlocked country in South East Asia. Due to the war for a long time till 1975 and the geographical barrier, the manufacturing industry has not developed in Laos. The Gross national income (GNI) per capita was only 500 USD in 2004, and 750USD in 2008 (ranking at 175th in 205 countries)[9]. The major industries are agriculture, hydropower, mining, and tourism. Approximately 80% of the population are engaged in subsistent agriculture in rural areas, which consisted of 42.8% of GDP component in 2006[10]. Considering this situation, agriculture based-product industry could be suitable for the development of Laos. In addition, Laos has a long history of using herbal medicine. There are various kinds of herb and medicinal plants around the country. About 8000–11000 flowering species were found in Laos. Some survey showed that there are many herbs and medicinal plants, some have not reported previously[11]. These medicinal plants have been used in food and traditional medicine.

However, these medicinal plants have been used base on personal experiences and scientific research data are often scarce. Since it has been generally accepted that the efficacy of herbal medicines can be attributed to the synergistic activity of various major and minor components of the herbs[12, 13], the identification of the components of herbal medicines is of great importance in controlling their quality and gaining a better understanding of their pharmacological effects. Consequently, the development of rapid and reliable methods for qualitative and quantitative determination of compounds in herbal medicine has become a significant and challenging issue. The development of methods to provide scientific data on medicinal plant are useful for the development of herbal products and boosting the industries in Laos as well as other developing countries, instead of only exportation of the medicinal plant or crude drug at low prices. Therefore, it is necessary to develop methods for simultaneous characterization of medicinal compounds from medicinal plants.

Another problem arose from the process for extracting of medicinal compounds. In some extraction process, a large amount of chemical such as strong acid and alkali has

been used, which could cause an adverse impact on the environment. In addition, low extraction yield, long extraction time, and acid residual in the product are also the problems to be concerned of. Accordingly, it is necessary to investigate for alternative extraction methods for sustainable development.

This research focuses on the development of methods for simultaneous characterization and effective extraction of the medicinal compounds in *Coscinium fenestratum*, an important medicinal plant in Lao P.D.R.

1.2 Literature Reviews

1.2.1 Lao Traditional Medicine and Medicinal Plants

In parallel with the use of modern medicine, Lao people have their own way of TM which is called "Ya Phurn Meuang Lao", that may date back to at least 2,500 years[14]. The first group of Lao people has an origin in the Southern part of China and has relied on agriculture since the ancient time. Their lives are in harmony with nature, especially with plants. It could be assumed that the Lao TM is one of the oldest medicines in the world. Unfortunately, the information were destroyed by war many times due to the invasion of foreign countries.

Today, Lao TM reflects a diversity of local tribal traditions, as well as knowledge of local environments[14]. Traditional remedies have a long-standing history in many Lao communities, and continue to provide useful tools for treating diseases. Many Lao people believed that TM are more efficient than the synthetic drugs sold in pharmacies[15]. Claudio O. Delang suggested three main reasons that Lao people rely on medicinal plants[16]. First, many people cannot afford to buy medicines in pharmacies. Second, pharmacies are often staffed by people with little training, while knowledge of medicinal plants is readily available from one’s neighbors, family members, traditional healers, and sellers. Third, people believe that Western medicines only cure the symptoms, not the causes of the illness, while TM addresses the causes of the illness. Thus, medicinal plants are not only popular in the countryside, where medical services are worse, but also in the capital city.

Laos has various medicinal plants and flower species that have been used in food and as TM. Silavanh indicated that there are more than 10,000 species of plants and animals found in Laos, of which 1,400 are only found in Laos[17]. However, many

medicinal plants have not been reported until recently. A. Libman *et al*[11]., conducted a field survey of commonly used medicinal plants in the district of Paksan, Bolikhamsai Province in Laos. They found that 55 species of plants, belonging to 49 genera in 31 families of vascular plants, are used in day-to-day medical therapy. Seven species have medicinal uses that overlap with uses reported in the literature. No medicinal uses have been previously reported for 31 of the species collected based on ethnobotanical field interviews, signifying that their uses may be unique to Laos. Nine of the 31 previously unreported species are mentioned as medicinal multiple times. This survey provided evidence that medicinal plants continue to represent an important asset to the health care in communities in Laos.

Lao government has a policy to promote the use of TM integrating with modern medicine. The national policy on TM was included in the National Drug Policy issued in 1998. In 1976, the Traditional Medicine Research Center (TMRC) was established by the ministry of health in order to study and incorporate the use of medicinal plants and traditional Lao medicines into the health care systems. TMRC is involved in ethnobotanical field study, plant collection and identification, and the cataloging of medicinal plants used in various parts of the country, as well as working with pharmaceutical factories in the development of drugs based on traditional Lao medicines.

Currently, there are more than 70 kinds of traditional medicine those were manufactured in pharmaceutical factories in Lao. Thirty herbal medicines were included on the national essential drug list that was issued in 2002[4]. They have been used for the treatment of malaria, pyrexia, diarrhea, diabetes and others. Some major medical plants are listed in **Table 1.1**.

In contrast, the medicinal plants and crude extract were heavily exported to surrounding countries such as China, Vietnam and Thailand recently[8]. Several foreign and local companies have been established to purchase medicinal plants from villagers and export in form of plants and crude extract. At present, China is one of the biggest trading partners of ASEAN. It is expected that the demand for medicinal plants and spices will further increase due to the China-ASEAN FTA that will take effect in 2015. With the increased demand for medicinal plants, the pressure on the environment as a result of over-harvesting is increasing. Laos is lack of information of medicinal plants

and it is difficult to realize the real value of the medicinal plants. For sustainable development, it is necessary to find the way how to utilize the medicinal plants effectively.

Table 1.1 Some important medicinal plants in Laos[18].

Disease	**Medicinal plant**
Diarrhea	*Coscinium fenestratum*
	Psidium guyava
	Caesalpiniaceae,
Diabetes	*Morordia charantia*
	Coscinium fenestratum
	Scoparia dulcis
Malaria	*Artemisia annua*
	Dichroa febrifuga
	Alcasia macrorrhiza
Pyrexia, Measles	*Scoparia dulcis*
	Strobilanthes cusia
	Cissanpelos parreira
Respiratory disease	*Belamcanda sinessis*
	Sesbania grandiflora
	Morus acidosa
Gastric ulcer	*Eleutherine subaphylla*
	Curcuma longa
	Oroxylum indicum
	Coscinium fenestratum
Dental carries	*Spilanthes acmella*
Rheumatism	*Suregada multiflorum*
Skin disease	*Mitragyna speciosa*
	Alocasia macrorrhiza
	Coscinium fenestratum
	Spilanthes acmella

1.2.2 *Coscinium fenestratum*

C. fenestratum is a medicinal plant belonging to family Menispermaceae, a woody climbing shrub with cylindrical stem, externally yellowish brown and internally yellow in color[19]. The stem yields a yellow dye, which is used either alone or in combination with turmeric and other coloring materials. **Figure 1.1** shows the photo of *C. fenestratum.*

Figure 1.1 Photos of *C. fenestratum*

C. fenestratum has been used as herbal medicine in Lao P.D.R, Vietnam, Thailand, India, and Sri Lanka for a long time. This plant has been used in the treatment of

diarrhea, inflammation, ulcers, skin disease, and diabetes mellitus[20]. Several biological effects have been attributed to the plant extracts, including antibacterial[21], antioxidant[22] , anti-diabetic[23], and hypotensive[24] activities. A total of 15 chemical compounds, primarily quaternary and tertiary protoberberine alkaloids, have been isolated previously[25-27].

C. fenestratum is also an important source of export-oriented berberine (BER) production in Laos[8, 28]. Numerous factories in many provinces (such as Bolikhamxay, Luang Prabang, Huaphanh, Khammuane, Savannakhet and Xekong) are extracting BER from the liana of *Coscinium sp.*. These factories are run by local companies, as well as foreign companies from China and Vietnam. These factories buy materials from local villagers at a price of around 500–1,000 Kip (US$0.05–0.1) per kilogram for raw *Coscinium sp*. (2007) and export a large quantity to China[8]. **Figure1.2** shows the BER extraction process in a factory in Laos. The plant was ground and macerated with sulfuric acid for 1 to 3 days, then sodium hydroxide was added to precipitate the impurities. After that hydrochloric acid was added for salt precipitation. A large amount of acid has been used for extraction of BER while the treatment of waste are remain as the concern issue. This process could cause adverse impact on the environment, gave low extraction yield and took a long time for extraction. In addition, acid residual in the product is one of the concerned issues. Moreover, over harvesting and unsustainable extraction of *C. fenestratum* is a major threat to wild biodiversity. In India and Sri Lanka, *C. fenestratum* has been listed as an endangered species[19].

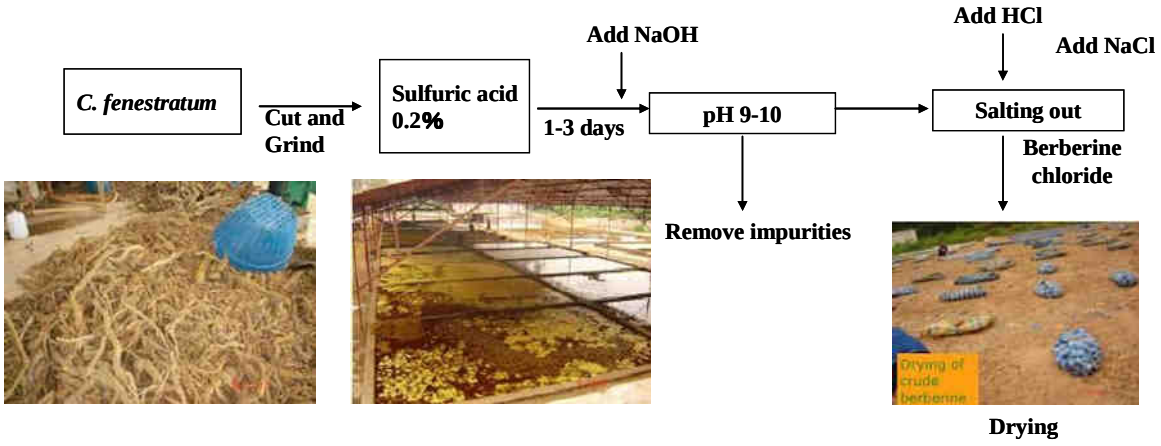


Figure 1.2 BER extraction process in factory in Laos[8]

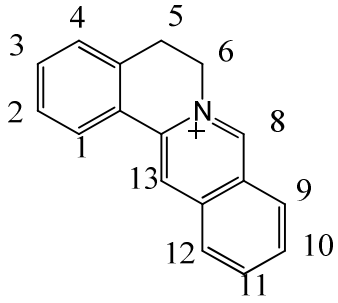
1.2.3 Protoberberine Alkaloids and Extraction of Alkaloids

Alkaloids form an important group of chemicals in medicine because of their high biological and pharmacological activities. Protoberberine alkaloids, which are isoquinoline alkaloids, have various activities such as antimicrobial[29], anti-inflammatory[30], antimalarial[31], and anti-diabetic[32] activities. Because of the potential medicinal applications of this group, some researchers have synthesized protoberberine alkaloids from their natural precursors[33]. However, they are very complex for cost-effective chemical synthesis, and therefore, medicinal plants remain the main source of protoberberine alkaloids. **Figure 1.3** shows some important protoberberine alkaloids.

Conventional alkaloid extraction methods are as follows: maceration; percolation with an organic solvent such as methanol, ethanol, or a strong acid; and Soxhlet extraction [33-35]. These extraction processes are time consuming and laborious; further, they require a large amount of solvent and have low extraction efficiency. Moreover, there is great concern about the adverse environmental impact of using a strong acid as a solvent. Alternative extraction methods for a rapid, simple, and selective extraction with lower solvent consumption have been developed recently; these include new techniques such as supercritical fluid extraction (SFE)[36], microwave-assisted extraction (MAE), and pressurized solvent extraction (PSE)[37] for extracting alkaloids. SFE is suitable for extracting non-polar and thermolabile compounds, but it requires complex optimization for extracting polar compounds. MAE and PSE can be applied to all types of solutes and solid matrices and they require fewer parameters to be optimized than SFE. In addition, MAE offers the advantage of performing multiple extractions simultaneously under moderate pressures [38].

1.2.4 Liquid Chromatography Mass Spectrometry (LC/MS)

NMR technique is a superior and widely used for the determination of chemical conformation and identification of compounds. However, it is necessary to have an appropriate amount (more than 1 mg) of pure material for measurement. On the other hand, it is often difficult and time consuming to purify a compound from a complex matrix because the concentration of the target compounds is usually extremely low in natural product. In contrast, mass spectrometry has a high sensitivity to detect the



Alkaloid	2	3	9	10	11
Berberine (BER)	O CH_2 O		OCH_3	OCH_3	
Palmatine (PAL)	OCH_3	OCH_3	OCH_3	OCH_3	
Jatrorrhizine (JAT)	OCH_3	OH	OCH_3	OCH_3	
Coptisine	O CH_2 O		O CH_2 O		
Columbamine	OH	OCH_3	OCH_3	OCH_3	
Thalifendine	O CH_2 O		OCH_3	OH	
Stepharanine	OH	OCH_3	OCH_3	OH	
Groendlandicine	OCH_3	OH	O CH_2 O		
Dehydrocorydalmine	OCH_3	OCH_3	OCH_3	OH	
Dehydrodiscretamine	OCH_3	OH	OCH_3	OH	
Dehydrocheilanthifoline	OH	OCH_3	O CH_2 O		
Demethylenberberine	OH	OH	OCH_3	OCH_3	
Epiberberine	OCH_3	OCH_3	O CH_2 O		
Pseudopalmatine	OCH_3	OCH_3		OCH_3	OCH_3
Pseudojatrorrhizine	OCH_3	OH		OCH_3	OCH_3
Pseudocoptisine	O CH_2 O			O CH_2 O	
Pseudoepiberberine	OCH_3	OCH_3		O CH_2 O	
Pseudocolumbamine	OH	OCH_3		OCH_3	OCH_3
Dehydrodiscretine	OCH_3	OH		OCH_3	OCH_3
Dehydrocoreximine	OH	OCH_3		OCH_3	OH
Thalifaurine	OCH_3	OH		O CH_2 O	

Figure 1.3 Chemical structure of some important protoberberine alkaloids[33]

compounds at low concentration. The combination use of mass spectrometry and gas or liquid chromatography is a powerful technique for detection of compounds in complex matrix. Especially, the combination of high-performance liquid chromatography and multi-stage mass spectrometry (HPLC-MS^n) is being used increasingly in pharmaceutical research and for the quality control in herbal medicine.

1.2.4.1 Liquid Chromatography

Liquid chromatography is a fundamental separation science in the field of life science and related field of chemistry. Unlike gas chromatography, which is unsuitable for nonvolatile and thermally fragile molecules, liquid chromatography can separate a very wide range of organic compounds, from small-molecule metabolites to peptides and proteins[39]. There are several types of retention mechanism exploited in analytical chromatography such as adsorption, phase partitioning, ion exchange, size exclusion (gel permeation), and biochemical affinity[40].

Recently, reversed-phase HPLC is the most widely used technique to separate and analyze compound in complex mixture. The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand such as ODS (Octa Decyl Silyl ($C_{18}H_{37}Si$)) in the stationary phase (**Figure 1.4**). Thus, the target hydrophobic compounds will have strong interaction with solid phase and retained longer than that of hydrophilic compounds. It may be categorized base on the adsorption and phase partitioning mechanism. The most common columns are packed with silica particles. The beads or particles are generally characterized by particle and pore size. Particle sizes generally range between 3 and 50 µm. Larger particles will generate less system pressure and smaller particles will generate more pressure. The smaller particles generally give higher separation efficiencies. Silica is the most common particle material. However, the chemical instability of silica in aqueous solutions at high pH has limited its application. Several techniques have developed to improve the stability of solid phase and the efficiency in separation using high purity of silica and end capping technique[41].

A medicinal plant typically contains a complex mixture of hundreds or more chemical compounds, e.g., alkaloids, glycosides, and flavonoids[42]. For analysis of such a complex mixture, analytical technique with strong separation capability is needed. Silica gel with chemically attached octadecylsilanol groups (C-18) is the most frequently used adsorbent in reversed-phase HPLC in herbal medicine analysis. Adsorbents with other alkyl groups, such as C-4, C-8 and C-30, can also be found in herbal products analysis. The longer the alkyl chain attached to the adsorbent, the higher the retention of the components. In herbal analysis, compounds belong to the same

structure class, including the isomers, often need to be separated in a single run. Therefore, to achieve sufficient resolution of analytes, long analysis times are always needed in the case of analysis of multiple structure similar compounds using conventional HPLC columns. Shortening the analysis time can be achieved by decreasing in the column size and the size of the adsorbent particles, or increasing in the eluant flow rate and temperature. However, the use of smaller columns, faster flow rates or higher temperature may sacrifice the resolution for time. Recently, interest in use of fast HPLC with 2 μm particle size has increased because of the dramatic increases in speed of analysis without losing the resolution and sensitivity.

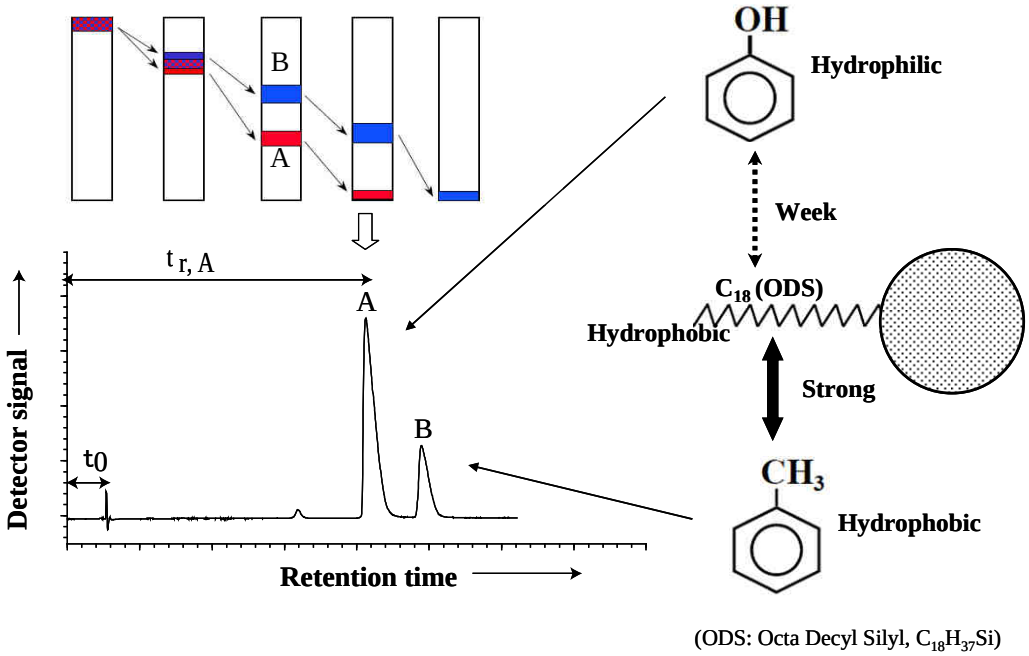


Figure 1.4 Retention mechanism in reversed-phase HPLC

1.2.4.2 Mass Spectrometry

The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z (mass to charge ratio) value. The mass analyzer uses electrical or magnetic fields, or combination of both, to move the ions to a detector that produces a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, it is the mass to charge ratio, and not only the mass, which is of importance, the analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield[43].

There are several types of mass analyzers such as quadrupole (Q), ion trap (IT), time-of-flight (TOF), sectors (B), Fourier transform ion cyclotron resonance (FTICR) and the combinations of them such as triple quadrupoles (QqQ), and Q(IT)-TOF. This research focuses on using mass spectrometers with quadrupole, and IT-TOF MS.

1.2.4.2.(a) Quadrupole Mass Spectrometer [44]

The quadrupole is the most widely used analyzer due to its ease of use, mass range covered, good linearity for quantitative work, resolution and quality of mass spectra, and relatively accessible price.

The quadrupole is composed of two pairs of metallic rods (**Figure 1.5**). One set of rod is at a positive electrical potential, and the other one at a negative potential. A positive ion entering the space between the rods will be drawn towards a negative rod. If the potential changes sign before it discharges itself on this rod, the ion will change direction. The principle of the quadrupole was first described by Paul and Steinwegen.

Ion traveling along z axis are subjected to the influence of a total electric field made up of a quadrupolar field superposed on the constant field resulting from the application of potential upon the rods:

$$\phi_0 = +(U - V\cos\omega t) \qquad (1)$$

$$-\phi_0 = -(U - V\cos\omega t) \qquad (2)$$

ϕ_0: Potential applied to the rods

$\omega = 2\pi\nu$: angular frequency , where ν is the frequency of the RF field

U : Direct potential

V : Zero-to-peak amplitude of RF voltage

The ions accelerated along the *z* axis enter the space between the quadrupole rods and maintain their velocity along this axis. However, they are submitted to acceleration along *x* and *y* that result the forces induce by electric filed.

From the equation of motion,

$$F_x = m\frac{d^2x}{dt^2} = -ze\frac{\partial\phi}{\partial x} \qquad (3)$$

$$F_y = m\frac{d^2y}{dt^2} = -ze\frac{\partial\phi}{\partial y} \qquad (4)$$

ϕ is function of ϕ_0

$$\phi_{(x+y)} = \phi_0 (x^2 - y^2)/r_0^2 = (x^2 - y^2)(U - V\cos\omega t)/r_0^2 \qquad (5)$$

Differentiating and rearranging the terms leads to the following equation of the movement (Paul equation):

$$\frac{d^2x}{dt^2} + \frac{2ze}{mr_0^2}(U - V\cos\omega t)x = 0 \qquad (6)$$

$$\frac{d^2y}{dt^2} + \frac{2ze}{mr_0^2}(U - V\cos\omega t)y = 0 \qquad (7)$$

The trajectory of an ion will be stable if the value of *x* and *y* never reach r_0, thus if it never hit the rods. To obtain the value of either *x* or *y* during the time, these equations need to be integrated. The following Mathieu equation is applied.

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u\cos 2\xi)u = 0 \qquad (8)$$

u stand for either *x* or *y*.

ξ is defined as $\xi = \frac{\omega t}{2}$ and thus $\xi^2 = \frac{\omega^2 t^2}{4}$

In the first term of the Paul equation (6) and (7), replacing t^2 by ξ^2 introduces a factor $\frac{\omega^2}{4}$. To compensate this factor, the whole equation must be multiplied by reverse, $\frac{4}{\omega^2}$. The following expressions were obtained.

$$a_u = a_x = -a_y = \frac{8zeU}{m\omega^2 r_0^2} \qquad (9)$$

$$q_u = q_x = -q_y = \frac{4zeV}{m\omega^2 r_0^2} \qquad (10)$$

For a given quadrupole, r_0 and $\omega = 2\pi\nu$ is maintained constant. *U* and *V* are the variables. For an ion of any mass , *x* and *y* can be determined during a time span as a function of *U* and *V*. Stability areas can be represented in an a_u, q_u diagram. In these areas the values of *U* and *V* are such that *x* and *y* do not reach values above or equal to r_0. **Figure 1.6** represents these stability areas. The upper part of this figure represents the stability areas along the x and y axis respectively. The overlay of these two diagrams

is represented in the lower part of the figure. The regions where the ion will have stable trajectories according to both the *x* and *y* axes are simultaneously identified.

From equation (9) and (10), the following equations are obtained.

$$U = a_u \frac{m}{z} \frac{\omega^2 r_0^2}{8e} \qquad (11)$$

$$V = q_u \frac{m}{z} \frac{\omega^2 r_0^2}{4e} \qquad (12)$$

By switching from one *m/z* to another results in a proportional multiplication of a_u and q_u, which means changing the scale of drawing in *U*, *V* coordinates; thus the triangular area A will change from one mass to another, like proportional triangles. **Figure 1.7** represents in a *U*, *V* diagram the areas A obtained with different masses.

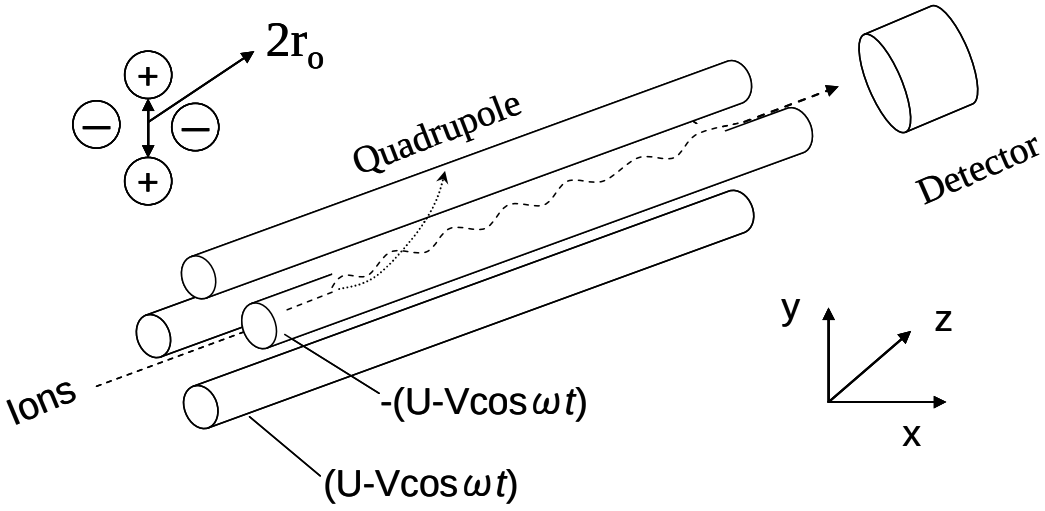


Figure 1.5 Structure of quadrupole MS instrument[44]

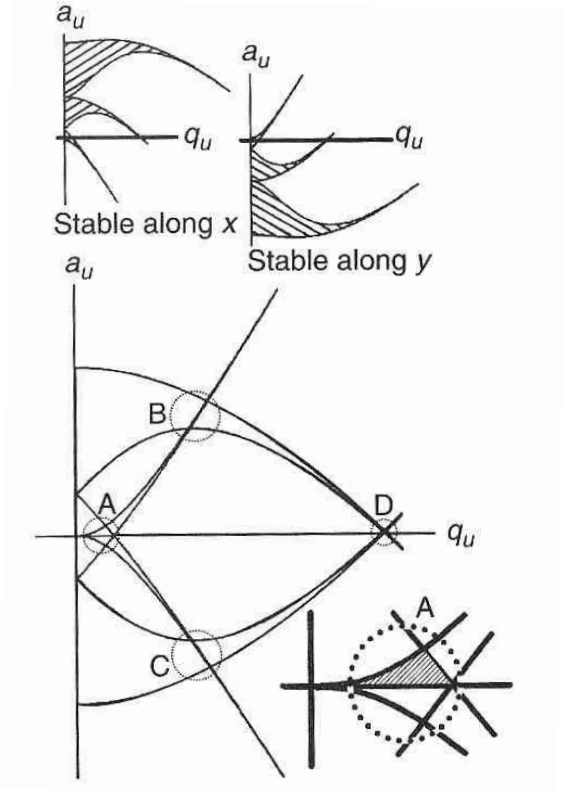


Figure 1.6 Stability areas for an ion along *x* or *y* (above) and along *x* and *y* (below)[44]. The area A is used commonly in mass spectrometers

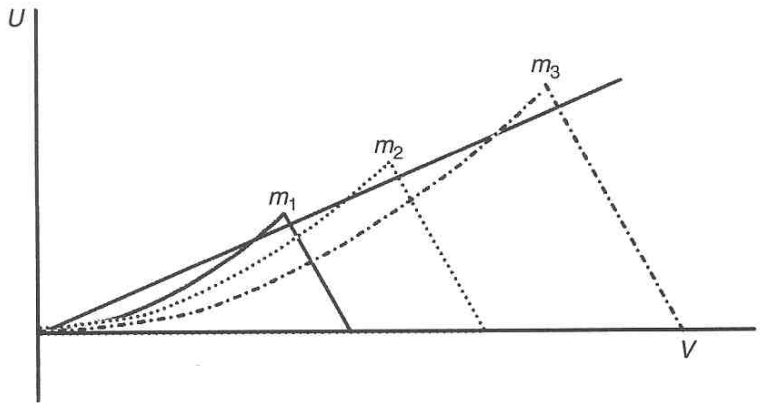


Figure 1.7 Stability areas as a function of *U* and *V* for ion with different mass[44].

Multistage mass spectrometer can be achieved by the combination of triple quadrupole system. The first quadrupole is used to select a first ion (precursor), which is fragmented in the collision cell. This is typically achieved in the collision cell by accelerating the ions in the presence of a collision gas (helium or argon). The energy of the collision with the gas can be varied to allow different degrees of fragmentaion. The resulting fragments are analysed by the second quadrupole, used either in selected ion

monitoring (SIM) mode or in scan mode. Study of mass spectral fragments can provide structural information. When using a single quadrupole instrument, it is possible to obtain fragmentation by using a technique called in source CID. The fragmentation takes place before the introduction of the ions into the optics of the mass spectrometer. This technique is useful if there is no chromatographic interference. With a triple quad system, the first quadrupole acts as a separation device, reducing the need for a perfect chromatographic separation.

1.2.4.2.(b) Ion Trap Mass Spectrometer (IT MS)[44]

The principle of the trap is to store the ions in a device consisting of a ring electrode and two end cap electrodes (**Figure 1.8**). The ions are stabilized in the trap by applying a RF voltage on the ring electrode. For maximum efficiency, the ions must be focused near the centre where the trapping fields are closest to the ideal and the least distorted maximizing resolution and sensitivity. In using the Mathieu equation to locate areas where ions of given masses have a stable trajectory, the equations are very similar to the ones used for quadrupole. However, in the quadrupole, ion motion resulting from the potential applied to the rods occurs in two dimensions, *x* and *y*, the *z* motion resulting from the kinetic energy of the ions when they enter the quadrupole field. In the ion trap, the motion of the ions under the influence of the applied potentials occurs in three dimensions, x, y and z. However, due to the cylindrical symmetry $(x^2 + y^2) = r^2$, it can also be expressed using *z*, *r* coordinates.

By ramping the RF voltage, or by applying supplementary voltages on the end cap electrodes, or by combination of both, it is possible to:

• destabilize the ions, and eject them progressively from the trap

• keep only one ion of a given *m/z* value in the trap, and then eject it to observe it specifically

• or keep only one ion in the trap, fragment it by inducing vibrations, and observe the fragments.

• repeat the last operation a few times to progressively fragment the ions. That is MS/MS[43]

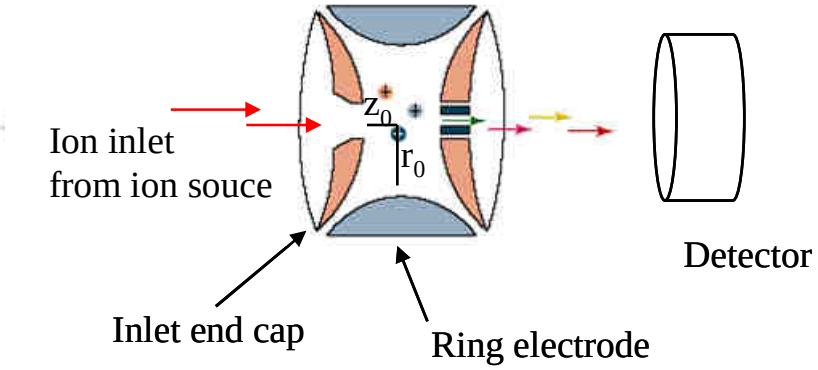


Figure 1.8 Schematic view of ion trap mass spectrometer

1.2.4.2.(c) Time-of-Flight Mass Spectrometer (TOF MS)[44]

In a TOF MS, Mass-to-charge ratio are determined by measuring the time that ion take to move through a field-free region between the source and the detector. Before it leaves the source, an ion with mass m and total charge $q = ze$ is accelerated by a potential V_s, its electric potential energy E_{el} is converted into kinetic energy E_k.

$$E_k = \frac{m\upsilon^2}{2} = qV_s = zeV_s = E_{el} \qquad (13)$$

The velocity of the ion leaving the source is given by rearranging the above equation as

$$\upsilon = \left(\frac{2zeV_s}{m}\right)^{1/2} \qquad (14)$$

After initial acceleration, the ion travels in a straight line at constant velocity to the detector. The time t need to cover the distance L before reaching the detector is

$$t = \frac{L}{\upsilon} \qquad (15)$$

Apply equation (14) to (15) and modify,

$$t^2 = \frac{m}{z}\left(\frac{L^2}{2eV_s}\right) \qquad (16)$$

Since the terms in parentheses being constant, m/z can be calculated from a measurement of t^2.

TOF MS analyzer has a wide mass range and can be very accurate in their mass measurements.

In spite of the enormous analytical potential of the combination of LC-(Q/IT) TOF-MS, not much has been reported on the analysis of herbal medicine[42]. This is may

be mainly due to the still high costs of the technique and to the initial poor quantitative capabilities of TOF instruments, which fall behind other more commonly used MS such as quadrupole or triple quardrupoles mass analyzers. However, the use of LC-(Q/IT)TOF-MS on herbal medicine analysis has increased quickly in last two years, which will, in the near future, revolutionized the field of herbal medicine analysis.

1.3 Objectives and Scope of This Study

The objective of this study is to develop a method for simultaneous characterization and effective extraction of medicinal compounds from *C. fenestratum*. The simultaneous characterization of compounds is based on the technique of liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS). The effective extraction of protoberberine alkaloids from *C. fenestratum* was studied using microwave-assisted extraction technique.

This study consisted of seven chapters in which the relationship among each chapter is shown in **Figure 1.9**. The summary of each chapter is given as follows:

Chapter 1

The background and literature reviews included medicinal plants in Laos, *C. fenestratum*, extraction and characterization methods, and the objectives of this study are introduced.

Chapter 2

The condition of extraction and separation of medicinal compounds in *C. fenestratum* using LC/MS were studied

Chapter 3

Simultaneous characterization of medicinal compounds in *C. fenestratum* by liquid chromatography hybrid ion trap time-of-flight mass spectrometry was studied. Compunds were categorized to 7 groups and their characterizations were discussed.

Chapter 4

Quantification of major alkaloids, variation of compounds in *C. fenestratum* from different areas of Laos, effect of plant size, and comparison with other protoberberine containing plants were studied.

Chapter 5

Microwave-assisted extraction of protoberberine alkaloids, BER, Palmatine (PAL), and jatrorrhizine (JAT) were studied using both in open (at atmospheric pressure) and closed vessel (under pressurized condition).

Chapter 6

The application of developed methods for extraction and characterization of medicinal compounds to other medicinal plants were studied.

Chapter 7

The general conclusions of this study are presented.

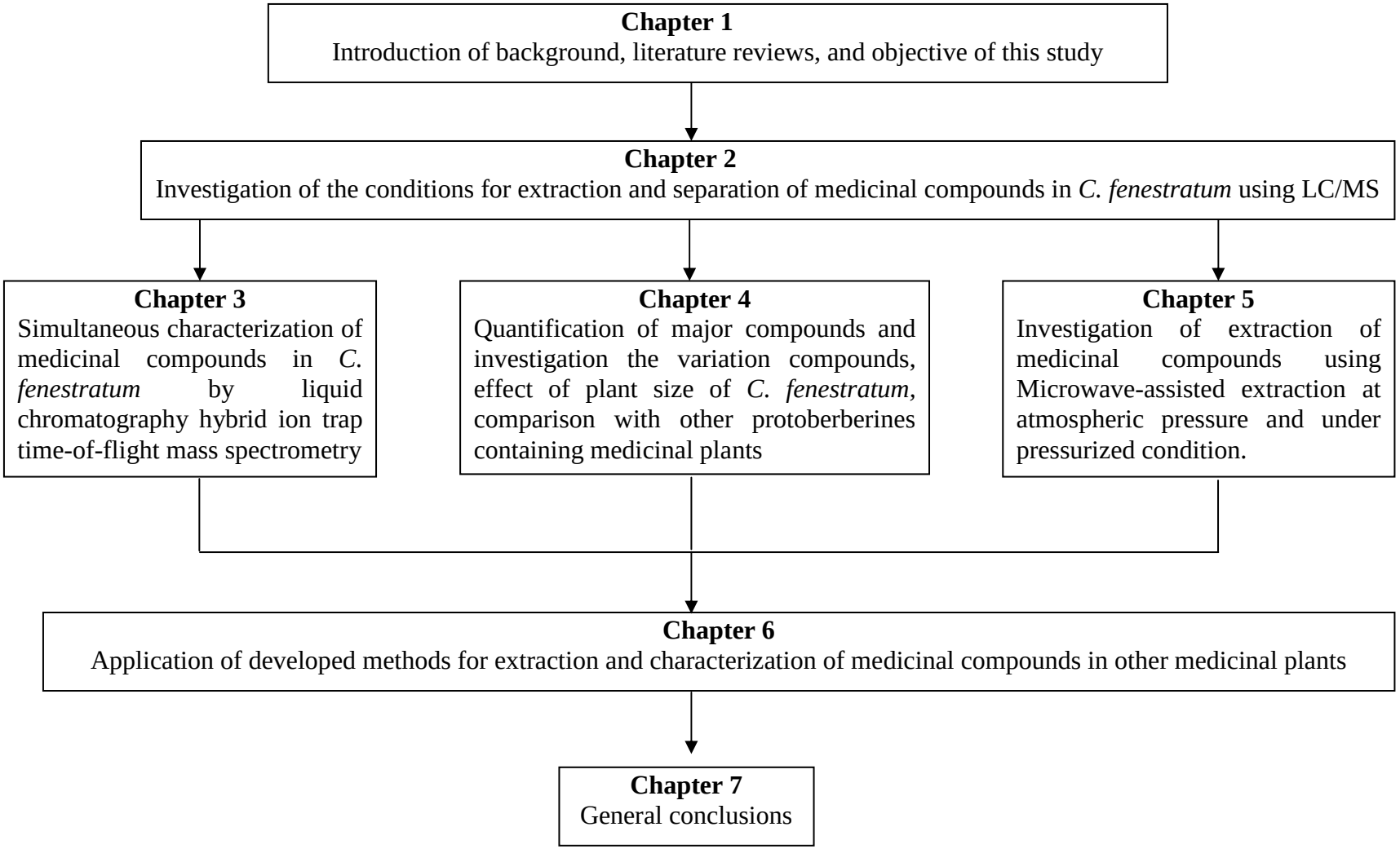


Figure 1.9 Schematic diagram of the structure of this thesis

CHAPTER 2

EXTRACTION AND SEPARATION OF COMPOUNDS IN *C. FENESTRATUM* BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

2.1 Introduction

To date, several methods have been developed to measure the principal component of *C. fenestratum*, BER; these include methods based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)[45, 46]. At least 15 chemical compounds, primarily quaternary and tertiary protoberberine alkaloids, have been isolated previously[25-27]. In herbal medicine, it has been generally accepted that the efficacy of herbal medicines can be attributed to the synergistic activity of various major and minor compounds of the herbs[12, 13]. Therefore, it is essential to develop a method for simultaneous analysis of the compounds in *C. fenestratum* in order to understand better the pharmacological effect and ensure the quality control of the herb. Moreover, there are no studies on the online analysis of oxoprotoberberine alkaloids by LC/MS, which show various pharmacological effects[47-49] and occur in several important medicinal plants such as *Coptis japonica* and *Phellodendron amurense*[48, 49] which widely used in Japanese and Chinese herbal medicine. In order to simultaneously analyze the compounds, it is necessary to investigate the condition for extraction and separation of the compounds.

In this chapter, the optimum condition for separation of compounds in *C. fenestratum* and selection of solvent for extraction were investigated. The effects of solvent such as MeOH, EtOH, water, and sulfuric acid on the extraction of *C. fenestratum* were studied.

2.2 Experimental

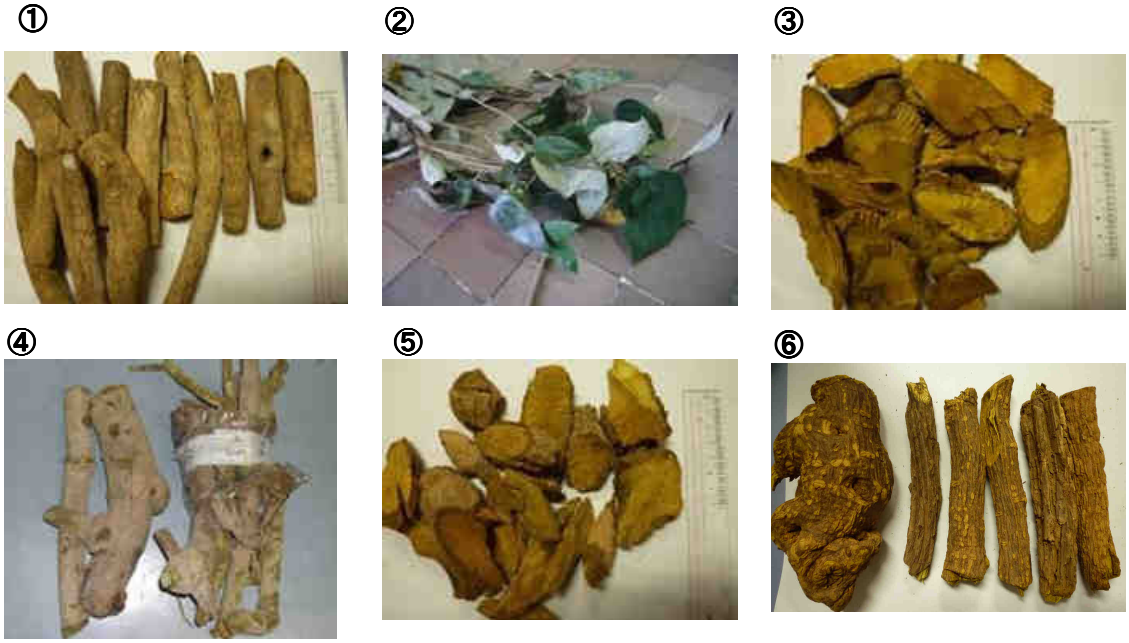
2.2.1 Materials

BER, PAL, JAT, methanol (LC/MS-grade), and formic acid (HPLC-grade) were purchased from Wako Pure Chemical Industries, Ltd., Japan. BER, PAL, JAT, and 20-hydroxyecdyson stock solution were prepared individually with methanol at the concentration 150 μg/mL, 42 μg/mL, 100 μg/mL, and 11.4 μg/mL, respectively.

Six batches of samples were obtained from different places in Vientiane province, Vientiane capital, Saysomboun and Louangphabang province as shown in this **Table 2.1**. Batch 1 and batch 4 were identified by Dr. Keooudone Rasphone (Pharmaceutical factory No 2, Laos). Batch 1 was used in this study.

Table 2.1 Six batches of sample obtained from different area

Sample	Obtained date	Place
Batch 1	Aug-06	Vientiane province
Batch 2	Dec-07	Nasaythong district, Vientiane capital
Batch 3	Dec-07	Morning market, Vientiane capital
Batch 4	May-08	Thulakhom Disctrict, Vientiane province
Batch 5	Jun-06	Market in Louangphabang province
Batch 6	Dec-07	Saysomboun province



2.2.2 Preparation and Extraction

The plants were ground by a blender (Wonder blender WB-1; Osaka Chemical Co. Ltd., Japan) and sieved to obtain powder of particle size under 250 µm. Extraction were conducted using Soxhlet extraction, extraction in water bath, and extraction using 0.1 % sulfuric acid aqueous solution. Soxhlet extraction (**Figure 2.1**) was performed by weighted 0.4 g of plant powder to extraction thimble and extracted with 80 mL of MeOH and EtOH in oil bath for 11 hours. In the extraction in water bath, the plant material 0.2 g were extracted with 40 mL of MeOH, EtOH, and water at 60˚C. Extraction using 0.1 % sulfuric acid aqueous solution was conducted at room temperature for 3 days. The extracted liquid was filtered with 0.45 µm membrane filter. The filtrate was transferred into 1.5 ml vial for LC/MS analysis and analyzed in triplicate.

Figure 2.1 Soxhlet apparatus

2.2.3 Analysis

HPLC/PDA-ESI/MS analysis was done by Shimadzu LC/MS 2010EV system (**Figure 2.2**). The LC system comprises a series of Shimadzu Prominence binary pump (LC-20AB), on-line vacuum degasser (DGU-20A3), autosampler (SIL-20A), column

oven (CTO-20A), photo diode-array detector (SPD-M20A). The separation was performed on ZORBAX Eclipse XDB-C18 Column (150 mm x 2.1 mm, 3.5 μm) (Agilent Technologies, Inc., USA). The mobile phase consisted of 0.1% formic acid aqueous solution and methanol. The optimum condition for analysis was investigated by isocratic elution and gradient elution containing methanol from 20% to 70%. The elution was performed at 0.2 mL/min with the sample injection 3 μL for qualification and 1μL for quantification. The column temperature was kept at 40˚C. UV spectra were scan from 200 to 470 nm. Peaks were simultaneously determined at 254 nm and 345 nm.

The mass spectrometer was fitted with electrospray ionization (ESI) interface. ESI was operated using scan mode and performed on both positive and negative ion in a single run with 1.5 s for each event time. The instrumental parameters were interface voltage 5 kV (positive mode), -4.5 kV (negative mode); detector voltage 1.65 kV (positive mode), 1.5 kV (negative mode); heat block temperature 200˚C, Curved Desolvation Line (CDL) temperature 250˚C, nitrogen nebulizing gas 1.5 L/min and m/z scan range 100–700. The chromatographic and mass spectrometric analyses were operated by LCMS solution Ver3.14 Software. The structure of MS spectrometer was shown in **Figure 2.3**.

Figure 2.2 Photography of LC/MS system

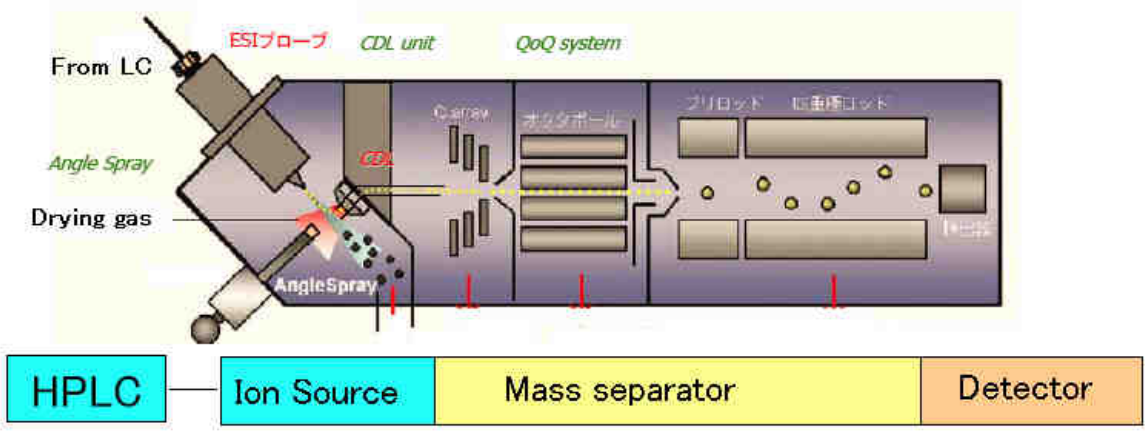


Figure 2.3 Structure of MS spectrometer [50]

2.3 Results and Discussion

2.3.1 Optimum Conditions for Separation of Compounds in *C. fenestratum*.

The separation of compounds in *C. fenestratum* was investigated on ZORBAX Eclipse XDB C-18 column. The column was chosen in this method development because of its capability to be used in wide range of pH with small tailing.

The optimum conditions were investigated using various conditions of mobile phase. **Figure 2.4** shows 4 representative results of investigating of optimum condition. The stem extract of *C. fenestratum* in methanol was injected directly to LC/PDA-ESI/MS without any further pre-treatment.

For isocratic mobile phase consisting of 70% MeOH, only one main peak was obtained. This indicated that most of the compounds were not retained in the column using this mobile phase condition. Reducing MeOH concentration to 35%, only 4 peaks were found. BER and PAL were not separated and the combined peak was observed at retention time 7.5 min. Using gradient elution contained 25–35% (0–20 min), 35% (20–40 min) and 25% (40–50 min) of methanol, JAT, BER and PAL were well separated and 12 peaks were detected. However, some minor compounds which were expected to be contained in *C. fenestratum* were not detected. It may be due to the low polarity of compounds that make them difficult to be eluted. Optimizing the gradient elution to 20–32% (0–20 min), 32–70% (20–35 min), 70% (35–40 min) and 25%

(40–50 min) of methanol, more than 20 peaks included minor compounds were observed. Therefore, this condition was considered to be the optimum condition for online analysis of *C. fenestratum*. The total ion chromatogram (TIC) from mass spectrometer using the optimum condition was shown in **Figure 2.5**. The retention time, maximum UV absorption wavelength and m/z of compounds were summarized in **Table 2.2**. Peak 4, 5, 7 and 8 exhibited four maximum absorptions at 220–240 nm, 260–280 nm, 340–350 nm, and 410–430 nm. This suggested that they were quaternary protoberberine alkaloids. The retention times, UV and mass spectra of peak 5, 7 and 8 are identical with those of JAT, BER, and PAL standard compounds. Consequently, they were identified as JAT, BER and PAL. $[M+H]^+$ and $[M+Na]^+$ were observed for compounds 10–20. The observation of $[M+Na]^+$ suggested that those compounds have many electron pairs. This is because binding a sodium ion with molecule requires the availability of several electron pair in the molecule. Considering this fact and the longer retention time, these compounds were suggested to be molecule with low polarity such as oxoprotoberberine alkaloids.

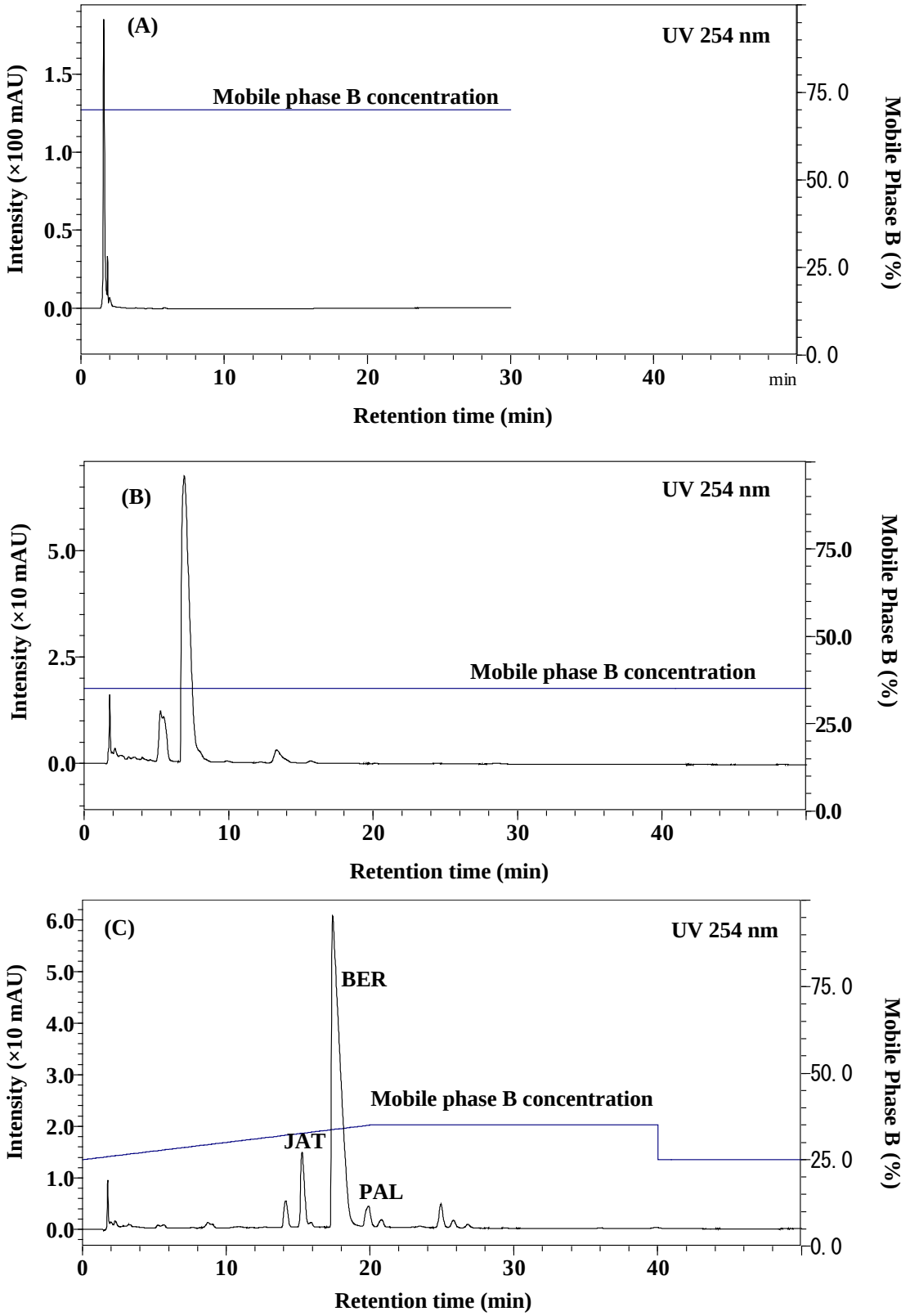


Figure 2.4 UV chromatogram of stem extract of *C. fenestratum* using different mobile phase concentration. (A) 70% MeOH; (B) 35% MeOH; (C) 25–35% MeOH; (D) 20–32–70% MeOH.

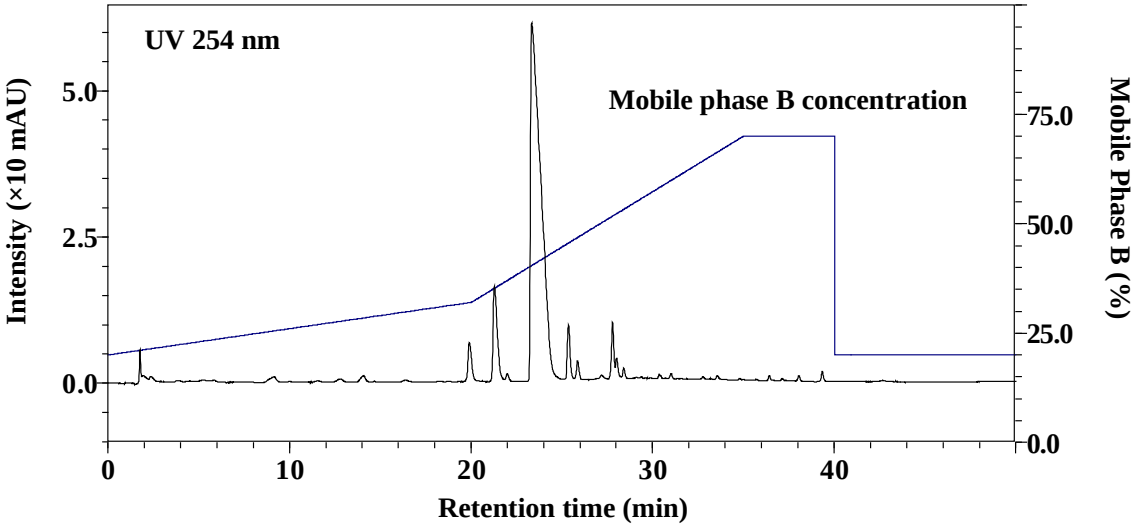


Figure 2.4 (*Continued*).

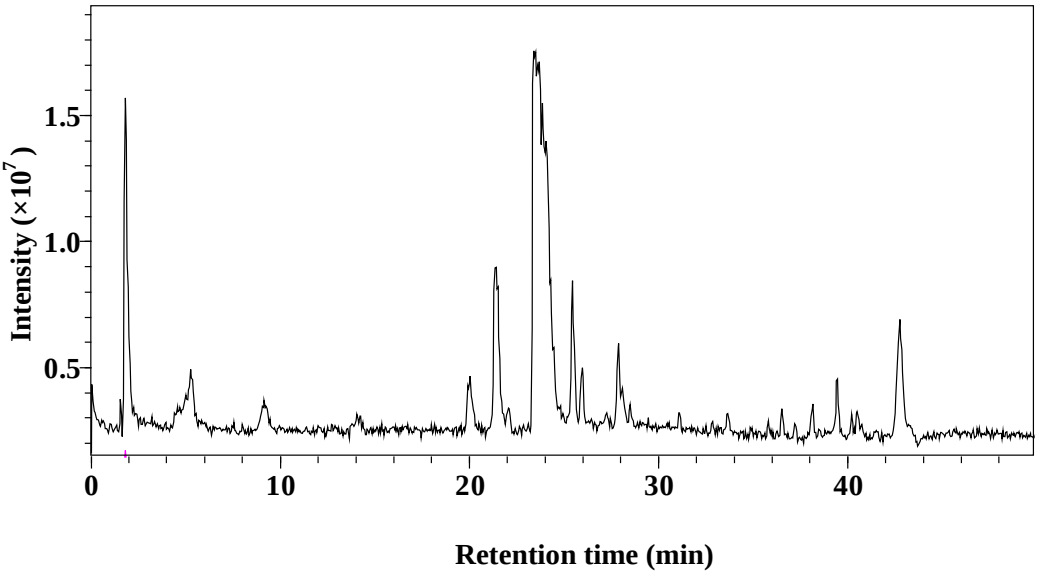


Figure 2.5 Total ion chromatogram (TIC) of stem extract of *C. fenestratum* using 20–32–70% MeOH as mobile phase

Table 2.2 UV and mass spectrum of compounds in *C. fenestratum*

Peak No	t_R (min)	UV λmax (nm)	HPLC-ESI-MS			Compound name
			$[M]^+$	$[M+H]^+$	$[M+Na]^+$	
1	5.26	222, 280	344			-
2	5.84	269, 304	342			-
2	9.14	221, 269, 300	356			-
3	14.06	206, 231, 264, 342, 411	352			-
4	19.91	228, 262, 342, 426	322			-
5	21.28	225, 272, 342, 426	338			Jatrorrhizine
6	21.99	240, 286, 337	338			-
7	23.35	228, 264, 345, 426	336			Berberine
8	25.36	225, 273, 342, 421	352			Palmatine
9	25.87	238, 286, 337	352			-
10	27.79	249		481	503	-
11	28.41	292, 318		314	336	-
12	31.01	202, 283, 369		342	364	-
13	32.78	202, 222, 333, 369		340	364	-
14	33.56	202, 282, 369		356	378	-
15	34.80	202, 222, 334, 370		354	376	-
16	35.71	204, 282, 369		370	392	-
17	36.43	202, 291, 317, 369		340	362	-
18	37.14	205, 221, 333, 369		368	390	-
19	38.05	206, 221, 342, 370		354	376	-
20	39.35	203, 224, 342, 369		352	374	-

2.3.2 Selection of Solvent for the Extraction of Compounds in *C. fenstratum*

Figure 2.6 shows the chromatogram of *C. fenestratum* extract using each solvent in water bath set at 60˚C for 180 min (extraction using 0.1% sulfuric acid was conducted at room temperature for 3 days). There is no significant different in the number of peaks found with each solvent condition. However, extraction using MeOH gave the higher peak area of major compounds compare to other solvent.

Table 2.3 shows the BER and PAL extraction yield by Soxhlet extraction for 8 h using MeOH, EtOH, and extraction using 0.1% sulfuric acid at room temperature. The extraction yield of BER using MeOH as solvent achieved 25.81 mg/ g-dry material and higher than extraction yield using EtOH (20.96 mg/g-dry material). Extraction using sulfuric acid at room temperature gave low extraction yield compared to that of Soxhlet extraction using MeOH and EtOH.

Figure 2.7 shows the summary of BER extraction yield by each extraction method. MeOH gave the highest extraction yield compared to EtOH and water. This could be the high dissociation of BER and PAL in MeOH rather than in EtOH and water.

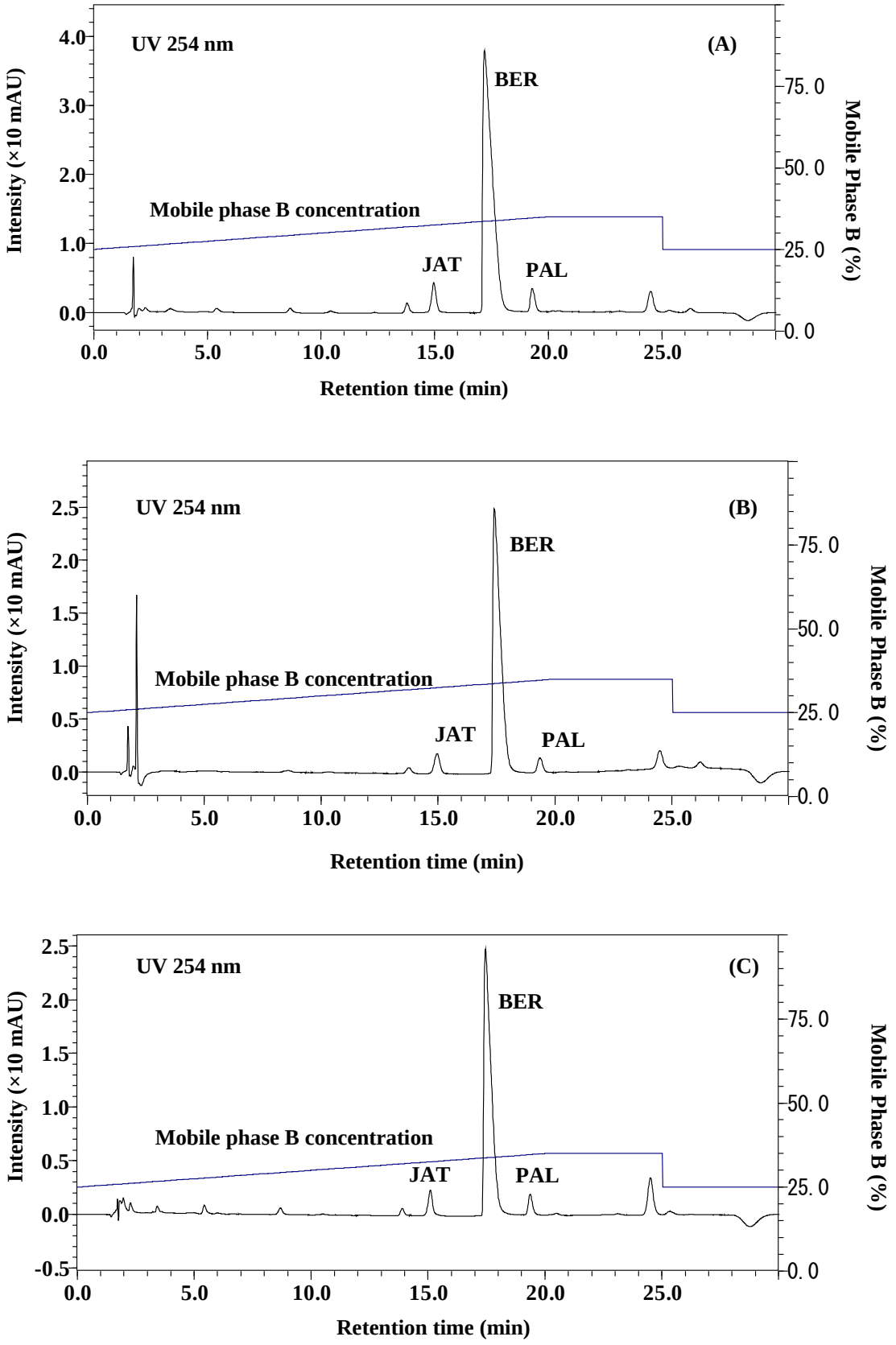


Figure 2.6 Chromatograms of stem extract of *C. fenestratum* using each solvent. (A) MeoH; (B) EtOH; (C) Water; (D) 0.1 % sulfuric acid.

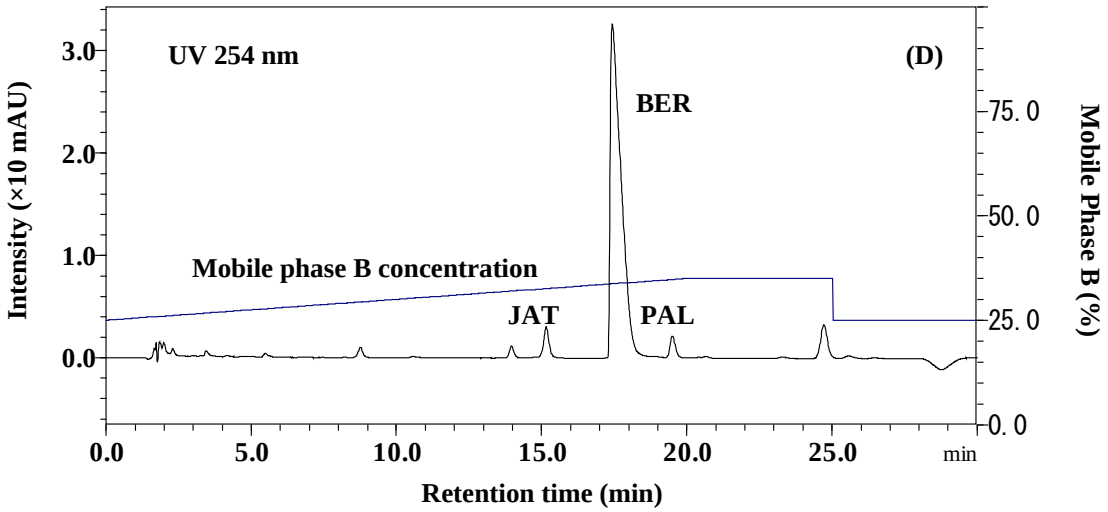


Figure 2.6 (*Continued*).

Table 2.3 Comparison of extraction yield using different solvent

	BER extraction yield (mg/g-dry material)	PAL extraction yield (mg/g-dry)
MeOH (Soxhlet extraction)	25.81	1.04
EtOH (Soxhlet extraction)	20.96	0.77
0.1% Sulfuric acid	19.24	0.62

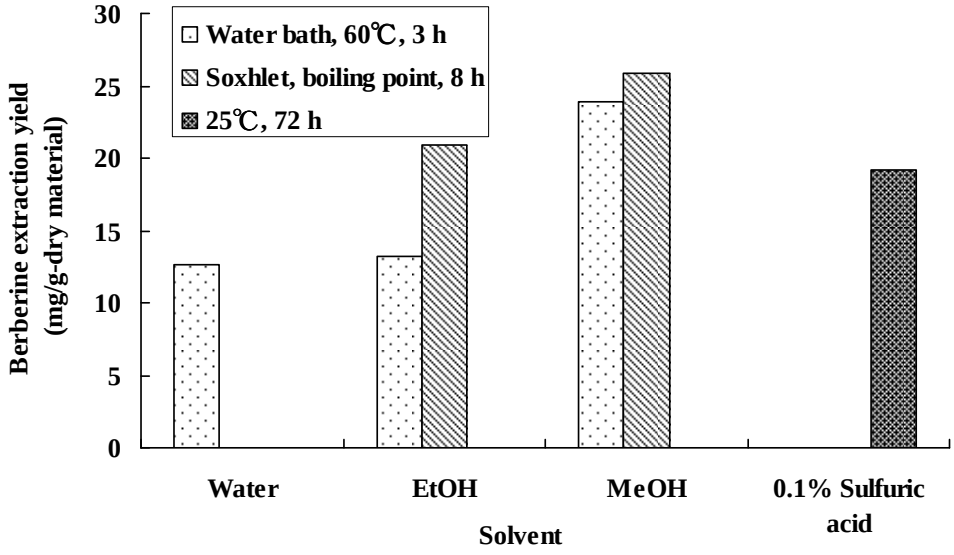


Figure 2.7 BER extraction yield by each extraction method

2.4 Conclusions

The separation conditions of compounds in *C. fenestratum* were investigated using liquid chromatography mass spectrometry. The optimum condition for separation compounds were studied on ZORBAX Eclipse XDB-C18 Column using 20–90% of MeOH and 0.1% formic acid aqueous solution as the mobile phase. More than 20 peaks were found and successfully separated in the stem extract of *C. fenestratum* using three step gradient condition of 20–32–70% MeOH. BER, JAT, PAL are the major compounds in *C. fenestratum*. MeOH gave higher extraction yield than EtOH and water in the extraction using water bath and Soxhlet extraction vessel.

CHAPTER 3

SIMULTANEOUS CHARACTERIZATION OF MEDICINAL COMPOUNDS IN *C. FENESTRATUM* BY LIQUID CHROMATOGRAPY HYBRID ION TRAP TIME-OF-FLIGHT MASS SPECTROMETRY

3.1 Introduction

The development of rapid and reliable methods for qualitative and quantitative determination of compounds in herbal medicine has become a significant and challenging issue. The combination of high-performance liquid chromatography and multi-stage mass spectrometry (HPLC-MS^n) is being increasingly used in pharmaceutical research and for quality control in herbal medicine, because of its superior sensitivity and selectivity[13, 51-54]. In these procedures, mass analyzers such as triple quadrupole or ion trap provide online mass information, which is useful in identifying the eluted components. However, both of these mass analyzers provide nominal mass values.

In contrast, time-of-flight mass spectrometry (TOF MS) provides higher accuracy and precision, which is remarkably efficacious in the identification of unknown compounds[2, 55-57]. The accurately measured mass values can be used to calculate candidate empirical formulae. Mass values obtained with a mass error of less than 5 ppm can significantly reduce the number of possible structures of the separated compounds. Accordingly, the combined use of ion trap MS^n for determining structural information and TOF MS for the obtaining accurate mass measurement offers a more powerful analytical approach for identifying the constituents of herbal medicines[2, 56].

The objective of this study is to develop method for the simultaneous characterization of known and unknown compounds in *C. fenestratum* by using liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS).

3.2 Experimental

Figure 3.1 shows the procedure of measurement and analysis. After the measurement, UV characteristic, molecular and fragment ion with accurate mass of each compound was analyzed. From the ion mass data, the chemical formulae were calculated and they were used to propose the chemical structure and the fragmentation pathway. The compounds were identified or tentatively identified by comparing with the fragmentation pathway of standard compounds and comparing with those reported in literatures.

3.2.1 Chemicals

BER, PAL, JAT, tetrahydroberberine (canadine), 20-hydroxyecdysone, methanol (LC/MS-grade) and formic acid (LC/MS-grade) were purchased from Wako Pure Chemical Industries, Ltd, Japan. Ultra-pure water was produced by Milli-Q Advantage system (Millipore Corp., USA). 8-Oxoberberine was synthesized from BER chloride by using a previously described procedure[58]. In brief, BER chloride 50 mg was dissolved in 20% KOH aqueous solution. The solution was refluxed at 80°C for 6 hours. The reaction mixture was extracted with CH_2Cl_2 (3 x 15mL). The combined CH_2Cl_2 extracts were dried, filtered, and evaporated to yield 8-oxoberberine. Stock solutions of BER (150 μg/ml), PAL (42 μg/ml), JAT (110 μg/ml), tetrahydroberberine (40 μg/ml), and 20-hydroxyecdysone (11.4 μg/ml) were prepared in methanol. A solution of 8-oxoberberine was prepared by dissolving 2.5 mg of the synthesized crystals in 50 ml of methanol. All the solutions were stored at 4ºC in the refrigerator. Standard solutions were prepared by diluting each stock solution in methanol.

3.2.2 Sample Preparation and Extraction

Stems of *C. fenestratum* (Batch 1) were obtained from Vientiane province, Laos in August 2006 and dried at room temperature.The plants were ground in a blender (Wonder blender WB-1; Osaka Chemical, Japan), and the powder was sieved using a 60-mesh sieve to obtain a powder with particle size less than 250 μm. Extraction was performed in a Soxhlet extraction vessel by adding 0.5 g of plant powder to the extraction thimble and extracting the powder using 100 ml methanol for 11 hours. The extracted liquid was filtered using a 0.45-μm membrane filter, and the filtrate was used for

LC/IT-TOF MS analysis.

3.2.3 LC/IT-TOF MS Analysis

LC/IT-TOF MS analyses were performed on an LC/IT-TOF MS system (Shimadzu Corp., Japan). The LC system consisted of a Shimadzu Prominence binary pump (LC-20AD), on-line vacuum degasser (DGU-20As), autosampler (SIL-20AC), column oven (CTO-20AC), and a photo diode-array detector (SPD-M20A). The separation was carried out on a ZORBAX Eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 μm) (Agilent technologies, Inc., USA). The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and methanol (B), was delivered at a flow rate of 0.2 ml/min by using the following gradient program: 20–32% (B) from 0–20 min, 32–70% (B) from 20–35 min, 70% (B) from 35–45 min and 20% (B) from 45–55 min. The sample injection volume was 2 μl. The column temperature was maintained at 40°C. The UV spectra were obtained by scanning the samples in the range of 200–470 nm, and the peaks were simultaneously determined at 254 nm.

The IT-TOF mass spectrometer was fitted with an electrospray ionization (ESI) interface as shown in **Figure 3.2**[59]. The LCMS-IT-TOF has been designed to maximize sensitivity and selectivity by optimizing the ion transport to the TOF analyzer and redefining the capability of the quadrupole ion trap. The ion trap is used to focus ions before ejection into the TOF as well as supporting MS^n analysis with effective precursor ion selection capabilities (resolution > 1,000 at 1,000 m/z). The detail information of the structure of this IT-TOF mass spectrometer can be found in the report of Taniguchi and Eizoh[60]. This LCMS-IT-TOF incorporates a host of new technologies and enhancements, including Compressed Ion Injection (CII) and Ballistic Ion Extraction (BIE). The CII method makes it possible to control the accumulation of ions before they are introduced into the ion trap. Adopted for the LCMS-IT-TOF, it effectively couples the LC system to the MS, dramatically increasing sensitivity. The use of BIE accelerates ions into the TOF instantly, and combined with the Dual-Stage Reflectron (DSR), achieves faster, more stable, and higher resolution spectra[59].

ESI-MS^n experiments were conducted in both positive and negative modes. The following instrumental parameters were used: interface voltage, +4.5 kV (positive mode), –3.5 kV (negative mode); heat-block temperature, 200°C; curved desolvation line (CDL)

temperature, 200°C; flow rate of nebulizing gas (N_2), 1.5 L/min; and pressure of drying gas (N_2), 100 KPa. Mass spectrometry was performed in the full-scan mode (MS^1) and automatic multiple-stage fragmentation-scan modes (MS^2–MS^4) over an *m/z* scan range of 100–700. In the automatic mode, all the ions were first accumulated in the octopole, and then, they were rapidly pulsed into the IT for MS^n analyses. The resulting ions were introduced into the TOF instrument for accurate mass determination. The ion-accumulation time was set at 30 ms. The following parameters for collision-induced dissociation (CID) were chosen: CID energy, 30%; collision-gas content, 50%. Argon was used for CID. The TOF-detector voltages for the positive and negative modes were 1.60 kV and 1.56 kV, respectively. Trifluoroacetic acid (TFA) sodium solution was used as the standard sample for calibrating the instrument. The chromatographic and mass-spectrometric analyses, including the prediction of chemical formulae were performed by using LCMS solution Ver3.41 software package (Shimadzu Corp., Japan).

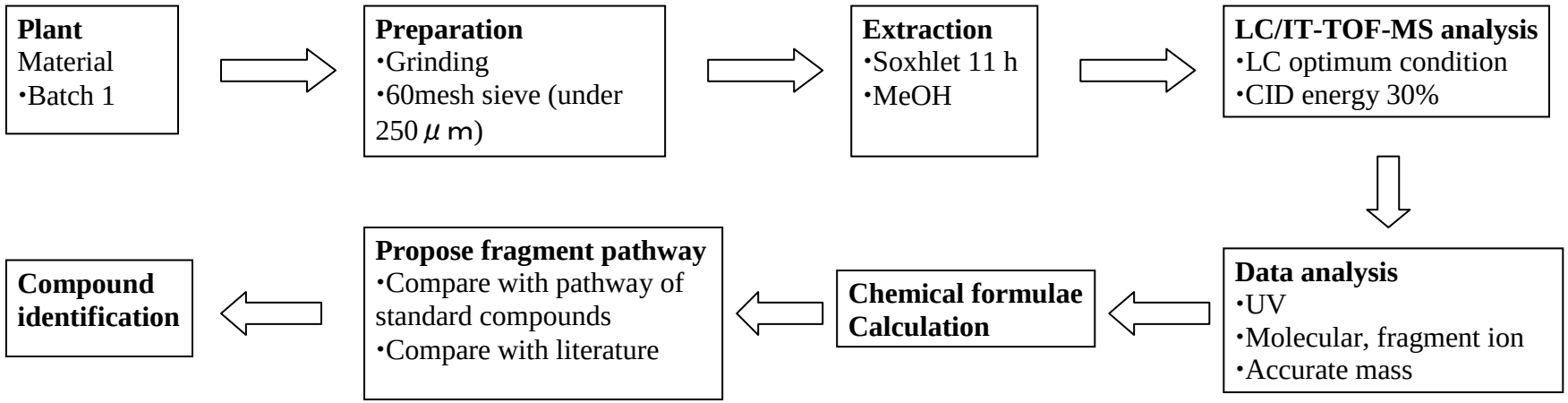


Figure 3.1 Procedure of measurement and analysis

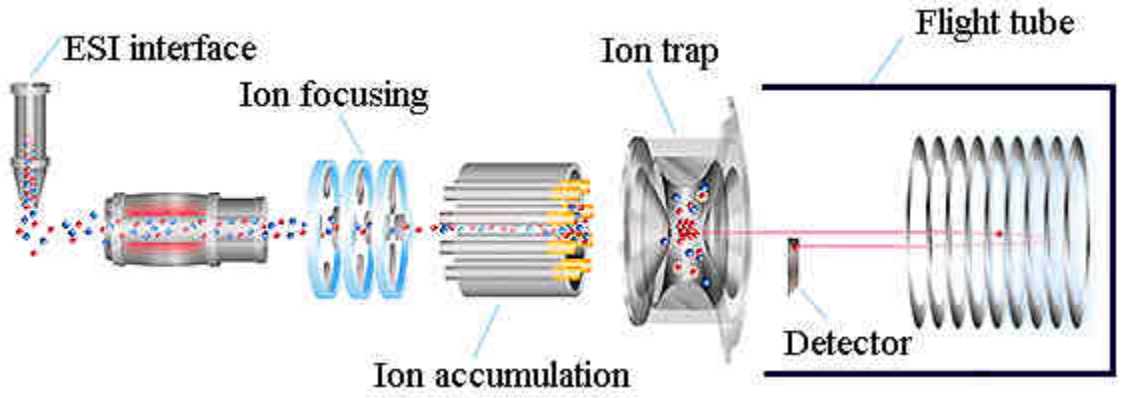


Figure 3.2 Structure of IT-TOF mass spectrometer

3.3 Results and Discussion

3.3.1 Online Analysis of Standard Compounds

The online analysis of 6 standard compounds was performed by using LC/IT-TOF MS under the optimum conditions described in the experimental section. As shown in **Table 3.1**, quaternary protoberberine alkaloids such as BER, PAL, and JAT exhibited maximum UV absorption at 4 wavelength ranges: 220–230 nm, 260–280 nm, 340–350 nm, and 420–430 nm. 8-Oxoberberine exhibited maximum UV absorption at 254 nm, 313 nm, 341 nm, and 369 nm. In contrast, 20-hydroxyecdysone exhibited maximum absorption at a single wavelength, 249 nm. In positive mode, BER, PAL, and JAT generated the molecular ion $[M]^+$ and the fragment ions $[M - {\cdot}CH_3]^{+\cdot}$, $[M - {\cdot}CH_3 - {\cdot}H]^+$, $[M - {\cdot}CH_3 - {\cdot}H - CO]^+$ in MS^2 and $[M - {\cdot}CH_3 - {\cdot}H - 2H]^+$ in MS^3. Tetrahydroberberine generated the protonated molecule $[M + H]^+$. $[M + H]^+$ was observed to undergo the Retro-Diels-Alder (RDA) fragmentation reaction when CID was applied in MS^2. 20-Hydroxyecdysone produced $[M + H]^+$ and the sodium adduct ion $[M + Na]^+$. The cleavage of water molecules from 20-hydroxyecdysone ($M - nH_2O$, n = 1–3) was also observed. In negative mode, JAT produced $[M - 2H + HCOOH]^-$ and 20-hydroxyecdysone produced $[M - H]^-$ and $[M - H + HCOOH]^-$. The molecular ion and the fragment ions of BER, PAL, JAT, and 20-hydroxyecdysone were consistent with those reported in previous studies[52, 61, 62].

$[M + H]^+$ and $[M + Na]^+$ were observed in the mass spectra of 8-oxoberberine in MS^1. **Figure 3.3** shows the fragmentation ion from the precursor ion at *m/z* 352.1169. This ion produced the fragments $[M + H - {\cdot}CH_3]^{+\cdot}$, $[M + H - {\cdot}CH_3 - {\cdot}H - CO]^+$, $[M + H - {\cdot}CH_3 - H_2O]^{+\cdot}$ in MS^2, $[M + H - {\cdot}CH_3 - {\cdot}CH_3]^+$ in MS^3, and $[M + H - {\cdot}CH_3 - {\cdot}CH_3 - CO]^+$ in MS^4, which were different from the corresponding ions of BER and tetrahydroberberine. Standard reference analysis was performed to establish the principal fragmentation pathways of quaternary protoberberines, tetrahydroprotoberberines, 8-oxoprotoberberines, and the steroid compound.

Table 3.1 Retention time (t_R), UV (λmax) and MS data of standard compounds.

Compound	t_R (min)	UV λmax	*m/z* [identity]		
			(+)ESI-MS	(–) ESI-MS	(+)ESI-MS^n fragments
Jatrorrhizine	23.05	225, 274, 343, 427	338.1390 $[M]^+$	382.1290 $[M–2H+HCOOH]^-$	323.1137, 322.1052, 320.0893, 307.0815, 294.1122
Berberine	25.45	226, 264, 346, 426	336.1236 $[M]^+$		321.0971, 320.0896, 318.0748, 292.0951
Palmatine	27.06	245, 273, 344, 425	352.1550 $[M]^+$		337.1293, 336.1228, 334.1061, 320.0911, 308.1288, 292.0954
Tetrahydro-berberine	21.76	285	340.1547 $[M+H]^+$		176.0712, 149.0593
20-Hydroxy-ecdysone	29.30	249	503.2982 [M+Na]+	525.3061 $[M–H+HCOOH]^-$	
			481.3164 $[M+H]^+$	479.2973 $[M–H]^-$	463.3050, 445.2931, 371.2195, 303.1997
			463.3059 $[M+H–H_2O]^+$		
			445.2952 $[M+H–2H_2O]^+$		
			427.2839 $[M+H–3H_2O]^+$		
8-Oxoberberine	41.19	224, 341, 369	374.0997 $[M+Na]^+$		
			352.1169 $[M+H]^+$		337.0947, 322.0696, 319.0821, 308.0927, 304.0615, 294.0757

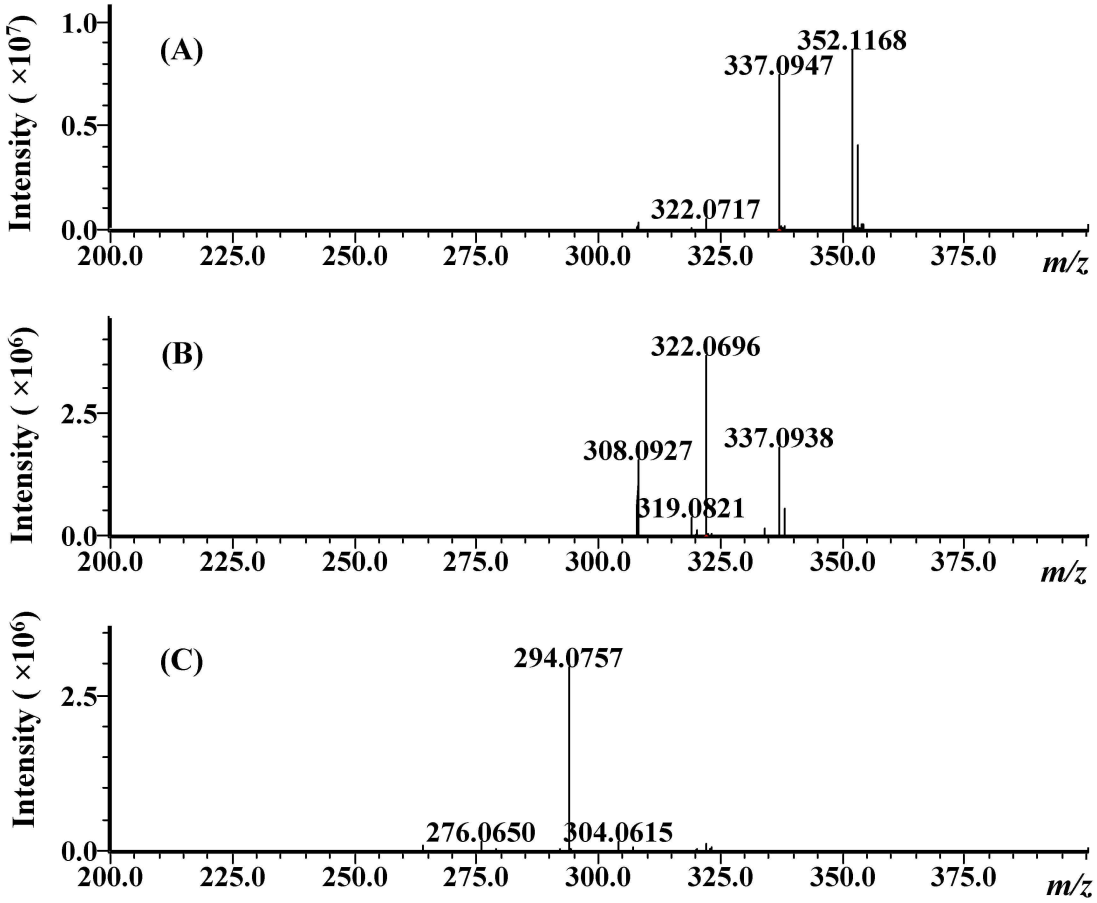


Figure 3.3 MS^n mass spectra of 8-oxoberberine obtained by IT-TOF. (A) MS^2 spectrum of ion at *m/z* 352.1169; (B) MS^3 spectrum of ion at *m/z* 337.0947; (C) MS^4 spectrum of ion at *m/z* 322.0696.

3.3.2 Simultaneous Characterization of Compounds from the Stem Extract of *C. fenestratum*

The stem extract of *C. fenestratum* was injected directly into LC/IT-TOF MS without any further pretreatment (**Figure 3.4** and **Table 3.2**). A total of 32 peaks were recorded, and the chemical structure of the compounds was characterized on the basis of the calculated accurate mass of the molecular ions, the protonated molecule, the fragment ions, the retention behavior, and the data from the UV spectra (**Table 3.2** and **Table 3.3**). The mass error of the measured value and the predicted value was less than 5 ppm for MS^1 and less than 10 ppm for most of the fragments in MS^2–MS^4 analysis. The empirical formulae obtained from the accurate mass values were compared to those in literature to identify the known compounds. The accurate fragmentation data was used to elucidate and identify the unknown compounds. These highly accurate mass values were useful in reducing the number of candidate chemical formulae and identifying the compounds easily and rapidly. It can be concluded that most of these compounds are quaternary alkaloids, 8-oxoprotoberberine alkaloids, tetrahydroprotoberberine alkaloids, along with a single steroid compound (**Table 3.2**). Compounds 1 and 2 were characterized as benzylisoquinoline alkaloids; compounds 3, 4, and 5, as aporphine alkaloids; compounds 6, 8, and 15, as tetrahydroprotoberberine alkaloids; compounds 7, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, and 20 as quaternary protoberberine alkaloids; compound 21 as a steroid compound; and compounds 23, 24, 25, 26, 27, 28, 29, 30, 31, and 32 were characterized as 8-oxoprotoberberine alkaloids. Compound 22 was tentatively identified as a benzyl tetrahydroisoquinoline alkaloid. **Figure 3.5**–**Figure 3.9** shows the representative of mass spectra and proposed fragmentation pathways of protoberberine compounds.

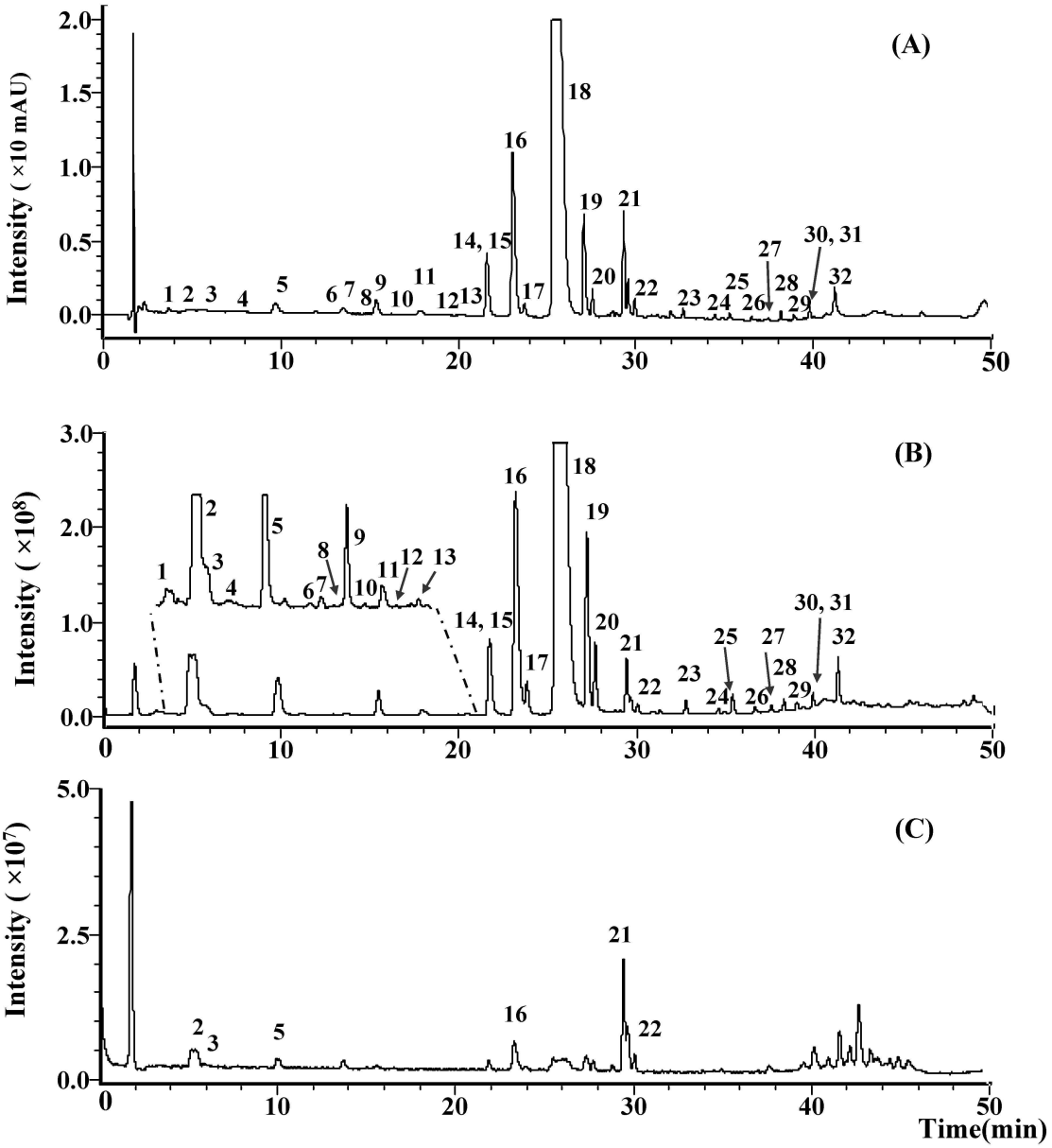


Figure 3.4 HPLC-PDA and total ion chromatogram of *C. fenestratum* (2 µl injected). (A) Monitored at 254 nm; (B) total ion chromatogram in positive mode; (C) total ion chromatogram in negative mode.

Table 3.2 Retention time (t_R), UV (λmax) and MS data of compounds from the stem extract of *C. fenestratum*.

Peak No	t_R (min)	Formula $[M]^+$ or $[M+H]^+$	UV λmax (nm)	Measured *m/z*	Predicted *m/z*	Error (ppm)	$[M+Na]^+$	(–) ESI *m/z* [identity]	Proposed compounds
1	3.20	$[C_{19}H_{24}NO_3]^+$	223, 275	314.1762	314.1756	1.91	–	–	Tembetarine derivative
2	4.85	$[C_{20}H_{26}NO_4]^+$	281	344.1846	344.1856	-2.91	–	388.1766$[M–2H+HCOOH]^-$, 434.1812$[M–2H+2HCOOH]^-$	Tembetarine
3	5.73	$[C_{20}H_{24}NO_4]^+$	217, 269, 302	342.1705	342.1700	1.46	–	386.1584$[M–2H+HCOOH]^-$	Magnoflorine
4	7.89	$[C_{20}H_{24}NO_4]^+$	222, 275	342.1686	342.1700	-4.09	–	–	*N,N*-demethyllindcarpine
5	9.71	$[C_{21}H_{26}NO_4]^+$	221, 269, 302	356.1858	356.1856	0.56	–	354.1709$[M–2H]^-$, 400.1774 $[M–2H+HCOOH]^-$	Menisperine
6	12.98	$[C_{19}H_{19}NO_4+H]^+$	–	326.1388	326.1387	0.31	–	–	Tetrahydrothalifendine
7	13.52	$[C_{20}H_{20}NO_5]^+$	231, 275, 342, 412	354.1342	354.1336	1.69	–	–	13-Hydroxyjatrorrhizine
8	15.03	$[C_{21}H_{25}NO_4+H]^+$	–	356.1852	356.1862	-2.81	–	–	Tetrahydropalmatine
9	15.37	$[C_{20}H_{18}NO_5]^+$	231, 264, 342, 411	352.1178	352.1179	-0.28	–	–	13-Hydroxyberberine
10	16.60	$[C_{18}H_{16}NO_4]^+$	–	310.1074	310.1074	0.00	–	–	Demethylenethalifendine
11	17.82	$[C_{19}H_{18}NO_4]^+$	232, 275, 342, 411	324.1227	324.1230	-0.93	–	–	Demethyleneberberine
12	18.80	$[C_{21}H_{22}NO_5]^+$	–	368.1501	368.1492	2.44	–	–	13-Hydroxypalmatine
13	20.31	$[C_{19}H_{18}NO_4]^+$	–	324.1227	324.1230	-0.93	–	–	Dehydrodiscretamine
14	21.60	$[C_{19}H_{16}NO_4]^+$	228, 262, 342, 426	322.1068	322.1074	-1.86	–	–	Thalifendine
15	21.78	$[C_{20}H_{21}NO_4+H]^+$	–	340.1546	340.1543	0.88	–	–	Tetrahydroberberine

16	23.05	$[C_{20}H_{20}NO_4]^+$	225, 272, 342, 426	338.1389	338.1387	0.59	–	382.1285$[M–2H+HCOOH]^-$	Jatrorrhizine
17	23.72	$[C_{20}H_{20}NO_4]^+$	240, 287, 310sh, 337	338.1384	338.1387	-0.89	–	–	Pseudojatrorrhizine
18	25.41	$[C_{20}H_{18}NO_4]^+$	228, 264, 346, 426	336.1229	336.1230	-0.30	–	–	Berberine
19	27.07	$[C_{21}H_{22}NO_4]^+$	225, 273, 342, 421	352.1544	352.1543	0.28	–	–	Palmatine
20	27.55	$[C_{21}H_{22}NO_4]^+$	239, 287, 310sh, 337	352.1547	352.1543	1.14	–	–	Pseudopalmatine
21	29.31	$[C_{27}H_{44}O_7+H]^+$	249	481.3162	481.3160	0.42	503.2979	525.3056$[M–H+HCOOH]^-$	20-Hydroxyecdyson
22	29.93	$[C_{18}H_{19}NO_4+H]^+$	292, 318	314.1374	314.1387	-4.14	336.1202	312.1239$[M–H]^-$, 358.1295$[M–H+HCOOH]^-$	1, 3-Dioxolo [4,5] isoquinoline-7-ol- 5,6,78-tetrahydro-6-[(methoxyphenyl)methyl]
23	32.66	$[C_{19}H_{19}NO_5+H]^+$	202, 283, 369	342.1331	342.1336	-1.46	364.1153	–	8-Oxodiscretamine
24	34.47	$[C_{19}H_{18}NO_5+H]^+$	202, 222, 333, 369	340.1173	340.1179	-1.76	362.1009	–	8-Oxodehydrodiscretamine
25	35.27	$[C_{20}H_{21}NO_5+H]^+$	202, 282, 369	356.1492	356.1492	0.00	378.1306	–	8-Oxoisocorypalmine
26	36.53	$[C_{20}H_{20}NO_5+H]^+$	202, 222, 334, 370	354.1324	354.1336	-3.39	376.1163	–	8-Oxojatrorrhizine
27	37.46	$[C_{21}H_{23}NO_5+H]^+$	204, 282, 369	370.1648	370.1649	-0.27	392.1470	–	8-Oxotetrahydropalmatine
28	38.16	$[C_{19}H_{17}NO_5+H]^+$	202, 291, 317, 369	340.1188	340.1179	2.65	362.1011	–	8-Oxotetrahydrothalifendine
29	38.89	$[C_{21}H_{21}NO_5+H]^+$	205, 221, 333, 369	368.1492	368.1492	0.00	390.1299	–	8-Oxopalmatine
30	39.79	$[C_{20}H_{19}NO_5+H]^+$	–	354.1332	354.1336	-1.13	376.1161	–	8-Oxotetrahydroberberine
31	39.96	$[C_{19}H_{15}NO_5+H]^+$	206, 221, 342, 370	338.1027	338.1023	1.18	360.0894	–	8-Oxothalifendine
32	41.19	$[C_{20}H_{17}NO_5+H]^+$	203, 224, 341, 369	352.1171	352.1179	-2.27	374.0997	–	8-Oxoberberine

Table 3.3 MS^n data in positive mode of compounds observed from the stem extract of *C. fenestratum*.

Peak No	HPLC/ESI-MS^n *m/z* (% base peak)
1	MS^2[314.1762]: 271.1322(32), 269.1142(100), 239.1144(7), 237.0890(18), 175.0767(39), 147.0758(15), 145.0650(32), 137.0559(18), 107.0496(23)
	MS^3[314.1762 → 269.1144]: 175.0732(78), 145.0645(100), 137.0603(44), 107.0493(64)
2	MS^2[344.1846]: 312.1734(5), 301.1450(11), 299.1299(67), 269.1203(8), 267.1000(19), 235.0793(17), 192.0999(6), 177.0898(6), 175.0748(100), 151.0781(9), 143.0485(36), 137.0624(36)
	MS^3[344.1846 → 175.0748]: 160.0481(38), 143.0490 (100)
3	MS^2[342.1705]: 311.1294(26), 299.1259(19), 297.1117(100), 279.097(17), 265.0845(53)
	MS^3[342.1705 → 297.1113]: 282.0889(22), 265.0848(100), 237.0951(18), 207.0779(9)
	MS^4[342.1705 → 297.1113 → 265.0877]: 250.0658(37), 237.0871(100), 207.0702(37), 191.0825(91)
4	MS^2[342.1686]: 297.1114 (100), 265.0842(56)
	MS^3[342.1686 → 297.1112]: 265.0851 (100)
	MS^4[342.1686 → 297.1112 → 265.0848]: 237.0900(100), 233.0613(37)
5	MS^2[356.1858]: 325.1408(14), 311.1282(100), 296.1044(12), 293.1223(6), 279.1018(16), 237.0919(16)
	MS^3[356.1858 → 311.1265]: 296.1037(100), 279.1029(18), 248.0860(6), 237.0929(32)
	MS^4[356.1858 → 311.1265 →296.1038]: 281.0804(100), 250.1026(85), 248.0793(36), 235.0791(63)
6	MS^2[326.1388]: 176.0706(100), 149.0597(32)
	MS^3[326.1388 →176.0710]: 176.058(100), 149.056(100)
7	MS^2[354.1342]: 339.1096(100), 338.1029(54), 310.1057(41)
	MS^3[354.1342 → 339.1092]: 338.1013(100), 324.084(8), 322.1064(21), 310.1071(48)
	MS^4[354.1342 → 339.1092 → 338.1013]: 336.0942(22), 323.0820(100), 320.0833(14), 306.0824(32)
8	MS^2[356.1852]: 192.1017(100), 177.0797(6)
	MS^3[356.1852 → 192.1017]: 177.0797(100)
	MS^4[356.1852 → 192.1017 → 177.0797]: 149.08 (100), 148.072(80)
9	MS^2[352.1178]: 337.0957(100), 336.0858(76), 308.0922(84)
	MS^3[352.1178 → 337.0957]: 336.0860(100), 334.0710(9.63), 308.0932(45)
	MS^4[352.1178 → 337.0957 → 336.0860]: 334.0707(100), 318.0741(47), 316.0581(35), 305.0598(5), 294.0741(7), 292.0584(6)
10	MS^2[310.1074]: 295.0818 (100)
	MS^3[310.1074 → 295.0839]: 267.087 (100), 266.079 (12)
	MS^4[310.1074 → 295.0839 → 267.087]: 250.084 (28), 238.0837(100)
11	MS^2[324.1227]: 309.0997(100), 308.0918(28), 280.0984(39)
	MS^3[324.1227 → 309.0997]: 308.0910(100), 294.0761(42), 280.0949(55)
	MS^4[324.1227 → 309.0997 → 308.0901]: 306.0751(100)
12	MS^2[368.1501]: 353.1239(100), 352.118(63), 324.1274(82)
	MS^3[368.1501 → 353.1254]: 352.1211[100], 324.1258(39)
	MS^4[368.1501 → 353.1254 → 352.1147]: 350.1015(100), 336.0865[93], 334.1066[33]
13	MS^2[324.1227]: 309.0981(100)
	MS^3[324.1227 → 309.0981]: 294.0738(100), 281.0997(35), 280.0872(12.27), 266.0794(19)
	MS^4[324.1227 → 309.0981→ 294.0738]: 266.0791(100), 238.0843(18)
14	MS^2[322.1068]: 307.0836(100)
	MS^3[322.1068 → 307.0836]: 292.0583(6), 279.0881(100), 278.0821(34), 251.0922(8), 250.0871(7)
	MS^4[322.1068 → 307.0836 → 279.0881]: 278.0813(100), 263.0615(7), 250.0871(35), 225.0758(15)
15	MS^2[340.1546]: 176.0706(100), 149.0597(5)
	MS^3[340.1546 → 176.0706]: 149.0614(100)
16	MS^2[338.1389]: 323.1141(100), 322.1077(56), 306.1091(6), 294.1125(69)
	MS^3[338.1389 → 323.1141]: 322.1074(42), 320.0903(43), 307.0824(100), 294.1133(63), 279.0919(19)
	MS^4[338.1389 → 323.1141→ 322.1074]: 321.1066(25), 320.0902(30), 307.0843(100)

17 MS^2[338.1384]: 338.1375(19), 323.1153(100), 322.1075(9), 294.1135(12)
MS^3[338.1384 → 323.1153]: 323.1152(22), 320.0903(5.48), 308.0908(82), 307.0819(81), 295.1204(100), 294.1122(82), 279.0888(89.59), 278.0989(10), 251.0916(7), 250.0845(8)
MS^4[338.1384 → 323.1153 → 295.1204]: 294.113(100), 279.0898(50), 278.089(17), 266.1108(6), 251.0924(11), 250.085(33)

18 MS^2[336.1229]: 321.0987(100), 320.0918(69), 292.0969(90)
MS^3[336.1229→ 321.0987]: 320.0895(100), 318.076(50.89), 292.0960(64)
MS^4[336.1229→ 321.0987 → 320.0895]: 318.0757(100)

19 MS^2[352.1544]: 352.1527(92), 337.1305(100), 336.1233(73.77), 320.1259(6), 308.1291(84)
MS^3[352.1544→ 337.1305]: 337.1278(36), 334.10536(55), 321.0990(33), 320.0922(100), 308.1288(71), 292.0976(20)
MS^4[352.1544 → 337.1305 → 320.0922]: 318.0755(100), 290.0819(6)

20 MS^2[352.1547]: 337.1275(30), 336.1217(100), 308.1270(31)
MS^3[352.1547 → 336.1217]: 334.1068(21), 320.0922(100), 292.0977(83)
MS^4[352.1547 → 336.1217 → 320.0922]: 318.0759(100), 305.0657(32), 292.0974(27), 290.0842(29), 274.0860(18), 262.0818(11)

21 MS^2[481.3162]: 445.2954(100), 371.2202(86), 303.1899(20)
MS^3[481.3162 → 445.2954]: 427.2855(77), 409.2824(15), 371.2212(100), 303.1982(10), 301.1771(10)

22 MS^2[314.1374]: 177.0556(100), 145.0277(15)
MS^3[314.1374 → 177.0556]: 145.0276(100), 117.0322(5)
MS^4[314.1374 → 177.0556 → 145.0276]: 117.0319(100)

23 MS^2[342.1331]: 327.1092(100), 326.1032(16), 312.0862(11), 310.1058(8), 178.0874 (31)
MS^3[342.1331 → 327.1092]: 326.1012(27), 312.0876 (27), 310.1046 (23), 308.0923(55), 296.0949(11), 178.0867(100), 176.0680(8)
MS^4[342.1331 → 327.1092 → 178.0867]: 163.0607 (100)

24 MS^2[340.1173]: 325.0951(100)
MS^3[340.1173 → 325.0951]: 310.0690(100)
MS^4[340.1173 → 325.0951→310.069]: 295.0475(100), 282.0743(41), 267.0524(21)

25 MS^2[356.1492]: 341.1260(61), 340.1204(10), 326.1008(20), 308.0942(5), 178.0873(28), 176.0702(100)
MS^3[356.1492 → 176.0702]: 161.0467(100), 133.0534(26),
MS^4[356.1492 → 176.0702 → 161.0467]: 133.0524(100)

26 MS^2[354.1324]: 339.1099(100), 324.0868(12), 310.1126(5)
MS^3[354.1324 → 339.1099]: 324.0861(100), 321.0912(6), 310.1050(33)
MS^4[354.1324 → 339.1099 →324.0861]: 309.0591(17), 296.0916(100), 281.0646(18)

27 MS^2[370.1648]: 355.1396(45), 354.1353(16), 340.1187(23), 324.1191(11), 192.1005(31), 190.0868(100)
MS^3[370.1648 → 190.0868]: 175.0622(96), 174.0533(79), 146.059(100)
MS^4[370.1648 → 190.0868 → 175.0622]: 174.0593(100)

28 MS^2[340.1188]: 325.0931(100), 324.0844(12), 310.0701(7), 176.0701(34),
MS^3[340.1188→ 325.0931]: 324.0856(26), 310.0694(26), 306.0761(43), 295.0818(12), 176.0706(100), 175.0674(6), 174.053(6)
MS^4[340.1188→ 325.0931→ 176.0706]: 174.0593(33), 149.0582(100)

29 MS^2[368.1492]: 353.1258(100), 338.1034(11)
MS^3[368.1492 → 353.1258]: 338.1028(100), 324.1214(29.51)
MS^4[368.1492 → 353.1258 → 338.1028]: 322.0704(6), 310.1064(100), 294.0748(9)

30 MS^2[354.1332]: 339.1086(39), 338.1059(11), 324.0854(13), 310.1092(5), 176.0718(35), 174.0559(100)
MS^3[354.1332 → 176.0706]: 174.0517(100), 118.0650(26), 116.0538(10)

31 MS^2[338.1027]: 323.0784(100)
MS^3[338.1027 → 323.0784]: 308.0548 (100)
MS^4[338.1027 → 323.0784 → 308.0548]: 280.0599(100), 252.0628(12)

32 MS^2[352.1171]: 337.0951(100), 322.0715(7)
MS^3[352.1171 → 337.0951]: 322.071(100), 319.0841(11), 308.0908(33)
MS^4[352.1171 → 337.0951 → 322.0710]: 304.0605(6), 294.0764(100), 276.0678(8)

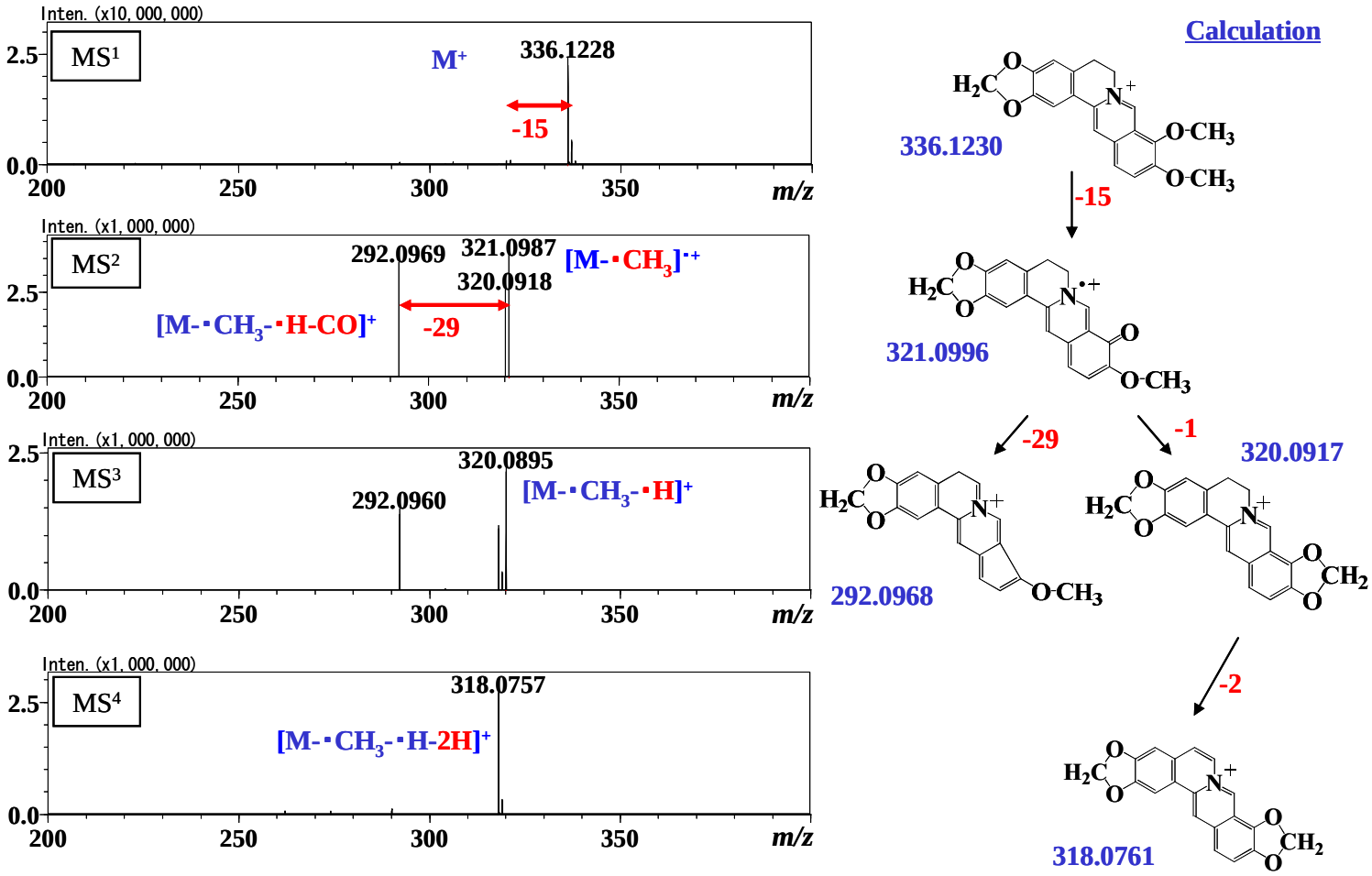


Figure 3.5 Mass spectra and proposed fragmentation pathway of berberine

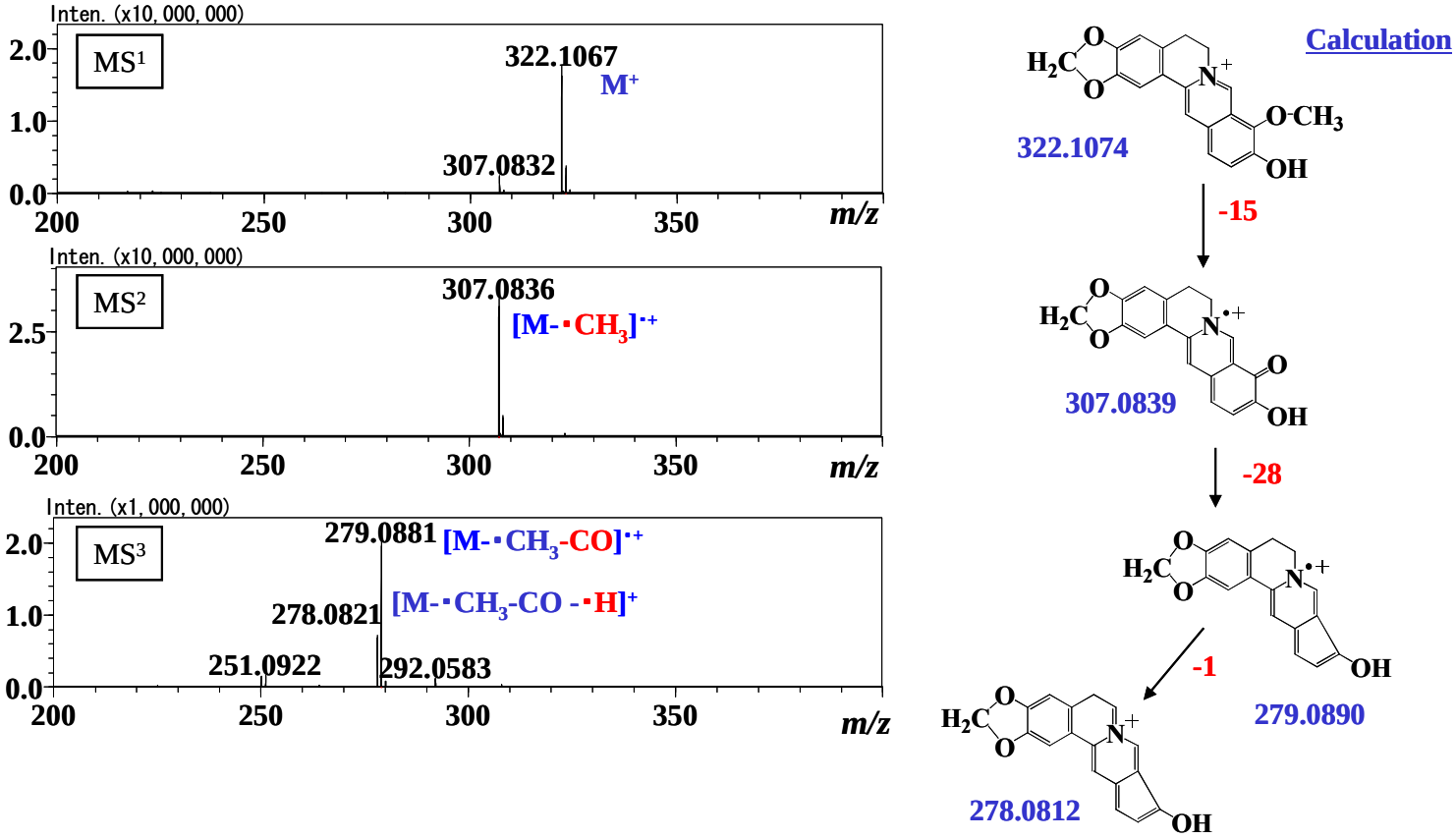


Figure 3.6 Mass spectra and proposed fragmentation pathway of thalifendine

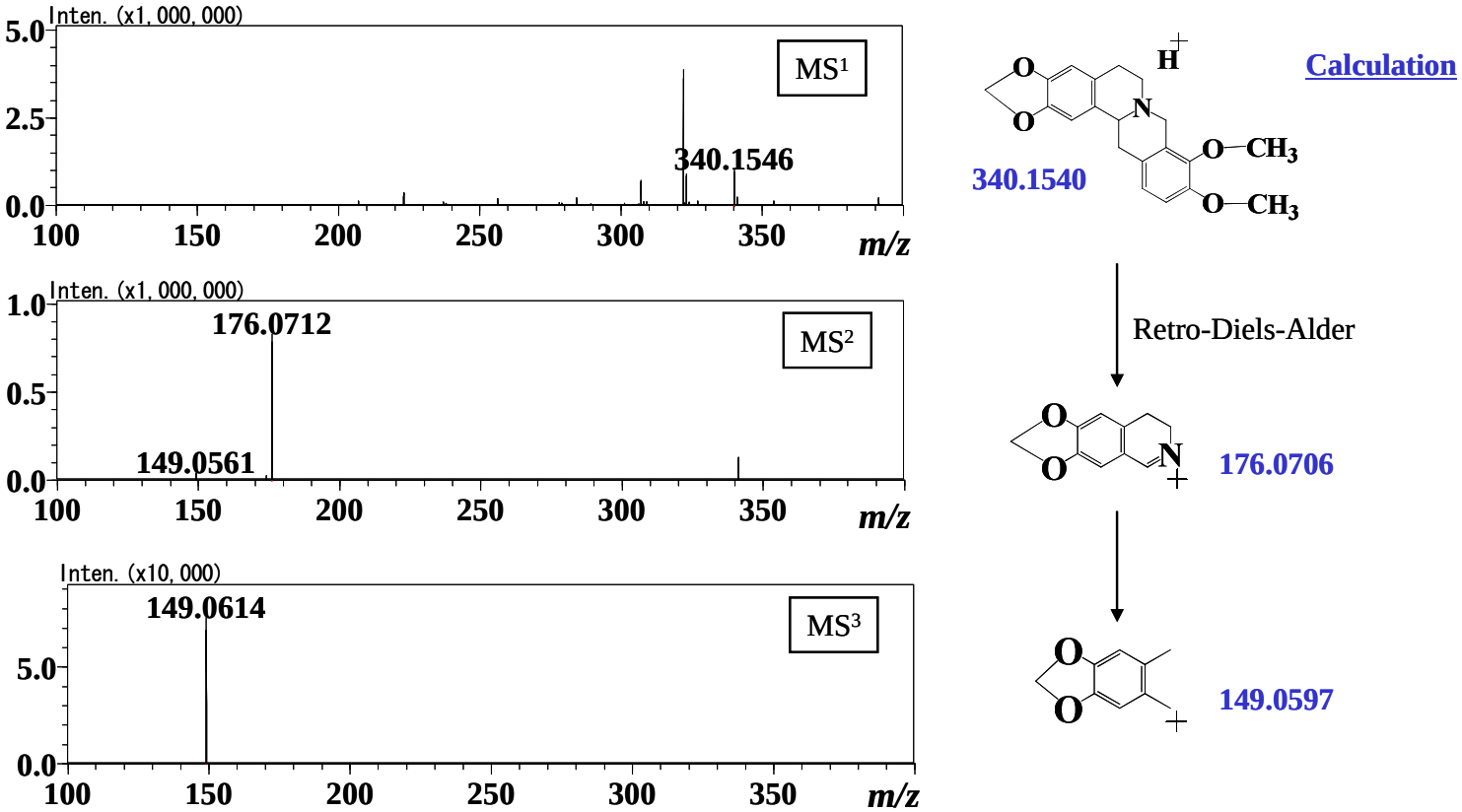


Figure 3.7 Mass spectra and proposed fragmentation pathway of tetrahydroberberine

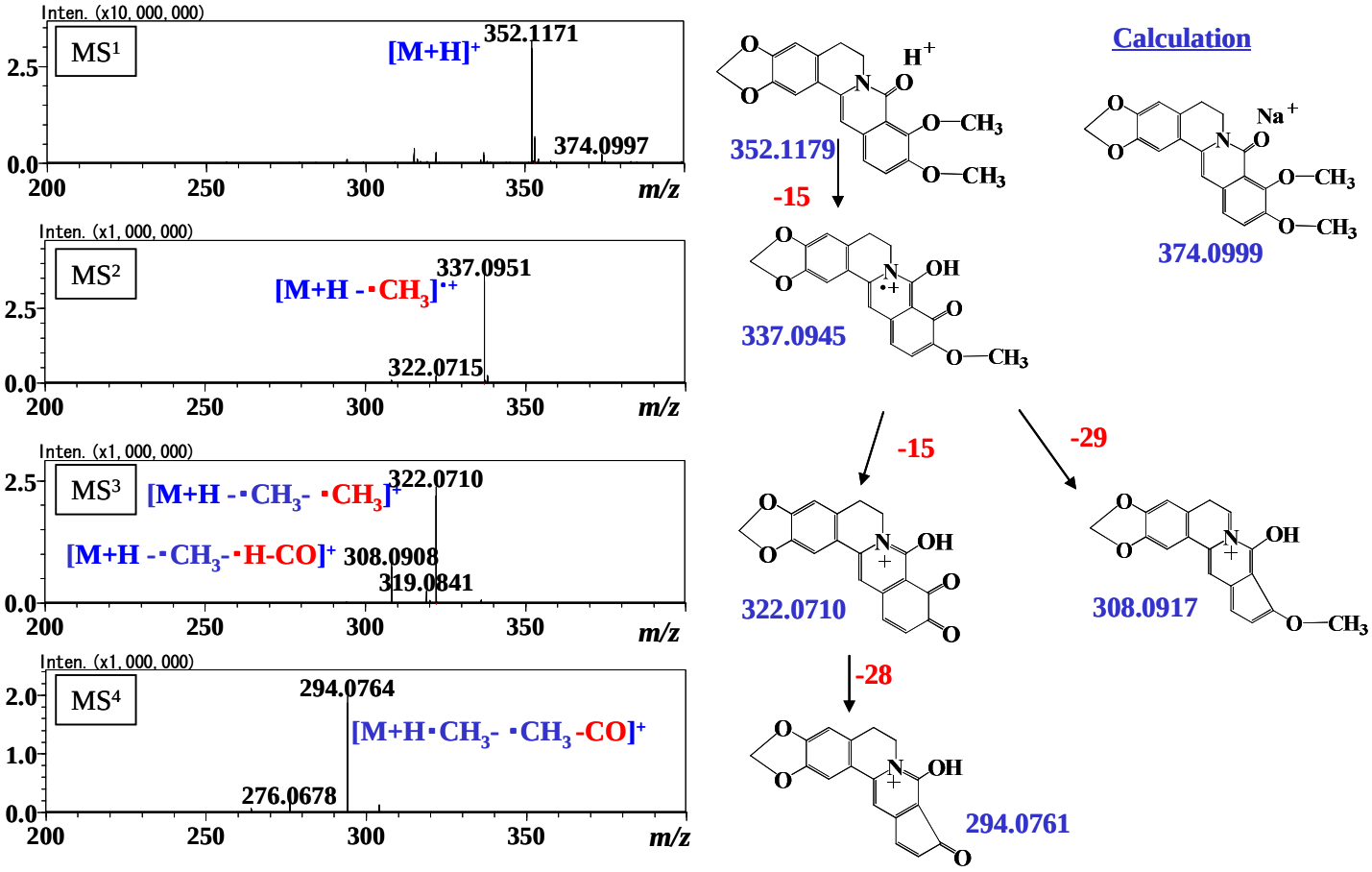


Figure 3.8 Mass spectra and proposed fragmentation pathway of 8-oxoberberine

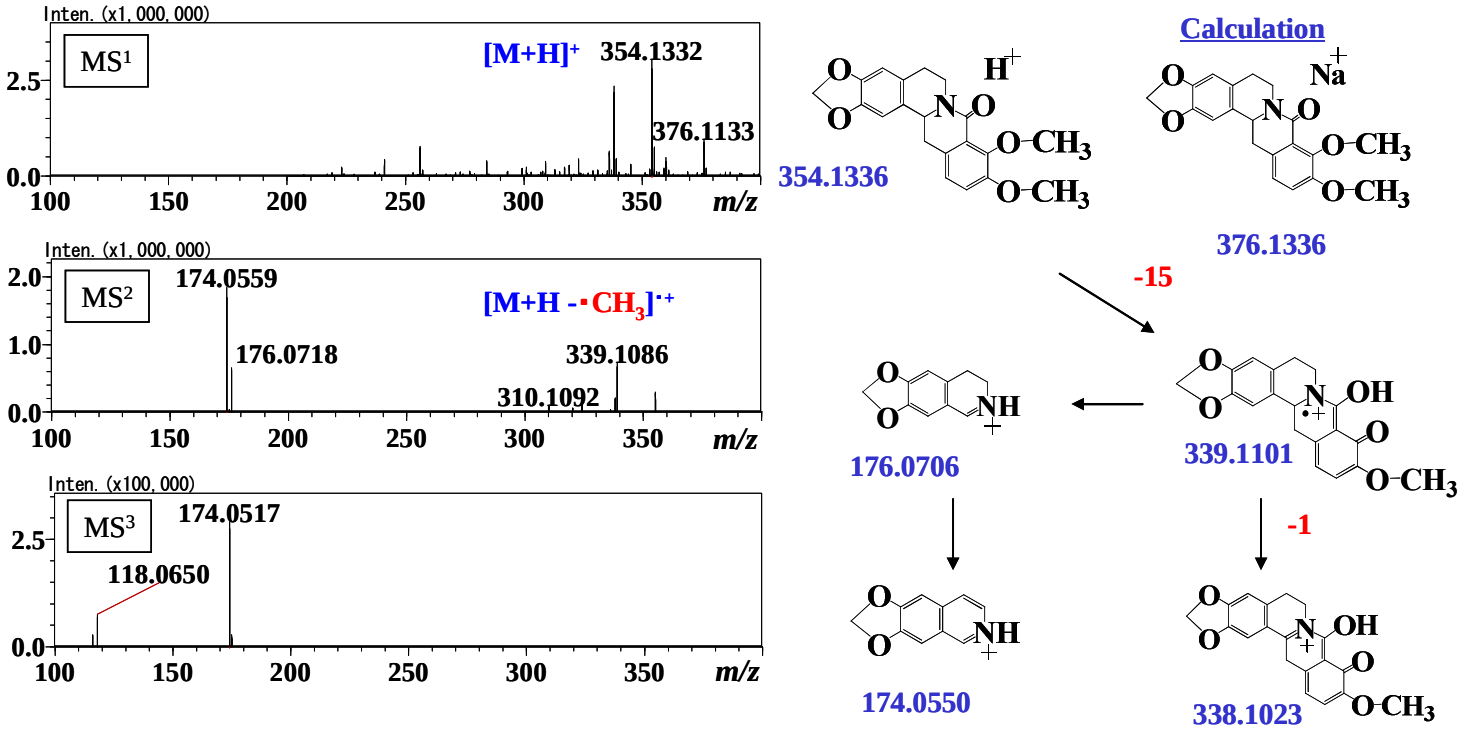


Figure 3.9 Mass spectra and proposed fragmentation pathway of 8-oxotetrahydroberberine

3.3.2.1 Quaternary Protoberberine Alkaloids

3.3.2.1.(a) Quaternary Protoberberine Alkaloids with Methoxy Groups at C-9 and C-10

Compounds 7, 9, 11, 16, 18, and 19 exhibited maximum absorption at 4 wavelength ranges, namely, 220–240 nm, 260–280 nm, 340–350 nm, and 410–430 nm, which suggested that they are quaternary protoberberine alkaloids[33]. Due to the low concentration of compound 12, the UV absorption of this compound could not be clearly monitored. However, it was characterized as a quaternary protoberberine compound from the fragmentation data (**Table 3.3**). The fragment ions of BER, PAL, JAT (**Table 3.2**) produced $[M - \cdot CH_3]^{+\cdot}$, $[M - \cdot CH_3 - \cdot H]^+$, $[M - \cdot CH_3 - \cdot H - CO]^+$ in MS^2 and $[M - \cdot CH_3 - \cdot H - 2H]^+$ in MS^3. The fragmentation pathways of these 3 compounds are proposed in **Scheme 1 (A)**. These fragmentation data indicated that the 3 alkaloids lost a methyl radical in MS^2 and then produced fragment A after the loss of the H atom and the ring-closure reactions in MS^3. Since the formation of such methylenedioxy group occurs only when 2 methoxy groups are vicinal to each other, fragment A can be used as a diagnostic ion[52]. Although the exact position of methoxy groups cannot be fully elucidated by MS^n analysis, methoxy groups are rarely found at positions other than C-2, C-3, C-9, C-10, and C-11[33]. The methoxy groups at C-9 and C-11 can be distinguished on the basis of differences in their UV-adsorption characteristics[33]. The retention times and the UV and mass spectra of compounds 16, 18, and 19 were identical to those of the standard compounds JAT, BER, and PAL. Consequently, compounds 16, 18, and 19 were identified as JAT, BER, and PAL, respectively. Compounds 7, 9, 11, and 12 followed the above mentioned fragmentation pathways, which indicated that they have 2 methoxy groups at C-9 and C-10. The mass of the $[M]^+$ ions of compounds 7, 9, and 12 were 16 Da (O) higher than the mass of the $[M]^+$ ions of JAT, BER, and PAL. These results suggest that these 3 compounds could be derivatives of JAT, BER, and PAL with an additional hydroxy group. In addition, the loss of water molecule from these compounds in MS^4 was also observed. The additional hydroxy groups was hypothesized to be at the C-13 position. This inference is based on the reasoning that the presence of the hydroxy group at C-5 would reduce the possibility of the formation of fragment A, while the presence of the hydroxy group at any other position could affect the formation of the fragment ions [52].

Thus, compounds 7, 9, and 12 were tentatively identified as 13-hydroxyjatrorrhizine, 13-hydroxyberberine, and 13-hydroxypalmatine, respectively. The lower elution times of compounds 7, 9, and 12 suggested that these compounds were more polar than JAT, BER, and PAL, and supported our hypothesis. The mass of the $[M]^+$ ion of compound 11 was 14 Da (CH_2) lower than the mass of the $[M]^+$ ion of BER; moreover, compound 11 generated fragment A, indicating the presence of 2 methoxy groups at C-9 and C-10. Therefore, compound 11 was identified as demethyleneberberine.

3.3.2.1.(b) Quaternary Protoberberine Alkaloids with Single Hydroxy Groups at C-9 or C-10

Compound 14 was characterized as quaternary protoberberine alkaloid, because it showed maximum UV absorption at 4 wavelength ranges, similar to that observed in case of BER. However, its fragmentation pathway was not similar to the pathway of the quaternary alkaloids with 2 methoxy groups at C-9 and C-10. Compounds 10, 13, and 14 produced $[M - \cdot CH_3]^{+\cdot}$ in MS^2, $[M - \cdot CH_3 - CO]^{+\cdot}$, $[M - \cdot CH_3 - CO - \cdot H]^+$, and $[M - \cdot CH_3 - \cdot CH_3]^+$ in MS^3. The generation of a single ion in MS^2 indicated that the $[M - \cdot CH_3]^{+\cdot}$ ion was highly stable. In addition, fragment A was not observed among the fragment ions of these compounds. Thus, these compounds were considered to be quaternary protoberberine alkaloids with a single hydroxy group at C-9 or C-10. Compound 14 generated an $[M]^+$ ion at *m/z* 322.1068, suggesting that it is thalifendine or its derivative, berberrubine. To characterize the differences in the behaviors of thalifendine and berberrubine, berberrubine was synthesized by using the method described by Y. Qin *et al.* [63]. The synthesized dark-red powder was dissolved in methanol (50 μg/ml) and analyzed using the same procedure. The berberrubine peak was the biggest peak (86% of total peak area; 345 nm) with a retention time of 25.34 min, which was close to the BER peak. The $[M]^+$ ion at *m/z* 322.1074 was observed as a base peak in the full-scan mode (MS^1) and at *m/z* 307.0852 in MS^2. In addition, a small peak (0.2% of total peak area) for an $[M]^+$ at *m/z* 322.1073 with a retention time of 21.64 min was observed, which was identical to the retention time of compound 14. The small peak was considered to be thalifendine produced as a byproduct from the reaction. Thus, compound 14 was identified as thalifendine[25]. The $[M]^+$ ion of compound 10 had *m/z* value of 310.1074, which was 14 Da (CH_2) lower than that of demethyleneberberine.

Compound 10 followed a primary fragment pathway that was similar to that of thalifendine. Therefore, compound 10 was identified as demethylenethalifendine or demethyleneberberrubine. Both demethylenethalifendine and demethyleneberberrubine are novel natural products that have been reported for the first time in this study. The molecular weight of compound 13 was 2 Da (2H) higher than that of thalifendine. These findings indicated that compound 13 had a methoxy and a hydroxy group at C-2 or C-3 and C-9 or C-10. Therefore, compound 13 was identified as dehydrodiscretamine or its derivative, e.g., jatrorrhizubine or stepharanine. The proposed fragmentation pathways of the quaternary protoberberines with OH groups at C-9 or C-10 are shown in **Scheme 1(B)**.

3.3.2.1.(c) Quaternary Protoberberine Alkaloids with Methoxy Groups at C-10 and C-11

The peaks for compounds 17 and 20 appeared close to those of JAT and PAL, respectively. Their $[M]^+$ ions (*m/z* 338.1384 and 352.1547) were identical to those of JAT and PAL. However, their UV spectra exhibited strong maximum absorption at 287 nm and shoulder at 310 nm, which is significantly different from the spectra of quaternary protoberberines with 2 methoxy groups at C-9 and C-10 (**Figure 3.10**). This spectrum is considered to be the characteristic of pseudoprotoberberine compounds with a methoxy group at C-11 instead of C-9[33]. Therefore, compound 17 was proposed to be pseudojatrorrhizine and compound 20 was proposed to be pseudopalmatine. Their fragment ions are similar to those of quaternary protoberberines with 2 methoxy groups at C-9 and C-10. However, there were some differences; for example, PAL and pseudopalmatine showed different base peaks in MS^2, and pseudojatrorrhizine generated an ion at *m/z* 295.1204 in MS^3, which was not observed in case of jatrorrhizine (**Table 3.3**). There are very few reports of online analysis of pseudoprotoberberine in the literature[64].

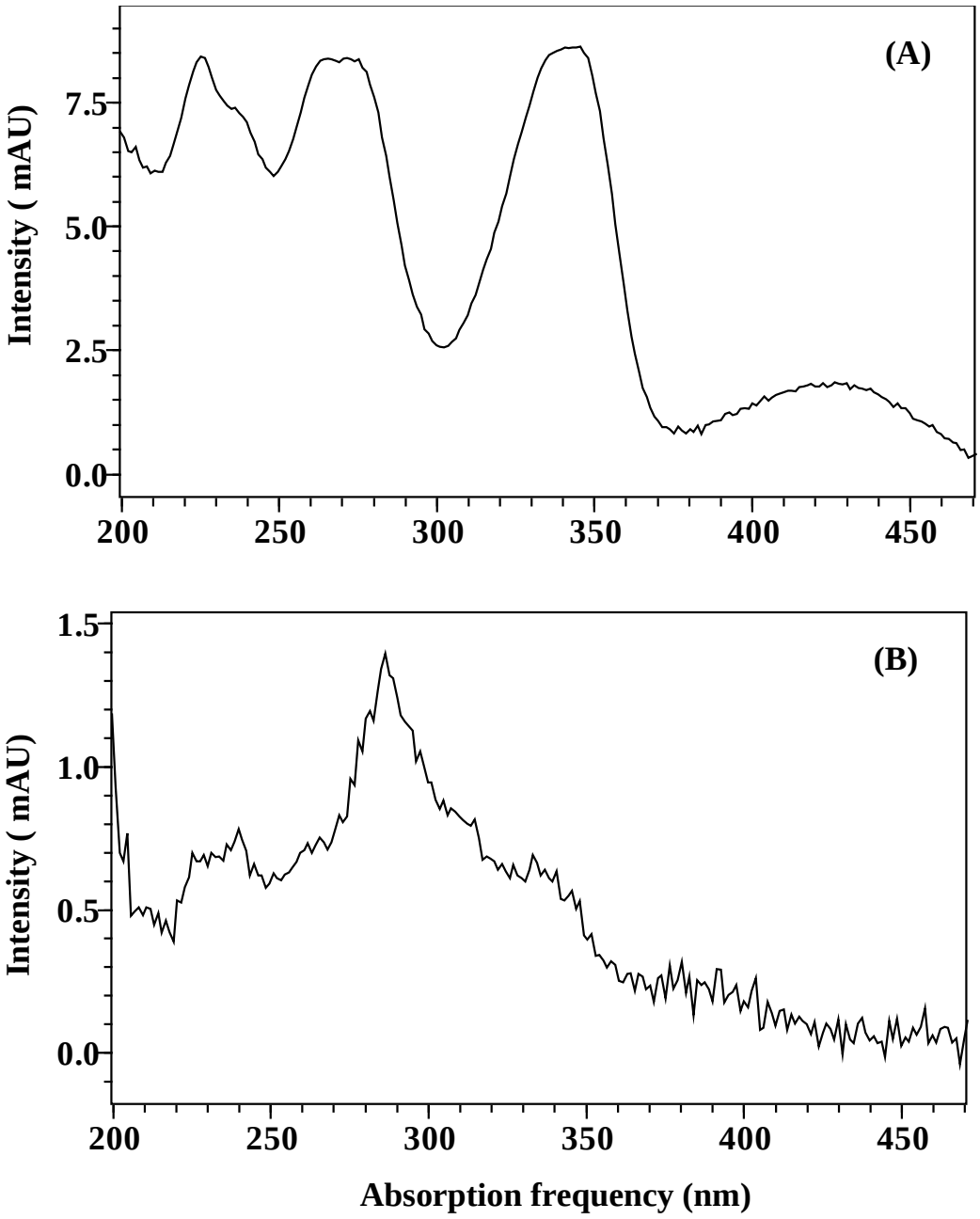
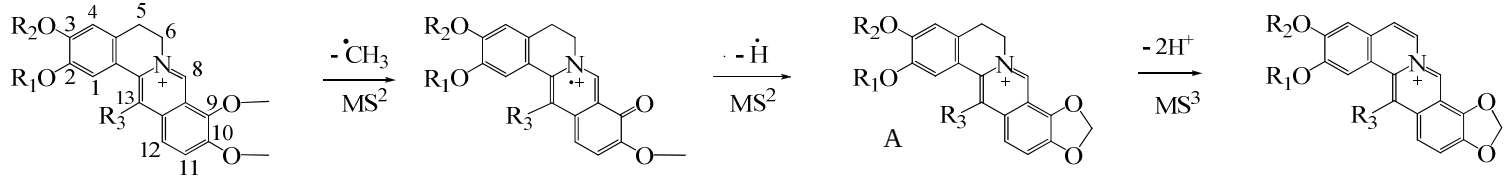


Figure 3.10 Comparison of UV adsorption characteristic of jatrorrhizine and pseudojatrorrhizine. (A) Jatrorrhizine;(B) Pseudojatrorrhizine

(A)

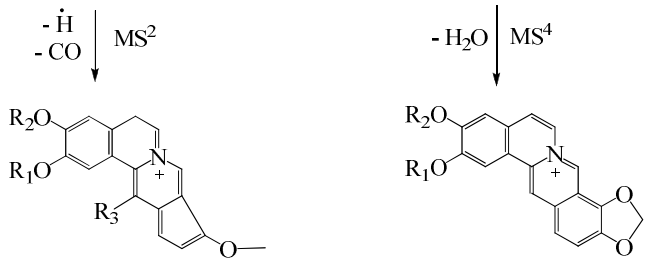


354.13 (7; R_1=CH_3, R_3=OH)
352.11 (9; R_1-R_2=CH_2, R_3=OH)
324.12 (11; R_1=R_2=OH, R_3=H)
368.15 (12; R_1=R_2=CH_3, R_3=OH)
338.13 (16; R_1=CH_3, R2=R_3=H)
336.12 (18; R_1-R_2=CH_2, R_3=H)
352.15 (19; R_1=R_2=CH_3, R_3=H)

339.10 (7; R_1=CH_3, R_3=OH)
337.09 (9; R_1-R_2=CH_2, R_3=OH)
309.09 (11; R_1=R_2=OH, R_3=H)
353.12 (12; R_1=R_2=CH_3, R_3=OH)
323.11 (16; R_1=CH_3, R2=R_3=H)
321.09 (18; R_1-R_2=CH_2, R_3=H)
337.13 (19; R_1=R_2=CH_3, R_3=H)

338.10 (7; R_1=CH_3, R_3=OH)
336.08 (9; R_1-R_2=CH_2, R_3=OH)
308.09 (11; R_1=R_2=OH, R_3=H)
352.11 (12; R_1=R_2=CH_3, R_3=OH)
322.10 (16; R_1=CH_3, R2=R_3=H)
320.08 (18; R_1-R_2=CH_2, R_3=H)
336.12 (19; R_1=R_2=CH_3, R_3=H)

336.08 (7; R_1=CH_3, R_3=OH)
334.06 (9; R_1-R_2=CH_2, R_3=OH)
306.07 (11; R_1=R_2=OH, R_3=H)
350.00 (12; R_1=R_2=CH_3, R_3=OH)
320.08 (16; R_1=CH_3, R2=R_3=H)
318.07 (18; R_1-R_2=CH_2, R_3=H)
334.10 (19; R_1=R_2=CH_3, R_3=H)



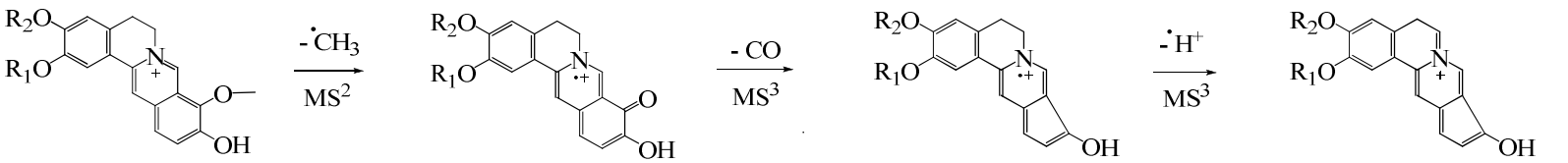
310.10 (7; R_1=CH_3, R_3=OH)
308.09 (9; R_1-R_2=CH_2, R_3=OH)
280.09 (11; R_1=R_2=OH, R_3=H)
324.12 (12; R_1=R_2=CH_3, R_3=OH)
294.11 (16; R_1=CH_3, R2=R_3=H)
292.09 (18; R_1-R_2=CH_2, R_3=H)
308.12 (19; R_1=R_2=CH_3, R_3=H)

320.08 (7; R_1=CH_3, R_2=H)
318.07 (9; R_1-R_2=CH_2)

334.10 (12; R_1=R_2=CH_3)

Scheme 1 Proposed fragmentation pathways of quaternary protoberberine alkaloids. (A) Compounds with 2 methoxy groups at C-9 and C-10; (B) Compounds with one hydroxy group at C-9 or C-10.

(B)



310.10 (10; R_1=R_2=H)
324.12 (13; R_1=CH_3, R_2=H)
322.10 (14; R_1-R_2=CH_2)

295.08 (10; R_1=R_2=H)
309.09 (13; R_1=CH_3, R_2=H)
307.08 (14; R_1-R_2=CH_2)

- ·CH_3 | MS^3

294.07 (13)
292.05 (14)

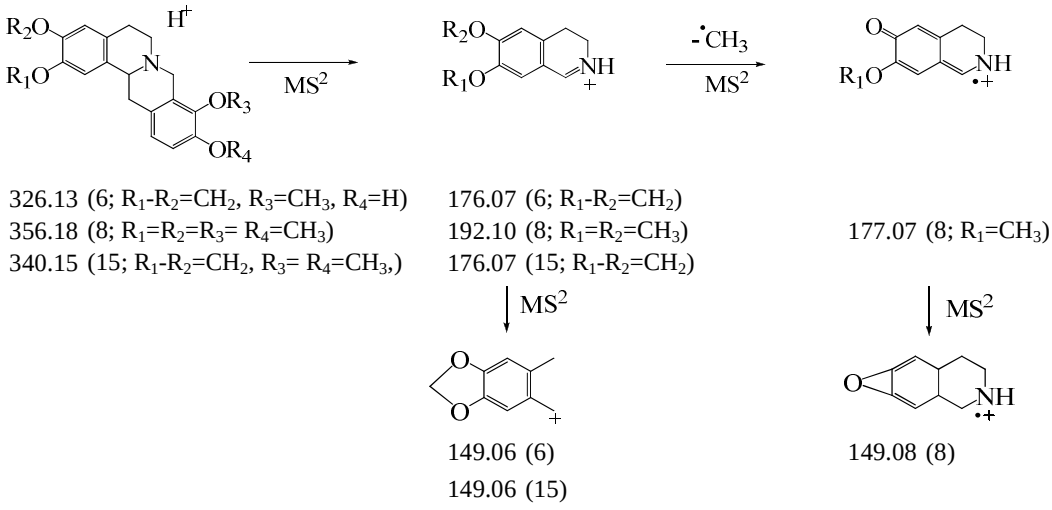
267.08 (10; R_1=R_2=H)
281.10 (13; R_1=CH_3, R_2=H)
279.08 (14; R_1-R_2=CH_2)

266.08 (10; R_1=R_2=H)
280.08 (13; R_1=CH_3, R_2=H)
278.08 (14; R_1-R_2=CH_2)

Scheme 1 *(Continued).*

3.3.2.2 Tetrahydroprotoberberine Alkaloids

Compounds 6, 8, and 15 generated protonated molecules ($[M + H]^+$) in the full-scan mode. The retention time, $[M + H]^+$, and the fragments of compound 15 (*m/z* 340.1546) were identical to those of tetrahydroberberine, which underwent the Retro-Diels-Alder (RDA) fragmentation reaction to produce an iminium ion at *m/z* 176.0706. Therefore, compound 15 was identified as tetrahydroberberine[26]. In case of compound 8, the mass of the $[M + H]^+$ ion at *m/z* 356.1852 and the fragment ion at *m/z* 192.1017 were 16 Da (CH_4) higher than the corresponding mass of tetrahydroberberine, and these mass matched with the corresponding mass of tetrahydropalmatine, which has been previously reported in *C. fenestratum*[45]. Therefore, compound 8 was identified as tetrahydropalmatine. The mass of the $[M + H]^+$ ion of compound 6 was 14 Da (CH_2) lower than the mass of the $[M + H]^+$ ion of tetrahydroberberine. Compound 6 generated fragment ions at *m/z* 176.0706 and *m/z* 149.0597, which were identical to the fragment ions of tetrahydroberberine, suggesting that it contains a methylenedioxy group at C-2 and C-3. Therefore, it was tentatively identified as tetrahydrothalifendine or tetrahydroberberrubine. The fragmentation pathways of tetrahydroprotoberberine are proposed in **Scheme 2**.



Scheme 2 Proposed fragmentation pathways of tetrahydroprotoberberines.

3.3.2.3 8-Oxoprotoberberine Alkaloids

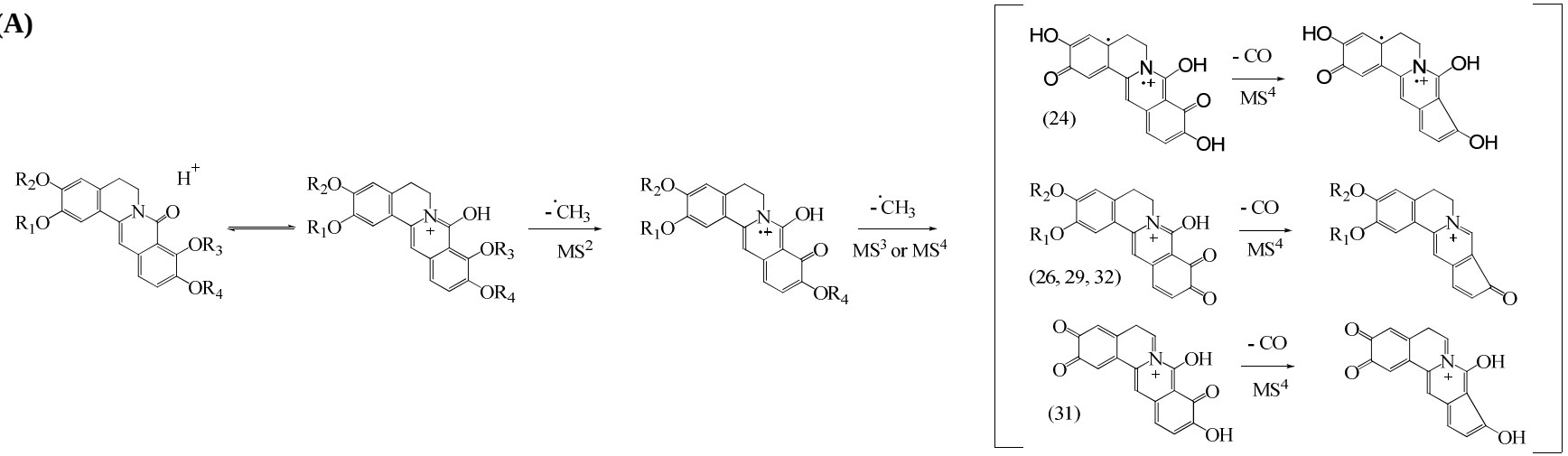
3.3.2.3.(a) 8-Oxodihydroprotoberberine Alkaloids

Compounds 24, 26, 29, 31, and 32 have similar UV absorption characteristics, with maximum absorption at wavelength ranges of 200–205 nm, 220–230 nm, 330–345 nm, and 365–370 nm. Compound 32 generated an $[M + H]^+$ ion at *m/z* 352.1171 (molecular formula, $C_{20}H_{17}NO_5$), and the fragments of this compound were identical to those of 8-oxoberberine. Therefore, compound 32 was identified as 8-oxoberberine[25]. The fragmentation patterns of compound 24 (*m/z*, 340.1173), 26 (*m/z*, 354.1324), 29 (*m/z*, 368.1492), and 31 (*m/z*, 338.1027) were similar to that of 8-oxoberberine. These findings indicated that the compounds 24, 26, 29, and 31 are 8-oxodihydroprotoberberine alkaloids. Compounds 26 and 29 generated $[M+H-{\cdot}CH_3]^{+\cdot}$ and $[M + H - {\cdot}CH_3 - {\cdot}H - CO]^+$ in MS^2 , $[M + H - {\cdot}CH_3 - {\cdot}CH_3]^+$ in MS^3 , and $[M + H - {\cdot}CH_3 - {\cdot}CH_3 - CO]^+$ in MS^4 . The *m/z* values of the $[M + H]^+$ ion and the corresponding fragment ions of compound 26 and 29 were 2 Da (2H) and 16 Da (CH_4) higher than the corresponding values for 8-oxoberberine. Therefore, compound 29 was identified as 8-oxopalmatine[25] and compound 26 was tentatively characterized as 8-oxojatrorrhizine or 8-oxodihydrocolumbamine (different positions of methoxy and hydroxy group at C-2 and C-3). Compound 26 was considered to be a novel natural product. The oxodihydroprotoberberines with methoxy groups at C-9 and C-10 showed loss of water molecule in MS^2. The fragments of compounds 24 (*m/z* 340.1173) and 31 (*m/z* 338.1027) showed only $[M + H - {\cdot}CH_3]^{+\cdot}$ ions at *m/z* 325.0951 and *m/z* 323.0784, respectively, in MS^2. These fragments were observed to be stable and were difficult to fragment further, indicating that they have only 1 methoxy group at C-9 or C-10. The molecular weight of compound 31 was 14 Da (CH_2) lower than that of 8-oxoberberine. Therefore, compound 31 was tentatively identified as 8-oxothalifendine. The molecular weight of compound 24 was 2 Da (2H) lower than that of compound 31. Therefore, it was tentatively identified as 8-oxodehydrodiscretamine or a derivative of 8-oxodehydrodiscretamine with a methoxy group at C-9 or C-10 and another one at C-2 or C-3. $[M + Na]^+$ was generated by all the 8-oxoprotoberberines (**Table 3.2**). The fragmentation pathways of 8-oxodihydroprotoberberine are proposed in **Scheme 3 (A)**.

3.3.2.3.(b) 8-Oxotetrahydroprotoberberine Alkaloids

Compounds 23, 25, 27, 28, and 30 showed similar fragmentation patterns. The fragment ions at *m/z* 176.07, 178.08, and 192.10, which were iminium ions with methylenedioxy groups, methoxy and hydroxy groups, and dimethoxy groups at C-2 and C-3, suggested the possibility of ring cleavage from the protoberberine skeleton. The fragmentation patterns of these compounds were similar to those of tetrahydroprotoberberine compounds, but $[M + H - \cdot CH_3]^{+\cdot}$, $[M + H - \cdot CH_3 - \cdot CH_3]^+$, and $[M + H - \cdot CH_3 - \cdot H]^+$ were also observed. The formation of these additional ions could be attributed to the effect of the oxygen atom attached at C-8, which made cleavage more difficult than that in case of tetrahydroprotoberberine. Therefore, compounds 23, 25, 27, 28, and 30 were considered to be 8-oxotetrahydroprotoberberine alkaloids. The iminium ions of compounds 28 and 30 generated ions at *m/z* 176.0706 and *m/z* 176.0718, suggesting that they had methylenedioxy groups at C-2 and C-3. The $[M + H]^+$ ions of compounds 28 and 30, which were observed at *m/z* 340.1188 ($C_{19}H_{17}NO_5$) and *m/z* 354.1332 ($C_{20}H_{19}NO_5$) were identical to those of 8-oxotetrahydrothalifendine and 8-oxotetrahydroberberine (8-oxocanadine), which have been previously isolated from *C. fenestratum* [25, 26]. Compounds 23 and 25 may possess hydroxy group and methoxy group at C-2 or C-3 due to their iminium ions, which were observed at *m/z* 178.0874 and *m/z* 178.0873. In case of compound 25, the $[M + H]^+$ ion observed at *m/z* 356.1492 was identical to the $[M + H]^+$ ion of 8-oxoisocorypalmine, which has been reported in *C. fenestratum* [15]. Compound 23 was characterized as 8-oxodiscretamine or a derivative of 8-oxodiscretamine with a methoxy group at C-9 or C-10. There are no reports of 8-oxodiscretamine alkaloids in literature. Therefore, compound 23 is considered to be a new compound. Compound 27 ($C_{21}H_{23}NO_5$) was identified as 8-oxotetrahydropalmatine. The proposed fragmentation pathways of 8-oxotetrahydroprotoberberines are summarized in **Scheme 3 (B)**.

(A)

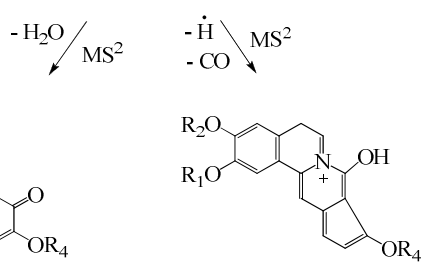


340.11 (24; R_1=R_3=CH_3, R_2=R_4=H)
354.13 (26; R_1=R_3=R_4=CH_3, R_2=H)
368.14 (29; R_1=R_2=R_3=R_4=CH_3)
338.10 (31; R_1-R_2=CH_2, R_3=CH_3, R_4=H)
352.11 (32; R_1-R_2=CH_2, R_3=R_4=CH_3)

325.09 (24; R_1=CH_3, R_2=, R_4=H)
339.11 (26; R_1=R_4=CH_3, R_2=H)
353.12 (29; R_1=R_2=R_4=CH_3)
323.08 (31; R_1-R_2=CH_2, R_4=H)
337.09 (32; R_1-R_2=CH_2, R_4=CH_3)

310.07 (24)
324.08 (26; R_1=, R_2=H)
338.10 (29; R_1=R_2=CH_3)
308.05 (31)
322.07 (32; R_1-R_2=CH_2)

282.07 (24)
296.09 (26; R_1=, R_2=H)
310.10 (29; R_1=R_2=CH_3)
280.05 (31)
294.07 (32; R_1-R_2=CH_2)

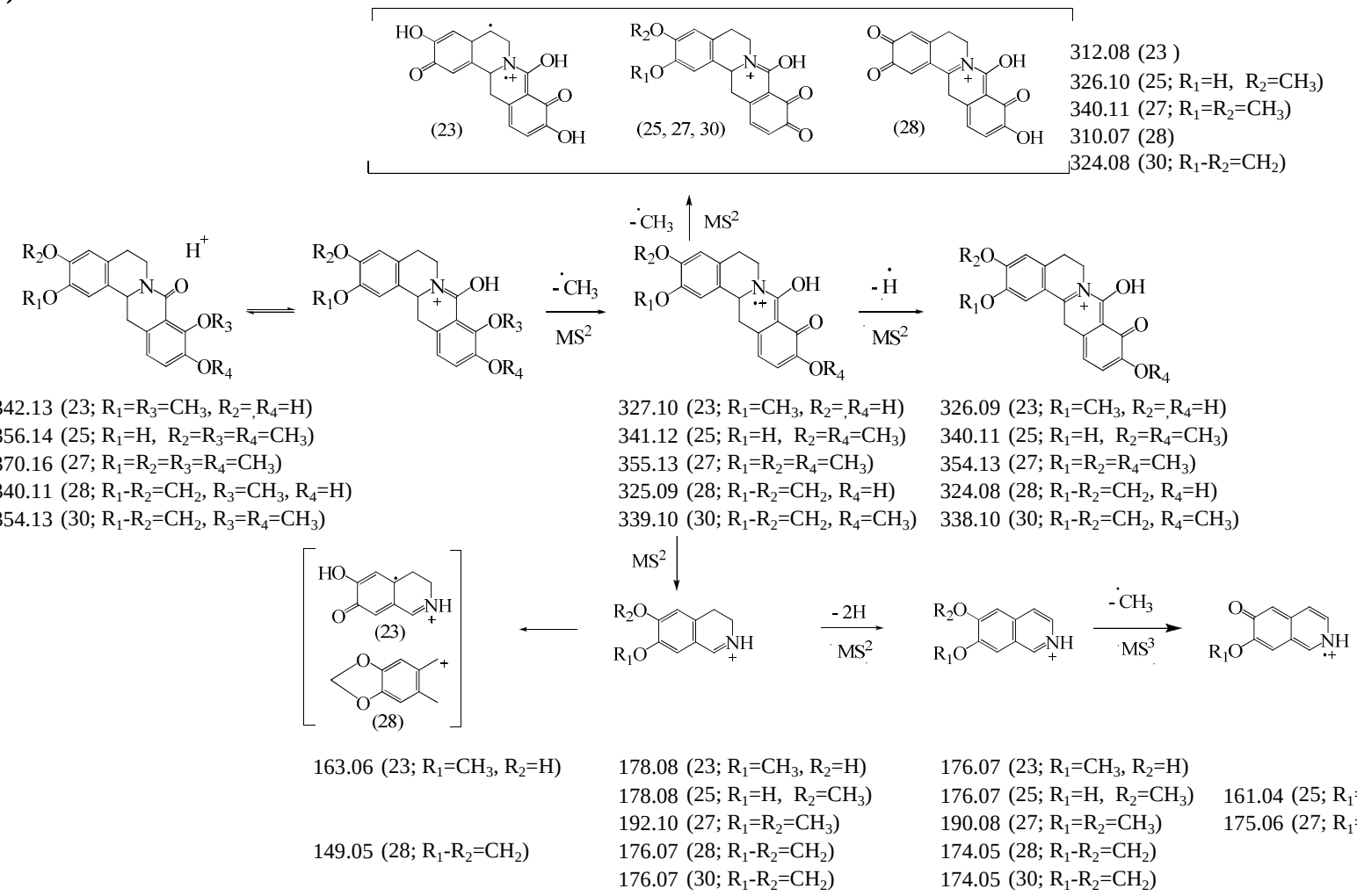


321.10 (26; R_1=R_4=CH_3, R_2=H)
335.11 (29; R_1=R_2=R_4=CH_3, R_2=H)
319.08 (32; R_1-R_2=CH_2, R_4=CH_3)

310.10 (26; R_1=R_4=CH_3, R_2=H)
324.12 (29; R_1=R_2=R_4=CH_3, R_2=H)
308.09 (32; R_1-R_2=CH_2, R_4=CH_3)

Scheme 3 Proposed fragmentation pathways of 8-oxoprotoberberines. (A) 8-oxodihydroprotoberberines; (B) 8-oxotetrahydroprotoberberines.

(B)



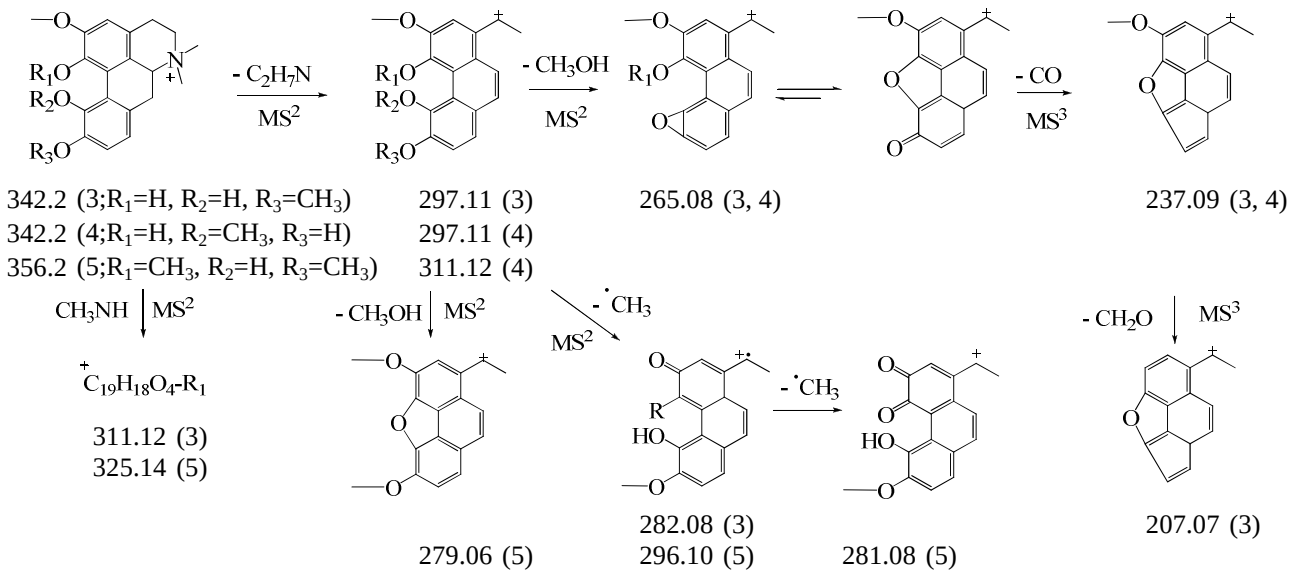
Scheme 3 *(Continued).*

3.3.2.4 Aporphine Alkaloids, Benzylisoquinoline Alkaloids, and a Steroid Compound

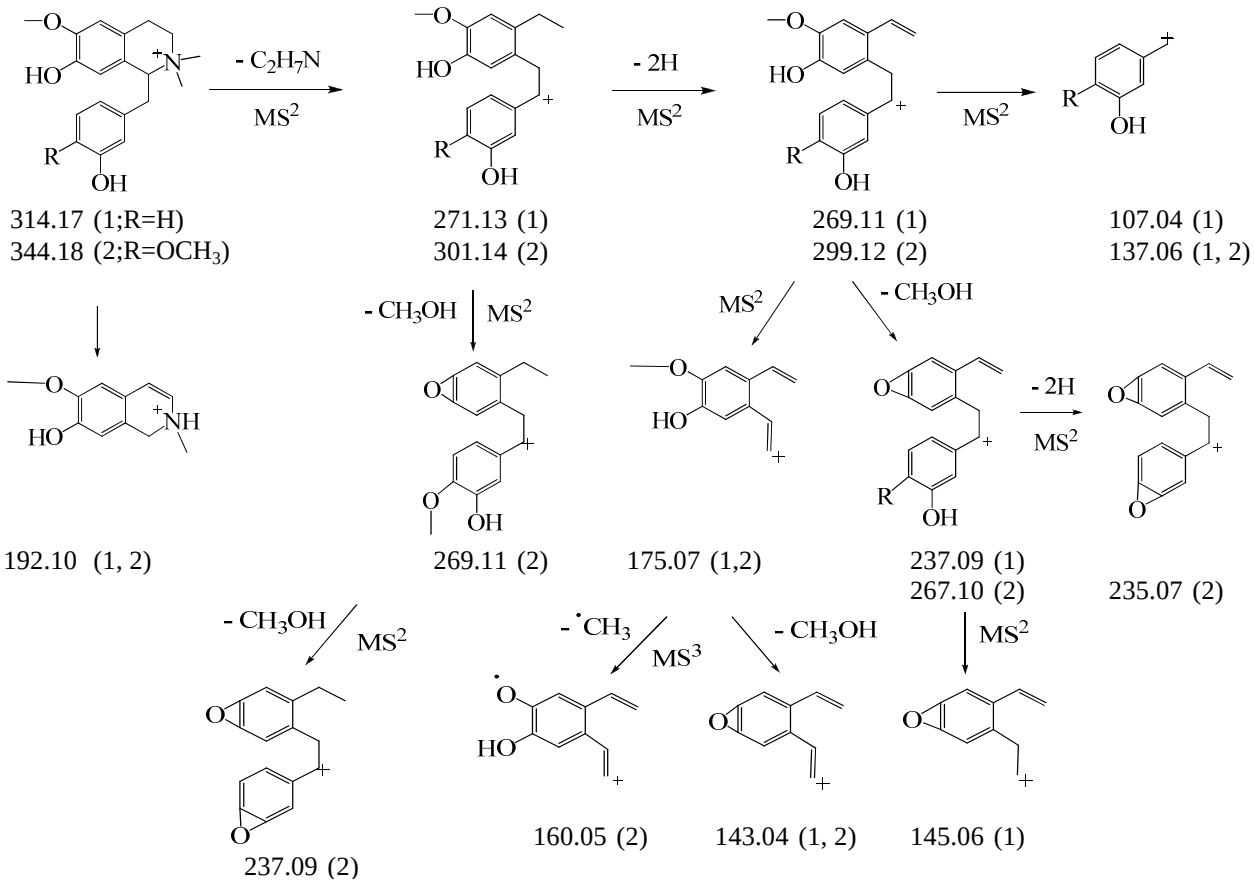
Aporphine alkaloids and steroid compounds have been suggested to be present in *C. fenestratum.* However, only *N,N*-demethyllindcarpine (MW 341), which is an aporphine alkaloid, has been actually reported in literature[27], and there are no reports of identification of steroid compounds. As shown in **Table 3.3**, compounds 3, 4, 5 showed molecular ions at *m/z* 342.1075, *m/z* 342.1686, and *m/z* 356.1858 and generated fragment ions $[M - (CH_3)_2NH]^+$ and $[M - (CH_3)_2NH - CH_3]^+$, and $[M - (CH_3)_2NH - CH_3OH]^+$ in MS^2. These findings are considered to be a characteristic of aphorphine alkaloids. The UV absorption spectra and the mass fragments of compound 3 and 5 were similar to those of magnoflorine and menisperine[52]. Thus, compounds 3 and 5 were tentatively identified as magnoflorine and menisperine, respectively. Compound 4 generated $[M]^+$ ions and fragments similar to those of magnoflorine, indicating that it is a derivative of magnoflorine, namely, *N,N*-demethyllindcarpine. Therefore, compound 4 was identified as *N,N*-demethyllindcarpine, which has been previously reported in *C. fenestratum*. The fragmentation pathways of aporphine alkaloids are proposed in **Scheme 4**.

Compound 2 produced $[M]^+$ ions at *m/z* 344.1846 in MS^1 and the fragment ion $[M - (CH_3)_2NH]^+$ as a major peak in MS^2. The formation of fragment ions at *m/z* 175.0748, *m/z* 151.0781, and *m/z* 143.0485 suggested a split in the skeleton structure. Compound 2 is considered to be a benzylisoquinoline alkaloid. The UV and mass spectra of this compound are similar to those of tembetarine[52]. Therefore, it was tentatively identified as tembetarine. The $[M]^+$ ion and principal fragments of compound 1 were 30 Da (CH_2O) lower than that of tembetarine. It was considered to be a derivative of compound 2. The fragmentation pathways of benzylisoquinoline alkaloids are proposed in **Scheme 5**.

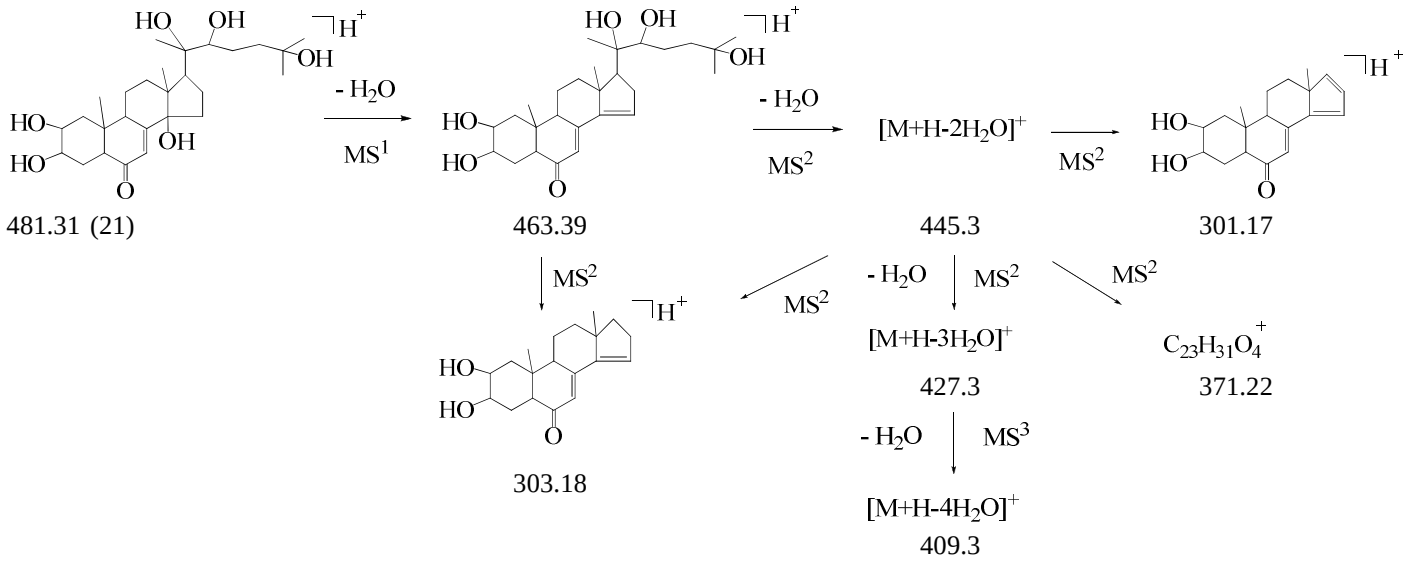
The retention time, UV spectra, and fragmentation pattern of compound 21 were identical to those of 20-hydroxyecdysone (**Table 3.2**), which lost up to 3 water molecules in MS^1. Accordingly, compound 20 was identified as 20-hydroxyecdysone. The fragmentation pathway is proposed in **Scheme 6**.



Scheme 4 Proposed fragmentation pathways of aporphine alkaloids



Scheme 5 Proposed fragmentation pathways of benzylisoquinoline alkaloids

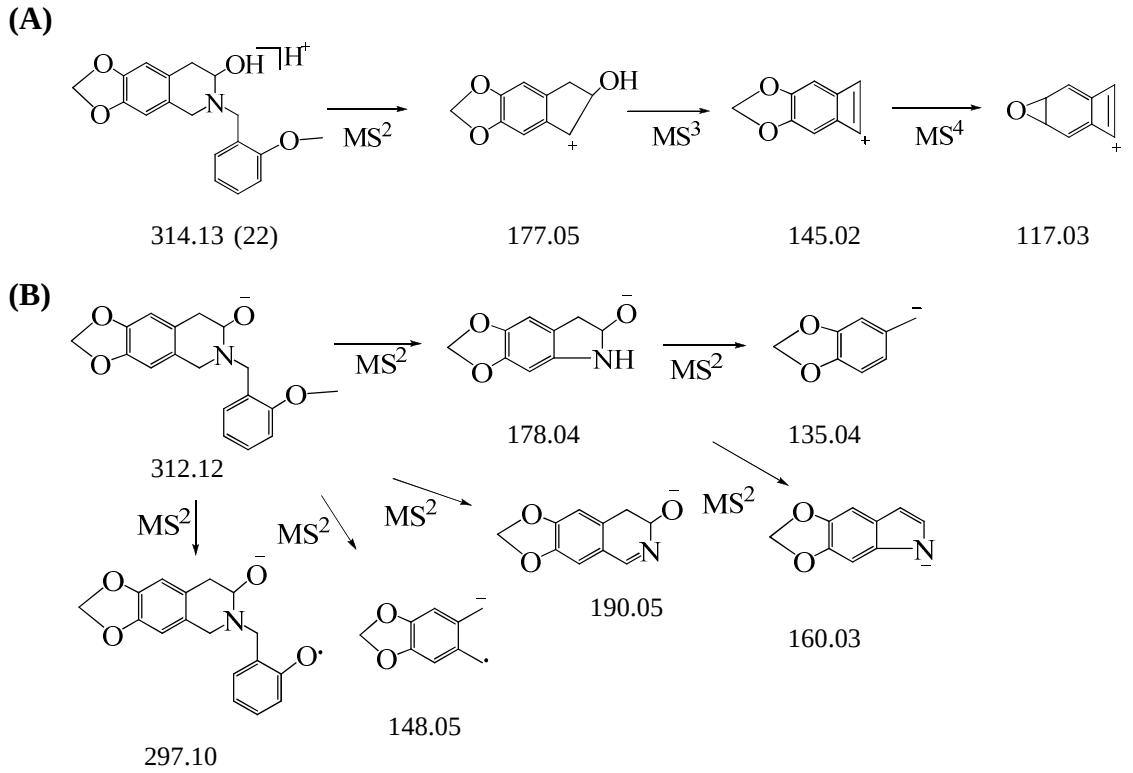


Scheme 6 Proposed fragmentation pathways of 20-hydroxyecdysone

3.3.2.5 Structural Identification of Compound 22 (1,3-Dioxolo[4,5-g]isoquinolin-7-ol,5,6,7,8-tetrahydro-6-[(methoxyphenyl)methyl]-)

Figure 3.11 shows the MS^n mass spectra of compound 22 in the positive and negative modes. In the full-scan mode, the spectrum showed the presence of an ion at *m/z* 314.1374 in the positive mode and at *m/z* 312.1249 in the negative mode. Using the accurate mass calculator software, the chemical formula $C_{18}H_{19}NO_4$ was obtained for the compound at *m/z* 314.1374. The fragments of the ion at *m/z* 314.1374, which was obtained in the positive mode, showed only 2 intensity peaks at *m/z* 177.0556 ($C_{10}H_9O_3^+$) and *m/z* 145.0277 ($C_9H_5O_2^+$) in MS^2, suggesting a split in the structural skeleton. The ion at *m/z* 177.0556 produced fragment ions at *m/z* 145.0276 and *m/z* 117.0322 ($C_8H_5O^+$). Considering the compounds found in *C. fenestratum*, most of them are alkaloids that have aromatic rings. There is high possibility that the compound 22 at m/z 314.1374 has aromatic rings. The number of rings or unsaturations of a molecule or of a fragment can be obtained by using the double-bond equivalent values (DBE ($C_cH_hN_nO_oS_s$)= c – (h/2) + (n/2) +1), which is calculated from the molecular formula of the ion[65]. The odd-electron radical cations have integer DBE values, whereas even-electron ions have half integer DBE values. The fragment ion at m/z 145.0277 ($C_9H_5O_2^+$, DBE 7.5) indicates that its possible structure contains a benzene ring (DBE 4) and 2 other rings (DBE 2), one of the rings has a double bond (DBE 1). This ion suggests that it was formed by losing of CH_3OH molecule and generated a double bond from the ion at m/z 177.0556 ($C_{10}H_9O_3^+$, DBE 6.5). The DBE 6.5 of $C_{10}H_9O_3^+$ supports the hypothesis that compound 22 has a benzene ring (DBE 4) and other 2 rings (DBE 2). These fragments indicated that the compound has a benzene ring connected to a ring from a methylenedioxy group and a ring that has a hydroxy group. In contrast, fragmentation of the ion at *m/z* 312.1249 in the negative mode principally yielded ions at *m/z* 178.0492 ($C_9H_8NO_3^-$) and *m/z* 135.0443 ($C_8H_7O_2^-$). The formula $C_9H_8NO_3^-$ indicated that compound 22 was a tetrahydroisoquinoline compound. Therefore, compound 22 was tentatively identified as a tetrahydroisoquinoline compound with a hydroxy group at C-3. The hydroxy group was considered to be at the C-3 position, because the possibility of the formation of ions at *m/z* 177.0556, *m/z* 145.0277, and *m/z* 117.0322 is lower if it was at any other position. The fragmentation pathway is proposed in **Scheme 7**. Although the position of the methoxy group in the phenyl ring could not be

determined, compound 22 was considered to be a novel natural product.



Scheme 7 Proposed fragmentation pathways of compound 22. (A) Positive mode; (B) Negative mode.

Among the 32 compounds characterized in this study, 12 compounds, namely, BER, PAL, JAT, thalifendine, tetrahydroberberine, tetrahydropalmatine, *N*,*N*-demethyllindcarpine, 8-oxoberberine, 8-oxopalmatine, 8-oxotetrahydrothalifendine, 8-oxoisocorypalmine, and 8-oxotetrahydroberberine have been isolated previously[25-27]. The other 20 compounds have been reported for the first time from *C. fenestratum* in this study. To our knowledge, compounds 10, 22, 23, and 26 are the novel natural products tentatively identified in this study. The structural conformation of these compounds will be confirmed by NMR spectroscopy in future work. However, some compounds that have been reported to be present in *C. fenestratum*, such as oxothaicanine (MW 385)[25] and β-sitosterol (MW 413), were not found, although the standard sample of β-sitosterol could be detected at a retention time of 38.76 min under the experimental conditions used in this study.

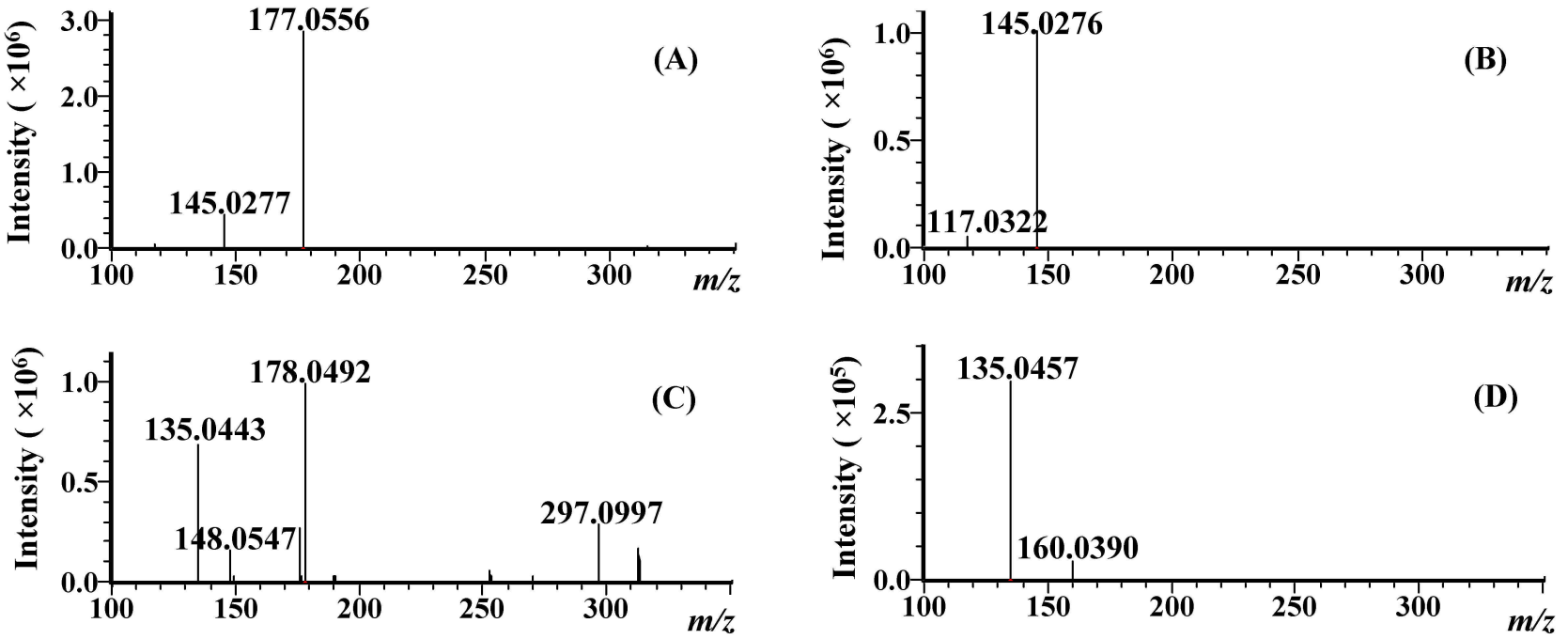


Figure 3.11 **MS^n data of peak No. 22. (A) MS^2 of ion at *m/z* 314.1374 ((+)ESI); (B) MS^3 of ion at 177.0556 ((+)ESI); (C) MS^2 of ion at *m/z* 312.1249 ((–)ESI); (D) MS^3 of ion at *m/z* 178.0492 ((–)ESI).**

3.4 Conclusions

In this study, the method for simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *C. fenestratum* by using LC/IT-TOF MS was developed. A total of 32 compounds were detected, of which 20 compounds, including 4 novel natural products, were identified or tentatively identified for the first time from *C. fenestratum*. This information may be useful for further studies on the pharmacological activities of the herb. The proposed method is an accurate and rapid method for characterizing various compounds in *C. fenestratum*. 8-oxoprotoberberines produced $[M + H]^+$ and $[M + Na]^+$ ions in MS^1 operated in the positive-ion mode. The fragmentation pathways of 8-oxoprotoberberines are different from those of quaternary protoberberines and tetrahydroprotoberberines. In addition, 8-oxotetrahydroprotoberberines generated iminium ions, which were formed by the cleavage of the protoberberine skeleton.

CHAPTER 4

QUANTIFICATION OF MAJOR PROTOBERBERINE ALKALOIDS AND A STEROID IN *C. FENESTRATUM*

4.1 Introduction

Several studies have investigated on the quantification of principle compound in *C. fenestratum*, BER, using TLC and liquid chromatography[45, 46]. The BER contents were reported from 1.0–3.5% of dry material[19, 46]. P. Rojsanga *et al*. studied berberine content in ten samples of dried stems purchased from different traditional drugstores from various parts of Thailand using TLC-densitometry[66]. Maceration with 80% ethanol, yields of the crude extract were in the range of 9.87–16.38% dry wt while berberine contents in the dried powder and in the crude extract were in the ranges of 1.71–2.89% wt./wt. and 11.84–18.45% dry wt., respectively. However, no simultaneous quantification of other multiple compounds together with berberine has been reported. In addition, the compounds in each part of the plant and the effect of plant size on the amount of the compounds have not been studied.

The objective of this study is to investigate the major compounds in each part of the plant, the variation of compounds in *C. fenestratum* collected from different six sites in Laos, the effect of plant size, and compare amount of major compounds with those of

other protoberberines containing medicinal plants such as *Coptis japonica* and *phellodendron amurense* which widely used in Japanese and Chinese herbal medicines.

4.2 Experimental

4.2.1 Materials

BER, PAL, JAT, EtOH, MeOH (LC/MS-grade), and formic acid (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd., Japan. Stock solutions of BER (150 mg/L), PAL (42 mg/L), and JAT (110 mg/L) were prepared in MeOH. All of the solutions were stored at 4°C. Standard solutions were prepared by diluting each stock solution in MeOH to a concentration of 10 to 150 mg/L (BER), 0.33 to 5.25 mg/L (PAL), and 0.69 to 11 mg/L (JAT). Ultrapure water produced by the Milli-Q Advantage vessel (Millipore, USA) was used in this study.

Six Batches of *C. fenestratum* obtained from Vientiane province,Vientiane municipality and Lunagphabang sa shown in **Table 2.1** (Chapter 2) were used in this study. Dried *C. japonica* (Lot No. 230604) and *P. amurense* (Lot No. 001205003) were purchased from a herbal medicine shop (Yatsume Kampo) in Tokyo, Japan.

4.2.2 Sample Preparation and Extraction

The plants were ground in a blender (Wonder blender WB-1; Osaka Chemical Co. Ltd., Japan), and the powder was sieved using a 60-mesh sieve to obtain a powder with particle size less than 250 µm. Extraction was performed in a Soxhlet extraction vessel by adding 0.5 g of plant powder to the extraction thimble and extracting the powder using 100 ml methanol for 11 hours. The extracted liquid was filtered using a 0.45-µm membrane filter, and the filtrate was used for LC/MS analysis.

4.2.3 Analysis

The quantification of BER, PAL, and JAT was performed using an LC/MS-2010EV apparatus (Shimadzu Corp., Japan). The separation was carried out in a ZORBAX Eclipse XDB column (3.5 μm, 2.1 × 150 mm) (Agilent Technology, Inc, USA). The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and methanol (B), was delivered at a flow rate of 0.2 mL/min by using the following gradient program: 20–32% (B) for 0–20 min, 32–70% (B) for 20–35 min, 70% (B) for 35–45 min, and 20% (B) for 45–55 min. The sample injection volume was 1 μL, and the column temperature was maintained at 40°C. The UV spectra were obtained by scanning the samples in the range of 200–470 nm, and the peaks were simultaneously determined at 249 and 345 nm. The amounts of BER, JAT, and PAL were calculated from the peak area at 345 nm and 20-hydroxyecdysone was calculated from peak area at 249 nm using the calibration line.

4.3 Results and Discussion

4.3.1 Compounds in Each Part of *C. fenestratum*

Figure 4.1 shows the total ion chromatogram of extract in each part of *C. fenestratum*. More than 20 peaks were obtained by total ion chromatogram of stem extract. The molecular weight of each compound was confirmed by a mass spectrometer. The major compounds were BER *(m/z,* 336), PAL (*m/z*, 352), JAT (*m/z,* 338), and thalifendine (*m/z*, 322), and the minor compounds included oxoberberine (*m/z*, 352), 20-hydroxyecdysone (*m/z,* 481), and compounds with *m/z* of 324. Most of the compounds are quaternary and tertiary protoberberine alkaloids as described in chapter 3. The correlation coefficient R^2 of calibration line at 345 nm of each compound was higher than 0.999.

The amount of main compounds in each part of *C. fenestratum* was investigated by Soxhlet extraction (**Figure 4.2**). BER is main compound in root, stem and bark. Leaves contained 20-hydroxyecdyson with the small amount of BER. However, berberine was not observed from the root and stem of sample in batch 6. Thus, the sample in batch 6 may be other species rather than *C. fenestratum* although it has similar sharp and color. This result suggested that the collector should pay attention on selection of the plant.

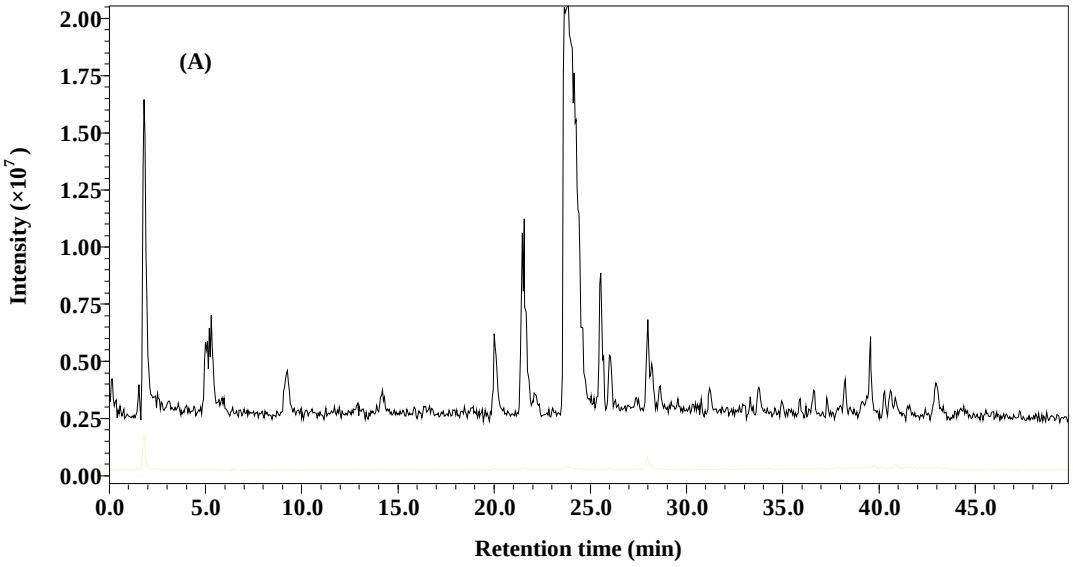
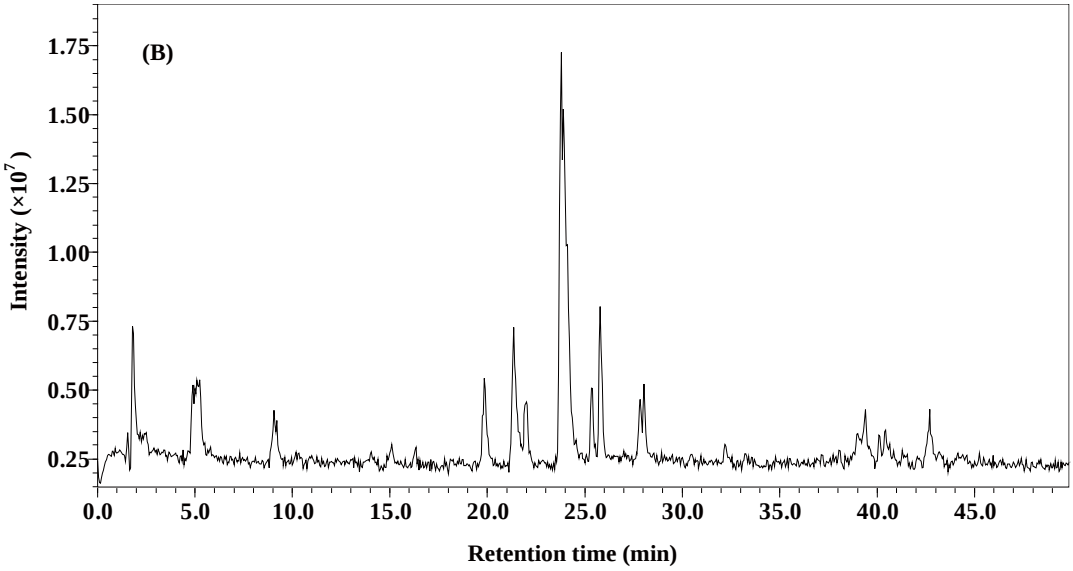


Figure 4.1 TIC chromatogram of each part of *C. fenestratum*. (A) Stem; (B) bark; (C) Leaves; (D) root; (E) root in batch 6.



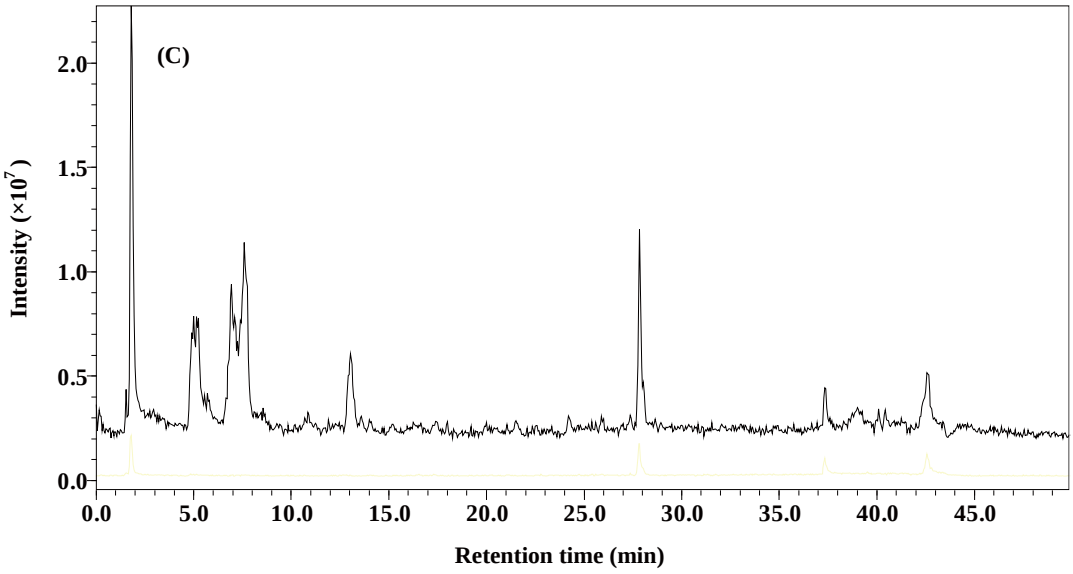
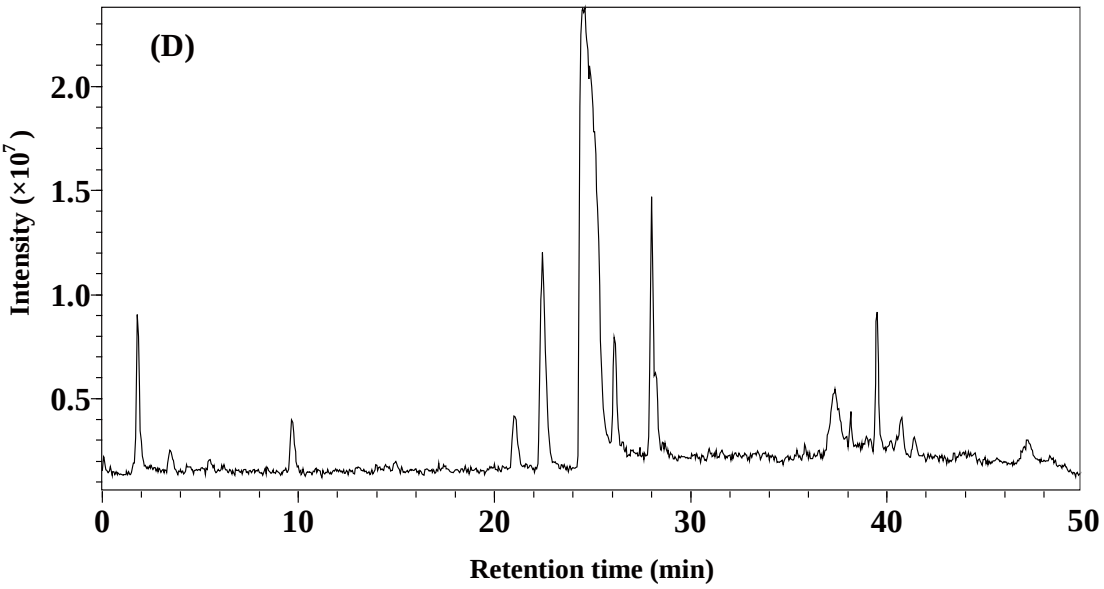


Figure 4.1 (*Continued*).



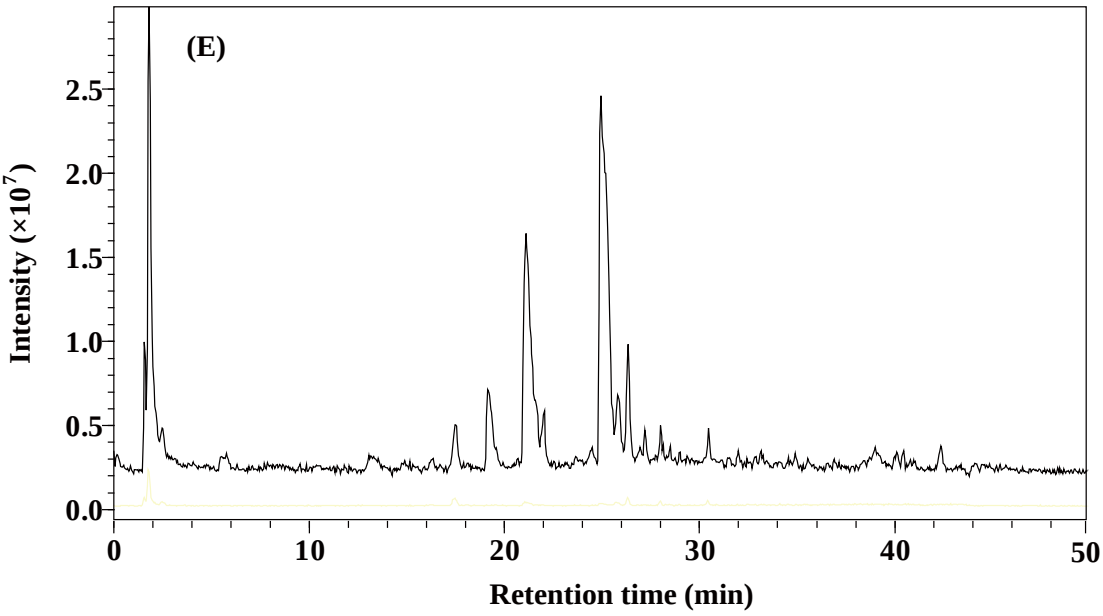


Figure 4.1 (*Continued*).

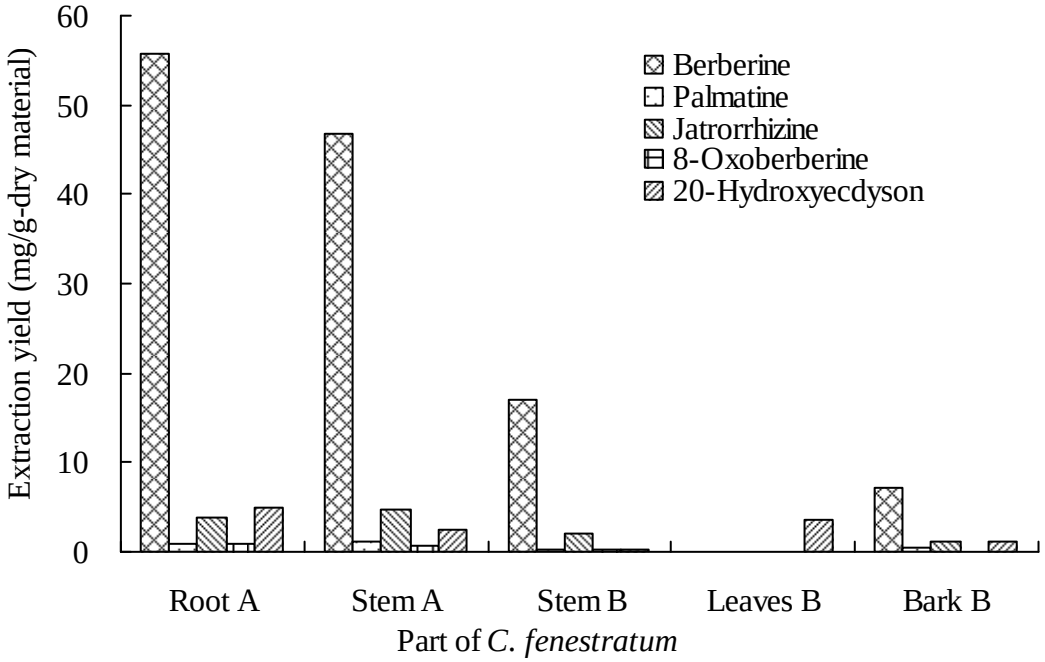


Figure 4.2 Amount of compounds in each part of *C. fenestratum*. (Root A, Stem A are from Batch 4; Stem B, Leaves B and Bark B are from Batch 2).

4.3.2 Variation of Compounds from Different Sites

Figure 4.3 shows the TIC peak area from mass spectrometer. A total of 43 peaks were observed from 5 samples obtained from different place of Laos as shown in **Table 2.1**. More than 30 compounds identical in each sample collected from different site. The peak area of each peak from TIC chromatogram was summarized in **Figure 4.4**. Variation in the amount of major compounds was clearly observed. However, the percentage of peak area of major compounds have similar trend. **Figure 4.5** shows the variation of percentage of the peak to total peak area. BER consisted of 52–65 % of total peak area, 10–15% for JAT, and 3–8 % for PAL. The peak intensity of pseudopalmatine and pseudojatrorrhizine were different among the 5 samples. The highest intensity and peak area of pseuodopalmatine and pseudojatrorrhizine were observed in batch 1, while psedojatrorrhine was mostly undetected in batch 2 and batch 4.

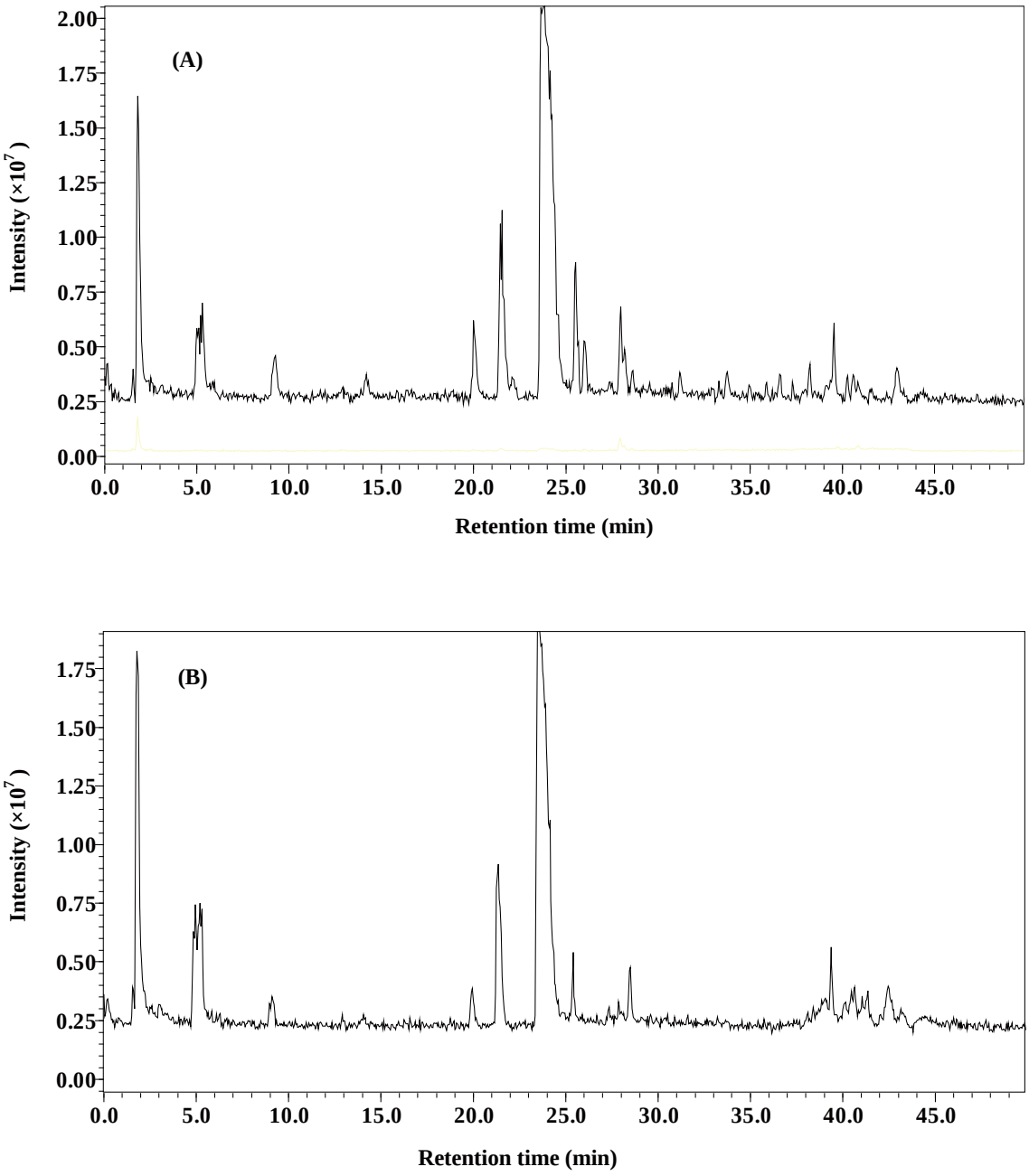


Figure 4.3 TIC chromatogram of *C. fenetratum* of each sample. (A) Batch 1; (B) Batch 2; (C) Batch 3; (D) Batch 4; (E) Batch 5.

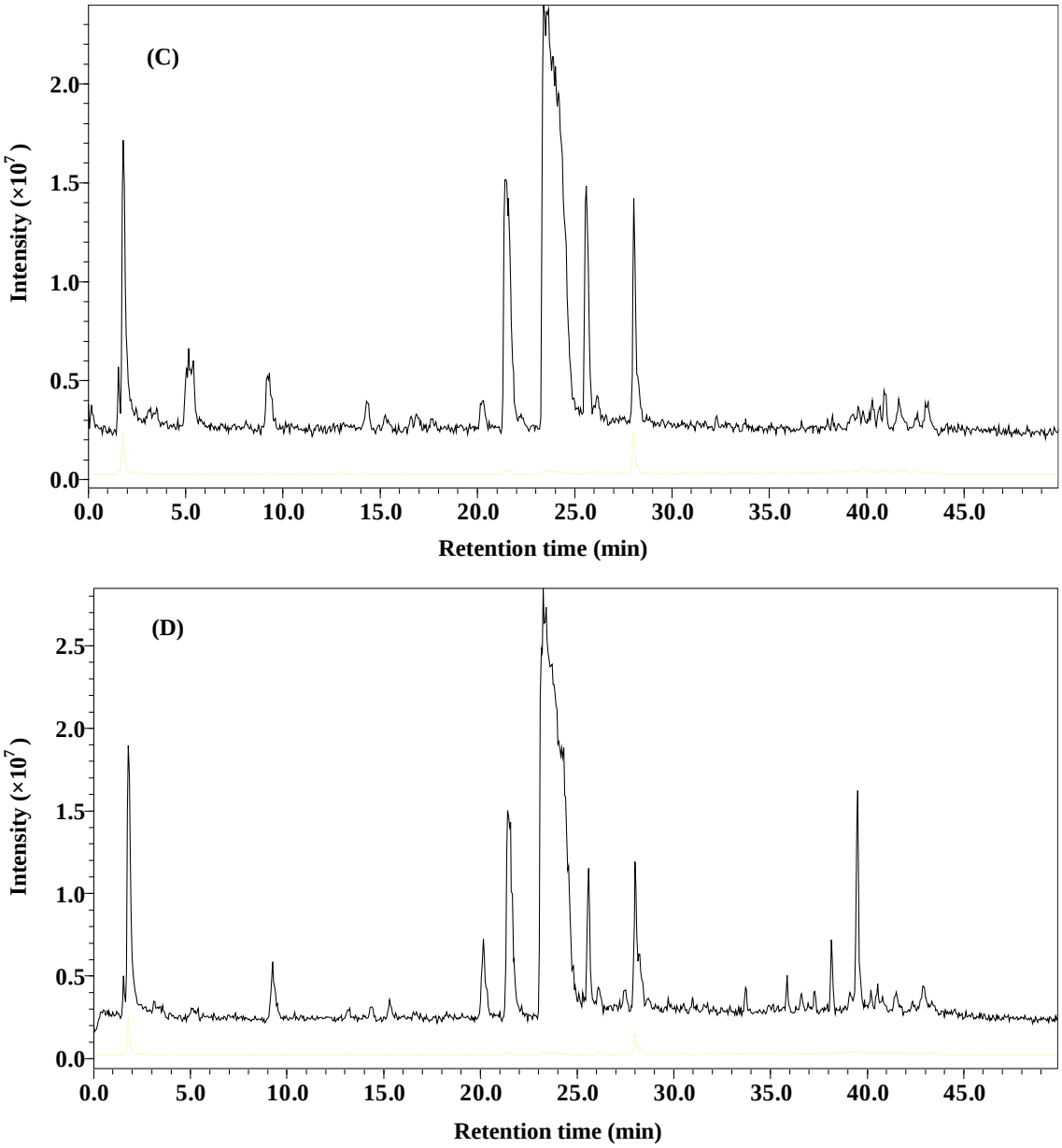


Figure 4.3 (*Continued*).

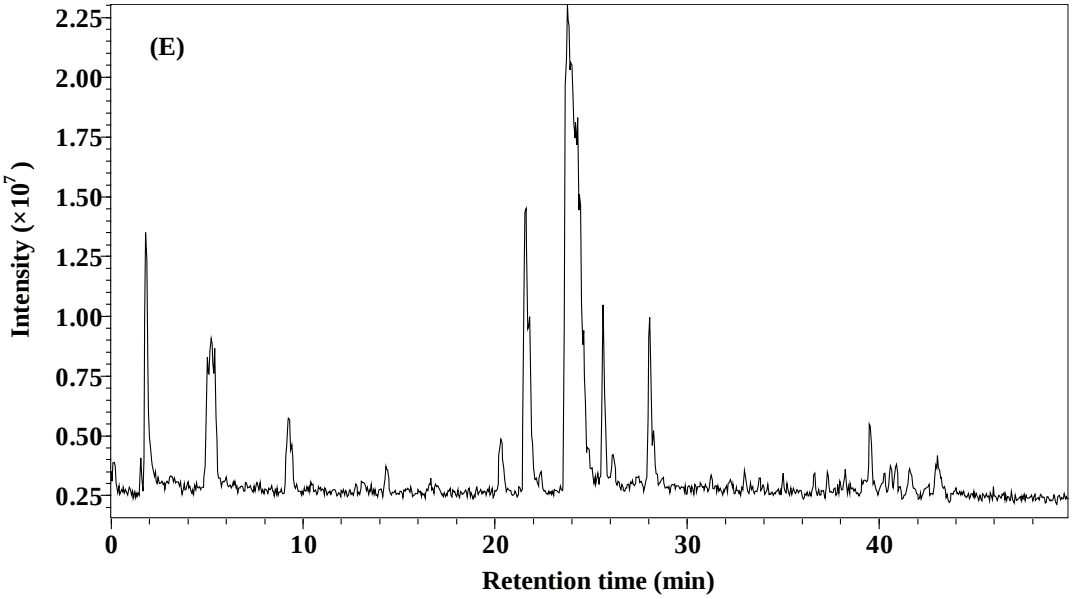


Figure 4.3 (*Continued*).

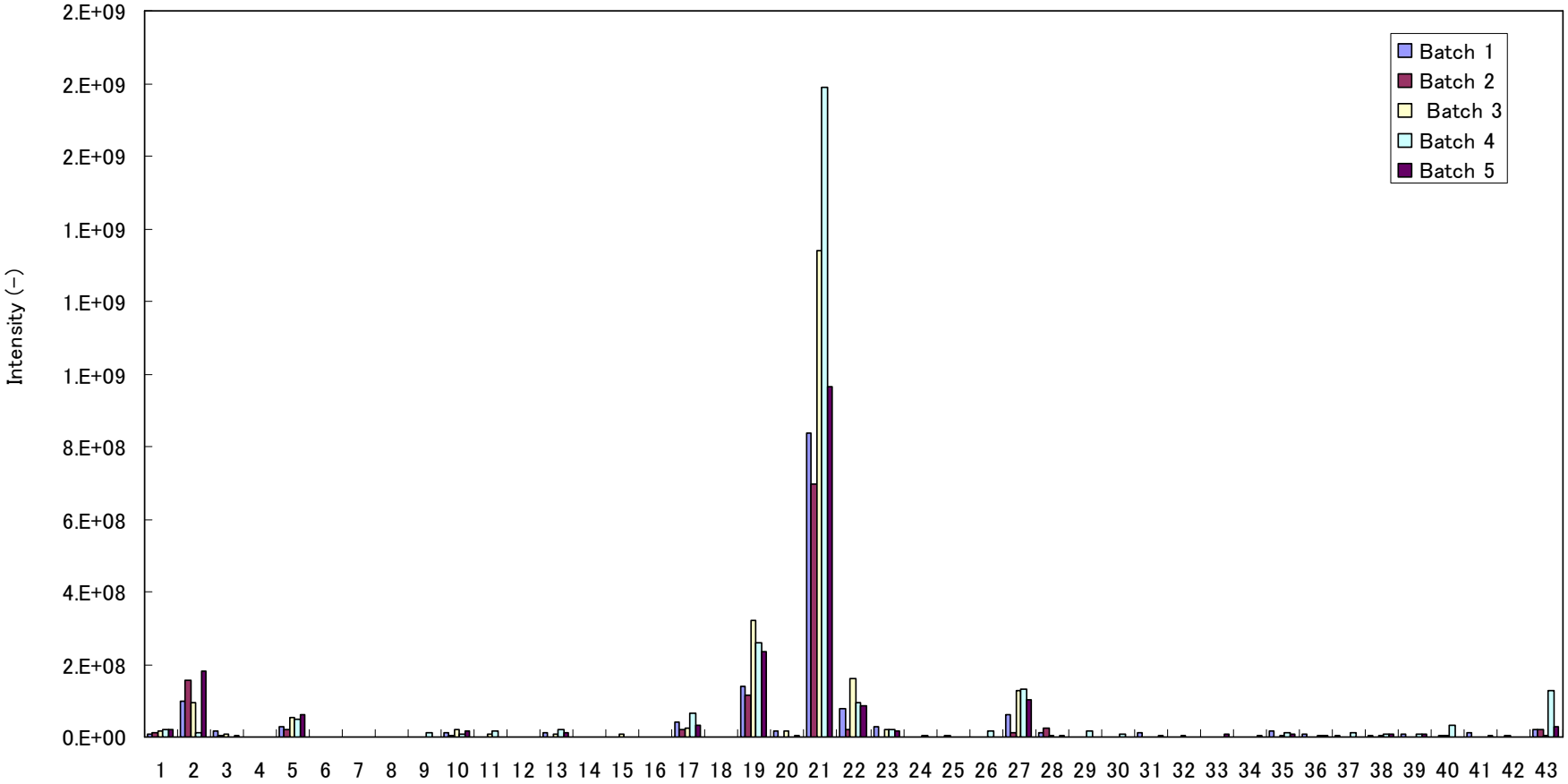


Figure 4.4 Peak area of each compound from TIC chromatogram.

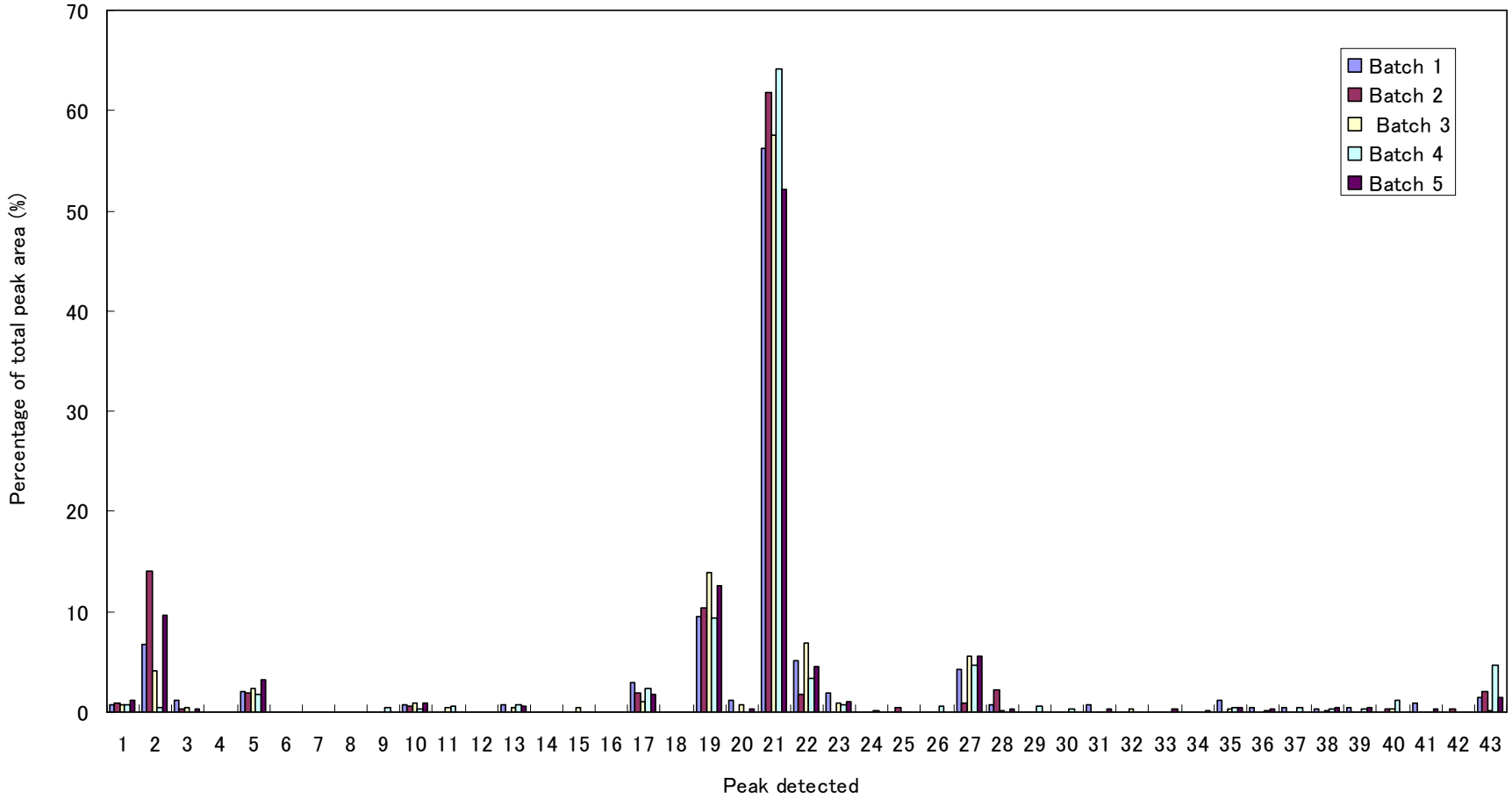


Figure 4.5 Percentage of each peak to total peak area of each sample.

4.3.3 Effect of Plant Size on Amount of the Compounds

Figure 4.6 shows the amount of BER (A), PAL, and JAT, oxoberberine and 20-hydroxyecdysone (B) in different size of *C. fenestratum*. The good correlation of medicinal compounds and size of the plant were observed for BER and JAT. The amount of BER and JAT were increased with the increase in diameter of *C. fenestratm* with the correlation coefficient R^2 0.83 and 0.74, respectively. Some correlation was also observed for oxoberberine and 20-hydroxyecdyson. But the correlations were smaller than those of beberine and jatrrizhine. This finding indicates that the sized of the plant is an important parameter that controls the amount of compounds in *C. fenestratum* and may be useful for the selection of the plant in practical use. The amount of PAL was increased from diameter of 15 to 30 mm. However, it was decreased significantly in the sample with the diameter of 40 ad 68 mm. Considering this result, PAL could be changed to other compounds. However, the conversion of PAL to other compounds is not found in literature.

Form this result, The plant size of *C. fenestratum* could be a factor to control the variation amount of compounds from different samples.

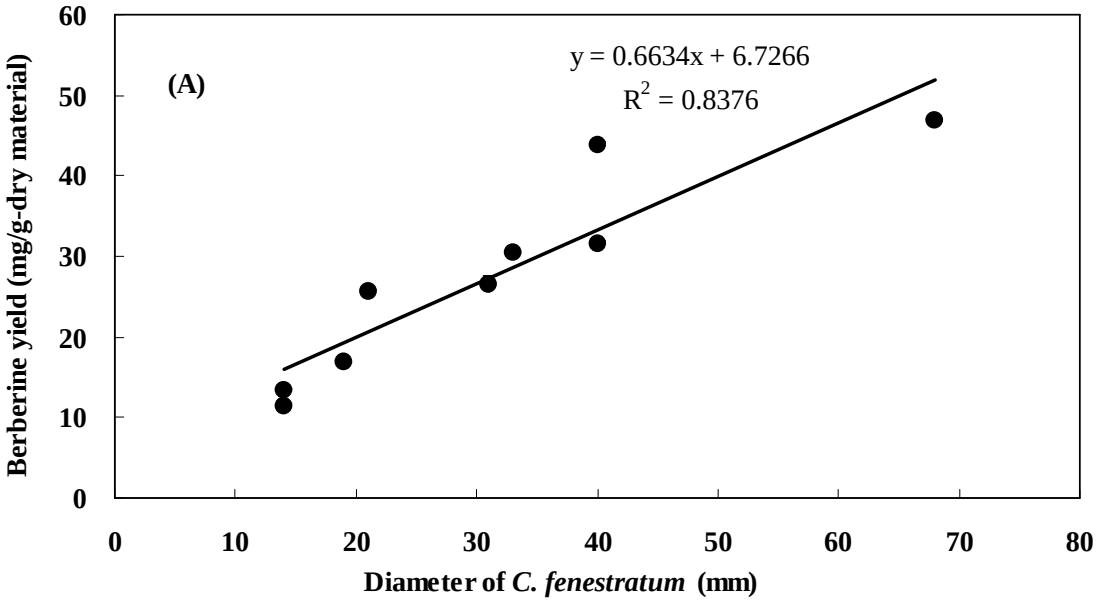


Figure 4.6 Amount of each compounds in different size of *C. fenestratum*. (A) BER; (B) PAL, JAT, 8-oxoberberine, and 20-Hydroxyecdysone.

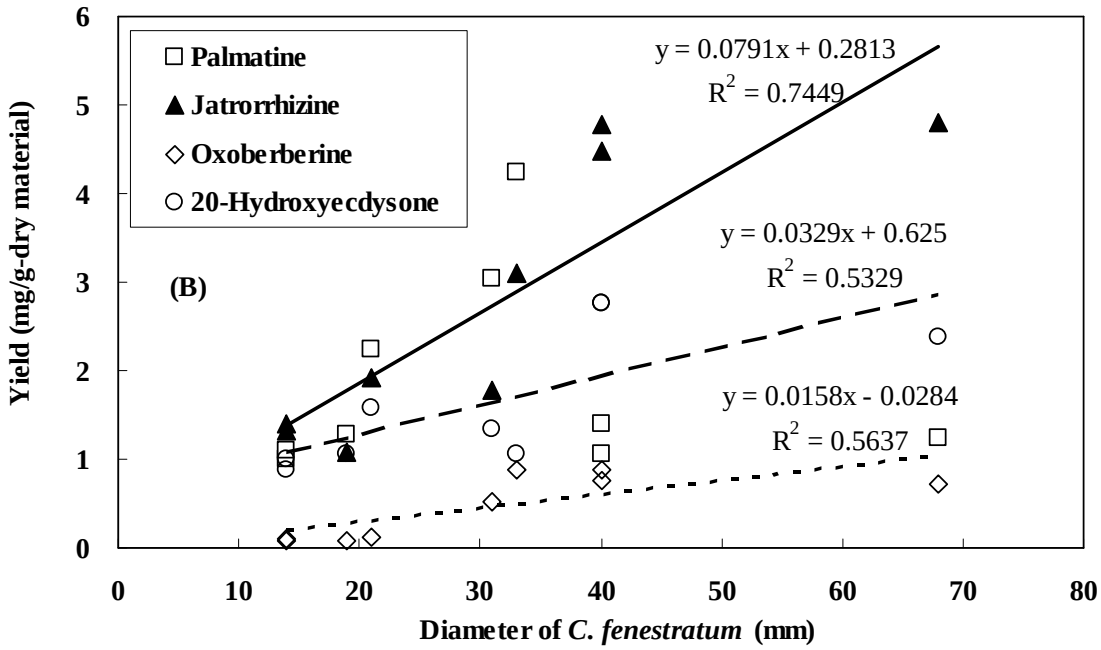


Figure 4.6 (*Continued*).

4.3.4 Comparison of Compounds in *C. fenestratum* with other Protoberberine Containing Medicinal Plants

Quantification of each compound was calculated from peak area obtained by PDA at 345 nm. **Table 4.1** shows the comparison of BER, PAL and JAT in *C. fenestratum* with those of other medicinal plants. The amount of BER and PAL in *C. fenestratum* were smaller than those in *C. japonica* and *P. amurense*. However, the amount of 8-oxoberberine in *C. fenestratum* is higher than those of *C. japonica* and *P. amurense*. *C. japonica* and *P. amurense* were cultivated in Japan, by cultivation or breed improvement, *C. fenestratum* may be achieved high amount of content as in *C. japonica*. In addition, there was some tendency of amount of BER, PAL and JAT between *C. fenestratum* and *C. japonica*. This suggested that *C.fenestratum* could be a potential medicinal plant that can be used as *C. japonica*.

Table 4.1 Comparison of major compounds in *C. fenestratum* with those of other medicinal plants

Plant name	BER (mg/g)	PAL (mg/g)	JAT (mg/g)	8-Oxoberberine (mg/g)
C. fenestratum	46.80 ± 0.38	1.23 ± 0.01	4.81 ± 0.03	0.72 ± 0.01
C. japonica	82.67 ± 0.17	3.48 ± 0.20	13.57 ± 0.05	0.24 ± 0.02
P. amurense	67.27 ± 0.31	6.36 ± 0.03	1.60 ± 0.04	0.06 ± 0.00

4.4 Conclusions

The amount of compounds in *C. fenestratum* were investigated and compared to those of other protoberberines containing medicinal plants. It was found that the amounts of compounds from different area varied from 1.8 % to 5.0 % of dry-material. However, there is no significant in the percentage of each compound to the total amount of compounds in *C. fenestratum*. The higher amount of BER, JAT were observed in the larger size of *C. fenestratum*, which indicates that the sized of the plant is an important parameter that controls the amount of compounds in *C. fenestratum* and may be useful for the selection of the plant in practical use. The amount of BER, PAL and JAT in *C. fenestratum* were also compared with those of *C. japonica* and *P. amurense*. Amount of BER, JAT and PAL were smaller than those in *C. japonica* and *P. amurense*. However, the amount of oxoberberine in *C. fenestratum* is higher than those in *C. japonica* and *P. amurense*. There was some tendency of amount of BER, PAL and JAT between *C. fenestratum* and *C. japonica*. This suggested that *C. fenestratum* could be a potential medicinal plant that can be used as *C. japonica*.

CHAPTER 5

MICROWAVE-ASSISTED EXTRACTION OF PROTOBERBERINE ALKALOIDS FROM *C. FENESTRATUM*

5.1 Introduction

Microwave energy causes molecular motion by the migration of ions and rotation of dipoles; therefore, microwave heating depends on the presence of polar molecules or ionic species. Microwave energy has been widely used for heating food materials, and recently, there has been an increasing interest in using microwave as a heat source for chemical synthesis [67], for the extraction of pollutants from the environment [38], and for the pyrolysis of wood [68]. MAE has been used for the extraction of some bioactive compounds from natural products, for example, for the extraction of tea polyphenols and caffeine from green tea leaves [69], glycyrrhizic acid from licorice root [70], and total triterpenoid saponins from *Ganoderma atrum* [71]. In most cases, the extraction time and organic solvent consumption with MAE was lower than those with conventional extraction methods. However, only a few studies have been performed on the extraction of alkaloids using MAE[72-74]. BER has been extracted using MAE only under atmospheric pressure[74], and the characteristics of MAE are not fully understood.

The objective of this study is to develop method for extracting protoberberine alkaloids, BER, PAL, and JAT from *C. fenestratum* by using MAE in both an open (at atmospheric pressure) and a closed vessel (under controlled pressure and temperature).

The solvent effects, extraction time, material and solvent ratio, and microwave output power for the extraction of protoberberine alkaloids in an open vessel were investigated. The possibility of significant reducing the solvent amount or using water as a solvent in order to develop a green process of MAE by using a closed vessel were also studied. The extraction yield obtained using MAE was compared to that obtained using conventional extraction methods.

5.2 Experimental

5.2.1 Materials

C. fenestratum (Batch 4*)* was obtained from Vientiane Province, Laos, in May 2008 and dried at room temperature. The plant was ground using a blender (Wonder blender WB-1; Osaka Chemical Co. Ltd., Japan) and the material was sieved using 32-mesh and 64-mesh sieves to obtain a powder with a particle size in the range of 500–1000 μm. This range of particle size was chosen to simulate the particle size that would be used in practice.

BER, PAL, JAT, EtOH, MeOH (LC/MS-grade), and formic acid (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd., Japan. Stock solutions of BER (150 mg/L), PAL (42 mg/L), and JAT (110 mg/L) were prepared in MeOH. All solutions were stored at 4°C. Standard solutions were prepared by diluting each stock solution in MeOH to a concentration of 10 to 150 mg/L (BER), 0.33 to 5.25 mg/L (PAL), and 0.69 to 11 mg/L (JAT). Ultrapure water produced by the Milli-Q Advantage vessel (Millipore, USA) was used in this study.

5.2.2 Microwave-Assisted Extraction (MAE)

The microwave apparatus Mars-X (CEM Corp., U.S.A.), as shown in **Figure 5.1 and Figure 5.2**, was used for MAE. It can be operated with an output power of up to 1600 W (2450 MHz). The temperature was measured using the fiber optic temperature probe RTP-300 Plus, and pressure was measured using the ESP-1500 Plus. The microwave apparatus automatically adjusted the radiated power when the

actual temperature reached the set temperature. The experiments were performed in both open and closed vessels.

Open-vessel MAE was performed in a 250 mL round-bottom flask connected to a water-cooled reflux condenser at its top. The optimum conditions were determined using different solvent concentrations of MeOH and EtOH in water at 60°C and the boiling point, at a microwave output power ranging from 50 W to 800 W, and with the ratio of plant material to solvent of 1.00 g to 10–60 mL. To compare the extraction time of MAE with that of extraction with an oil bath, the extraction in two modes: with and without pre-heating were performed. Extraction without pre-heating was performed using directly radiated microwaves on solutions containing plant material. The temperature increased, but it was maintained at 60°C. For extraction with pre-heating, the plant material was added to a solvent pre-heated to 45°C, and then exposed the sample to radiated microwaves until a temperature of 60°C was reached after 2 min, following which the temperature was controlled at 60°C. The extraction liquid was cooled for 4 min before filtration.

Closed-vessel MAE was performed in a Teflon-made GreenChem vessel (CEM Corp., U.S.A.) with a capacity of 100 mL, which could operate under a pressure of up to 1.38 MPa (200 PSI). The extraction was performed using two vessels: one as a control and the other as test. Due to safety considerations, the output power was set at 300 W. The temperature and pressure in the control vessel were measured. The *C. fenestratum* material (1.00 g) was extracted in 40 mL of solvent. The extraction vessel was cooled for 20 min before filtration.

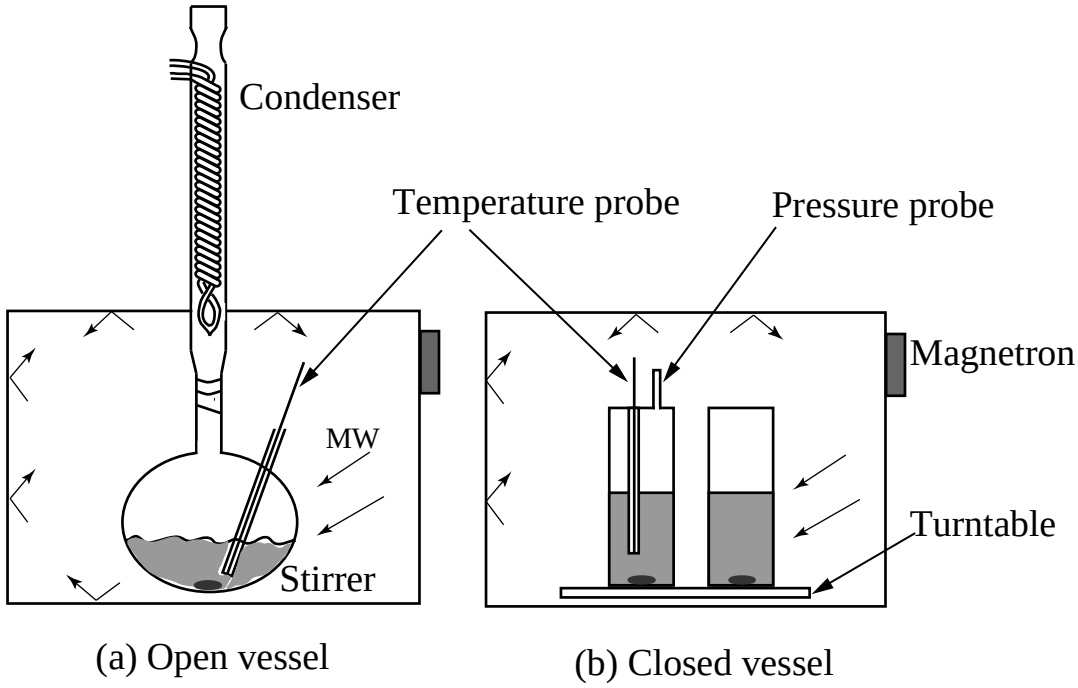


Figure 5.1 Structure of the microwave apparatus

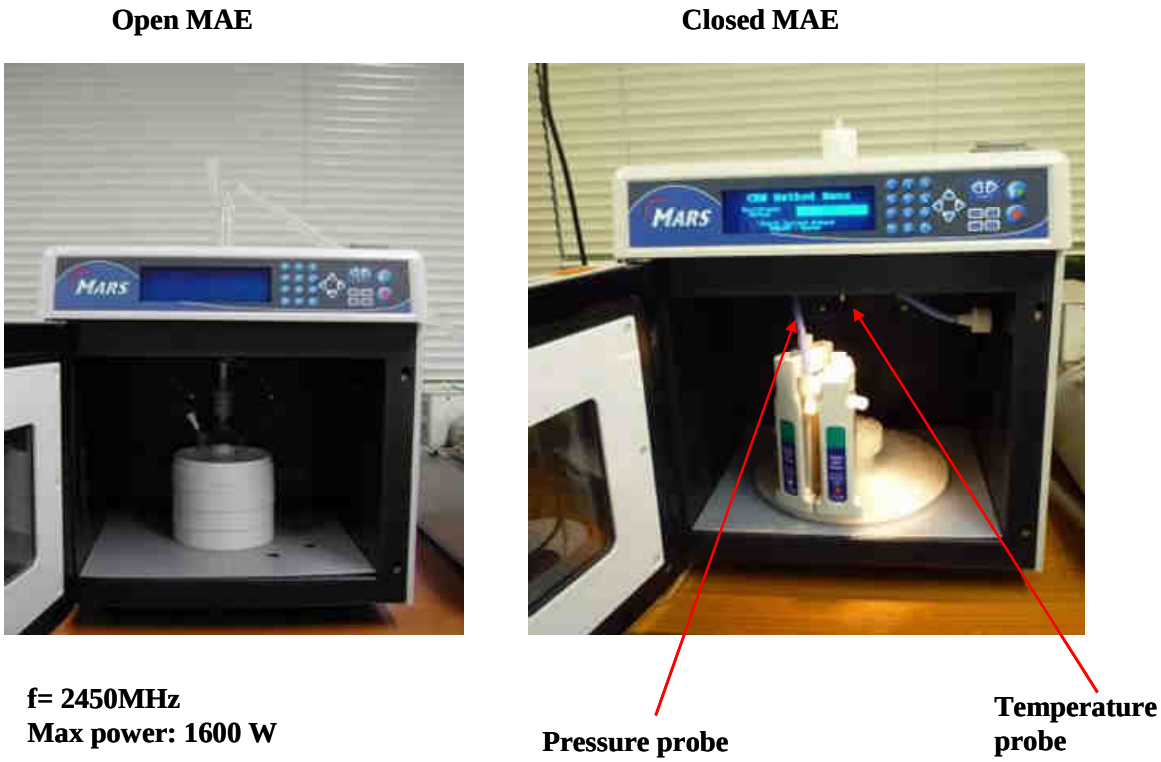


Figure 5.2 Photos of MAE in Open and Closed MAE

5.2.3 Conventional Extraction Methods

Soxhlet extraction was performed using the Soxhlet apparatus by adding 1.00 g of plant powder to the extraction thimble and extracting with 100 mL MeOH for 8 h and 24 h.

Thermal extraction was carried out using the same flask in an oil bath with a magnetic stirrer and the fiber optic temperature probe used in MAE (**Figure 5.3**). Extraction was performed in two modes, as in the case of MAE. In the experiment with pre-heating, a flask containing 40 mL of the solvent was pre-heated in an oil bath set at 80°C. The plant powder (1.00 g) was added to the heated solvent when the temperature reached 45°C, and the flask was placed in the oil bath. The temperature in the flask increased and reached 60°C in 2 min. The flask was moved up and down to maintain the temperature at 60°C. Plant material (1.00 g) was added to the solvent in the flask, and the flask was placed in the oil bath set at 60°C for the experiment without pre-heating.

Extraction by sulfuric acid aqueous solution was performed using 1.00 g of the powder mixed with 40 mL of a 0.2% sulfuric acid aqueous solution in a 100-mL flask. The solution was stirred at 100 rpm and incubated at room temperature for 10 min to 24 h. After filtration, the pH of the solution was adjusted from 1.40–1.60 to 5.50–5.70 using a 25% ammonia solution.

All the extracted samples were filtered through filter paper No.101 (Toyo Roshi Kaisha, Japan). The filtrate was diluted five times in MeOH, and then filtered using a 0.45-μm membrane filter (Toyo Roshi Kaisha, Japan) for liquid chromatography mass spectrometry (LC/MS) analysis.

5.2.4 Analysis

The quantification of BER, PAL, and JAT was performed using an LC/MS-2010EV apparatus (Shimadzu Corp., Japan). The separation was carried out in a ZORBAX Eclipse XDB column (3.5 μm, 2.1 × 150 mm) (Agilent Technologies, Inc., U.S.A.). The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and methanol (B), was delivered at a flow rate of 0.2 mL/min by using

the following gradient conditions: 20–32% (B) for 0–20 min, 32–70% (B) for 20–35 min, 70% (B) for 35–45 min, and 20% (B) for 45–55 min. The sample injection volume was 1 µL, and the column temperature was maintained at 40°C. The UV spectra were obtained by scanning the samples in the range of 200–470 nm, and the peaks were simultaneously determined at 345 nm. The amounts of BER, JAT, and PAL were calculated from the peak area at 345 nm by using the calibration line.

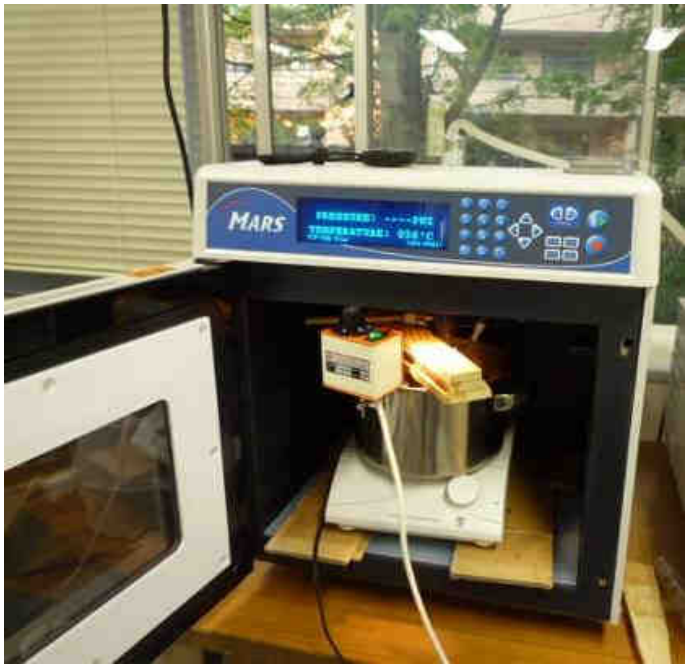


Figure 5.3 Photo of extraction using oil bath.

5.3 Results and Discussion

5.3.1 Extraction of Protoberberine Alkaloids by Open-vessel MAE

The nature of the solvent is important to be considered in MAE. As with the other techniques, the solvent should efficiently solubilize the analytes of interest without significantly extracting the matrix material. In addition, the microwave-absorbing properties of the solvent are of great importance as sufficient heating is required. The ability of a specific substance to convert electromagnetic energy into heat is determined by the loss tangent, tanδ (tanδ = $\varepsilon''/\varepsilon'$, where $\varepsilon''$is the

dielectric loss, indicative of efficiency when electromagnetic radiation is converted into heat, and ε′is the dielectric constant describing the polarizability of molecules in an electric field). In general, solvents can be classified as high microwave-absorbing (tanδ > 0.5), medium microwave-absorbing (tanδ = 0.1–0.5), and low microwave-absorbing (tanδ < 0.1) [67]. EtOH (tanδ, 0.941), MeOH (tanδ, 0.659), water (tanδ, 0.123), and alcoholic aqueous solutions were chosen as the solvents in this study because protoberberine alkaloids are relatively polar organic compounds.

Figure 5.4 shows the BER extraction yield after changing the ratio of solvent to water. Microwaves were radiated at 300 W for 15 min for extraction at boiling point. The temperature reached the boiling point in 3 min for solvents that contained more than 40% of EtOH or MeOH. Water, 20% EtOH, and 20% MeOH required 3.5–4.0 min to reach the boiling point. If pure solvents are used, MeOH is superior to EtOH and water due to the high dissociation of BER in MeOH. However, the extraction yields obtained using EtOH aqueous solutions were higher than those obtained using MeOH aqueous solutions. This could be because the boiling point of EtOH aqueous solutions (79–90°C) is higher than that of MeOH aqueous solutions (66–90°C). Moreover, the use of 60% EtOH can achieve an extraction yield higher than that of MeOH. Considering the pharmaceutical toxicity of MeOH, 60% EtOH is a suitable solvent that can also reduce the amount of EtOH used for the extraction process. The extraction yield using EtOH aqueous solution at 60°C was lower than the extraction yield at boiling point. This result indicated that a higher extraction yield can be achieved by increasing the extraction temperature.

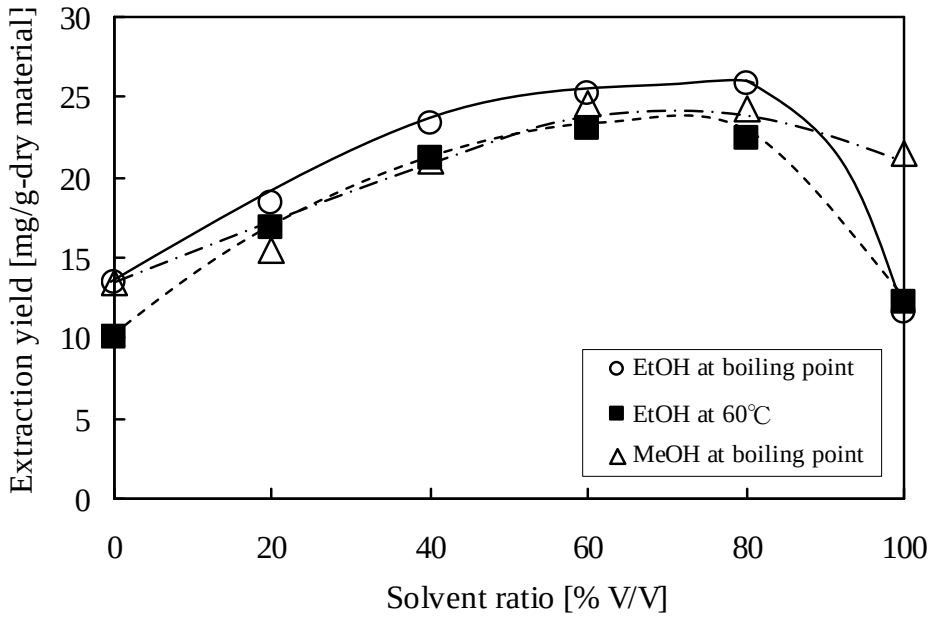


Figure 5.4 Effect of the solvent ratio on the extraction yield of BER.

Figure 5.5 shows the effect of the ratio of plant material to solvent volume on the extraction yield at 300 W for 15 min. The extraction yield by MAE increased as the amount of solvent increased from 10 to 40 mL; however, no difference in the yield was observed for solvent amounts greater than 40 mL. Thus, 40 mL of 60% EtOH was considered to be the optimum solvent volume.

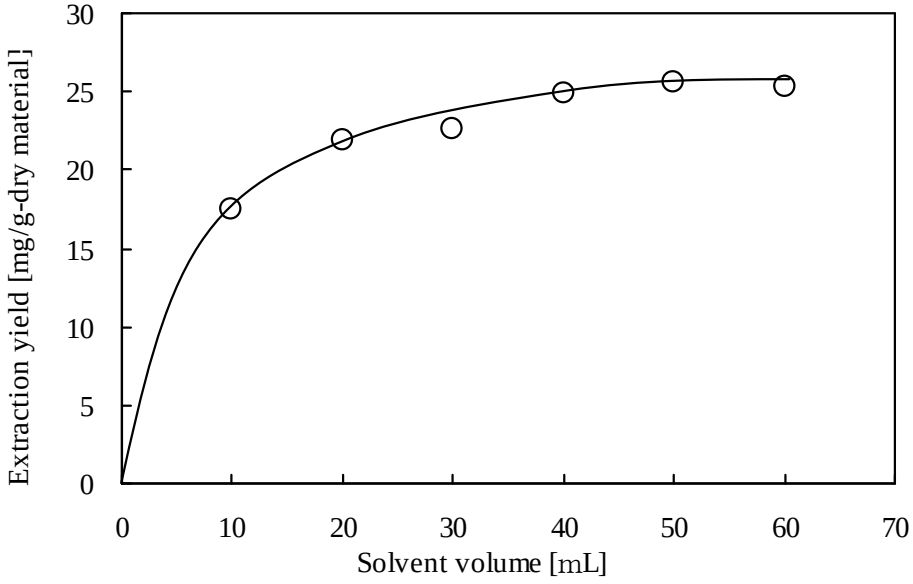


Figure 5.5 Effect of the ratio of plant material to solvent volume on the extraction yield of BER

The effect of the microwave output power on the extraction yield using 60% EtOH for 15 min is shown in **Figure 5.6**. The extraction yield increased when an output power of 50 W to 200 W was applied. The increased yield in this output power range was considered to be a result of the increase in temperature. The temperature was increased by only 4°C from 23°C to 27°C for 50 W and by only 15°C from 23°C to 38°C for 100 W. However, no significant difference was observed for microwave power greater than 200 W. Considering the time required for the extraction, a microwave power of 300 W was chosen as optimum for the extraction. It took 4 min to reach the boiling point when 200 W was applied, whereas it took only 2 min at 300 W.

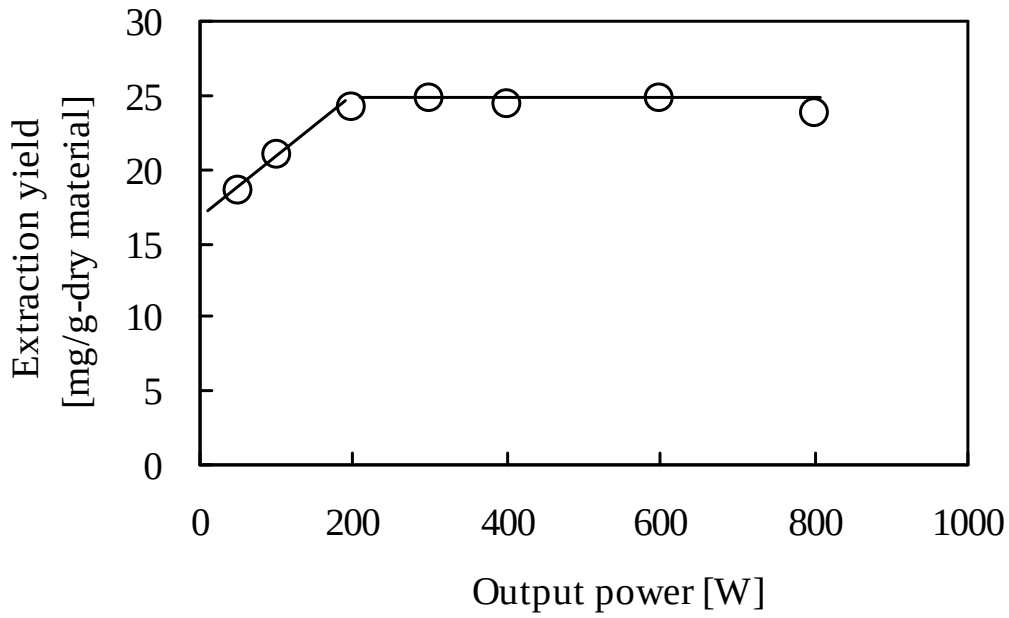


Figure 5.6 Effect of microwave output power on the extraction yield

Figure 5.7 shows the effect of the extraction time on open-vessel MAE compared to those extractions using oil bath and 0.2% sulfuric acid. The BER extraction yield with MAE at 60°C was 20.23 mg/g-dry material in 5 min and it reached 23.10 mg/g-dry material in 15 min. The extraction yield by MAE was higher than that of extraction by oil bath, and extraction by 0.2% sulfuric acid aqueous solution. The extraction of PAL and JAT was observed to have the same tendency as

that of BER (data not shown). However, when comparing MAE to extraction with pre-heating in an oil bath, the former gave the same extraction yield as the latter. This suggested that there was no significant difference in the BER extraction yield from *C. fenestratum* between open-vessel MAE and an oil bath when the temperature was controlled at 60°C, although a specific effect of microwaves on plant material has been found in a previous report[75]. This effect was observed when the plant contained water. Microwaves were believed to interact selectively with the free water molecules present in the glands and vascular vessels, leading to rapid heating and temperature increase, followed by rupture of the wall and release of essential oils into the solvent.

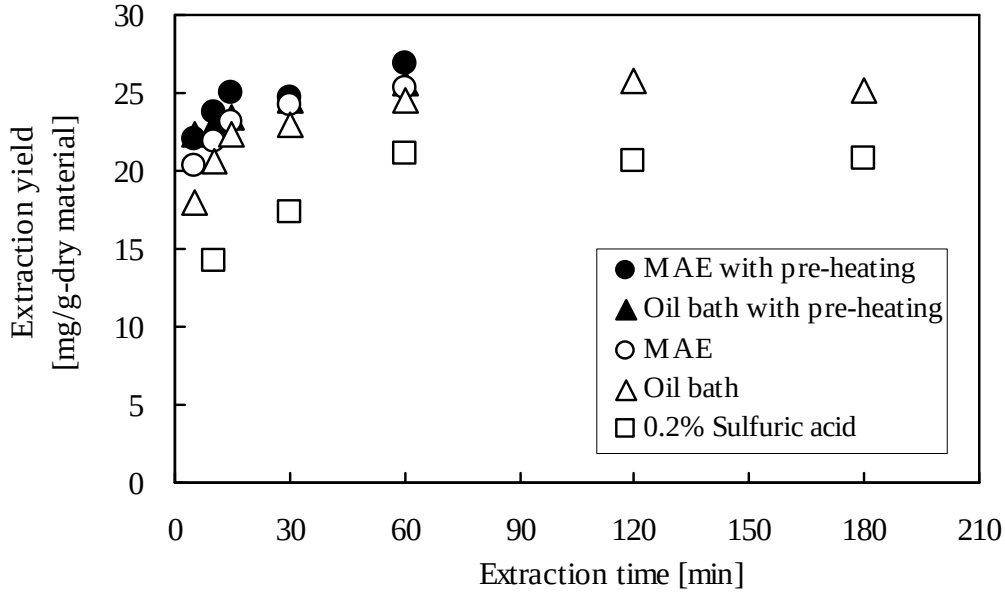


Figure 5.7 Effect of the extraction time on extraction yield

5.3.2 Extraction of Protoberberine Alkaloids by Closed-vessel MAE

The solutions of 60% EtOH, 20% EtOH, 10% EtOH, 0.03 M NaCl, and water were used to investigate the extraction of protoberberine alkaloids in a closed vessel. The use of water as a solvent and EtOH as an additive could reduce the amount of organic solvent, and water can be used as a green solvent for extracting protoberberine alkaloids. A solution of 0.03 M NaCl was chosen for this study because of its high microwave-absorbing ability and its potential to reduce the extraction time. Temperature was set according to the following conditions: 25 to

100°C for 0–4 min, 100°C to the desired temperature (100, 120, 140, 160, and 170°C) for 4–11 min, and maintaining the temperature for 4 min. The ability to convert microwave energy to heat is decreased when the temperature increases; therefore, it is more difficult to reach a temperature above 170°C with water. A temperature of 170°C was reached in 13 min by microwave radiation at 300 W. **Figure 5.8** shows the temperature as the radiation time. **Figure 5.9** shows the photos of plant material after extraction. The color of plant material changed from yellow to dark-gray when increasing temperature from 100°C to 170°C. This observation was significant when using water, but not observed when using 60% EtOH. **Figure 5.10** shows the photos of the extracted liquid by each solvent. The liquid changed from light yellow to dark-yellow when increasing the temperature.

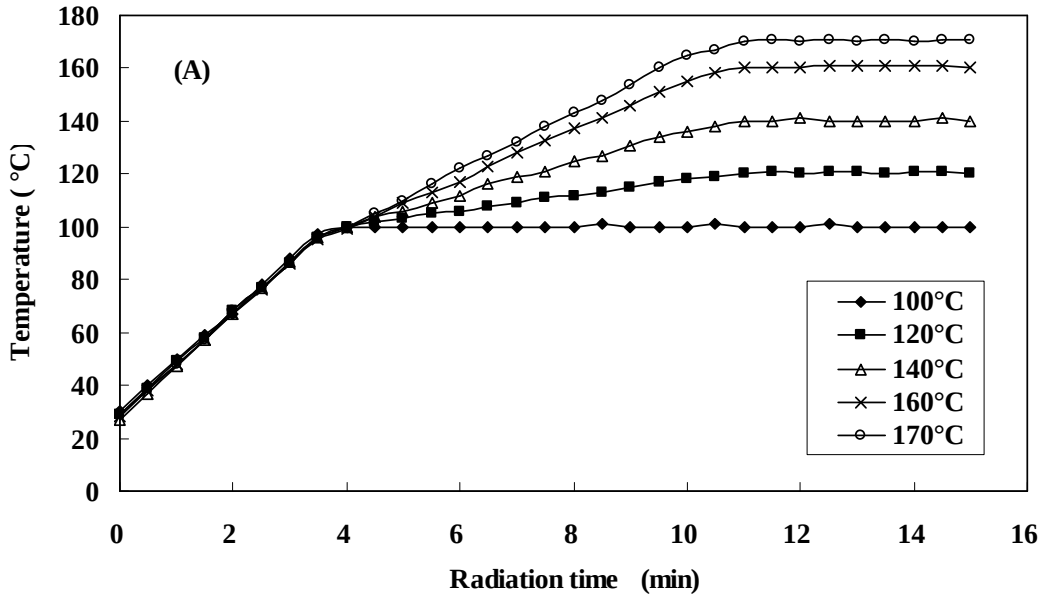
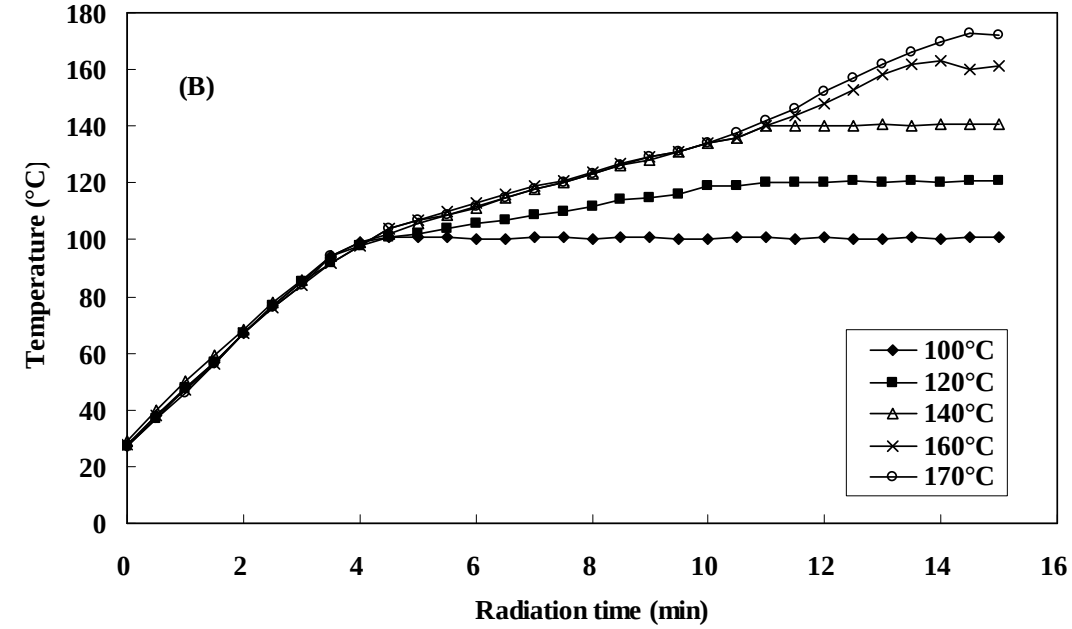


Figure 5.8 Temperature behavior using different solvent. (A) 0.03M NaCl; (B) Pure water; (C) 10% EtOH; (D) 20% EtOH; (E) 60% EtOH.



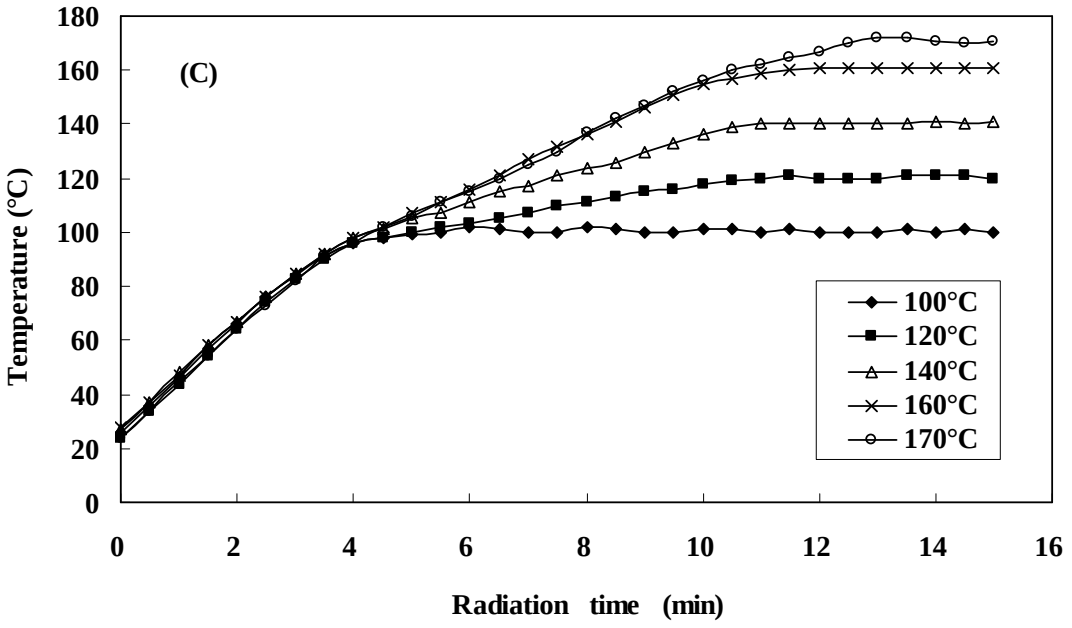
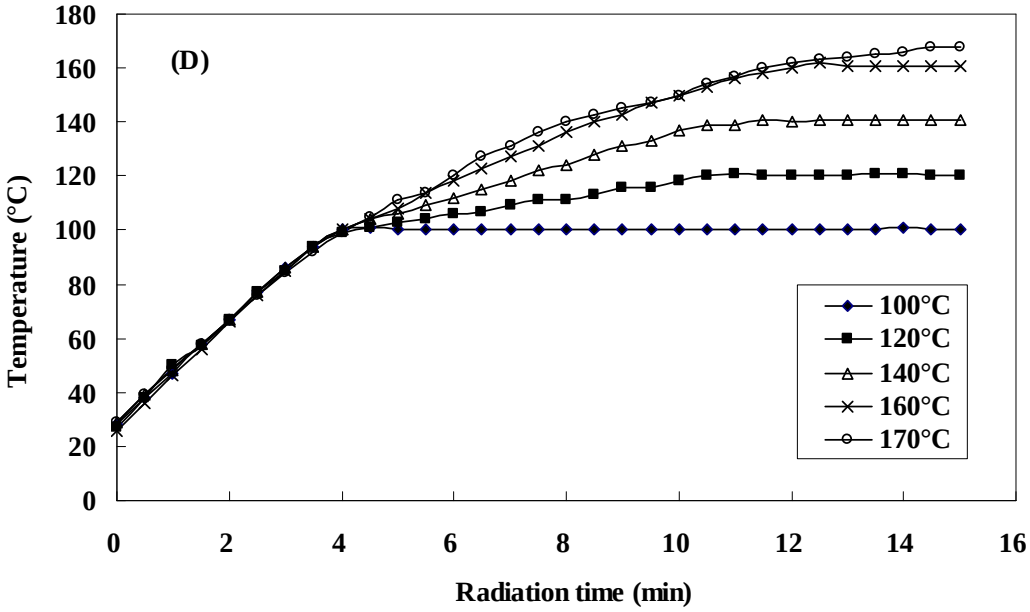


Figure 5.8 (*Continued*).



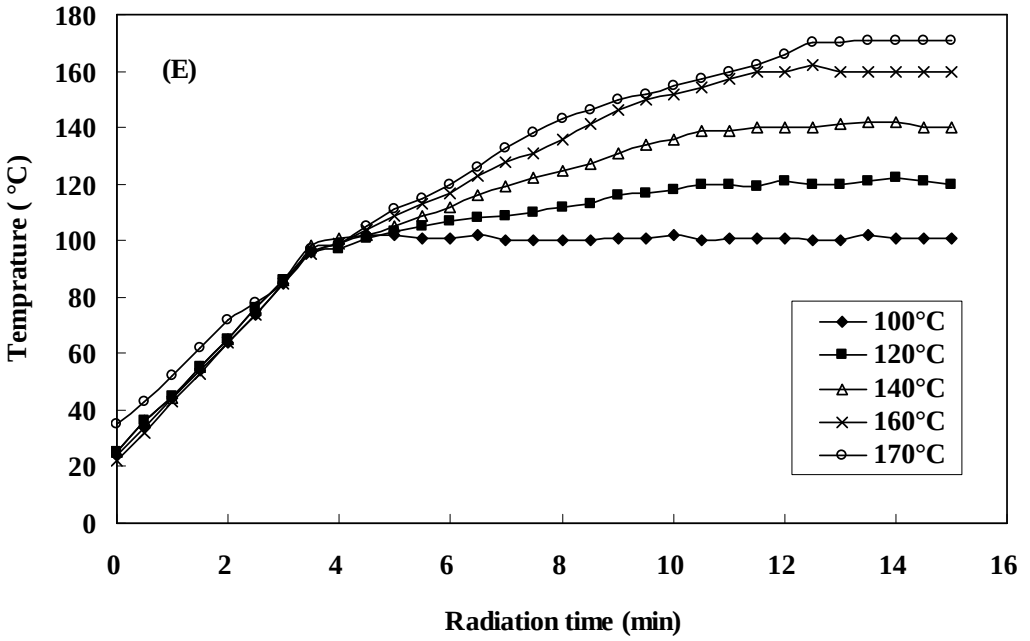


Figure 5.8 (*Continued*).

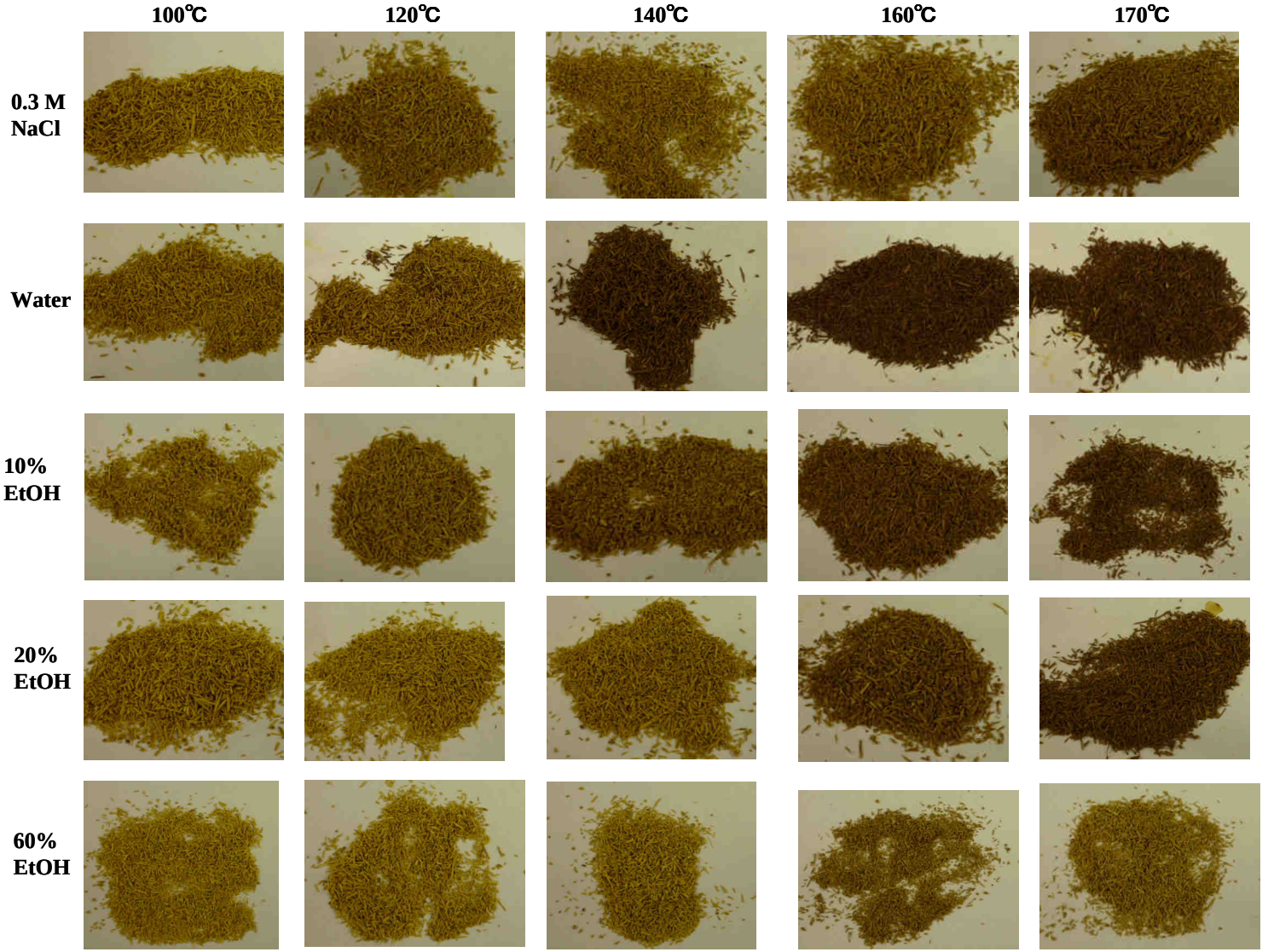
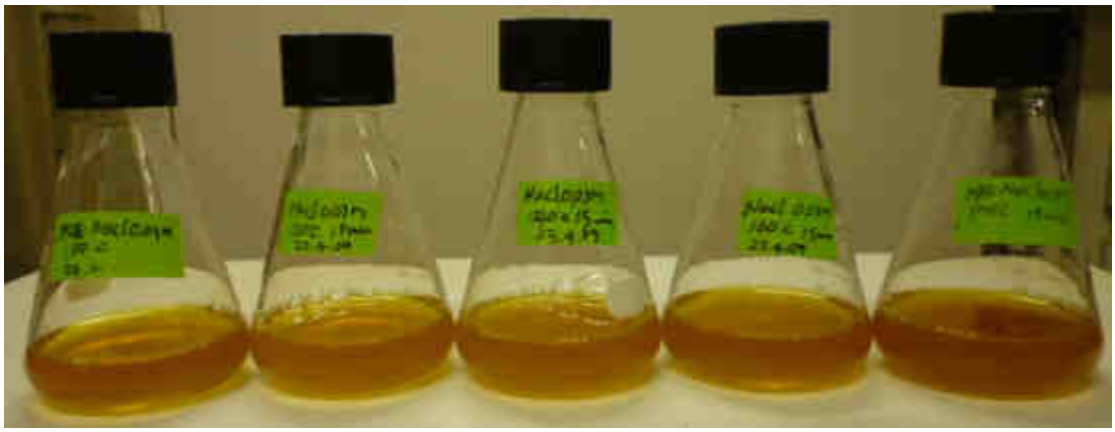
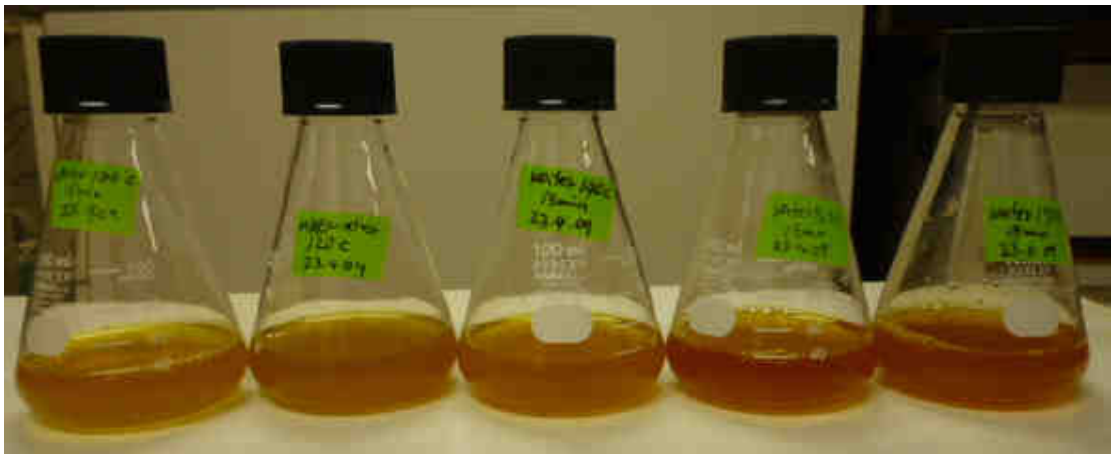


Figure 5.9 Photos of plant material after extraction by each solvent



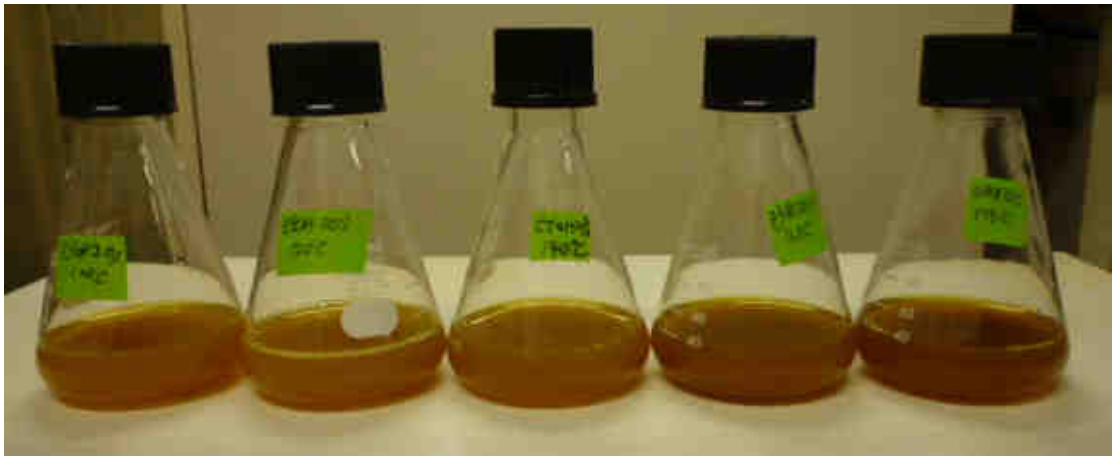
(A)



(B)

(C)

Figure 5.10 Photos of extracted liquid by each solvent at different temperature. (A) 0.03 M NaCl aqueous solution; (B) water; (C) 10% EtOH; (D) 20% EtOH; (E) 60% EtOH.



(D)

(E)

Figure 5.10 (*Continued*).

5.3.2.1 Effect of the Solvent on the Extraction of Berberine by Closed-vessel MAE

Figure 5.11 shows the extraction yield of BER, JAT, and PAL in a closed vessel at a temperature from 100 to 170°C using different solvents. The BER extraction yield increased with an increase in temperature from 100 to 160°C. Extraction by 60% EtOH gave a superior extraction yield compared to other conditions. The BER extration yield obtained using 60% EtOH at 160°C was the highest, with a maximum extraction yield of 27.35 mg/g-dry material. Using water as a solvent, the extraction yield increased significantly from 13.30 mg/g-dry material at 100°C to 20.36 mg/g-dry material at 160°C. This result suggests that the BER extraction yield by using water can be increased by increasing the extraction temperature. However, the extraction yield decreased at 170°C, which indicated BER degradation at this temperature. This result is in agreement with that of Y. Sakai *et al*[76].. This study reports BER degradation over 160°C when extracting BER from the Amur cork tree with steam. The addition of 10% and 20% ethanol resulted in a greater extraction yield than that of water at temperatures between 100ºC and 140ºC. However, no significant difference was observed at 160°C. The extraction yield of JAT and PAL showed the same tendency as that of BER; but, the highest extraction yields of JAT (2.36 mg/g-dry material) and PAL (0.73 mg/g-dry material) were obtained at 170°C. No degradation of JAT and PAL was observed for extraction at high temperatures, except during extraction using water at 170°C. Although 0.03 M NaCl is a higher microwave-absorbing solvent than water, the extraction yield of BER, JAT, and PAL using 0.03 M NaCl were lower than those obtained using water. This could be because protoberberine alkaloids are less soluble in NaCl solution than in water. **Table 5.1** shows the pH of solutions after cooling to room temperature. In the extraction using water, there was a lower pH than that of the solvent using an additive. Lower pH was observed at the increasing of extraction temperature.

5.3.2.2 Effect of Extraction Time on the Extraction of Berberine Using Water and 20% EtOH

Figure 5.12 shows the effect of the extraction time on extraction yield when using water and 20% EtOH at 140°C and 160°C. Most of the extraction yields at

160°C were higher than those at 140°C and the extraction yield increased with an increase in the extraction time up to 20 min. The highest extraction yield by water was 20.31 mg/g-dry material at 160°C and a total radiation time of 15 min. However, the BER yield by water at 160°C decreased for extraction times longer than 15 min, which indicates BER degradation with increased extraction time at high temperatures. Extraction by 20% EtOH gave the highest extraction yield (21.95 mg/g-dry material) at 160°C for a total radiation time of 20 min and 21.94 mg/g-dry material at 140°C for a total radiation time of 30 min. The BER degradation at 160°C for 30 min was observed to be of the same level as that with water. However, no decrease was observed in the extraction yield by using 20% EtOH at 140°C for a total radiation time from 11 to 30 min. Therefore, adding 20% EtOH could reduce or prevent BER degradation at high temperatures (140°C). The extraction of BER using 20% EtOH at 160°C should not exceed 20 min, otherwise the degradation of BER occurs.

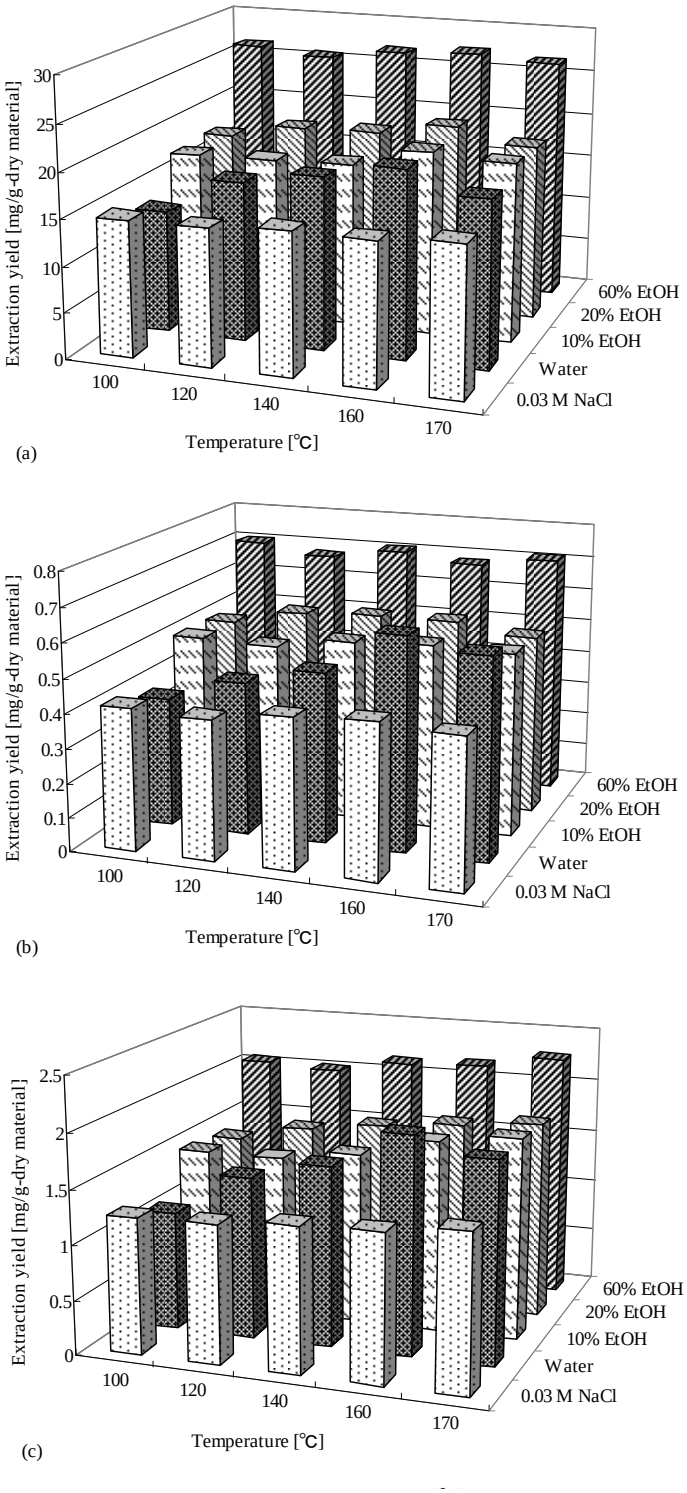


Figure 5.11 Extraction yield of major compounds obtained using closed-vessel MAE. (a) BER; (c) PAL; (c) JAT

Table 5.1 pH of each extraction solution

Temperature (℃)	100	120	140	160	170
0.03 M NaCl	5.48	5.25	5.11	4.71	4.57
Water	4.94	4.58	4.29	4.00	3.91
10% EtOH	5.51	5.12	4.86	4.45	4.32
20% EtOH	5.80	5.71	5.31	4.64	4.46
60% EtOH	6.56	6.52	6.28	5.64	5.56

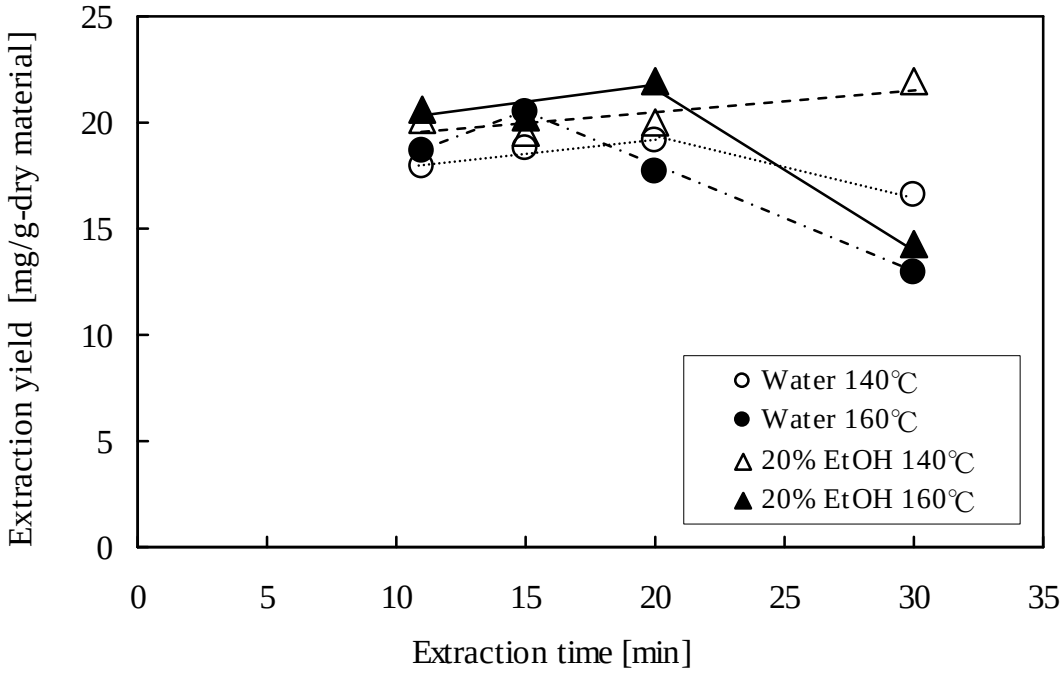


Figure 5.12 Effect of the extraction time on extraction yield in a closed vessel

5.3.3 Effect of Temperature on Berberine Degradation by MAE

No degradation was observed for open-vessel MAE. However, BER degradation was observed at high temperatures, especially at temperatures above 160°C for extraction times more than 20 min in a closed vessel.

Figure 5.13 shows the chromatogram of the extraction by MAE using water as the solvent at 170°C. BER degradation and a product compound eluted at 16.65 min were observed. The product compound showed *m/z* at 324, which indicated the reduction of a C atom from BER. The peak area of the new product could be used as

an indicator of BER degradation. **Figure 5.14** shows the peak area of the by-product compound at 16.65 min in each extraction condition. The peak area of extraction using water increased as the temperature increased from 120°C to 170°C. Interestingly, with the addition of additives such as NaCl and EtOH, the peak area of the product compound was smaller than that of water.

The differences in BER degradation between water and water with additive could be due to the differences in the microwave-absorbing ability of each solvent. Microwave could be absorbed by the plant material, causing specific heating and generating higher temperatures in the plant material than in the surrounding bulk solution. Ethanol and NaCl aqueous solutions absorb a higher amount of microwaves than pure water. This characteristic of EtOH and NaCl aqueous solution could reduce the quantity of electromagnetic waves directly absorbed by the plant material and decrease the specific heating of the plant material compared to water. An experiment to determine the effect of plant material on the degradation of BER at 180°C (data not shown) using cellulose powder as a model of plant material was also conducted. With a total radiation time of 17 min (the temperature reached 180°C after 15 min of radiation), the degradation of BER with a 509.3 mg/L (20.37 mg/ 40 mL) initial concentration in water was 3.96% for the experiment without cellulose and 28.2% for the experiment with 1.00 g cellulose. This result also supported the above hypothesis that the specific heating of plant material by microwaves could induce BER degradation at high temperatures.

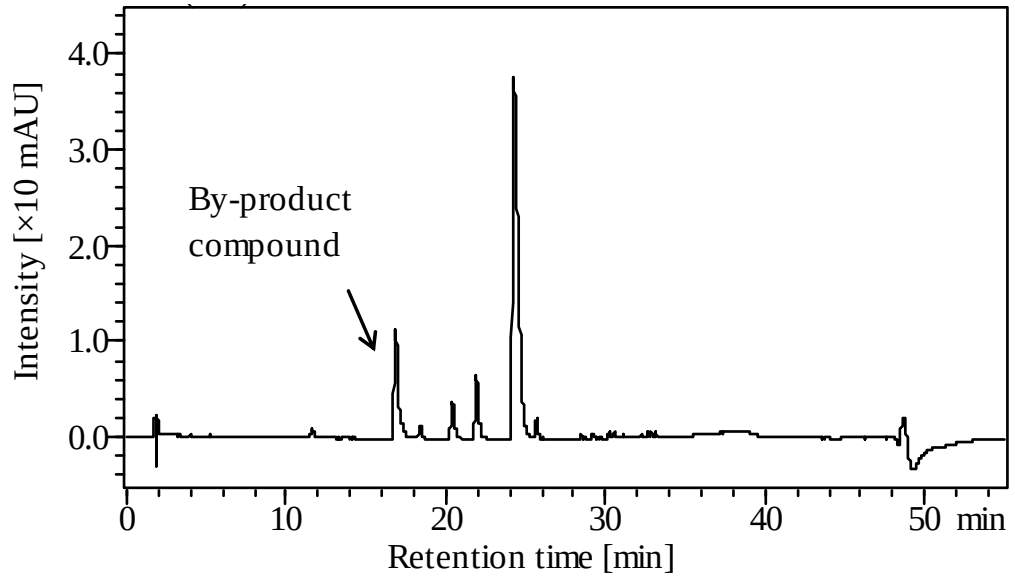


Figure 5.13 UV Chromatogram of extracted liquid obtained by closed-vessel MAE using water at 170°C (at 345 nm).

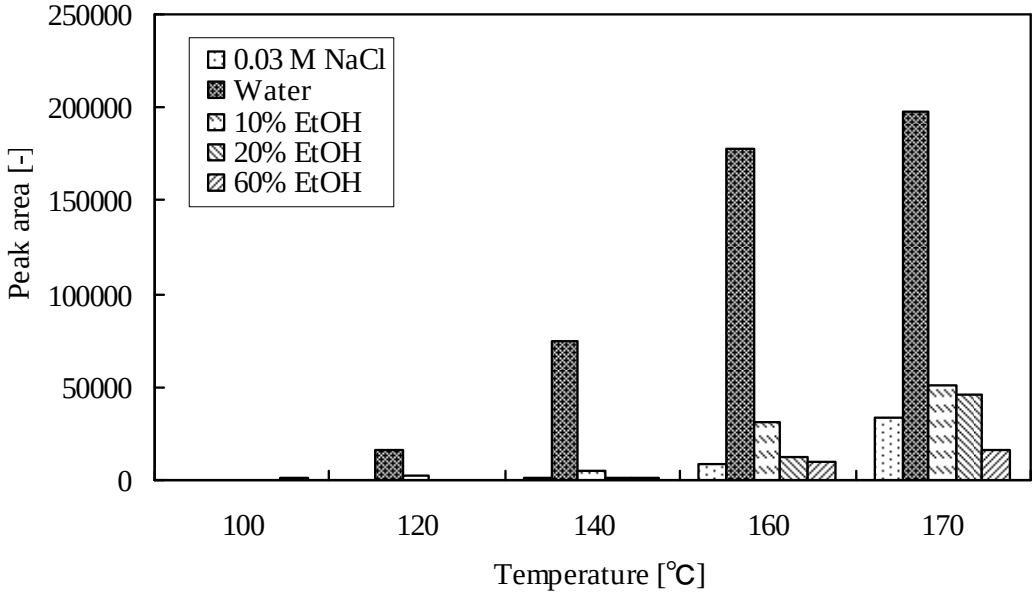


Figure 5.14 Peak area of compound retention at 16.65 min.

5.3.4 Comparison of MAE with Conventional Extraction Methods

Figure 5.15 shows the comparison of the maximum extraction yield according to each extraction method in this study. MAE in both an open and closed vessel using 60% EtOH at 160°C for 15 min gave a higher BER extraction (25.20 and 27.35 mg/g) than extractions by Soxhlet using methanol for 8 h (24.37 mg/g) and by 0.2% sulfuric acid for 24 h (21.82 mg/g). Using water as a solvent, increasing the BER extraction yield from 12.85 mg/g (Oil bath at 100°C) to 20.31 mg/g was achieved by increasing the temperature to 160°C by MAE in a closed vessel. The extraction yield using water was 84% of that of the 8 h Soxhlet extraction using MeOH and at the same level as that of extraction by 0.2% sulfuric acid for 24 h. The increased extraction yield by water at high temperatures may be because of the change in electric permittivity (dielectric constant and electric loss), which depends on frequency and temperature[77]. According to the equation

($D = 5321/T + 233.76 - 0.9297T + 0.001417T^2 - 0.0000008292T^3$, where T is absolute temperature in Kelvin degree) proposed by Akerof and Oshry[78], the dielectric constant (representative of polarity) of water was decreased from 80.2 at 20° C to 39.8 at 170° C (430～840 MHz). The electric constant decreases with increasing frequency; thus, the electric constants of water at 2450 MHz used in this study were considered to be lower than the above values and were close to those of methanol ($\varepsilon' = 32.6$) and ethanol ($\varepsilon' = 24.3$) at 20° C (2450 MHz) [79]. This characteristic allows water to act as an effective solvent for the extraction of protoberberine alkaloids at high temperatures. MAE could significantly shorten the extraction time with a high extraction yield and reduce the amount of solvent used for the extraction of protoberberine alkaloids from *C. fenestratum*.

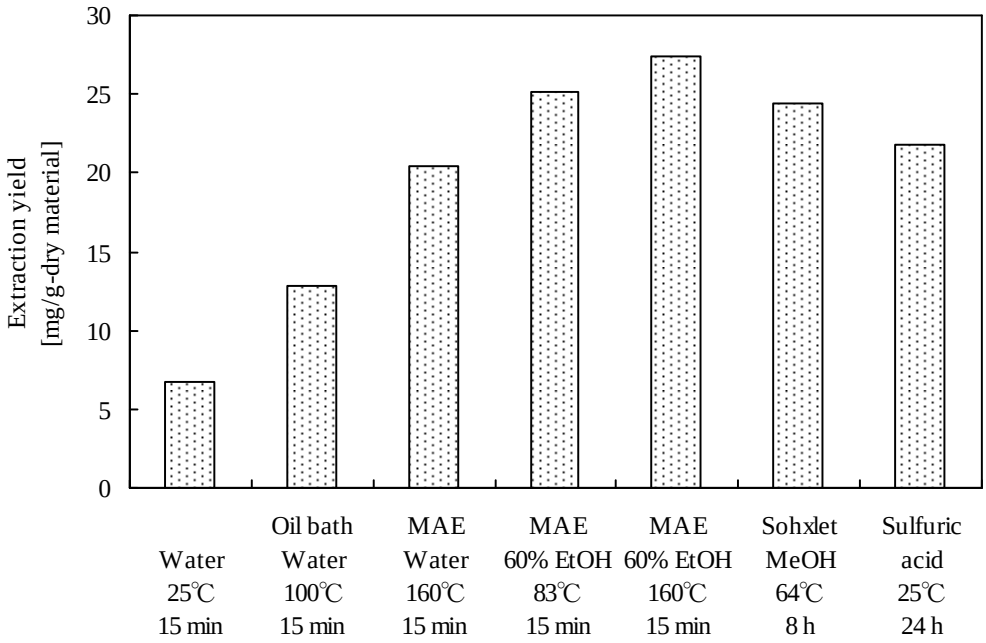


Figure 5.15 Comparison of MAE with conventional extraction methods for the extraction of BER.

5.4 Conclusions

An effective method for extracting protoberberine alkaloids from *C. fenestratum* by MAE was successfully developed. Extraction using optimum conditions (60% EtOH, material/solvent ratio 1.00 g:40 mL, microwave output power 300 W) gave a shorter time (15 min) with a higher BER extraction yield compared to extractions by Soxhlet using MeOH for 8 h and by 0.2% sulfuric acid for 24 h. The highest extraction yield was achieved at 160°C for BER and 170°C for JAT and PAL. Using water as a solvent, extraction at 160°C for 15 min total radiation time can increase the extraction yield to the same level as that of extraction by 0.2% sulfuric acid for 24 h. However, BER degradation at high temperatures is observed above 160°C. Additives such as EtOH or NaCl could reduce or prevent this degradation. MAE is more effective than the conventional extraction methods with regard to the extraction time and extraction yield, which could reduce energy consumption. It is an environmentally friendly process that can reduce the amount of organic solvent used

or could use water as a green solvent; it eliminates the need for using a strong acid for the extraction process.

CHAPTER 6

APPLICATION OF EXTRACTION AND ANALYSIS METHOD FOR SIMULTANEOUS CHARACTERIZATION OF COMPOUNDS IN OTHER MEDICINAL PLANTS

6.1 Introduction

There are many medicinal plants in Laos that have not been studied. Among many medicinal plants in Laos, Kiderm is one of the medical plants that have been used for treatment diabetes mellitus. A pharmaceutical company in Laos is trying to develop products for diabetes. According to the researcher in the pharmaceutical company, it was observed the blood glucose level lowering when applying tea of this plant to patients with type 2 diabetes. However, the scientific name and the compounds in Kiderm remain unknown.

One of the objectives of this study is to apply the developed methods for the extraction and analysis of compounds in Kiderm. The specification of main compound in Kiderm is performed by using liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/MS-IT-TOF) that described in Charpter 3.

C. japonica and *P. amurense* have been widely used in Japanese and Chinese herbal medicine. However, HPLC and LC/MS/MS analyses of these plants have been restricted to the investigation of the major quaternary protoberberine compounds such as BER, PAL, JAT, coptisine, and epiberberine[62, 80]. However, for the quality control of these herbs, it is essential to develop a technique for accurate simultaneous determination of both major and minor compounds. The second objective in this study

is to apply the developed simultaneous analysis method to analyze the compounds in *C. japonica* and *P. amurense,* and compare with those of *C. fenestratum*.

6.2 Experimental

6.2.1 Materials and Chemicals

Roots of Kiderm (**Figure 6.1**) was purchased from the medicinal plant shop in Natham village, Pakngum district, Vientiane province, Laos. Chemicals used in this study were described in Chapter 3 and Chapter 4.

6.2.2 Extraction

To investigate the effect of the solvent, dry roots of Kiderm were ground and sieved to obtain powder in the range of 500–1000 μm. The powder 0.5 g was weight to the flask and extracted in 100 mL of water, MeOH, EtOH and 60% EtOH aqueous solution in water bath at 60°C for 2 hour. The extracted liquid was filtered with 0.45 μm membrane filter. The filtrate was transferred into 1.5 mL vial for LC/MS analysis. The area of each peak from chromatogram was compared.

For the structural characterization of main compounds, a weight of Kiderm roots 500 g was extracted in hot MeOH. The extracted liquid was filtered using filter No.101 (Toyo Roshi, Japan). The filtrated liquid was evaporated using evaporator. The extract residue 1.6 mg was dissolved in 16 mL methanol (100 ppm). The filtrate was transferred into 1.5 mL vial for LC/MS-IT-TOF analysis.

For the simultaneous characterization of compounds in *C. japonica* and *P. amurense*, Sample of *C. japonica* and *P. amurense* described in Chapter 4 were used in this study and proceed in the same way with that of *C. fenestratum.* A weight of 0.5g of each dried material was extracted in 100 mL methanol using Soxhlet extraction. Extracted liquid was diluted 4 times for *C. japonica* and 2 times for *P. amurense* in methanol before LC/IT-TOF MS analysis.

(A)

(B)

Figure 6.1 Photos of root (A) and leaves of Kiderm.

6.2.3 HPLC and LC/IT-TOF MS Analysis

HPLC and LC/IT-TOF MS analysis were performed in the same way as described in Chapter 2 and Chapter 3. However, the m/z scan range was 100–1000 for the investigation of compounds in Kiderm.

6.3 Results and Discussion

6.3.1 Extraction of Compounds in Kiderm by thermal extraction and MAE

Figure 6.2 shows chromatogram of extraction using water in water bath for 2 hours. More than 16 peaks were observed. **Figure 6.3** shows the peak area of each compounds observed in Kiderm using each solvent. Water and 60% EtOH gave higher extraction yield than the extractions by pure MeOH and EtOH. This suggested that the compounds in Kiderm are hydrophilic. Main peak and other several PDA peaks show the maximum UV absorption around 270–280 nm indicates that the compounds could be flavonoid compounds in the group of flavones, flavonols, dihydrochalcones, catechins and flavan-3-ols. Peak 11 shows major ions at *m/z* 740 and 745 in positive mode, and *m/z* 721 in negative mode. The ions at *m/z* 745 $[M + Na]^+$ and *m/z* 721 $[M - H]^-$ indicate that the molecular weight of main component is 722.

MAE in 15 min gave similar result to those of extraction by water bath for 2 hours. The peak is similar (**Figure 6.4**). This suggested that MAE can also be applicable for extraction compounds in Kiderm.

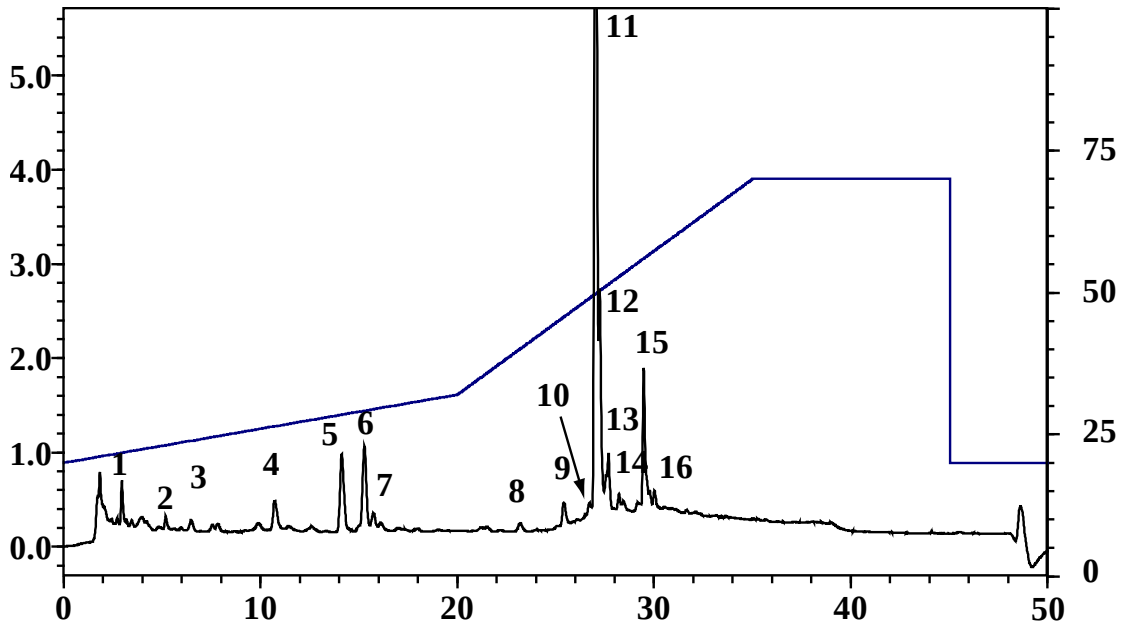


Figure 6.2 UV chromatogram of Kiderm by thermal extraction using water (at 279 nm).

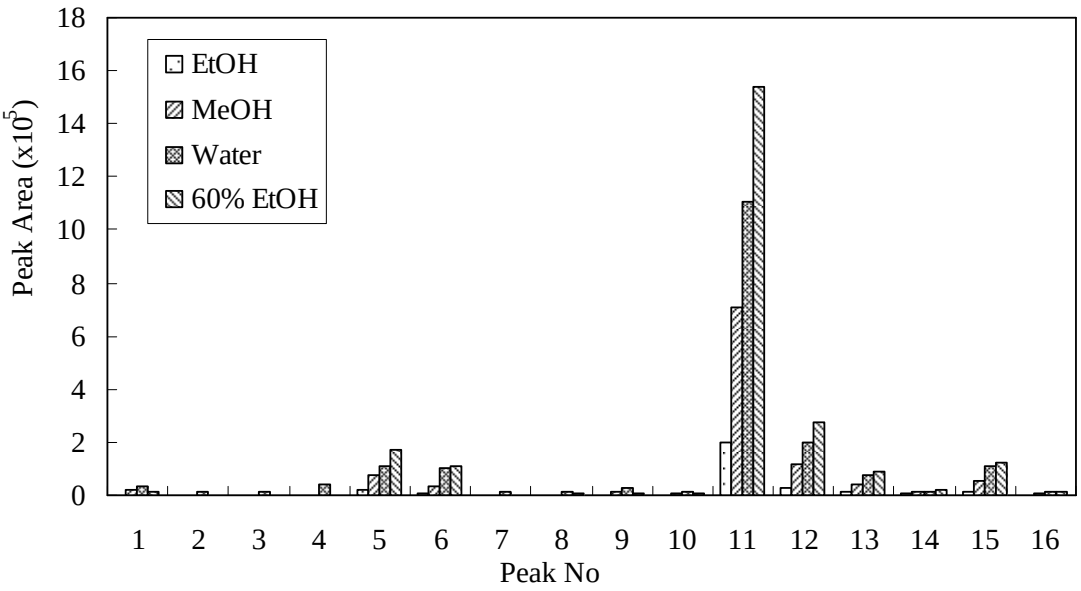


Figure 6.3 Peak area of each compound in Kiderm by thermal extraction.

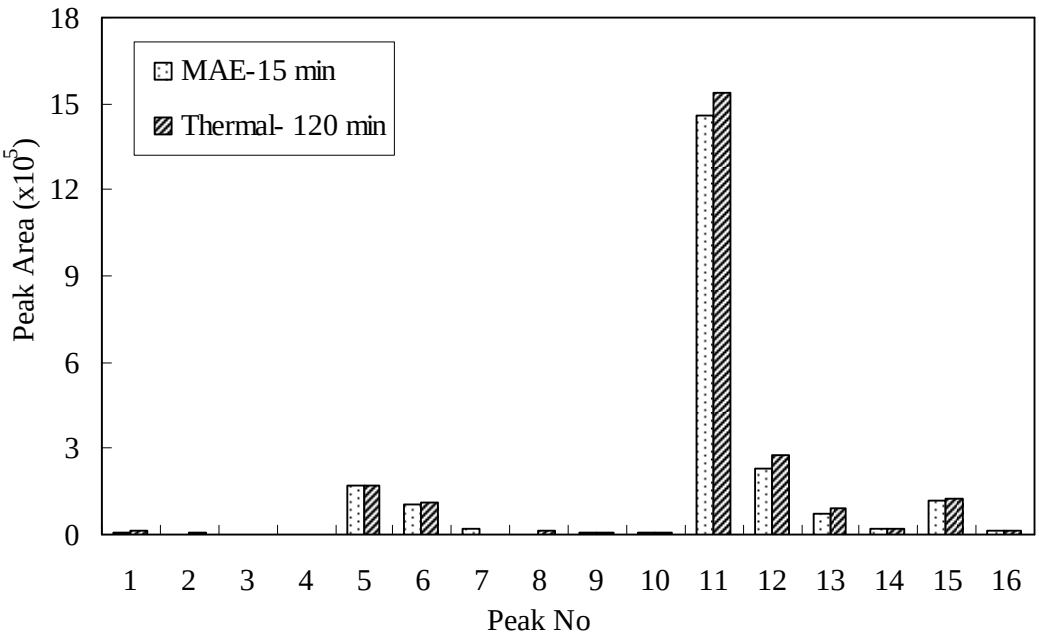


Figure 6.4 Comparison of MAE and thermal extraction.

6.3.2 LC/MS-IT-TOF analysis

Figure 6.5 shows the chromatogram obtained by LC/IT-TOF MS. Mass data of each peak were summarized in **Table 6.1**. **Figure 6.6** and **Figure 6.7** shows the mass fragments of the main peak obtained from positive and negative mode, respectively. In negative mode, *m/z* at 721.1783 in MS^1 was observed as the main ion indicating the deprotonated molecule $[M - H]^-$. In positive mode, sodium adducted ion $[M+Na]^+$ at 745.1707 was observed. This result indicates that molecular weigh of the compound is 722.18. Fragmentation of ion at *m/z* 721.1783 gave ion at 541.1340 which was 180.0443 lower than the precursor ion (721.1783). The loss of 180 da from a molecular could be due to the loss of glucoside ($C_6H_{12}O_6$). However, calculation of $C_6H_{12}O_6$ gave exact mass at 180.0634 which shows 106 ppm higher than 180.0443. This result suggested the low possibility that the molecule has glucoside. Instead, $C_9H_8O_4$ (exact mass 180.0423) was proposed as potential fragment which loss from the molecular structure. This lost fragment could be caffeic acid. Ion at m/z 361.0883 also indicated the cleavage of this fragment from the molecule. The strong intensity of ion at m/z 299.0861 suggested the skeleton structure of the compounds. From this result, the main compound in Kiderm was considered to be a flavonoid compound with two molecules of caffeic acid. Compounds 5, 6, 11, and 15 could have similar basic structure, since they produced similar fragment ions (**Table 6.1**). The compounds could be similar to flavonoids that were reported to have efficacy in lowering blood glucose, and aldose reductase inhibitory activity[81-83]. However, the accurate position of functional group of the flavonoids can not be determined by spectrometry. Thus, the accurate structure should be confirmed by NMR spectroscopy in further work.

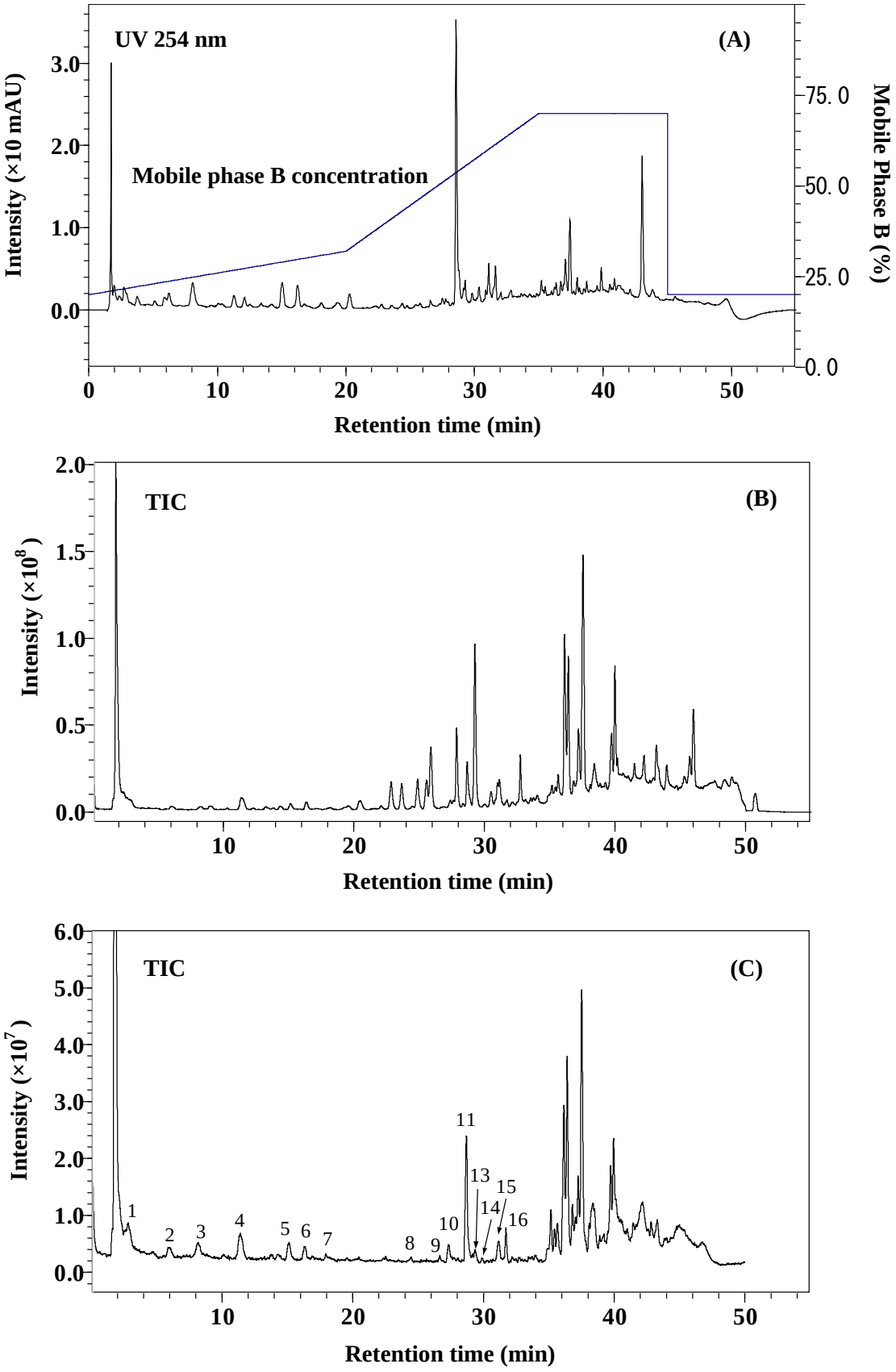


Figure 6.5 Chromatogram of extract of Kiderm using LC/IT-TOF MS. (A) at 254 nm; (B) TIC in positive ESI; (C) TIC in negative ESI.

Table 6.1 Mass data of some compounds in Kiderm

Peak No	Rt	MS^1 *m/z*	MS^2 *m/z* / MS^3 *m/z* (% base peak)
1	2.82	359.0993	197.0442(100), 179.0345(31), 135.0447(9)
2	5.90	443.1904	397.1098
3	8.23	373.1143	193.0473(100), 149.0583(37) /149.0628(100)
4	11.35	403.1241	357.1189(100), 195.0698(60), 179.0561(45)
5	15.16	541.1352	361.0889(8), 317.1048(14), 299.0899(63), 281.0791(13), 273.1132(38), 237.0930(13), 197.0452(30), 179.0375(17)
6	16.41	541.1342	361.0918(43.54), 343.0765(16), 317.1041(25), 299.0936(8), 273.1116(100), 255.0968(10), 197.0476(16), 179.0361(7)
7	17.94	403.1249	371.0987(100), 223.0629(71), 179.0554(43)
8	24.39	525.1380	361.0883(62), 343.0863(13), 317.0969(13), 273.1108(100)
9	26.59	555.1487	375.1068(54), 357.0991(19), 331.1204(100), 325.0686(7), 299.0861(7), 197.0430(9)
10	27.32	389.0853	275.0909 / 219.0646
11	28.58	721.1783	541.1340 / 361.0936(12), 343.0863(7.58), 317.1019(15), 299.0918 (83), 281.0817(18), 273.1111(35), 255.0982(7), 237.0908(19), 197.0441(35), 179.0347(15)
12	-		
13	29.34	719.1644	539.1148(13), 521.1066(42), 495.1286(8), 359.0767(58), 341.0661(58), 323.0589(15), 297.0767(10), 279.0668(13)
14	29.84	717.1436	519.0918(100), 321.0360(14) / 339.0492(17), 321.0425(100), 279.0278(9)
15	31.16	705.1811	541.1351 / 361.0884(8), 343.0783(8), 317.1046(23), 299.0914(100), 281.0835(31), 273.1119(68), 255.0969(13), 237.0923(16), 197.0447(51), 179.0357(26)
16	31.70	343.0823	161.0242 / 133.0286

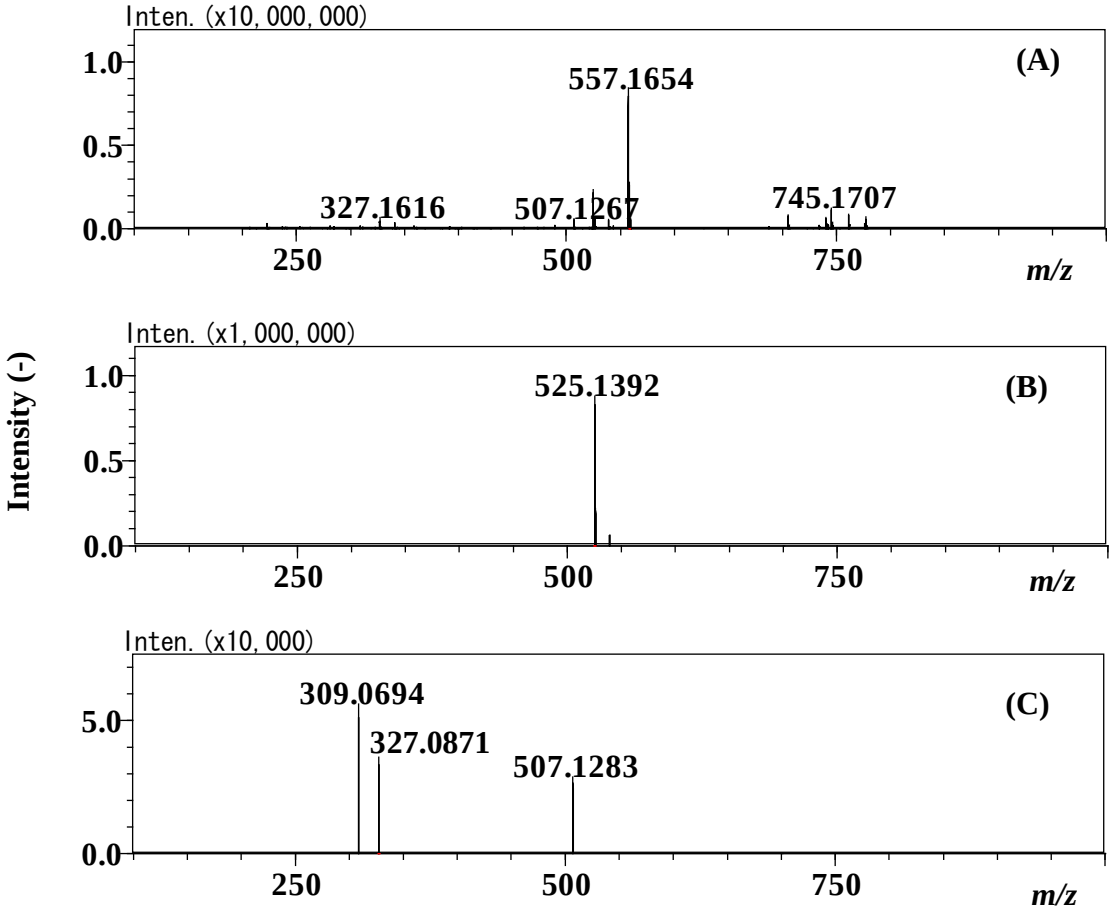


Figure 6.6 Mass spectrum of compound at 29.00 min in positive ESI. (A) MS^1; (B) MS^2 of *m/z* at 745.1707; (C) MS^3 of *m/z* at 525.1392.

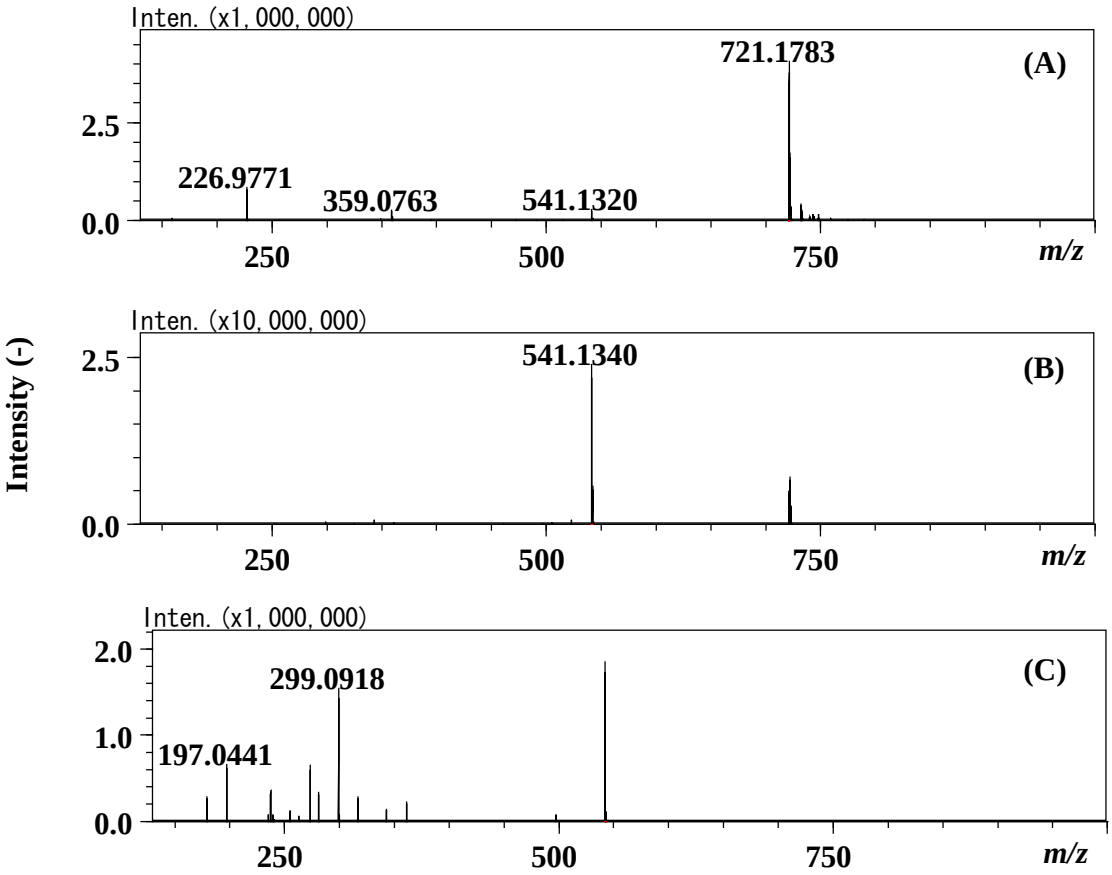


Figure 6.7 Mass spectrum of compound at 29.00 min in negative ESI. (A) MS^1; (B) MS^2 of ion at m/z 721.1783; (C) MS^3 of ion at m/z 541.1340.

6.3.3 Comparison of Compounds in *C. fenestratum* with Those in *C. japonica* and *P. amurense*.

Figure 6.8 shows the total ion chromatogram of the extract of *C. japonica* and *P. amurense* There were 11 compounds in *C. japonica* (compounds 1, 3, 5, 8, 9, 11, 14, 16, 18, 19, and 32) and *P. amurense* (compounds 3, 5, 8, 9, 11, 14, 15, 16, 18, 19, and 32) that were identical to those in *C. fenestratum*. These compounds were the tembetarine derivative, magnoflorine, menisperine, tetrahydropalmatine, 13-hydroxyberberine, demethyleneberberine, thalifendine, tetrahydroberberine, JAT, BER, PAL, and 8-oxoberberine. All these compounds, except for the tembetarine derivative, demethylenberberine, 13-hydroxyberberine, have been previously isolated from *C. japonica* and *P. amurense*. 8-Oxoberberine was detected in both *C. japonica* and *P. amurense* at a retention time of 41.19 min. Thus, the technique can be applied to characterize oxoprotoberberine compounds in other medicinal plants or herbal medicines. This evidence also suggests that *C. fenestratum* can be potentially used as alternative for *C. japonica* and *P. amurense*, although further studies on the biological activities of other minor compounds may be required.

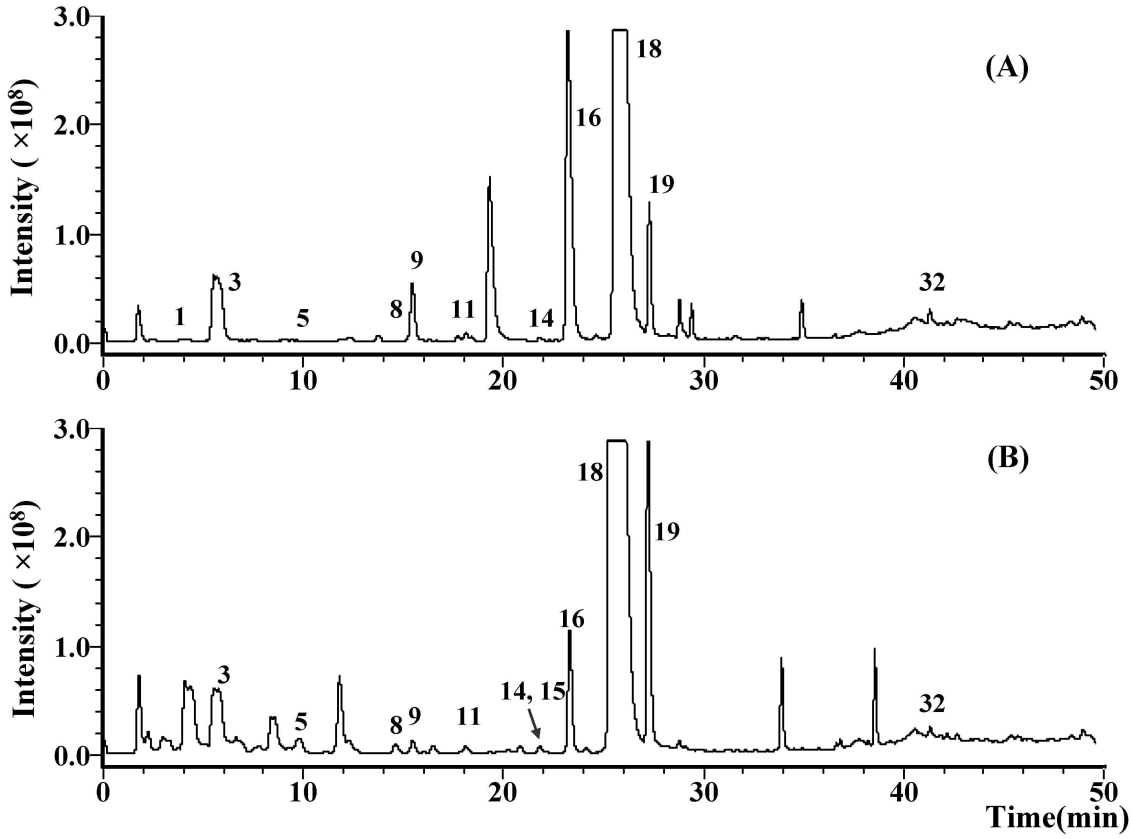


Figure 6.8 (+)ESI Total ion chromatogram of *C. japonica* and *P. amurense* (2µl injected). (A) *C. japonica;* (B) *P. amurense.*

6.4 Conclusions

Compounds in Kiderm can be extracted using polar solvent such as water and 60% EtOH. Most of compounds are hydrophilic. The main compound was proposed to be the flavonoids compounds. At least 4 compounds could be flavoinoid with two molecules of caffeic acid. The glucose level lowering effect of Kiderm could be due to the effect of the compounds that similar with those of flavonoids that reported previously. MAE gave similar result to those of extraction by thermal but reduce the extraction time.

There were 11 compounds in *C. japonica* and *P. amurense* that were identical to those in *C. fenestratum*. The method using LC/IT-TOF MS can also be applied to identify 8-oxoberberine in other medicinal plants such as *C. japonica* and *P. amurense*.

This study suggests that LC/IT-TOF MS has a great potential in the simultaneous analysis of secondary metabolites in medicinal plants as well as herbal medicines.

CHAPTER 7

CONCLUSIONS

7.1 General Conclusions

The purpose of this study is to develop method for simultaneous characterization and for effective extraction of medicinal compounds form *C. fenestratum*. The simultaneous characterization of compounds is based on hyphenate liquid chromatography and ion trap time-of-flight mass spectrometry (LC/IT-TOF MS). The effective extraction of protoberberine alkaloids from *C. fenestratum* were studied using microwave-assisted extraction (MAE) technique.

The optimum condition for separation of the compounds in *C. fenestratum* was investigated using liquid chromatography mass spectrometry (LC/MS). The experiment was performed on ZORBAX Eclipse XDB-C18 Column using 20–70% of MeOH and 0.1% formic acid aqueous solution as the mobile phase. More than 20 peaks were found and successfully separated in the stem extract of *C. fenestratum* using 3 step gradient condition of 20–32–70% MeOH. BER, JAT, and PAL are the major compounds in *C. fenestratum*. MeOH gave higher extraction yield than EtOH and water in the extraction using water bath and Soxhlet extraction vessel, and extraction by sulfuric acid at room temperature.

The method for simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *C. fenestratum* by using LC/IT-TOF MS was developed as described in chapter 3. A total of 32 compounds were found, of which 20 compounds, including 4 novel natural products, were

identified or tentatively identified for the first time from *C. fenestratum*. The information may be useful for further studies on the pharmacological activities of the herb. The proposed method is an accurate and rapid method for characterizing various compounds in *C. fenestratum*. 8-oxoprotoberberines produced $[M + H]^+$ and $[M + Na]^+$ ions in MS^1 operated in the positive-ion mode. The fragmentation pathways of 8-oxoprotoberberines are different from those of quaternary protoberberines and tetrahydroprotoberberines. In addition, 8-oxotetrahydroprotoberberines generated iminium ions, which were formed by the cleavage of the protoberberine skeleton.

In chapter 4, the quantification of major compounds in *C. fenestratum* were investigated and compared to those of other protoberberines contained medicinal plants. Variation of compounds in *C. fenestratum* from different area were found. The amount of BER and JAT was higher in larger size of *C. fenestratum*, which indicates that the plant size is an important parameter for determination of the amount of compounds in the plant. The amount of BER, PAL and JAT in *C. fenestratum* were also compared with those of *C. japonica* and *P. amurense*. Amount of BER, JAT and PAL were smaller than those in *C. japonica* and *P. amurense*. There was some tendency of amount of BER, PAL and JAT between *C. fenestratum* and *C. japonica*. This suggested that *C.fenestratum* could be a potential medicinal plant that can be used as *C. japonica*.

In chapter 5, the effective method for extracting protoberberine alkaloids from *C. fenestratum* by MAE was investigated. Extraction using optimum conditions (60% EtOH, material/solvent ratio 1.00 g:40 mL, microwave output power 300 W) gave a shorter time (15 min) with a higher BER extraction yield compared to those of extractions by Soxhlet using MeOH for 8 h and using 0.2% sulfuric acid for 24 h. The highest extraction yield was achieved at 160°C for BER and 170°C for JAT and PAL. Using water as a solvent, extraction at 160°C for 15 min total radiation time can

increase the extraction yield to the same level as that of extraction by 0.2% sulfuric acid for 24 h. However, BER degradation at high temperatures is observed above 160°C. Additives such as EtOH or NaCl could reduce or prevent this degradation. MAE is more effective than the conventional extraction methods with regard to extraction time and extraction yield, which could reduce energy consumption. It is an environmentally friendly process that can reduce the amount of organic solvent used or could use water as a green solvent; it eliminates the need for using a strong acid for the extraction process.

In chapter 6, the application of the developed methods in other medicinal plants was investigated. Using LC/IT-TOF MS, several compounds could be flavonoid compounds with two molecules of caffeic acid. MAE gave similar result to those of extraction by thermal but reduce the extraction time. For the comparison compounds in *C. fenestratum* with other protoberberines containing medicinal plants, 11 compounds in *C. japonica* and *P. amurense* were found to be identical to those in *C. fenestratum*. The method using LC/IT-TOF MS can also be applied to identify 8-oxoberberine in other medicinal plants such as *C. japonica* and *P. amurense*. This study suggests that LC/IT-TOF MS has a great potential in the simultaneous analysis of secondary metabolites in medicinal plants as well as herbal medicines.

In conclude this research provide basic information for the further development of herbal product relating to *C. fenestratum*. The simultaneous characterization method is useful for quality control of herbal medicine and gaining better understanding of the pharmacological of herbal medicines. The further contribution of this research were summarize in **Figure 7.1**.

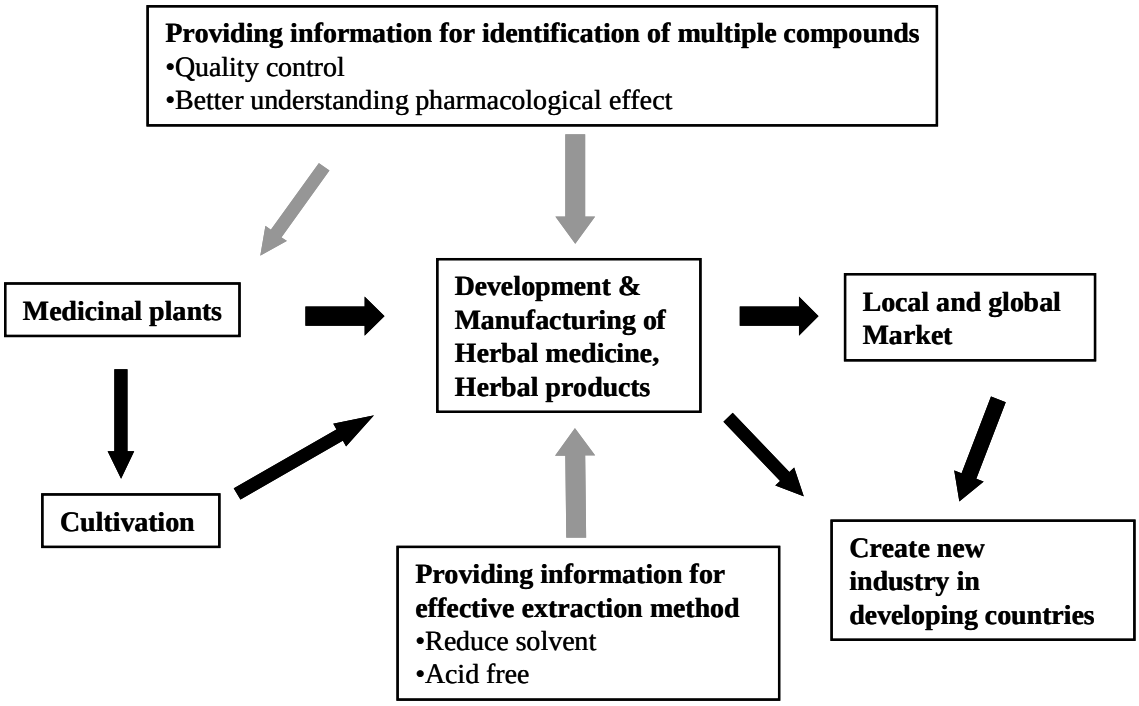


Figure 7.1 Further contribution of this research

7.2 Recommendation for Further Study

This research provides basic research data for the simultaneous identification and quantification of the compounds in *C.fenestratum* using LC/MS and LC/IT TOF MS; and the methods for extraction protoberberine alkaloids using MAE. The compounds found in this study are useful for the quality control of the herbal medicine, and offer a scientific base data for gaining a better understanding of pharmacological effect of the plants. However, the accurate chemical structure of several compounds can not determined by current approach using mass spectrometry.

MAE gave high extraction yield in a short time compare to conventional extraction method such as Soxhlet extraction and extraction using sulfuric acid. It was also found that MAE using water at 160˚C can achieved same level of extraction yield as that of

using sulfuric acid. However, the degradation of compounds was observed which could reduce the target compounds. Thus, the author suggest the following issue for further study to maximize the value of this research for contribution in the development of industry using medicinal plants in Laos as well as in the development of sciences and technologies.

1) Purify and determine the chemical structure those tentatively identified in this study by NMR spectroscopy.
2) Investigate for reducing the BER degradation in MAE when using water as a solvent.
3) Investigate how to scale up the MAE to be applicable in industry level.
4) The finding of berberine degradation in MAE also offers an idea that it may be an effective way for the chemical synthesis of berberine derivatives.
5) It is interesting that *C. fenestratum* contained various kinds of 8-oxoprotoberberine alkaloids, while only oxoberberine is found in *C. japonica* and *P. amurense*. The further study for the enzyme in *C. fenestratum* could provide information for the differences and valuable for the production of various 8-oxoprotoberberine alkaloids.

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LIST OF ORIGINAL PUBLICATIONS

Ⅰ. International Journal Papers

1. Phengxay Deevanhxay, Makoto Suzuki, Nariaki Maeshibu, He Li, Ken Tanaka, Sachio Hirose, "Simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *Coscinium fenestratum* by liquid chromatography hybrid ion trap time-of-flight mass spectrometry", *Journal of Pharmaceutical and Biomedical Analysis* **2009**, 50,(3), 413–425

2. Phengxay Deevanhxay, Makoto Suzuki, Nariaki Maeshibu, Sachio Hirose, "Microwave-assisted extraction of protoberberine alkaloids from *Coscinium fenestratum*", *Journal of Chemical Engineering of Japan* [in print]

Ⅱ. International Conferences

1. Nariaki Maeshibu, Phengxay Deevanhxay, Makoto Suzuki, Ken Tanaka, Keoudone Rasphone, Sachio Hirose, "Online analysis of compounds in Lao medicinal plant-Kiderm", Regional Symposium on Chemical Engineering, 16th Regional symposium on chemical engineering, Abstract accepted, (December, **2009**, Manila, Philippines)

2. Makoto Suzuki, Phengxay Deevanhxay, Nariaki Maeshibu, Sachio Hirose Pressurized microwave-assisted extraction of protoberberine alkaloids from *Coscinium fenestratum*, Regional Symposium on Chemical Engineering, 16th Regional symposium on chemical engineering, Abstract accepted, (December, **2009**, Manila, Philippines)

3. Phengxay Deevanhxay, Makoto Suzuki, He Li, Sachio Hirose, "Online analysis of bioactive compounds in *Coscinium fenestratum* from Lao P.D.R", 15th Regional symposium on chemical engineering, 133–138 (December, **2008**, Kuala Lumpur, Malaysia)

4. P. Deevanhxay, M. Suzuki, H. Li, S. Hirose, "Effective method for extracting

protoberberines from *Coscinium fenestratum* by Microwave-assisted extraction", 12 th Asian Pacific Confederation of Chemical Engineering Congress, Vol5–6, 162 (August, **2008**, Dalian,China)

Ⅲ. Domestic Conferences

1. Phengxay Deevanhxay, "Extracting protoberberines from *Coscinium fenestratum* by microwave-assisted extraction", The First Energy-GCOE CDP Forum, (March, **2009**, Tokyo)

2. Phengxay Deevanhxay, Makoto Suzuki, He Li, Sachio Hirose, "Extracting protoberberines by microwave-assisted extraction", 73 th Annual Meeting of Japan Society of Chemical Engineers , 99 (March, **2008**, Hamamatsu)