การพิสูจน์เอกลักษณ์และฤทธิ์ทางชีวภาพของพืชสกุลแอสพารากัส

นายธีระวัฒน์ บุญโสม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

IDENTIFICATION AND BIOACTIVITIES OF PLANTS IN THE GENUS *ASPARAGUS*

Mr. Teerawat Boonsom

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacognosy

Department of Pharmacognosy and Pharmaceutical Botany

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Thesis Title	IDENTIFICATION AND BIOACTIVITIES OF PLANTS IN THE GENUS *ASPARAGUS*
By	Mr. Teerawat Boonsom
Field of Study	Pharmacognosy
Thesis Advisor	Associate Professor Suchada Sukrong, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Professor Kittisak Likhitwitayawuid, Ph.D.)

..... Thesis Advisor

(Associate Professor Suchada Sukrong, Ph.D.)

..... Examiner

(Associate Professor Boonchoo Sritularak, Ph.D.)

..... Examiner

(Sornkanok Vimolmangkang, Ph.D.)

..... External Examiner

(Associate Professor Kornkanok Ingkaninan, Ph.D.)

ธีระวัฒน์ บุญโสม: การพิสูจน์เอกลักษณ์และฤทธิ์ทางชีวภาพของพืชสกุลแอสพารากัส. (IDENTIFICATION AND BIOACTIVITIES OF PLANTS IN THE GENUS *ASPARAGUS*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, 99 หน้า.

พืชในสกุลแอสพารากัสเป็นพืชที่มีประโยชน์ สามารถใช้เป็นยาและเป็นอาหารที่ให้ผลทางยาหรือมีประโยชน์ต่อสุขภาพ รากสามสิบ (*Asparagus racemosus*) เป็นสมุนไพรที่นิยมใช้อย่างแพร่หลายและมีองค์ประกอบทางเคมีเป็นสารในกลุ่มสเตียรอยด์แซโพนินที่พบในปริมาณสูง ได้แก่ สารชาทาวาริน I-IV ซึ่งมีฤทธิ์คล้ายฮอร์โมนเอสโทรเจน รากแห้งใช้เป็นสมุนไพร เรียก "รากสามสิบ" ในปัจจุบันพืชที่ออกฤทธิ์คล้ายฮอร์โมนเอสโทรเจนกำลังอยู่ในความนิยม จึงทำให้มีโอกาสปนด้วยสมุนไพรชนิดอื่น การระบุชนิดของ *A. racemosus* ทำได้ค่อนข้างยากเนื่องจากลักษณะภายนอกมีความคล้ายคลึงกับพืชชนิดอื่นๆ ในสกุลเดียวกัน ดังนั้นการระบุชนิดของ *A. racemosus* จึงเป็นสิ่งจำเป็น ในการวิจัยนี้สามารถเพิ่มปริมาณดีเอ็นเอในส่วนยีนแม็ททูเรสเค (แม็ทเค) ของพืชสกุลแอสพารากัสได้ทั้ง 8 ชนิด พบว่าความยาวของลำดับนิวคลีโอไทด์ของยีนแม็ทเค มีขนาดเท่ากัน คือ 1557 คู่เบส เมื่อวิเคราะห์ลำดับนิวคลีโอไทด์ของพืชสกุลแอสพารากัสทั้ง 8 ชนิด พบความแตกต่างของลำดับนิวคลีโอไทด์ทั้งหมด 10 ตำแหน่ง และ *A. racemosus* แสดงลำดับนิวคลีโอไทด์ที่แตกต่างจาก *Asparagus* ชนิดอื่นๆ จึงนำมาสู่การพัฒนาเทคนิคพีซีอาร์-อาร์เอฟแอลพีเพื่อใช้แยก *A. racemosus* ออกจากต้นอื่น นอกจากนี้ยังสามารถใช้เทคนิคพีซีอาร์-อาร์เอฟแอลพีในการตรวจสอบเครื่องยาสมุนไพรที่เรียก รากสามสิบ ได้

เมื่อศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ความเป็นพิษต่อเซลล์ของสารสกัดหยาบจากส่วนรากของพืชสกุลแอสพารากัส 8 ชนิด พบว่าสารสกัดหยาบทั้งหมดมีฤทธิ์ในการต้านอนุมูลอิสระน้อย ที่น่าสนใจคือสารสกัดหยาบจากรากต้นโปร่งฟ้าแสดงความเป็นพิษต่อเซลล์มะเร็งช่องปากชนิด KB และเซลล์มะเร็งปอดชนิด NCI-H187 ปานกลาง ด้วยค่า IC_{50} เท่ากับ 19.59 และ 32.60 ไมโครกรัมต่อมิลลิตร ตามลำดับ ข้อมูลที่ได้เป็นการรายงานครั้งแรกถึงความเป็นพิษต่อเซลล์มะเร็งจากรากโปร่งฟ้า

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต.....

สาขาวิชา.....เภสัชเวท..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2554.....

5376579333 : MAJOR PHARMACOGNOSY
KEYWORDS : ASPARAGACEAE / *ASPARAGUS RACEMOSUS* / *MAT*K GENE / PCR-RFLP / PHYLOGENETIC RELATIONSHIP / ANTIOXIDANT ACTIVITY / CYTOTOXICITY

TEERAWAT BOONSOM : IDENTIFICATION AND BIOACTIVITIES OF PLANTS IN THE GENUS *ASPARAGUS*. ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., 99 pp.

The genus *Asparagus* represents highly valuable plant species having therapeutic and nutraceutical importance. The plant *Asparagus racemosus* is one of the most widely used sources of phytoestrogens because of its high content of the steroidal saponins, shatavarins I–IV, in roots. The dry root of *A. racemosus*, known as “Rak-Sam-Sip” in Thai, is one of the most popular herbal medicines. Recently, the interest in plant-derived estrogens has increased tremendously, making *A. racemosus* particularly important and a possible target for fraudulent labeling. However, the identification of *A. racemosus* is generally difficult due to its similar morphology to other *Asparagus* spp. Thus, accurate authentication of *A. racemosus* is essential. In this study, 1,557-bp nucleotide sequences of the maturase K (*mat*K) gene of eight *Asparagus* taxa were analyzed. Ten polymorphic sites of nucleotide substitutions were found within the *mat*K sequences. *A. racemosus* showed different nucleotide substitutions to the other species. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the *mat*K gene was developed to discriminate *A. racemosus* from others. Ten commercially crude drugs called “Rak-Sam-Sip” were also analyzed by PCR-RFLP technique.

Root extracts from eight *Asparagus* species were evaluated for antioxidant and cytotoxicity. All extracts exhibited weak antioxidant activity. Interestingly, crude extract of *A. setaceus* showed significant cytotoxic activity against KB cells and NCI-H187 cells with IC_{50} of 19.59 and 32.60 µg/ml, respectively. This is the first time that cytotoxicity of *A. setaceus* has been reported.

Department: Pharmacognosy and Pharmaceutical Botany Student’s Signature……....

Field of Study: ……Pharmacognosy…… Advisor’s Signature……

Academic Year: …2011……

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my thesis advisor, Assoc. Prof. Suchada Sukrong, Ph.D. at The Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for her invaluable help, suggestions, patience, and constant encouragement throughout the course of this study. I am most grateful for her teaching and advice, not only the research methodologies but also many other methodologies in life. I would not have achieved this far and this thesis would not have been completed without all the support that I have always received from her.

In addition, I would like to express heartfelt respect and sincere gratitude to Assoc. Prof. Thatree Phadungcharoen, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, and Assoc. Prof. Kornkanok Ingkaninan, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, for kindness in collecting specimens and suggestions in my investigation. I also thank Assist. Prof. Dr. Jessada Denduangboripant, Department of Biology, Faculty of Science, Chulalongkorn University, for his valuable advice and encouragement.

Deepest gratitude is also due to the thesis committee for their important and constructive suggestions and crucial reviews for my thesis.

Moreover, I would like to thank Prof. Dr. Kittisak Likhitwitayawuid, Assoc. Prof. Surattana Amnuoypol, Assoc. Prof. Boonchoo Sritularak, Sornkanok Vimolmangkang, and all of teachers in The Department of Pharmacognosy and Pharmaceutical Botany for helpful discussion and valuable suggestions for improvements.

I am grateful for financial support provided by National Nanotechnology Center (NANOTEC), NSTDA, Ministry of Science and Technology, Thailand, The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR 1166I) and Chulalongkorn University Centenary Academic Development Project.

I thank everyone in the Department of Pharmacognosy and Pharmaceutical Botany and other persons whose names have not been mentions here for helping me get through the difficult times, and for all the emotional support, entertainment, and friendships.

Finally, and most important, I take deepest pleasure in thanking my parents, my grandmother, and my sister who always love, care, understand and support me. This thesis and this degree are dedicated to them.

CONTENTS

Page

CHAPTERS Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent (part per 100); percentage
/	per
18S rDNA	18S ribosomal RNA gene
AFLP	amplified fragment length polymorphism
ARMS	amplification refractory mutation system
A, T, G, C	nucleotides containing the base adenine, thymine, guanine and cytosine, respectively
bp	base pairs
^{o}C	degree celcius
CI	consistency index
cm	centimeter
cpDNA	chloroplast DNA
EDTA	ethylenediamine tetra acetic acid
h	hour
HCl	hydrochloric acid
ITS	internal transcribed spacer
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
kb	kilobase
*mat*K gene	gene encoding maturase K
min	minute
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
MP	maximum parsimony
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
ng	nanogram

PAUP	phylogenetic analysis using parsimony
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction- restriction fragment length polymorphism
pH	the negative logarithm of the concentration of hydrogen ions
RAPD	random amplified polymorphic DNA
*rbc*L gene	gene encoding the large subunit of the ribulosebiphosphate carboxylase
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RI	retention index
RNA	ribonucleic acid
SCAR	sequence characterized amplified regions
SD	standard deviation
spp.	species
TBR	tree-bisection-reconnection
Tris	tris (hydroxymethyl) aminomethane
*trn*K gene	gene encoding $tRNA^{Lys}$
*trn*L-*trn*F spacer	non-coding intergenic spacer between *trn*L and *trn*F in the chloroplast genome
U	unit
UPGMA	unweighted pair group method with arithmetic averages
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviole

CHAPTER I

INTRODUCTION

The genus *Asparagus* (Asparagaceae) includes over 300 species widely distributed in temperate and tropical regions (Clifford and Conran, 1987). Some species have economic value as ornamentals (*A. densiflorus*) or are noted for its highly prized vegetable (*A. officinalis*) (Riccardi *et al*., 2011). Several *Asparagus* species of Asia and Africa have significant medicinal properties (Nwafor and Okwuasaba, 2003) due to the presence of steroidal saponins and sapogenins in various parts of the plants (Negi *et al*., 2010). Currently, medicinal plants play a vital role to preserve our health. The genus *Asparagus* represents highly valuable plant species having therapeutic and nutraceutical importance (Shasnay *et al*., 2003). The leaves and the tuberous roots of *Asparagus* are medically important in several diseases (Vijay *et al*., 2009). In India, it has been used in Ayurvedic medicine as an anti-diarrhea and anti-tuberculosis remedy (Sharma *et al*., 2000). The two major steroidal saponins, shatavarins I and IV, from the roots of *A. racemosus* were isolated (Ravikumar *et al*., 1987; Joshi *et al*., 1988; Hayes *et al*., 2008) and have been recognized for its phytoestrogenic properties (Gopumadhavan *et al*., 2005). Several studies have demonstrated other medicinal effects of dry roots of *A. racemosus* as an adaptogen (Bhattacharya *et al*., 2000) and an immunoadjuvant (Gautam *et al*., 2004). In Thai traditional medicine, *A. racemosus* (Rak Sam Sip) is one of the most popular herbal plants (Bopana and Saxena, 2007) since its dry roots are well known as an anti-inflammatory, an aphrodisiac and a galactagogue (Pongboonrod, 1950).

In some countries, *A. officinalis* has been used as an anti-cancer herbal medicine for a long time (Zhong *et al*., 1999). Pharmacological studies on this plant have demonstrated anti-inflammatory (Jang *et al*., 2004), cytotoxic (Shao *et al*., 1997), antimutagenic (Tang and Gao, 2001), and antifungal activities (Shimoyamada *et al*., 1990). *A. cochinchinensis* was historically believed to possess numerous therapeutic properties, including anticancer and anti-inflammatory activities (Huang, 1993; Lee *et*

al., 2009). The compounds from roots of *A. acutifolius* demonstrated antifungal activity against the human pathogenic yeasts *Candida albicans*, *C. glabrata* and *C. tropicalis* (Sautour *et al*., 2007). *A. oligoclonos* have been used as cough and asthma remedies in far eastern countries of Korea, and cytotoxic activity against 5 human cell lines (Yang *et al*., 2004). The dried root of *A. pubescens* has a long history of traditional medicinal. It is used for family planning and for the treatment of various gastrointestinal disorders, including peptic ulcer, diarrhea, anti-nociceptive and anti-inflammatory (Hutchinson and Dalziel, 1968; Nwafor *et al*., 2003). Therefore, other plants from genus *Asparagus* could be explored for biological activities such as antioxidant and cytotoxicity to access a plant's suitability for its potential applications, particularly as natural medicine.

Recently, interest in plant-derived estrogens has increased tremendously, making *A. racemosus* particularly important. However, the trading economics of roots of *A. racemosus* is strongly affected by the presence of less-effective substitutes such as *A. officinalis* (Bopana *et al*., 2007). Moreover, the dry root of *A. racemosus* is similar in appearance to those of other *Asparagus* spp. (e.g. *A. setaceus*, *A. aethiopicus*, *A. officinalis*, *A. filicinus*, *A. densiflorus*, *A. umbellatus* and *A. densiflorus*) and cannot be distinguished from other *Asparagus* spp. by morphology alone. Because of substitution, the origin of any medicinal products claimed to be derived from *A. racemosus* should be confirmed to prevent misuse – a modern DNA technique based on molecular markers has proved to be effective for this purpose (Yang *et al*., 2001). Many molecular biological methods have been used for DNA analysis of medicinal plants, including randomly amplified polymorphic DNA (RAPD) (Cui *et al*., 2003), amplified fragment length polymorphism (AFLP) and sequence characterized amplified region (SCAR) (Choi *et al*., 2008), multiplex amplification refractory mutation system (ARMS) (Park *et al*., 2006), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Wang *et al*., 2007). PCR-RFLP is possibly the most commonly used routine technique as it is simple, rapid and reliable (Yang *et al*., 2004), especially for commercial products (Aranishi *et al*., 2005).

Presently, DNA barcoding, a technique based on the analysis of short standardized regions of DNA had been proposed for species identification. A Consortium for the Barcode of Life (CBOL) Plant Working Group analyze 7 leading candidate plastid DNA regions including *mat*K gene, *atp*F-*atp*H spacer, *rbc*L gene, *rpo*B gene, *rpo*C1gene, *psb*K-*psb*I spacer, and *trn*H-*psb*A spacer for a standard plant barcode. The 2-locus combination of *rbc*L and *mat*K as the plant barcode has been recommended (A Consortium for the Barcode of Life Plant Working Group, 2009). Due to their high substitution rates, maturase (*mat*K) gene sequences of the chloroplast DNA have been widely employed as a powerful tool to identify the botanical origin of herbal drugs and to examine inter- and intra-specific phylogenetic relationships (Ohsako *et al*., 2000).

In the present study, nucleotide sequences of *mat*K of eight *Asparagus* species collected in Thailand were analyzed. The phylogenetic relationship among these species was studied. PCR-RFLP was developed based on a partial *mat*K region for rapid identification of the medicinally phytoestrogenic species, *A. racemosus*, and for application in analysis of crude drugs in local markets. In addition, the antioxidant activities of eight *Asparagus* species were evaluated on DPPH radical scavenging, ferric reducing antioxidant power (FRAP) and superoxide anion scavenging (NBT). In addition, cytotoxic activity against human cell line KB (human epidermoid carcinoma of oral cavity), MCF-7 (breast adenocarcinoma) and NCI-H187 (human small cell lung cancer) of root extracts of *Asparagus* species were determined.

CHAPTER II

LITERATURE REVIEW

2.1 Botanical description of the genus *Asparagus*

The Flora of China (Chen and Tamanian, 1753) describe the characteristics of the genus *Asparagus* as follows;

"Herbs perennial or subshrubs, dioecious or hermaphroditic, usually with short rhizomes. Main stems erect or climbing, generally branched, with cladodes (leaflike stems) in axils of main stems and branches. Cladodes borne in clusters, rarely solitary, green, flat, 3-angled, or subterete. Leaves appressed to stem, not green, scalelike, base spurred, spurs often extended into spines. Inflorescence an axillary cluster of flowers, rarely a solitary flower, sometimes a raceme or umbel. Pedicel articulate, subtended by membranous bracteoles. Perianth campanulate or subglobose; segments free or occasionally connate at base. Stamens 6; filaments usually adnate to perianth segments in varying degrees; anthers dorsifixed. Ovary 3-loculed; ovules few per locule. Fruit a berry. Seeds 1 to few."

Seven *Asparagus* species exist in Thailand according to Smittinand (2001) (Table 2.1). Over 300 are species widely distributed in temperate and tropical regions (Clifford and Conran, 1987). Samples of *Asparagus* species are shown in Appendix A.

Table 2.1 The list of *Asparagus* species existing in Thailand.

No.	Scientific name	Thai name
1	*A. acerosus* Roxb.	จั่นดิน
2	*A. filicinus* Buch.-Ham.	พอควายมิ ม้าสามต๋อน
3	*A. officinalis* L.	หน่อไม้ฝรั่ง
4	*A. plumosus* Baker	สนราชินี
5	*A. racemosus* Willd.	จ๋วงเครือ เตอสีเบาะ ผักชีช้าง ผักหนาม สามร้อยราก สามสิบ
6	*A. setaceus* (Kunth.) Jessop	จามจุรีแผง โปร่งฟ้า
7	*A. sprengeri* Regel (Synonym *A. aethiopicus* L.)	ปริก

2.2 *Asparagus* species and biological activities

The literature survey revealed that the steroidal saponins are the main biologically active constituents of genus *Asparagus*. Chemical constituents and bioactivities of the *Asparagus* species are summarized in Table 2.2.

Table 2.2 Chemical constituents and biological activities of the different parts of *Asparagus* species.

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. adscandens Roxb.	fruits	Asparanin A **[1]**	Saponins	Cytotoxicity	Sharma, Chand and Sati, 1982a; Liu *et al*., 2009
	fruits	Asparanin B **[2]**	Saponins	Fertility enhancer	Sharma *et al*., 1982a; Bhutani *et al*., 2010
	roots	Asparanin D **[3]**	Saponins	-	Sharma *et al*., 1982b
	fruits	Asparoside A **[4]**	Saponins	Antifungal	Sharma *et al*., 1982a; Belhouchet *et al*., 2008
	fruits	Asparoside B **[5]**	Saponins	Antifungal	Sharma *et al*., 1982a; Belhouchet *et al*., 2008
	roots	Asparoside C **[6]**	Saponins	-	Sharma *et al*., 1982b

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
	roots	Asparoside D [7]	Saponins	-	Sharma *et al*., 1982b
	leaves	Oligofurostanoside I [8]	Saponins	-	Sharma and Sharma, 1984
	leaves	Oligofurostanoside 2 [9]	Saponins	-	Sharma and Sharma, 1984
A. adscandens Roxb.	roots	Oligospirostanoside [10]	Saponins	Immunomodulator	Sharma *et al*., 1982b; Honda *et al*., 2003
	leaves	Spirostanoside 1 [11]	Saponins	-	Sharma and Sharma, 1984
	leaves	Spirostanoside 2 [12]	Saponins	-	Sharma and Sharma, 1984
	roots	Steroid glycoside-I [13]	Saponins	-	Tandon, Shukla and Thakur, 1990
	roots	Steroid glycoside-II [14]	Saponins	-	Tandon *et al*., 1990

Table 2.2 (Continued)

A. cochinchinensis Merr.	roots	Asp-IV **[15]**	Saponins	-	Konishi and Shoji, 1979
	roots	Asp-V **[16]**	Saponins	-	Konishi and Shoji, 1979
	roots	Asp-VI **[17]**	Saponins	-	Konishi and Shoji, 1979
	roots	Asp-VII **[18]**	Saponins	-	Konishi and Shoji, 1979
	Whole plant	Methylprotodioscin **[19]**	Saponins	Cytotoxicity	Hiang *et al*., 1988; Ke and Xinsheng, 2003
	roots	Oligofurostanoside **[20]**	Saponins	-	Hiang *et al*., 1988
	roots	Pseudoprotodioscin **[21]**	Saponins	Anti-inflammatory	Kawano *et al*., 1975; Tetsuro *et al*., 2011
A. curillus Wall.	fruits	Oligofurostanoside **[22]**	Saponins	-	Sharma, Sati and Chand, 1983

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. curillus Wall.	roots	Oligofurostanoside I [23]	Saponins	-	Sharma, Sati and Chand, 1982c
	roots	Oligofurostanoside II [24]	Saponins	-	Sharma, Sati and Chand, 1982d
	fruits	Oligofurostanoside II [25]	Saponins	-	Sharma *et al*., 1983
	roots	Oligofurostanoside 7 [26]	Saponins	-	Sati and Sharma, 1985
	roots	Oligofurostanoside 8 [27]	Saponins	-	Sati and Sharma, 1985
	roots	Spirostanol glycoside [28]	Saponins	-	Sati and Sharma, 1985
	roots	Spirostanol glycoside-I [29]	Saponins	-	Sharma, Sati and Chand, 1982
	fruits	Spirostanol glycoside-I [30]	Saponins	-	Sharma *et al*., 1983c

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. curillus Wall.	roots	Spirostanol glycoside-2 [31]	Saponins	-	Sharma, Sati and Chand, 1982c
	fruits	Spirostanol glycoside-II [32]	Saponins	-	Sharma *et al*., 1983
	roots	Spirostanol glycoside-III [33]	Saponins	-	Sharma, Sati and Chand, 1982c
A. dumosus Baker	Whole plant	Chacotrioside [34]	Saponins	Cytotoxicity	Nakano *et al*., 1989; Charles *et al*., 2009
	Whole plant	Dumoside [35]	Saponins	-	Nakano *et al*., 1989
	Whole plant	Methylprotodioscin [36]	Saponins	Cytotoxicity	Aquino *et al*., 1986; Ke and Xinsheng, 2003

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. dumosus Baker	Whole plant	Trigonelloside [37]	Saponins	-	Bogacheva, Kiselev and Kogan, 1976
A. filicinus Buch.-Ham.	roots	Aspafilioside A [38]	Saponins	Cytotoxicity	Ding and Yang, 1990; Jia-Jun *et al*., 2010
	roots	Aspafilioside B [39]	Saponins	Cytotoxicity	Ding and Yang, 1990; Jia-Jun *et al*., 2010
	roots	Aspafilioside C [40]	Saponins	-	Ding and Yang, 1990
	roots	Asp-IV [41]	Saponins	-	Chongren, Xingcong and Yi, 1990
	roots	Asp-IV [41]	Saponins	-	Chongren, Xingcong and Yi, 1990

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. filicinus Buch.-Ham.	Whole plant	Filicinin A **[42]**	Saponins	-	Sharma and Thakur, 1996
	Whole plant	Filicinin B **[43]**	Saponins	-	Sharma and Thakur, 1996
	Whole plant	Filicinoside A **[44]**	Saponins	-	Sharma and Thakur, 1994
	Whole plant	Filicinoside B **[45]**	Saponins	-	Sharma and Thakur, 1994
	Whole plant	Filicinoside C **[46]**	Saponins	-	Sharma and Thakur, 1996
	Whole plant	Filicinoside D **[47]**	Saponins	-	Sharma and Thakur, 1996

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. filicinus Buch.-Ham.	roots	22-Methoxysaponin **[48]**	Saponins	-	Ding and Yang, 1990
A. gobicus Ivanova ex Grubov.	roots	-	Norlignans	Cytotoxicity	Yang *et al*., 2004
A. gonocladus Baker	Aerial parts	-	Saponins	-	Mandloi and Sant, 1981
A. officinalis L.	roots	Asparagoside A **[49]**	Saponins	Cytotoxicity	Gorynu, Krokhmalyuk and Kintya, 1976; Huang, Lin and Kong, 2008
	roots	Asparagoside B **[50]**	Saponins	-	Gorynu, Krokhmalyuk and Kintya, 1976

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. officinalis L.	roots	Asparagoside D [51]	Saponins	-	Goryanu and Kintya, 1976
	roots	Asparagoside F [52]	Saponins	-	Goryanu and Kintya, 1977
	roots	Asparagoside G [53]	Saponins	-	Goryanu and Kintya, 1976
	roots	Asparagoside H [54]	Saponins	-	Goryanu and Kintya, 1977
	roots	Asparagoside I [55]	Saponins	-	Gorynu, Krokhmalyuk and Kintya, 1976
	shoots	Asparasaponin I [56]	Saponins	-	Kawano, Sato and Sakamura, 1977
	shoots	Asparasaponin II [57]	Saponins	-	Kawano, Sato and Sakamura, 1977

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. officinalis L.	fruits	Capsanthin **[58]**	Carotenoids	Antioxidant	Deli *et al.*, 2000; Hiroshi *et al.*, 1998
	fruits	Capsorubin **[59]**	Carotenoids	Antioxidant	Deli *et al.*, 2000; Takashi *et al.*, 2001
	fruits	Capsanthin 5,6-epoxide **[60]**	Carotenoids	Antioxidant	Deli *et al.*, 2000; Takashi *et al.*, 2001
	roots	Officinalisnin I **[61]**	Saponins	-	Kawano *et al.*, 1975
	roots	Officinalisnin II **[62]**	Saponins	-	Kawano *et al.*, 1975
	seeds	Protodioscin **[63]**	Saponins	Cytotoxicity	Shao *et al.*, 1997
	Bottom cut	Saponin AS-1 **[64]**	Saponins	-	Shimoyamada *et al.*, 1990

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. officinalis L.	fruits	Spirostanol glycoside **[65]**	Saponins	Immobilization of human spermatozoa	Pant *et al*., 1998
	roots	-	Steroids	Cytotoxicity	Velavan *et al*., 2007
A. plumosus Baker var. Nanus Hort	leaves	Furostanol glycoside 1 **[66]**	Saponins	-	Sati and Pant, 1985a
	leaves	Furostanol glycoside 2 **[67]**	Saponins	-	Sati and Pant, 1985a
	leaves	Spirostanol glycoside-I **[68]**	Saponins	-	Sati and Pant, 1985b
	leaves	Yamogenin 2 **[69]**	Saponins	Molluscicidal	Sati and Pant, 1985b; Mortada, 1998
	leaves	Yamogenin 3 **[70]**	Saponins	Molluscicidal	Sati and Pant, 1985b; Mortada, 1998

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. pubescens Baker	roots	-	-	Antinociceptive and antiinflammatory	Nwafor *et al*., 2003
A. racemosus Willd.	roots	Asparinin A **[71]**	Saponins	-	Hayes *et al*., 2008
	roots	Asparagamine A **[72]**		Antioxidant	Sekine *et al*., 1994; Wiboonpun *et al*., 2004
	roots	Immunoside **[73]**	Saponins	Immunomodulator	Hayes *et al*., 2008; Honda *et al*., 2003
	roots	8-Methoxy-5,6,4´-trihydroxy-isoflavone-7-*O*-β-D-glucopyranoside **[74]**	Flavonoids	-	Saxena *et al*., 2001
	Leaves	Quercetin-3-glucoronide **[75]**	Flavonoids	-	Rastogi *et al*., 1969

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. racemosus Willd.	roots	Racemofuran [76]		Antioxidant	Wiboonpun *et al*., 2004
	Fruits	Racemoside A [77]		-	Mandal *et al*., 2006
	Fruits	Racemoside B [78]		-	Mandal *et al*., 2006
	Fruits	Racemoside C [79]		-	Mandal *et al*., 2006
	roots	Racemosol [80]	Phenanthrene	Antioxytocic, Antioxidant	Sekine *et al*., 1997; Wiboonpun *et al*., 2004
	roots	Shatavarin I [81]	Saponins	Antifungal	Hayes *et al*., 2008; Belhouchet *et al*., 2008
	roots	Shatavarin IV [82]	Saponins	Immunomodulator	Ravikumar *et al*., 1987; Hayes *et al*., 2008; Gautam *et al*., 2009

Table 2.2 (Continued)

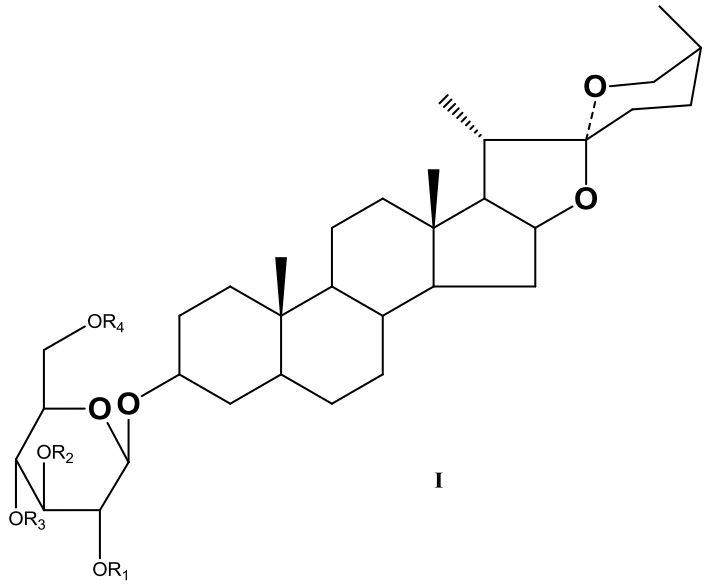
Asparagus species	Parts used	Compound	Category	Activity	Reference
A. racemosus Willd.	roots	Shatavarin V [83]	Saponins	-	Hayes *et al*., 2008
	roots	Shatavarin VI [84]	Saponins	-	Hayes *et al*., 2008
	roots	Shatavarin VII [85]	Saponins	-	Hayes *et al*., 2008
	roots	Shatavarin VIII [86]	Saponins	-	Hayes *et al*., 2008
	roots	Shatavarin IX [87]	Saponins	-	Hayes *et al*., 2008
	roots	Shatavarin X [88]	Saponins	-	Hayes *et al*., 2008
	roots	-	-	Gastroduodenal ulcer protective	Sairam *et al*., 2003
	roots	-	-	Immunostimulant, antihepatotoxicity	Muruganandan *et al*., 2001

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. racemosus Willd.	roots	-	-	Antitussive, antibacterial	Mandal *et al*., 2000
	roots	-	-	Cytotoxicity, antioxidant, antimicrobial	Potduang *et al*., 2008
A. sprengeri Regel	roots	Sprengerinin A [89]	Saponins	-	Sharma and Kumar, 1993
	roots	Sprengerinin B [90]	Saponins	-	Sharma and Kumar, 1993
	roots	Sprengerinin C [91]	Saponins	-	Sharma and Kumar, 1993
	roots	Sprengerinin D [92]	Saponins	-	Sharma and Kumar, 1993

Table 2.2 (Continued)

Asparagus species	Parts used	Compound	Category	Activity	Reference
A. stipularis Forssk	roots	Tigogenin-3-*O*-dixylogluside [93]	Saponins	-	Tandon *et al*., 1990
A. tenuifolius Gilib	roots	Asparagoside C [94]	Saponins	-	Dong and Panova, 1996; Panova, Nikolov and Zong, 1982
	roots	Asparagoside D [95]	Saponins	-	Dong and Panova, 1996; Panova *et al*., 1982
	roots	Asparagoside E [96]	Saponins	Antifungal	Dong and Panova, 1996; Panova *et al*., 1982; Belhouchet *et al*., 2008



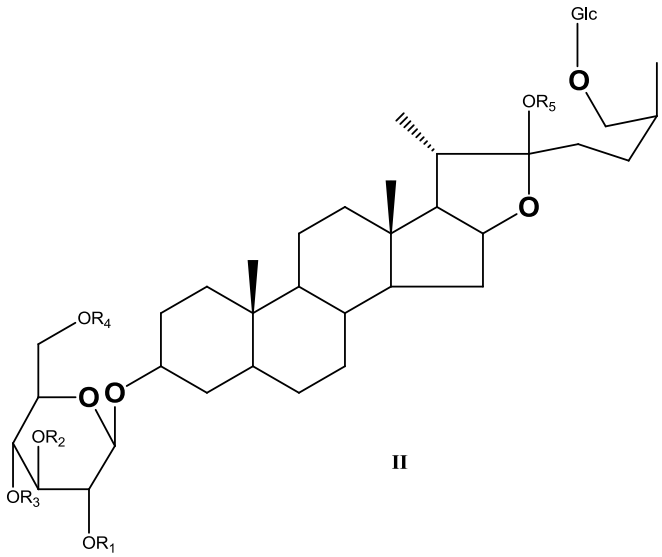
I

Compound	R1	R2	R3	R4
[1] Asparanin A	Glc	H	H	H
[2] Asparanin B	Glc	H	Rha	H
[3] Asparanin D	Glc	H	Ara	Rha
[10] Oligospirostanoside	H	H	Ara	Rha
[28] Spirostanol glycoside	Glc	H	Rha	Ara
[29] Spirostanol glycoside-I	H	H	Ara	H
[30] Spirostanol glycoside-I	H	H	Rha	H
[31] Spirostanol glycoside-2	Rha	H	Ara	H
[32] Spirostanol glycoside-II	Glc	H	Rha	H
[37] Trigonelloside	Xyl	H	Xyl	H
[42] Filicinin A	H	H	Glc ^{6}-Xyl ^{4}-Gal	H
[43] Filicinin B	H	H	Glc ^{6}-Xyl ^{2}-Glc ^{4}-Gal	H
[48] 22-Methoxysaponin	H	H	Xyl	H

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species

Compound	R1	R2	R3	R4
[49] Asparagoside A	H	H	H	H
[50] Asparagoside B	H	H	H	H
[51] Asparagoside D	H	Glc	H	H
[52] Asparagoside F	H	Glc	Glc-^{4}Xyl	H
[64] Saponin AS-1	Glc	H	Xyl	H
[65] Spirostanol glycoside	Glc	H	Rha	H
[68] Spirostanol glycoside-I	H	H	Ara	H
[69] Yamogenin 2	Rha	Rha	H	H
[70] Yamogenin 3	H	Glc	Rha	H
[73] Immunoside	Rha	H	Rha	H
[79] Racemoside A	-	-	^{1}Glc-^{4}Glc ^{1}Rha-^{6}Glc-	^{1}Glc-^{6}Glc
[78] Racemoside B	-	-	-	^{1}Glc-^{6}Glc ^{1}Rha-^{6}Glc
[79] Racemoside C	-	-	-	^{1}Rha-^{4}Glc ^{6}Rha-^{4}Glc
[82] Shatavarin-IV	Rha	H	Glc	H
[83] Shatavarin V	Rha	H	Glc	H
[85] Shatavarin VIII	Glc	H	Ara	Glc
[87] Shatavarin IX	Glc	Glc	H	H
[88] Shatavarin X	Rha	H	Glc(6-OAc)	H
[94] Asparagoside C	H	Glc	H	H

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



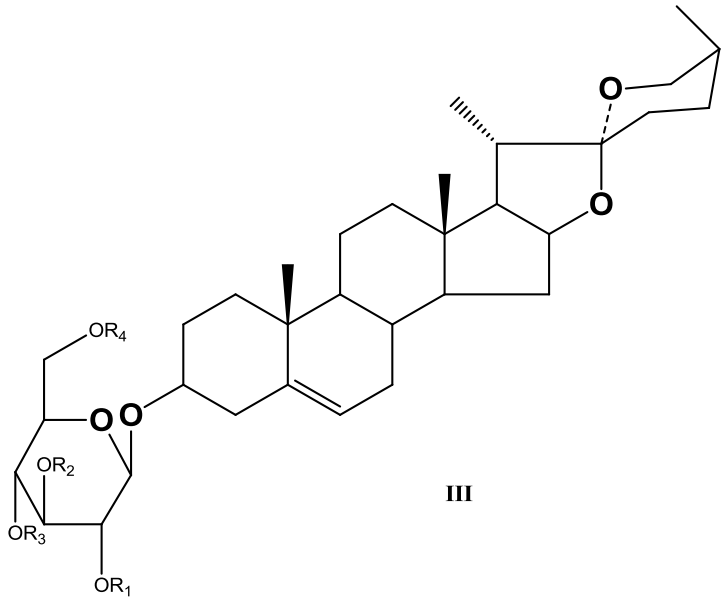
II

Compound	R1	R2	R3	R4	R5
[4] Asparoside A	Glc	H	Rha	H	CH_3
[5] Asparoside B	Glc	H	Rha	H	H
[6] Asparoside C	Glc	H	Ara	Rha	CH_3
[7] Asparoside D	Glc	H	Ara	Rha	H
[15] Asp-IV	H	H	Xyl	H	CH_3
[16] Asp-V	H	H	H	Rha	CH_3
[17] Asp-VI	H	H	Xyl	Rha	H
[18] Asp-VII	Glc	H	Xyl	Rha	CH_3
[22] Oligofurostanoside	H	H	Rha	H	H
[23] Oligofurostanoside I	Glc	H	Ara	H	CH_3
[24] Oligofurostanoside II	Glc	H	Ara	H	H
[25] Oligofurostanoside II	Glc	H	Rha	H	CH_3
[26] Oligofurostanoside 7	Glc	H	Rha	Ara	CH_3
[27] Oligofurostanoside 8	Glc	H	Rha	Ara	H
[40] Aspafilioside C	H	H	Xyl	Ara	H
[44] Filicinoside A	H	H	H	H	CH_3
[45] Filicinoside B	H	H	H	H	H

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)

Compound	R1	R2	R3	R4	R5
[46] Filicinoside C	H	H	Glc ^{6}-Xyl ^{4}-Gal	H	CH_3
[47] Filicinoside D	H	H	Glc ^{6}-Xyl ^{4}-Gal	H	H
[50] Asparagoside B	-	-	-	-	H
[53] Asparagoside G	H	Glc	Glc	H	CH_3
[54] Asparagoside H	H	Glc	Glc-^{4}Xyl	H	CH_3
[55] Asparagoside I	Rha	-	Xyl	H	H
[61] Officinalisnin I	Glc	H	H	H	H
[62] Officinalisnin II	Glc	H	Xyl	H	H
[81] Shatavarin I	Glc	H	Rha	H	H
[96] Asparagoside E	H	Glc	H	H	H

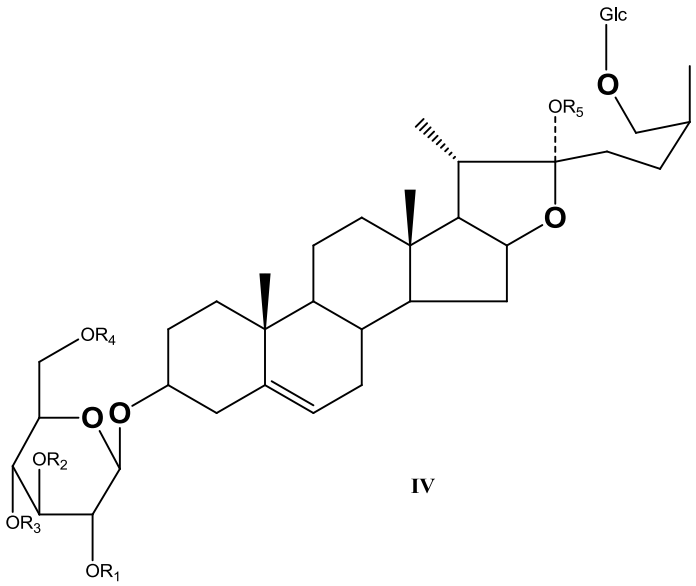
Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



III

Compound	R1	R2	R3	R4
[8] Oligofurostanoside I	H	H	H	Rha
[9] Oligofurostanoside 2	H	H	Rha	Rha
[89] Sprengerinin A	H	H	Xyl	H
[90] Sprengerinin B	H	H	H	Rha
[91] Sprengerinin C	Rha	H	Xyl	H
[92] Sprengerinin D	Rha	H	H	Rha

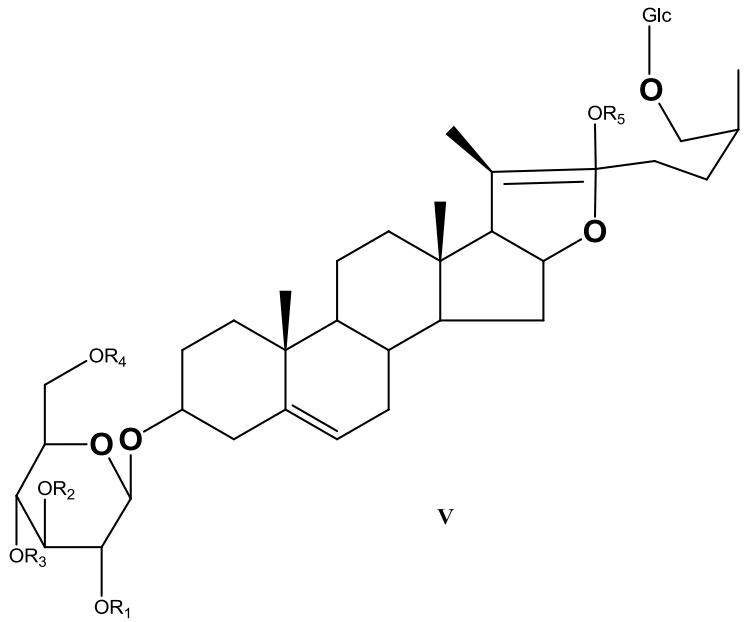
Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



IV

Compound	R1	R2	R3	R4	R5
[11] Spirostanoside 1	H	H	Rha	Rha	CH_3
[12] Spirostanoside 2	H	H	Rha	Rha	H
[56] Asparasaponin I	H	H	Rha	H	-
[57] Asparasaponin II	H	H	Rha	H	-
[63] Protodioscin	Rha	H	Rha	H	CH_3
[66] Furostanol glycoside 1	Rha	Rha	H	H	CH_3
[67] Furostanol glycoside 2	Rha	Rha	H	H	H
[93] Tigogenin-3-*O*-dixylogluside	Rha	H	Rha	H	H

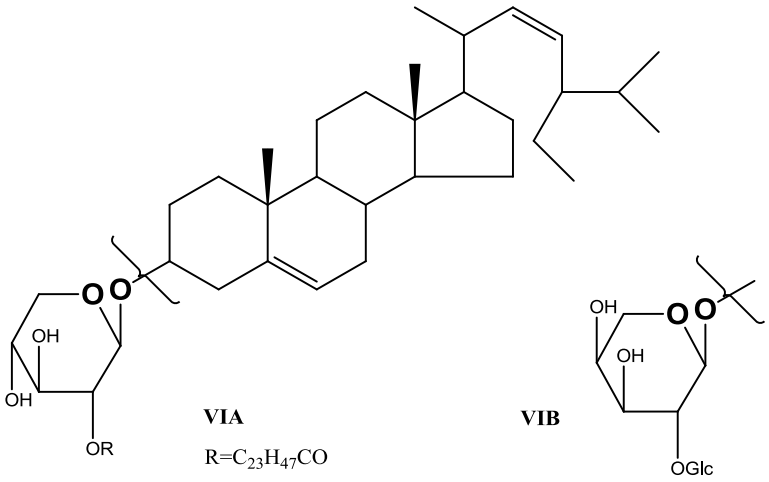
Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



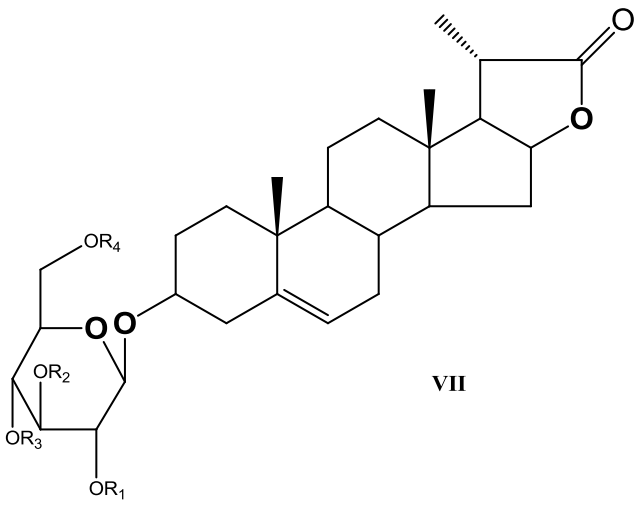
V

Compound	R1	R2	R3	R4	R5
[19] Methylprotodioscin	H	H	Rha	H	-
[20] Oligofurostanoside	H	H	Rha	H	H
[21] Pseudoprotodioscin	Rha	H	Rha	H	-

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)

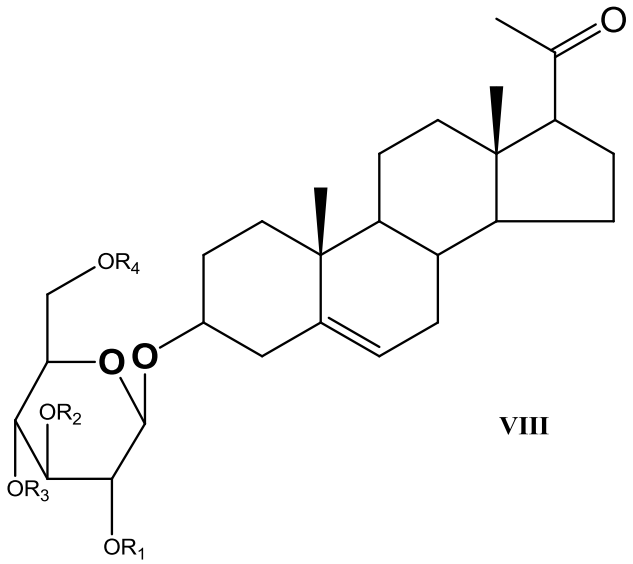


Compound	R1	R2	R3	R4	R5
[13] Steroid glycoside-I	$C_{27}H_{47}CO$	-	-	-	-
[14] Steroid glycoside-II	Glc	-	-	-	-



Compound	R1	R2	R3	R4
[35] Dumoside	Rha	H	Rha	H

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



VIII

Compound	R1	R2	R3	R4
[34] Chacotrioside	Rha	H	Rha	H

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)

Major structural types

I = Spirostan-3-ol

II = 3β, 26-Dihydroxy-22α methoxy furostane-3-ol

III = 3 β, 25*R*-Spirost-5-en-3-yl

IV = 22-Hydroxylfurost-5-en-3-yl

V = Furosta-5,20(22)-dien-3-yl

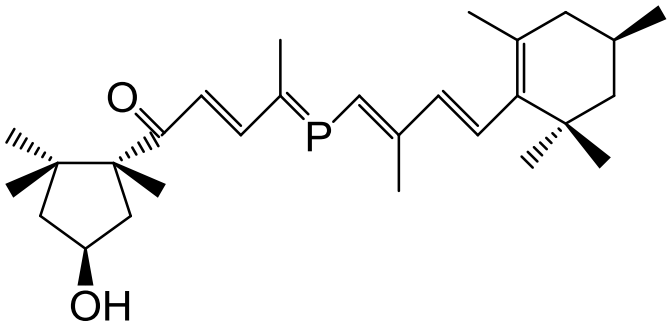
VI = Stigmasterol

VII = Pregn-5-en-20-carboxylic acid

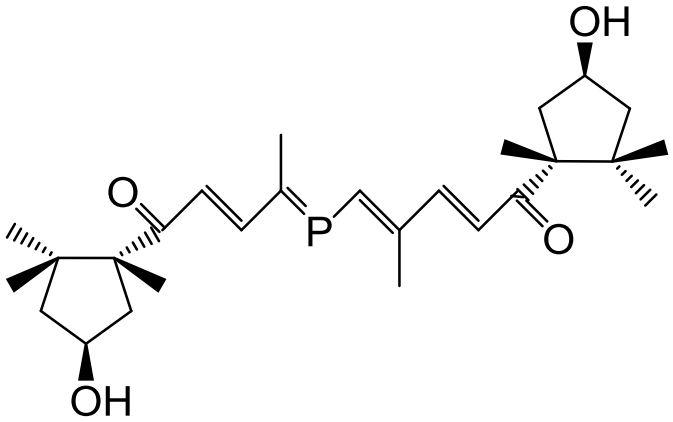
VIII = Preg-5-en-3-yl

Substitute groups

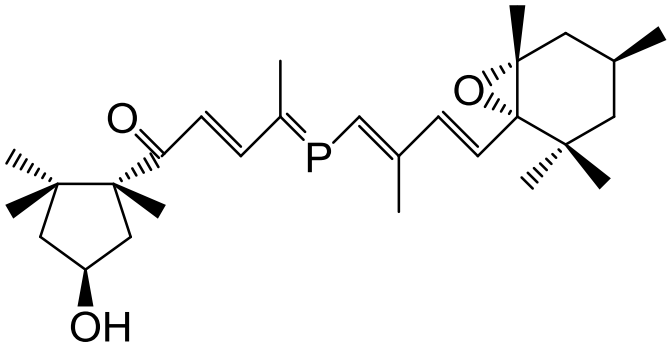
Xyl = Xylose, Glc = Glucose, Rha = Rhamnose, Ara = Arabonose, Gal = Galactose



[58] Capsanthin

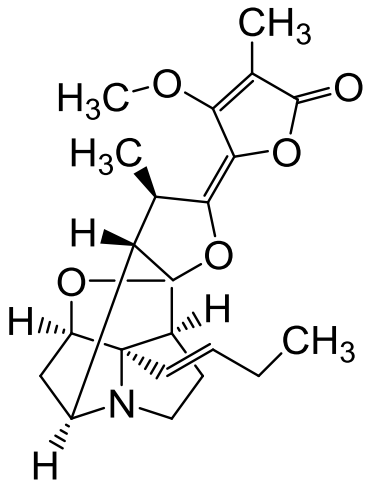


[59] Capsorubin

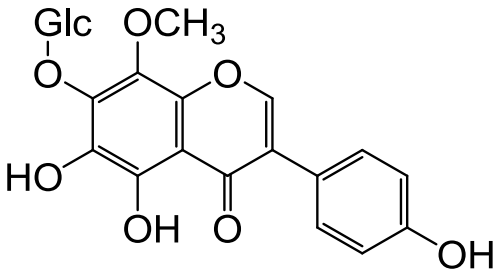


[60] Capsanthin 5,6-epoxide

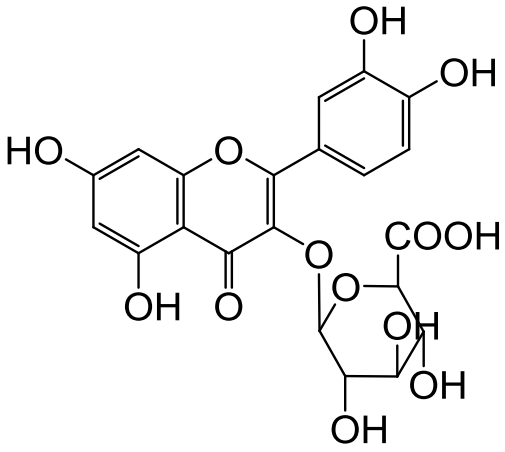
Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



[72] Asparagamine A

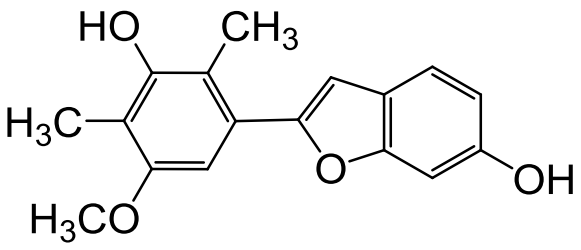


[74] 8-Methoxy-5,6,4´-trihydroxy-isoflavone-7-*O*-β-D-glucopyranoside

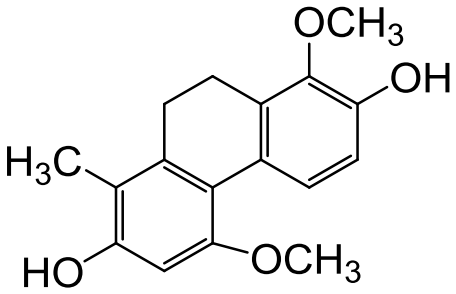


[75] Quercetin-3-glucoronide

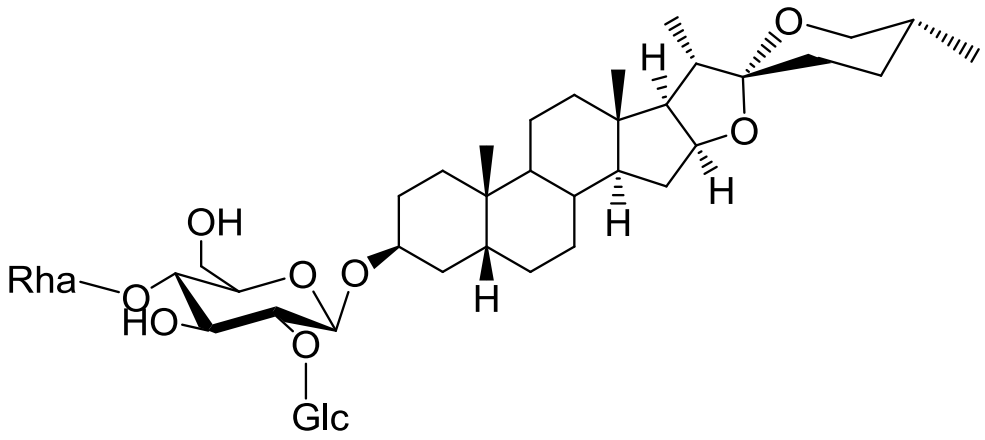
Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



[76] Racemofuran

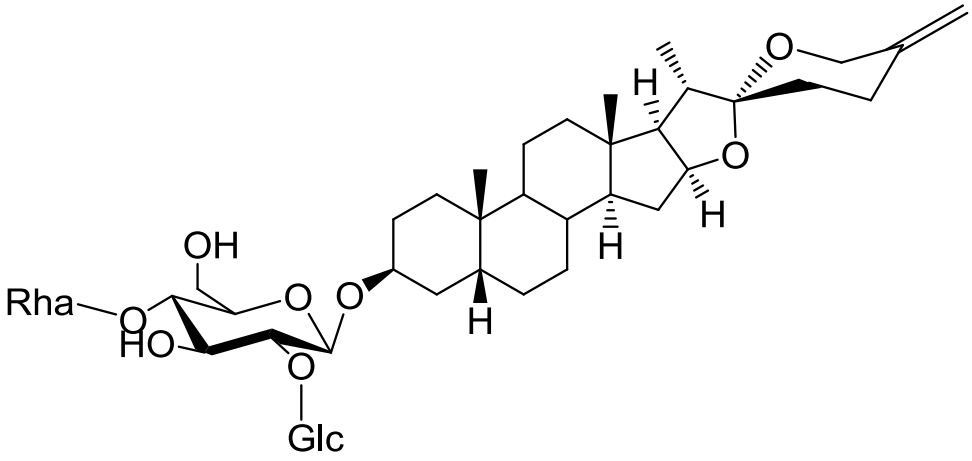


[80] Racemosol



[84] Shatavarin VI

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)



[85] Shatavarin VII

Figure 2.1 Structures of compounds previously isolated from *Asparagus* species (continued)

2.3 Molecular identification techniques

Counterfeits of medicinal materials degrade the effect of medicinal plants and result in cases in which patients do not recover or even die. Thus, authentication is an essential step for successful and reliable clinical application. In general, the authentication of medicinal plant is solved using methods including morphology, microscopy, taxonomy, physical and chemical analysis and DNA technology.

In recent years, the rapid development of molecular biology techniques has led to their application in medicinal plants authentication. The uniqueness of DNA in plant species can be used for taxonomic identification. This method has proven specific, stable, convenient and also accurate. Furthermore, a tiny amount of sample is always sufficient for DNA analysis. Nowadays identifications of medicinal plants by DNA molecular markers are mainly performed, namely, DNA fingerprinting, DNA sequencing and DNA genechip.

DNA fingerprinting, Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS) analyses were used as an efficient identification method for Rhizoma Rhei (Yang *et al*., 2004). Random amplified polymorphism DNA (RAPD) analysis was carried out to determine the genetic variation of the *Panax* genus (Cui *et al*., 2003) and to distinguish the three medicinal species, *Echinacea angustifolia* DC., *E. pollida* Nutt., and *E. purpurea* (L.) Moench (Wolf *et al*., 1999; Nieri *et al*., 2003). Multiplex amplification refractory mutation system (MARMS) was applied to the identification of *Panax* species (Zhu *et al*., 2004). Several studies used PCR-RFLP analysis and successfully for identification of many plants (Xu *et al*. 2001; Wang *et al.* 2007; Liu *et al.* 2007).

The DNA sequencing is one of the most informative techniques for the molecular systematic studies because nucleotide sequences directly reflect genetic information alteration. The rates and patterns of changes affect the evolution of genes and the organisms. Moreover, DNA sequences can be used for constructing the molecular phylogeny of related organisms. DNA sequencing provides highly robust, reproducible,

and informative data set, and can be adapted to different levels of discriminatory potential by choosing appropriate genomic target regions (Weising *et al*., 2005). DNA sequencing, the 5S-rRNA spacer region sequences were used to identify Bulbus Fritillariae cirrhosaes (Li *et al*., 2003), Herba Epimedii (Sun *et al*., 2004) and Radix Adenophorae (Zhao *et al*., 2003). Single-nucleotide polymorphism (SNP) analysis was used to identify Rhizoma Curcumae Longae (Sasaki *et al*., 2004). Based on rDNA ITS sequences of *Dendrobium officinale* Kimura et Migo and the other 37 species of *Dendrobium*, a pair of allele-specific diagnostic primers was designed to distinguish *D. officinale* Kimura et Migo from other *Dendrobium* species (Dong *et al*., 2003).

DNA genechip, a DNA microarray for detecting processed medicinal *Dendrobium* species was constructed by incorporating the ITS1-5.8S-ITS2 sequences if 16 *Dendrobium* species on a glass slide (Zhang *et al*., 2003). For complicated situations, applying various methods together is necessary in order to conclusively confirm an identification or authentication.

Plants have three kinds of genomes, the chloroplast genomes (cpDNA) in addition to the nuclear (nDNA) and mitochondrial (mtDNA) genomes. The mtDNA is rarely used in molecular markers of plant due to its structure, size, and gene order are various depending on plant species (Kress *et al*., 2005). The nDNA and cpDNA are commonly used to investigate in the molecular systematics and taxonomy of plants. The nDNA has more complexity and repetitive properties. The cpDNA is well suitable for evolutionary and phylogenetic studies because it is a relative abundant component of total DNA. In addition, it contains primarily single copy genes, and has conservative rate of nucleotide substitution. The most common targets in cpDNA are *rbc*L, *ndh*F, *trn*H-*psb*A, *trn*K, and *mat*K while the most common genes in nDNA is nuclear ribosomal gene; 18S rDNA, an internal transcribed spacer (ITS2), and finally the 26S rDNA (Kress *et al*., 2005; Weising *et al*., 2005). In recent years, the *trn*K gene and the included maturase (*mat*K) gene sequences of the chloroplast DNA (cpDNA) have been widely employed as a powerful tool to identify the botanical origin of herbal drugs and to examine inter- and intra-

specific phylogenetic relationships, due to their high substitution rates (Sugita *et al*., 1985).

2.4 Natural phytoestrogens, *Asparagus racemosus*

Numerous clinical studies provide evidence that synthetic hormones increase the risk of endometrial cancer, breast cancer, venous thromboembolic events and gall-bladder disease in women. Research on the relationship between hormone replacement therapy (HRT) and the risk of endometrial cancer has been carried out in 1995, among others, by Grady et al. (Grady *et al*., 1995). They confirmed a significant increase of risk associated with estrogens used for long periods, lasting several years after stopping of the use of them. The awareness that HRT is associated with undesirable effects has increased an interest in phytoestrogens, i.e. plant-derived estrogens. The studies show that they simulate the effects of estrogen in some tissues, while in others they act antagonistically. According to many experts, substances blocking the binding of estrogen in the tissues, i.e. compounds of the agonists or antagonist activity may act preventively, particularly in postmenopausal women, in whom endogenous estrogens are recognized as a potential carcinogen. Another type of medical intervention in the female sex hormone homeostasis involves the use of synthetic drugs on the function of the partial estrogen receptor antagonists, known as selective estrogen receptor modulators (SERM) such as raloxifene. Natural compounds classified as phytoestrogens have a similar physiological function and they have a safety advantage over synthetic drugs.

Natural therapies using phytoestrogens are receiving increased attention as dietary components that can affect several aspects of human health and are beneficial in countering the manifestations of postmenopausal state (Adlercreutz *et al*., 1992; Agnusdei *et al*., 1995). The traditional system of Indian medicine has cited several plants that are useful in the management of menopausal syndrome. In the system of Ayurvedic medicine, an extract from the root of *A. racemosus* has also been used to increase milk secretion during lactation. The test results confirming its ability to enhance

lactation are not clear. Joglekar, Ahuja and Balwani (1967) observed an increase in milk secretion after administration of *A. racemosus* in the form of Ricalex® tablets. *A. racemosus* is also a plant known for its phytoestrogenic properties and for use as a hormone modulator (Mayo, 1998). In studies on rats, Rao (Rao, 1981) showed the inhibitory action of *A. racemosus* on DMBA-induced mammary carcinogenesis. A diet containing 2% of root extract of this plant, obtained by extraction with chloroform and methanol (1:1) led to a significant decrease in the incidence of this cancer as well as the decrease in the average number of tumors. Research of *A. racemosus* capsules (EveCare®) confirmed their efficacy in the treatment of dysfunctional uterine bleeding (DUB) (Nevrekar, Bai and Khanna, 200). DUB is regular or acyclic bleedings from the uterus with no organic cause such as neoplasia, inflammation of the endometrium, submucous fibroids or polyps. Dysfunctional uterine bleedings may occur throughout the reproductive period. 20% of them are observed in adolescents, 40% occur at the age of 18-45, the other 40% - in premenopausal stage. The study included 70 women with DUB in the age group of 20-45 years. In the study group, 63 women had achieved a regularized menstrual cycle. It can be attributed to the local healing of the endometrium stimulated by endometrial microvascular thrombosis caused by high doses of phytoestrogens. Other studies conducted on a group of 40 women confirmed the effectiveness of EveCare capsules in the treatment of dysmenorrhoea and pre-menstrual syndrome (PMS) (Swarup and Umadevi, 1998).

2.5 Antioxidant activity

Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke (Prior and Cao, 2000). The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark (Mathew and Abraham, 2006). There are many synthetic antioxidants in use. It is reported, however, they have several side effects (Ito

et al., 1983), such as risk of liver damage and carcinogenesis in laboratory animals (Gao *et al*., 1999; Williams *et al*., 1999; Osawa and Namiki, 1981). There is therefore a need for more effective, less toxic and cost effective antioxidants. Medicinal plants appear to have these desired comparative advantages, hence the growing interest in natural antioxidants from plants. Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems.

ROS including hydroxyl radical, superoxide radical, hydrogen peroxide and singlet oxygen are often generated from exogenous factors or byproducts of biological reactions (Cerutti *et al*., 1991). ROS have been found to play an important role in several diseases such as inflammatory injury, ageing, atherosclerosis, cardiovascular disease and cancer (Halliwell *et al*., 1997). Living organisms have promoted complex antioxidant systems to neutralize ROS and to reduce damage. These antioxidant systems contain enzymes such as superoxide dismutase (SOD) and catalase (CAT), macromolecules such as albumin, and ferritin. Small molecules including ascorbic acid, alpha-tocopherol, carotenoids, polyphenols, reduced glutathione (GSH), uric acid, methionine and bilirubin (Yu *et al*., 1994). A dominant scavenger of plants may serve as a possible preventive intervention for free radical-mediated diseases (Gupta *et al*., 2006; Miguel, 2010). When oxygen traps single electron, it becomes unstable and thus very reactive, since it generates harmful chain reactions against many biological molecules. The extreme toxicity of oxygen is related to its high capability of generating free radicals and in turn destroying many major biological molecules. They can attack on lipids and proteins and destroy membranes. ROS can damage DNA and lead to mutation and chromosomal damage. Oxidized cellular thiols abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Valko *et al*., 2006). ROS can attack various substrates in body and contribute to development of

chronic diseases. For example, oxidatively modified LDL has been hypothesized to be a causative agent in the development of cardiovascular diseases (Touyz, 2004).

2.6 Cytotoxic activity

Plant-derived compounds have always been an important source of medicines for various diseases and have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxicity and cancer chemopreventive effects (Gonzales and Valerio, 2006). Recently there is considerable scientific and commercial interest in the continuing discovery of novel anticancer agents of plant origin (Cragg and Newman, 2005) and such investigations targeting plant products have recently regained prominence with the increasing understanding of their biological significance and increasing recognition of the origin and function of their structural diversity (Conforti *et al*., 2008). Cancer is the second leading cause of death and its incidence has increased dramatically worldwide (Madhusudan and Middleton, 2005). Cancer is a general term applied to malignant diseases characterized by rapid and uncontrolled abnormal cells formation which may mass together to form a growth or proliferate throughout the body, and it may progress until it causes death.

Some famous selected examples used to represent the importance of those plants based on human observation, trial and error, religious advices and from various generations' accumulated experiences, which should never neglected or classified as unscientifically based treatment. The medicinal plants derived from folklore are huge, *Vinca rosea* (Sun and Zeng, 2005), *Podophyllum peltatum* (Gordaliza *et al*., 1994) and *Taxus* spp (Wani *et al*., 1980) are selected examples. These plants and many others lead to discover important drugs including vincristine, vinblastine, podophyllotoxin, 10-hydroxy-camptothecin and Taxol (Wani *et al*., 1980; Coker *et al*., 2003).

CHAPTER III

MOLECULAR ANALYSIS OF THE GENUS *ASPARAGUS* BASED ON *mat*K SEQUENCES AND ITS APPLICATION TO IDENTIFY *A. RACEMOSUS*, A MEDICINALLY PHYTOESTROGENIC SPECIES

3.1 Introduction

In Thai traditional medicine, *A. racemosus* (Rak Sam Sip) is one of the most popular herbal plants (Bopana *et al*., 2007) since its dry roots are well known as an anti-inflammatory, an aphrodisiac and a galactagogue (Pongboonrod, 1950). In India, it has been used in Ayurvedic medicine as an anti-diarrhea and anti-tuberculosis remedy (Sharma *et al*., 2000). Steroidal saponins from the roots of *A. racemosus* were isolated (Figure 3.1) (Ravikumar *et al*., 1987; Joshi *et al*., 1988; Hayes *et al*., 2008) and have been recognized for its phytoestrogenic properties (Gopumadhavan *et al*., 2005).

Recently, interest in plant-derived estrogens has increased tremendously, making *A. racemosus* particularly important. However, the trading economics of roots of *A. racemosus* is strongly affected by the presence of less-effective substitutes such as *A. officinalis* (Bopana *et al*., 2007). Moreover, the dry root of *A. racemosus* is similar in appearance to those of other *Asparagus* spp. (e.g. *A. setaceus*, *A. aethiopicus*, *A. officinalis*, *A. filicinus*, *A. densiflorus*, *A. umbellatus* and *A. densiflorus*) and cannot be distinguished from other *Asparagus* spp. by morphology alone. Because of substitution, the origin of any medicinal products claimed to be derived from *A. racemosus* should be confirmed to prevent misuse.

In this chapter, nucleotide sequences of *mat*K of eight *Asparagus* species collected in Thailand were analyzed. The phylogenetic relationship among these species was studied and compared with a previous phylogeny constructed from *pet*D–*rpo*A intergenic sequences. Additionally, PCR-RFLP was developed based on a partial *mat*K region for rapid identification of the medicinally phytoestrogenic species, *A. racemosus*, and for application in analysis of crude drugs in local markets.

3.2 Materials and methods

3.2.1 Plant materials

Thirty-six specimens of eight *Asparagus* taxa were collected from various regions of Thailand (Table 3.1). All specimens were identified by Assoc. Prof. Thatree Phadungcharoen, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand. Herbarium voucher specimens were prepared and kept at the Museum of Natural Medicines, Faculty of Pharmaceutical Science, Chulalongkorn University. The collected living plants were then cultivated in the Medicinal Plant Garden of the Faculty of Pharmaceutical Science, Chulalongkorn University. For crude drug analysis, ten commercial crude drug samples claimed to be *A. racemosus* (i.e. C1–C10) were purchased from various traditional drug stores (Table 3.2).

3.2.2 Genomic DNA extraction

Total genomic DNA was extracted from 50–100 mg of fresh leaves of the plant specimens or commercially available crude drug samples. Extraction of the DNA utilized DNeasy™ Plant Mini Kit (Qiagen, Germany) was according to the manufacturer's protocol. Briefly, fresh leaves or crude drug samples were ground into fine powder in liquid nitrogen by a mortar and a pestle, and suspension and lysis buffers were then added. The lysate was applied to a QIAshredder spin column to remove precipitates and cell debris by centrifugation. The flow-through fraction was applied to a DNeasy mini spin column. The spin column was centrifuged, washed and eluted with double deionized water. DNA quantity and quality were determined by agarose gel electrophoresis.

Table 3.1 Plant materials and their accession numbers for the *mat*K gene.

Species	Place of collection (Thailand, Province)	Date of collection	Voucher no.	Genbank accession
A. racemosus	Phetchabun	2010.07	SS-KI-080753	AB646501
Willd.	Bangkok	2010.09	SS-SS-020953	
	Trat	2010.09	SS-TB-020953	
	Prachin Buri	2010.09	SS-TB-050953	
	Chiang Mai	2010.09	SS-TB-070953	
	Nong Khai	2010.09	SS-TB-120953	
	Kanchanaburi	2010.08	SS-KI-060853	
	Rayong	2010.09	SS-KI-270953	
	Mae Hong Son	2010.09	SS-KI-280953	
A. aethiopicus	Phetchabun	2010.09	SS-TP-110953	AB646503
L. (Synonym	Bangkok	2010.09	SS-TP-110953-1	
A. sprengeri	Prachuap Khiri Khan	2010.11	SS-TP-191153	
Regel)	Bangkok	2010.11	SS-TP-191153-2	
	Bangkok	2010.11	SS-TP-191153-3	
A. densiflorus	Rayong	2010.11	SS-TP-221153	AB646508
(Kunth.)	Bangkok	2010.12	SS-TB-031253-1	
Jessop	Bangkok	2010.12	SS-TB-031253-2	
	Kanchanaburi	2011.01	SS-TB-030154	
A. densiflorus	Prachin Buri	2010.11	SS-TP-231153-1	AB646506
cv. Myers	Bangkok	2010.11	SS-TP-231153-2	

Table 3.1 (Continued)

Species	Place of collection (Thailand, Province)	Date of collection	Voucher no.	Genbank accession
A. filicinus	Chiang Mai	2010.11	SS-TB-081153-1	AB646505
Buch.-Ham.	Chiang Mai	2010.08	SS-TB-161053-2	
	Kanchanaburi	2010.08	SS-TB-250853	
A. officinalis L.	Bangkok	2010.07	SS-TP-030753	AB646504
	Chiang Mai	2010.11	SS-TB-081153	
A. setaceus	Chiang Mai	2010.07	SS-TB-080753-1	AB646502
(Kunth.)	Chiang Mai	2010.07	SS-TB-080753-2	
Jessop	Bangkok	2010.07	SS-TB-080753-3	
	Bangkok	2010.07	SS-TB-080753-4	
	Prachin Buri	2010.08	SS-TP-170853-1	
	Prachin Buri	2010.08	SS-TP-170853-2	
	Prachin Buri	2010.08	SS-TP-170853-3	
A. umbellatus	Bangkok	2010.11	SS-TP-221153-1	AB646507
Royle	Kanchanaburi	2010.11	SS-TP-221153-2	
	Bangkok	2010.12	SS-TB-031253-4	

Table 3.2 Details of commercially available crude drugs analyzed.

Crude drug	Abbr.[a]	Purchased location (Thailand, Province)	Claimed species	Detected species
Rak Sam Sib	C1	Khonkaen	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C2	Bangkok	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C3	Bangkok	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C4	Chiang Mai	*A. racemosus*	*A. officinalis* or *A. filicinus*
Rak Sam Sib	C5	Khonkaen	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C6	Bangkok	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C7	Mae Hong Son	*A. racemosus*	*A. officinalis*
Rak Sam Sib	C8	Khonkaen	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C9	Kanchanaburi	*A. racemosus*	*A. racemosus*
Rak Sam Sib	C10	Rayong	*A. racemosus*	*A. racemosus*

[a]Abbreviation for commercially available crude drug claimed as *A. racemosus*.

3.2.3 PCR amplification and sequence determination of the *mat*K gene

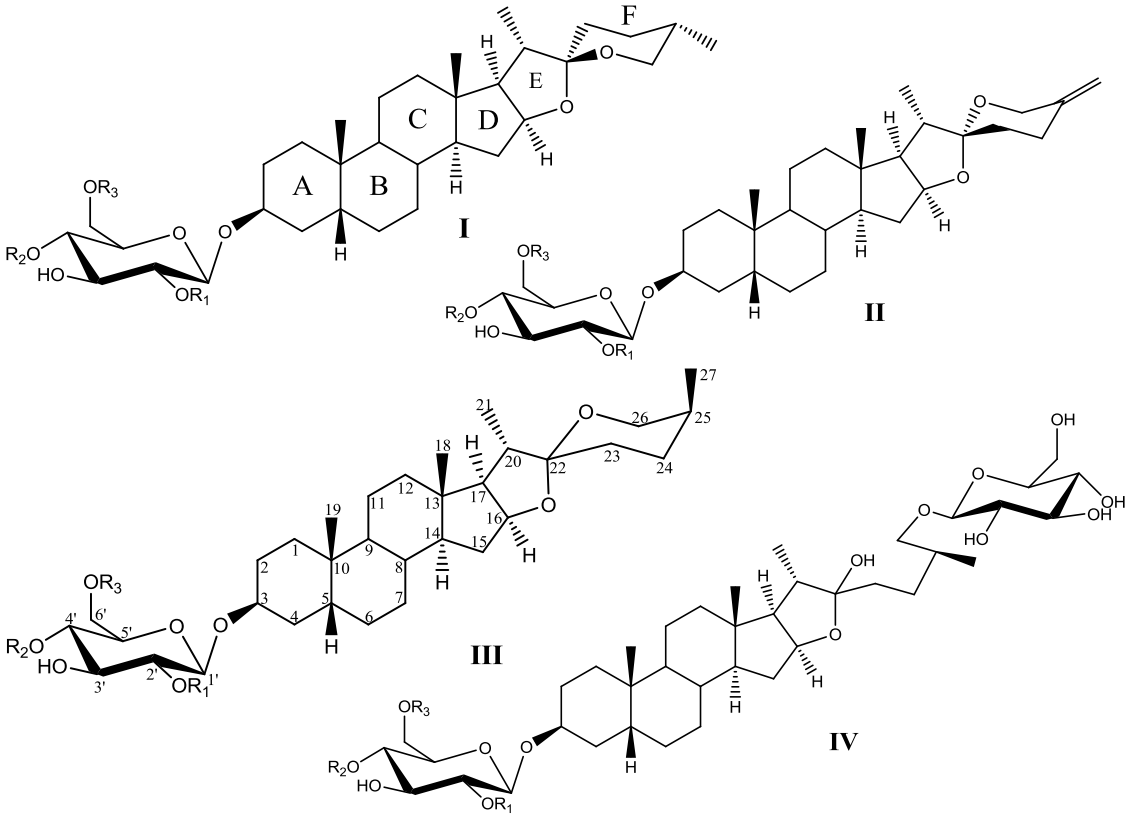
The complete *mat*K region of the *Asparagus* samples was amplified with a primer pair of **a** (5′-ATC TGT TGA TTG GAC TTC CTG TTT C-3′) and **c** (5′-GTG GAT TCA AAC ACT ACA TTA CGG G-3′) (Figure 3.2). The PCR amplification was performed in 50 µL of reaction mixture, consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, 1.5 U *Taq* Polymerase (Promega, USA), and 10–100 ng of total DNA as a template. PCR amplifications were carried out in a C1000^{TM} Thermal Cycler (Bio-Rad, USA) using cycling conditions of a hot start at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min, and final extension at 72°C for 10 min. PCR products were purified by using a High Pure PCR purification kit (Roche, USA). The obtained *mat*K sequences were assembled for their consensus sequences. Sequence alignments were constructed using CLUSTALW program. The *mat*K gene sequences were submitted to DDBJ/EMBL/GenBank nucleotide sequence databases with their accession numbers (Table 3.1).

3.2.4. Phylogenetic analysis

Based on the complete *mat*K sequence alignment, molecular phylogenetic trees were constructed using PAUP* version 4.0 beta10 program (Sinauer Assoc. Inc., USA). Maximum parsimony (MP) analysis was performed using a branch-and-bound searching method. In addition to the sequences of eight *Asparagus* taxa determined in the present study, *Cordyline stricta* (HM640647) also belonging to the family Asparagaceae was used as an outgroup. Bootstrap (1000 replications) statistic-supporting analysis was performed to estimate the confidence of the result topologies of the MP trees found. The intergenic region between *pet*D and *rpo*A (*pet*D–*rpo*A) intron sequences of five *Asparagus* species (*A. setaceus* AB177753, *A. racemosus* AB177750, *A. officinalis* AB177746, *A. filicinus* AB177740 and *A. densiflorus* AB177737) and *Convallia keiskei* (AB177757) were also retrieved from GenBank nucleotide database to construct a *pet*D–*rpo*A phylogenetic tree and compare with the *mat*K phylogeny.

3.2.5. PCR-RFLP analysis

The partial *mat*K region of eight *Asparagus* taxa was amplified with a pair of **a** (5´-ATC TGT TGA TTG GAC TTC CTG TTT C-3´) and **b** (5´-CAT ATG GAT GGG ATG GGG TAT TAG-3´) primers (Fig. 2). The PCR amplification was performed in 50 µL of reaction mixture, consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, 1.5 U *Taq* Polymerase (Promega) and 10–100 ng total DNA as a template. PCR amplifications were carried out in a C1000^{TM} Thermal Cycler (Bio-Rad) by the cycling conditions of a hot start at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min, and final extension at 72°C for 10 min. Aliquots of 10 µL of PCR purified products were digested with *Bss*KI (BioLabs Inc., USA) at 60°C for 2 h in a total volume of 20 µL. According to this method, ten commercial crude drug samples with the name 'Rak Sam Sib' purchased from traditional drug stores (Table 3.2) were also examined.



Compound	Aglycone types	R1	R2	R3
Shatavarin I	IV	-D-Glc	-L-Rha	H
Immunoside	III	-L-Rha	-L-Rha	H
Asparinin A	III	-D-Glc	-	H
Shatavarin IV	III	-D-Glc	-L-Rha	H
Shatavarin V	III	-L-Rha	-D-Glc	H
Shatavarin VI	I	-D-Glc	-L-Rha	H
Shatavarin VII	II	-D-Glc	-L-Rha	-
Shatavarin VIII	III	-D-Glc	-L-Ara	-D-Glc
Shatavarin IX	III	-D-Glc	-D-Glc	H
Shatavarin X	III	-L-Rha	-D-Glc (6-OAc)	H

Figure 3.1 Structures of furostanol and spirostanol glycoside isolated from *A. racemosus* roots, divided in four major structural types (**I** = 5**β**-spirost-25(27)-en-3**β**-ol, **II** = 5**β**-spirostan-3**β**-ol, **III** = 25*S*-5**β**-spirostan-3**β**-ol, **IV** = 25*S*-5**β**-furostan-3**β**,22**α**,26 triol) (Hayes *et al*., 2008).

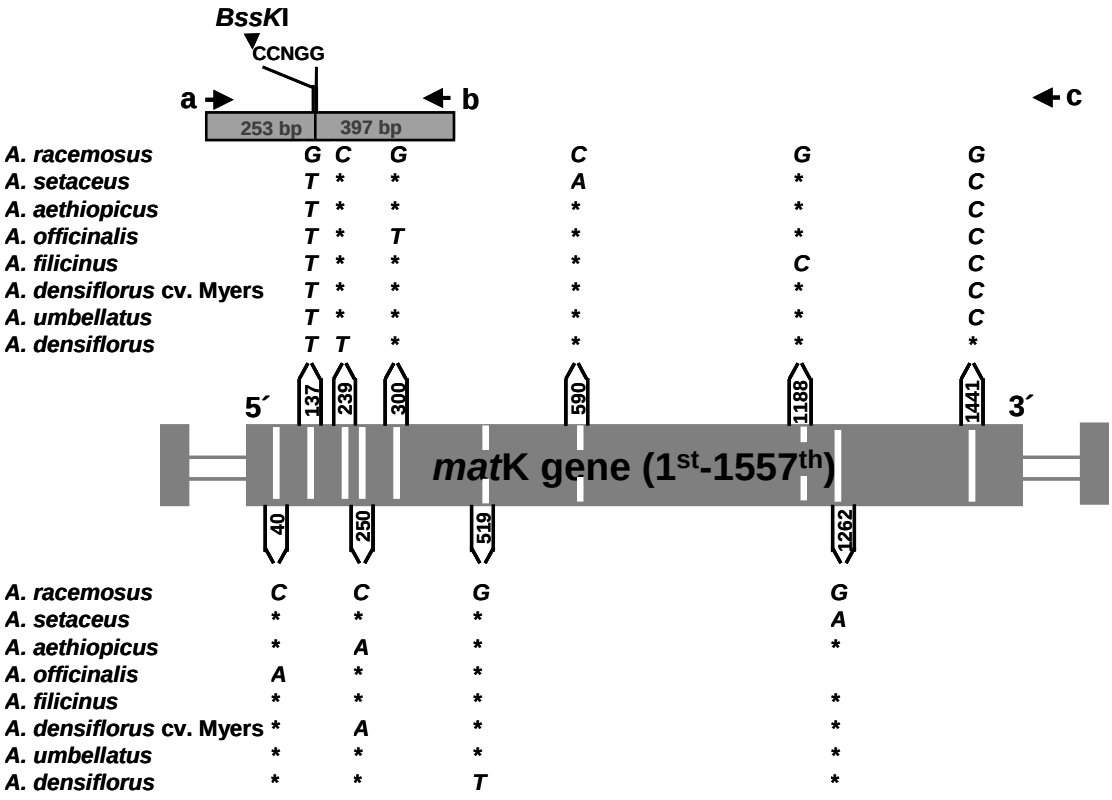


Figure 3.2 Structure and comparison of *mat*K gene sequences among eight *Asparagus* sp. Base substitutions are presented at the nucleotide positions 40, 137, 239, 250, 300, 519, 690, 1188, 1262, and 1441 respectively. An asterisk indicates a nucleotide similar to *A. racemosus*. Nucleotide substitution at position 137 was applied to PCR-RFLP analysis. The restriction enzyme site of *Bss*KI (5´ CCNGG 3´) used for identifying *A. racemosus* from another seven *Asparagus* spp. is indicated by a down-direction arrow. The partial *mat*K PCR product of *A. racemosus* was digested with *Bss*KI and resulted in two fragments of 397 and 253 bp. Sharp arrows indicate orientation and approximate position of the primers (a, b and c = primer).

3.3 Results and discussion

Asparagus racemosus, a medicinally phytoestrogenic species, was separated from other *Asparagus* spp. by a molecular technique. DNA amplification and sequencing was used to obtain the complete *mat*K gene sequences of eight *Asparagus* taxa. The *mat*K gene is an ORF embedded in the intron of the *trn*K gene coding the transfer RNA for lysine (tRNALys^{UUU}) (Sugita *et al*., 1985) and has been successfully used for identification in many medicinal plants such as the genera *Panax* (Zuo *et al*., 2011), *Sabia* (Sui *et al*., 2011) and *Dendrobium* (Asahina *et al*., 2010). This is the first report on full sequences of the *mat*K gene of *Asparagus* spp. in Thailand. The entire *mat*K sequences of eight taxa were found to be 1,557 bp in length. The alignment of the full-length *mat*K gene among the eight species and base substitution is shown in Figure B (Appendix B). Only ten nucleotide-substitutions were observed among the eight taxa (Figure 3.2). Our findings agree well with the suggestion of Fukuda et al. (2005) that the degree of genetic differentiation among species of the genus *Asparagus* is small, even though these species are morphologically diverse. For instance, although *A. aethiopicus* and *A. densiflorus* cv. Myers are morphologically distinct, their sequences were identical, and only one nucleotide different from *A. umbellatus* at position 250 of the *mat*K gene. Moreover, the *mat*K sequences of *A. filicinus*, *A. setaceus* and *A. officinalis* were slightly different from each other, but still relatively similar. Notably, the *mat*K sequence of the phytoestrogenic species, *A. racemosus*, was 2 and 3 bp different from those of *A. umbellatus* and *A. densiflorus*, respectively. Compared with the sequence of *A. racemosus*, those of *A. filicinus*, *A. setaceus* and *A. officinalis* had 4 base substitutions; and the species-specific nucleotide site for *A. racemosus* was found at position 137. Within *Asparagus* spp., the *mat*K nucleotide sequences from all plant specimens of such taxa were confirmed to be identical to each other, even though they were collected from different locations. The identical sequences of the plant samples within each species therefore indicated intra-specific stability of the *Asparagus mat*K gene. Differences in nucleotide sequences have been successfully used in several previous studies for identification of medicinal plants (Vongsak *et al*., 2008). Since no

nucleotide polymorphism was found among the *mat*K sequences of different samples from the same species, any base difference between the eight *Asparagus mat*K sequences observed in this study could be utilized to improve the morphological authentication practice.

According to Clifford and Conran (1987), the genus is largely classified into three subgenera (*Asparagus*, *Protasparagus* and *Myrsiphyllum*) based on species morphology. The members of the subgenus *Asparagus* are dioecious with unisexual flowers, while *Protasparagus* and *Myrsiphyllum* include only hermaphroditic plants, being discriminated by differences in floral morphology and number of ovules. Later molecular systematic studies of *Asparagus* using RFLP analysis of cpDNA (Lee *et al*., 1997) and nuclear DNA internal transcribed spacer (ITS) regions (Stajner *et al*., 2002) confirmed the separation of the subgenus *Asparagus* from *Protasparagus* and *Myrsiphyllum*. Furthermore, the phylogeny of *Asparagus* conducted by Fukuda et al. (2008), which analyzed sequence polymorphisms of the non-coding cpDNA regions for 26 taxa, also revealed the monophyletic clade of the subgenus *Asparagus*, although with low bootstrap support. The results also showed that one of the two *Myrsiphyllum* species analyzed was a sister species to all other *Asparagus* taxa. Most recently, Kubota et al. (2012) explained interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* spp. by molecular phylogeny of five non-coding cpDNA regions. They suggested that dioecious species were derived from a single evolutionary transition from hermaphroditism in *Asparagus*. Unfortunately, the taxonomic relationships among the subgenera of *Asparagus* and their evolutionary histories remain unresolved.

To clarify the phylogenetic relationship among the eight *Asparagus* taxa in the present study, a MP analysis was performed and produced six equally most parsimonious trees (MPTs) of 80 steps, with a consistency index (CI) of 0.9875 and a retention index (RI) of 0.8750. From our molecular phylogenetic study using the *mat*K gene, the semistrict consensus tree of the six MPTs (Figure 3.3A) revealed that all *Asparagus* spp. were monophyletically grouped together, and two sister pairs with more

than 50% bootstrap supports were found: a pair of *A. filicinus* and *A. filicinus* AB029805 and a pair of *A. racemosus* and *A. densiflorus* with 63 and 59% bootstrap values, respectively. The *pet*D–*rpo*A nucleotide data set gave only one MPT (Figure 3.3B) and showed a similar tree topology. The molecular phylogeny potentially divided the eight *Asparagus* species into two clades, following the taxonomic suggestion of Fukuda et al. (2008): the subgenus *Asparagus* clade of Eurasian species (*A. setaceus*, *A. officinalis* and *A. filicinus*), and the subgenus *Protasparagus* of Southern African species (*A. racemosus*, *A. densiflorus*, *A. umbellatus* and *A. densiflorus* cv. Myers). *A. aethiopicus* was the only taxon not grouped specifically with any other species. This result agrees well with the previously published plastid *pet*B intron and *pet*A–*rpo*A intergenic spacer phylogenetic tree of Fukuda et al. (2008). To investigate the genetic diversity among populations as well as the relatedness to their chemical compositions, further study such as with AFLP and RAPD markers should be used to survey the whole-genome information of different *Asparagus* germplasm.

Although the degree of genetic differentiation among the complete *mat*K gene sequences of the eight *Asparagus* spp. was small, the specific nucleotide site of *A. racemosus* found in the present study (Figure 3.2) could be further developed to a simple, rapid molecular authentication method. In Thailand, *A. racemosus* is often commercially packed as small pieces of dry roots, which no longer bear the original features of the plant. The trade and economics of *A. racemosus* is therefore greatly affected since the morphology of its dry roots is similar to those of other *Asparagus* spp. The PCR-RFLP authentication has previously been shown to be suitable for many medicinal plants. Thai medicinal remedies always contain a variety of herbal ingredients, and the DNA of these herbs is usually highly degraded into small pieces due to oxidative and hydrolytic processes during preservation and extraction (Mizukami *et al*., 2000). Taking into account the difficulty of amplifying long DNA fragments due to the degeneration of genomic DNA during preparation or storage of crude drugs, we designed a new primer set (primer **a** and **b**) for amplification of short regions.

For PCR-RFLP authentication of *A. racemosus*, the *Bss*KI restriction enzyme was shown to specifically digest such species-specific polymorphic sites found in the *mat*K region. The partial *mat*K PCR product (650 bp partially amplified) of *A. racemosus* was digested with *Bss*KI and resulted in two fragments of 397 and 253 bp, whereas no digested fragment was found from the other seven *Asparagus* spp. (Figure 3.4). Therefore, the PCR-RFLP authentication method for *A. racemosus* could be applied to any commercial crude drugs claimed to be derived from *A. racemosus*. Ten commercially available crude drug samples of *A. racemosus* with the name "Rak Sam Sib" purchased from various places in Thailand were examined using this PCR-RFLP technique (Figure 3.5A and 3.5B). The PCR products of eight samples including three from Khonkaen (C1, C5 and C8), three from Bangkok (C2, C3 and C6), one from Kanchanaburi (C9), and one from Rayong (C10) were digested by *Bss*KI (Figure 3.5B) – and the PCR-RFLP pattern of the eight samples matched that of *A. racemosus*, suggesting that they were *A. racemosus*. In contrast, the PCR fragments of the other two samples (commercial samples C4 and C7 purchased in Chiang Mai and Mae Hong Son, respectively) were not digested by *Bss*KI (Figure 3.5B). The results indicated that C4 and C7, which are traded by the same name, "Rak Sam Sib", were not *A. racemosus*. The *mat*K gene of C4 and C7 were amplified and sequenced. The results showed that the nucleotide sequence of C4 was 99% matched with that of *A. officinalis* and *A. filicinus*. It was likely that C4 was derived from them with the local name of "Rak Sam Sib". The sequence of sample C7 was 100% matched with *A. officinalis*. Since dry roots of *A. racemosus* have a similar appearance to *A. officinalis* (Bopana *et al*., 2007) and processed as crude drugs, their distinguishing characteristics may disappear and they are very easily mixed up. From our results, in the Thai traditional drug markets, there is the presence of adulterants of, or substitutes for, *A. racemosus*. Thus, accurate identification of the herbal drugs is a prerequisite for clinical use and pharmacological research. The PCR-RFLP methods described in this study could be applied for high levels of quality control of *A. racemosus* raw materials.

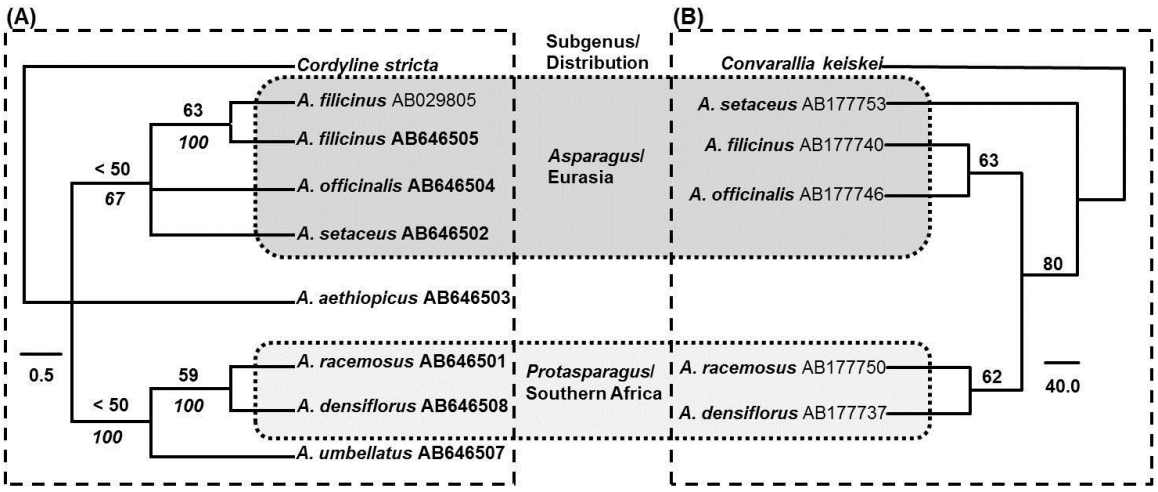


Figure 3.3 A semistrict consensus tree of six equal maximum parsimony (MP) trees resulting from phylogenetic analysis of the genus *Asparagus* based on *mat*K gene sequences. A single MP *mat*K phylogeny (A) is compared with the MP tree of five *Asparagus pet*D–*rpo*A intron sequences retrieved from GenBank (B). Numbers above branches indicate bootstrap percentages from the statistic-supporting analysis. Italic numbers under branches show percentages of semistrict congruency among the parsimonious trees.

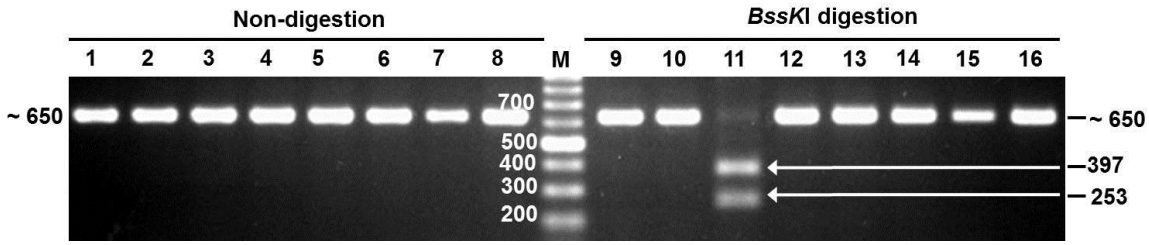


Figure 3.4 PCR-RFLP analysis on the partial *mat*K gene using a restriction enzyme *Bss*KI. Agarose gel of the PCR products of non-digestion (lanes 1–8) and digested with *Bss*KI (lanes 9–16) shows the new 253 and 397 bp fragments specifically for *A. racemosus* (indicated by arrows). Lane 1, 9 = *A. officinalis*; lane 2, 10 = *A. setaceus*; lane 3, 11 = *A. racemosus*; lane 4, 12 = *A. densiflorus*; lane 5, 13 = *A. densiflorus* cv. Myers; lane 6, 14 = *A. aethiopicus*; lane 7, 15 = *A. umbellatus*; and lane 8, 16 = *A. filicinus*. M = VC 100 bp plus DNA ladder.

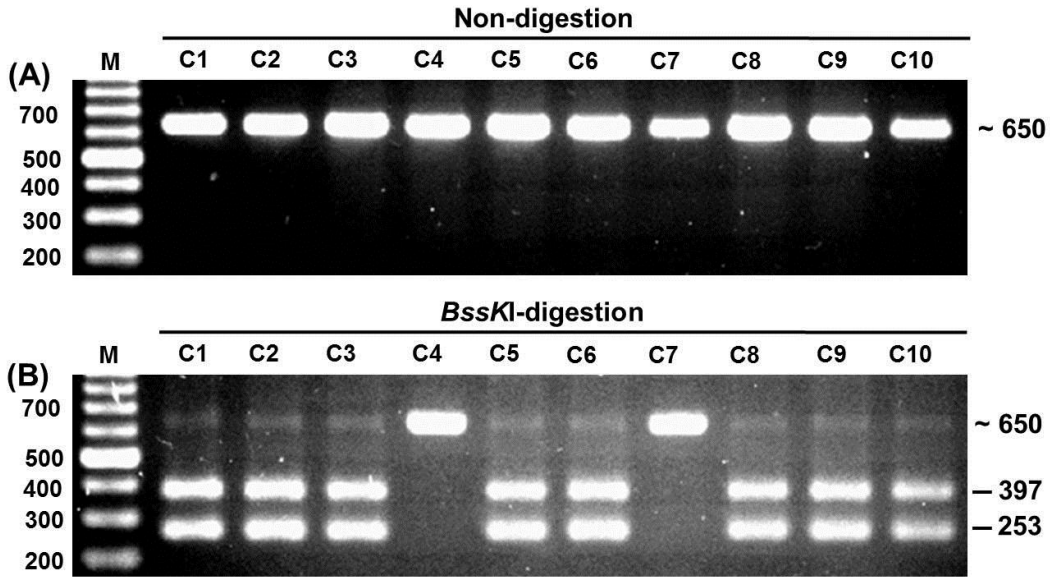


Figure 3.5 Authentications of ten commercially available crude drugs (C1–C10) claimed to be *A. racemosus*, using PCR-RFLP. (A) Agarose gel profile of PCR products before digestion with *BssK*I. (B) Agarose gel profile of PCR products after digestion with *BssK*I, showing the resulting fragments. M = VC 100 bp plus DNA Ladder.

3.4 Conclusion

In conclusion, we reported the molecular phylogenetic study of eight species of *Asparagus* based on the *mat*K gene and the implication for authentication. The *mat*K gene sequences provided valuable information for identification and barcoding of these *Asparagus* spp. Moreover, we successfully developed a PCR-RFLP method for the rapid and reliable authentication of the medicinally phytoestrogenic species, *A. racemosus*, both in fresh samples and also in commercially available crude drugs.

CHAPTER IV

EVALUATION OF ANTIOXIDANT AND CYTOTOXIC ACTIVITIES FROM ROOT EXTRACTS OF *ASPARAGUS* SPECIES

4.1 Introduction

The roots of a large number of *Asparagus* species have long been used in folk medicine (Khaliq-Uz-Zaman *et al*., 1998). *Asparagus* extracts and isolated compounds have revealed a wide range of biological activities. For example, the studies of *A. officinalis* have demonstrated anti-inflammatory (Jang *et al*., 2004), cytotoxic (Shao *et al*., 1997), antimutagenic (Tang, 2001), and antifungal activities (Shimoyamada *et al*., 1990). *A. cochinchinensis* was historically believed to possess numerous therapeutic properties, including anticancer and anti-inflammatory activities (Huang, 1993; Lee *et al*., 2009). In addition, *Asparagus* cultivar Ramada and Grande were found to possess antioxidant activity (Rodriguez *et al*., 2005). The genus *Asparagus* could be explored for antioxidant and cytotoxic activities for potential applications, particularly as natural medicine.

In this chapter, the antioxidant properties of eight *Asparagus* species were evaluated for DPPH radical scavenging, ferric reducing antioxidant power (FRAP) and superoxide anion scavenging (NBT) properties. In addition, cytotoxic activities against human cell lines KB (human epidermoid carcinoma of oral cavity), MCF-7 (breast adenocarcinoma) and NCI-H187 (human small cell lung cancer) of root extracts of *Asparagus* species were determined.

4.2 Materials and methods

4.2.1 Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Nitro blue tetrazolium (NBT), Riboflavin, L-methionine, Ethylenediaminetetraacetic acid (EDTA), 2,4,6-tri (2-pyridyl)-s-triazine

(TPTZ), Iron (III) chloride hexahydrate, Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), L-Ascorbic acid, Rutin and (+)-Catechin were purchased from Sigma Chemical Co., Ltd. All other chemicals used were of analytical grade.

4.2.2 Plant materials

Eight *Asparagus* species were collected from various regions of Thailand (Table 4.1). All specimens were identified by Assoc. Prof. Thatree Phadungcharoen of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand.

Table 4.1 Plant materials used in this study.

Species	Place of collection (Thailand, Province)	Voucher no.
A. racemosus Willd.	Phetchabun	SS-KI-020853
A. aethiopicus L.	Phetchabun	SS-TP-110953
A. densiflorus (Kunth.) Jessop	Rayong	SS-TP-211153
A. densiflorus cv. Myers	Prachin Buri	SS-TP-201153
A. filicinus Buch.-Ham.	Chiang Mai	SS-TB-080853
A. officinalis L.	Bangkok	SS-TP-030753
A. setaceus (Kunth.) Jessop	Chiang Mai	SS-TB-080753
A. umbellatus Royle	Bangkok	SS-TP-221153

4.2.3 Extract preparation

The dried roots of plant materials (50 g of each) were extracted with 200 ml of methanol for 72 h. The extraction was repeated three times. The obtained methanolic

extracts were filtered and evaporated using a rotary evaporator to give a dried crude extract. The dried extract was stored at -20°C until used.

4.2.4 Antioxidant activity determination

4.2.4.1 DPPH radical scavenging assay

DPPH radical scavenging activity of the methanol extracts was assessed according to the method reported by Gyamfi (1999) with slight modifications. Briefly, a 20 µl solution of the crude extract, at different concentrations diluted twofold (25-400 µg/ml) in DMSO, was mixed with 180 µl of 0.1 mM DPPH in methanol. The mixture was shaken and allowed to stand at room temperature in the dark for 30 min. Blank solutions were prepared with each test sample solution (20 µl) and 180 µl of methanol while the negative control was 180 µl of 0.1 mM DPPH solution plus 20 µl of methanol. Trolox and Ascorbic acid, well-known antioxidants, were used as the reference standard. Three replicates were made for each test sample. Thereafter, the absorbance of the assay mixture was measured at 517 nm against each blank with a UV-spectrophotometer. Lower absorbance of the reaction mixture indicated higher radical-scavenging activity. The inhibition ratio was calculated according to the following equation: % inhibition = [(absorbance of control-absorbance of sample)/ absorbance of control] x 100.

4.2.4.2 Superoxide anion scavenging assay

Measurement of superoxide radical scavenging activity was carried out according to the method of Dasgupta (2004). The assay was based on the capacity of the test sample to inhibit the reduction of nitroblue tetrazolium (NBT) in the riboflavin-light-NBT system. A 96-well microplate was used. The reaction mixture (120 µl) in each well contained 40 µl of 1.5 M sodium phosphate buffer, 2 µl of 120 µM riboflavin, 20 µl of 78 mM methionine, 20 µl of 600 µM EDTA, 20 µl of 450 µM NBT and 30 µl of sample solution. The production of blue formazan was monitored by measuring the increase in absorbance at 570 nm after a 10-min illumination with a fluorescent lamp. The entire

reaction proceeded in a closed box lined with aluminum foil. A similar reaction mixture was kept in the dark and served as the blank. Trolox and ascorbic acid were used as reference standard. Three replicates were made for each test sample. The inhibition ratio was calculated according to the following equation: % inhibition = [[(absorbance of control in dark-absorbance of sample in dark)/ absorbance of control in dark]-[(absorbance of control in light-absorbance of sample in light)/ absorbance of control in light]] x 100.

4.2.4.3 Ferric reducing antioxidant power (FRAP) assay

This assay was determined according to the method of Benzie and Strain (1996) with slight modifications. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) with 16 ml glacial acetic acid and brought to 1 litre with distilled water. The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. A total of 175 µl of working FRAP reagent was added to each well in a 96-well. 25 µl of sample was then added to each well. Each sample was run in triplicate. After addition of sample to the FRAP reagent, reading at 595 nm was performed after 4 min using a Microplate Reader. Trolox, a reference standard, was used to perform the calibration curves. The reducing power ability was expressed in µM Trolox equivalents (TE) in milligrams per gramme sample.

4.2.5 Total phenolic and flavonoid contents

Total phenolic contents were measured by Folin-Ciocalteu assay (Singleton, Orthofer, and Lamuela-Raventos, 1999). Briefly, 2.32 ml of distilled water, 40 µl of sample, and 200 µl of Folin-Ciocalteu reagent were added to a 15 ml centrifuge tube. The contents were mixed and allowed to stand for 8 min at room temperature. Next, 600 µl of 7% sodium carbonate solution was added and allowed to stand at room temperature for 60 min, and then absorbance was recorded at 760 nm. Total phenolic

contents were standardized against catechin and expressed as milligrams per millilitre of catechin equivalents. The linearity range for this assay was determined as 7.8125-250 µg/ml of catechin (R^2= 0.9966)

The total flavonoid content of *Asparagus* was determined using a modified method by Xu and Chang (2007) and used rutin as a standard. Extracts or standard solutions (1 mL) were mixed with 5% $NaNO_2$ solution (0.3 mL). After standing for 5 min, the mixture was combined with 10% $AlCl_3$ solution (0.3 mL). 1 M NaOH (2 mL) was added to the mixture 5 min later. The absorbance of the solutions at 510 nm was then measured. The results were expressed as mg rutin equivalent (mg rutin/g extract). The linearity range for this assay was determined as 15.625-250 µg/ml of rutin (R^2= 0.9962)

4.2.6 Cytotoxicity assay

The cytotoxicity assay against three cancerous human-cell lines including KB (oral human epidermal carcinoma), NCI-H187 (human lung cancer) cells and MCF-7 (breast cancer) cells, was done in this study by the Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC). The test was performed using resazurin microplate assay method (REMA) (Brien *et al.,* 2000), with ellipticine, doxorubicin and tamoxifen as positive controls. The samples were diluted to 50 µg/ml for maximum final test concentration. The protocols are according to bioassay laboratory guideline (Bioassay laboratory protocol 01, 2009). Percent inhibition of cell growth is calculated by the following equation: % Inhibition = $[1-(FU_T/FU_C)] \times 100$, FU_T and FU_C are the mean fluorescent units from treated and untreated conditions, respectively. Dose response curves are plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Prosoftware (Molecular Devices, USA).

4.2.7 Statistical analysis

All results were expressed as mean ± SD (n = 3). Differences between groups were tested by one-way ANOVA. The significance of difference was calculated by Duncan's test and *p* values <0.05 were considered to be significant.

4.3 Results and discussion

4.3.1 Antioxidant activity, total phenolic and total flavonoid contents

The generation of radical oxidative species involves either radical processes or different potential redox systems. The solubilities of antioxidant compounds determine their effective antioxidant activities in either aqueous or lipid systems (Sanchez-Gonzalez *et al*., 2005). Antioxidant activities, including DPPH radical scavenging, superoxide radical scavenging and reducing power ability, of the root extracts of *Asparagus* species were evaluated. DPPH method is one of the most popular methods in natural antioxidant studies (Moon and Shibamoto, 2009). It is well accepted that the DPPH radical scavenging activity by antioxidants is attributable to their hydrogen-donating ability (Chen and Ho, 1995). DPPH radical scavenging activity of methanol extracts from roots of *Asparagus* species and Trolox and ascorbic acid, as the reference standard, were in a dose-dependent manner (Table 4.2). DPPH radical scavenging activity of different *Asparagus* species reflected antioxidant potency through IC_{50} values. A lower value of IC_{50} indicates a higher antioxidant activity. Among them, the extract of *A. setaceus* exhibited the highest DPPH radical scavenging activity followed by *A. densiflorus*, *A. aethiopicus*, *A. officinalis*, *A. umbellatus, A. filicinus* and *A. racemosus*. The IC_{50} values of Trolox and ascorbic acid were 5.96 and 17.82 µg/ml, respectively. Substances which are able to perform this reduction can be considered as antioxidant and radical scavengers (Hinneburg *et al*., 2006). The hydrogen atoms or electrons donation ability of the corresponding extract will be measured from the bleaching of violet colored methanol solution of DPPH. This interaction depends on the structural conformation of the bioactive compounds present in the plant extracts, among which the hydroxyl groups of flavonoids are highly favorable (El-Sayed, 2009). Although

A. racemosus extracts exhibit far less antioxidant activity than Trolox and ascorbic acid when tested *in vitro* with the DPPH method, numerous studies have reported their substantial antioxidant effect when tested *in vivo* (Bhatnagar *et al*., 2005; Sairam *et al*., 2003; Kamat *et al*., 2000).

Wu et al. (2008) reported that the superoxide radical is biologically important because it can be decomposed to form a stronger oxidative species such as singlet oxygen and hydroxyl radicals. Superoxide radicals are known to be very harmful to cellular components as a precursor of many ROS. For example, in presence of metal ions, the superoxide radical would further produce a highly reactive hydroxyl radical (MacDonald-Wicks *et al*., 2006). Superoxide anion can reduce nitrobluetetrazolium (NBT) to form blue formazan. Low intensity of the blue color is observed if a scavenger of is superoxide anion present. The superoxide scavenging activities of methanol extracts from root of *Asparagus* species were compared (Table 4.2). All plant extracts inhibited the formation of reduced NBT in a dose-related manner. The IC_{50} value of the root extracts increased in the following order: *A. officinalis*, *A. densiflorus* cv. Myers, *A. setaceus*, *A. umbellatus*, *A. aethiopicus*, *A. densiflorus*, *A. filicinus* and *A. racemosus*. The IC_{50} value of Trolox and ascorbic acid were 7.05 and 49.89 µg/ml, respectively. *A. officinalis* showed the potent superoxide anion scavenging activity.

Table 4.2 DPPH and superoxide radical scavenging activities of methanol extracts from roots of *Asparagus* species.

Asparagus species	IC_{50} (μg/ml)	
	DPPH radical scavenging activities	Superoxide radical scavenging activities
A. officinalis	279.84 ± 2.32[c]	58.45 ± 4.65[b]
A. densiflorus cv. Myers	97.82 ± 1.78[b]	62.96 ± 3.78[b]
A. racemosus	>500[e]	487.68 ± 4.91[e]
A. setaceus	70.04 ± 3.42[b]	86.16 ± 4.43[c]
A. aethiopicus	276.78 ± 2.46[c]	351.81 ± 4.58[d]
A. umbellatus	293.05 ± 3.14[c]	98.13 ± 3.21[c]
A. filicinus	311.08 ± 4.76[d]	468.82 ± 3.65[e]
A. densiflorus	244.68 ± 4.87[c]	362.87 ± 4.34[d]
Trolox	5.96 ± 1.24[a]	7.05 ± 1.45[a]
Ascorbic acid	17.82 ± 1.32[a]	49.89 ± 1.96[b]

Different letters (a-e) within the same column indicate significant difference at the level of $p<0.05$ by Duncan test.

Many studies have demonstrated that the reducing power in plant extracts was highly related with their antioxidant activities (Chang *et al*., 2002). The reducing properties have been shown to exert antioxidant action by breaking the free radical chain through the donation of hydrogen atom (Gordon, 1990). We analyze reducing power by FRAP assay, which is simple, fast and reproducible (Wong *et al*., 2006). The FRAP is versatile and can be readily applied to both aqueous and alcohol extracts of different plants (Oonsivilai *et al*., 2008). The antioxidant potential of *Asparagus* extracts was estimated from their power to reduce the TPTZ-Fe^3 complex to TPTZ-Fe^2 complex. In this study, the reducing power of the extract from roots of *Asparagus* species was calculated as Trolox equivalent (TE) (Table 4.3). The reducing power ranged from 2.85

to 35.42 mM TE/g dry weight. *A. densiflorus* cv. Myers extract showed the highest reducing power ability (35.42 mM TE/g dry weight), followed by *A. setaceus*, *A. officinalis*, *A. umbellatus*, *A. densiflorus*, *A. aethiopicus*, *A. filicinus* and *A. racemosus*. *A. setaceus* extract showed antioxidant activity when subjected to DPPH and FRAP assay. It could be explained that *A. setaceus* extract had the ability to reduce both radicals and ferric ions. Also, all extracts showed antioxidant activity differently which depend on the components in each extract. Previous researchers reported that phenolic acids and flavonoids are the source of antioxidant activity in plants (Cook and samman, 1996).

Velioglu et al. (1998) has suggested that the total phenolic contents of plant materials are related with their antioxidant activities. Total phenolic contents showed variation in *Asparagus* extracts, and the range was from 85.49 to 152.09 mg catechin /g DW (Table 4.3). *A. setaceus* had the highest total phenolic contents, followed by *A. densiflorus* cv. Myers, *A. umbellatus*, *A. aethiopicus*, *A. officinalis*, *A. densiflorus* and *A. filicinus*, whereas *A. racemosus* had the lowest content. It is noteworthy that, apart from the phenolic compounds, there may be other secondary metabolites in plants that could be responsible for antioxidant activity. As a result, a total phenolic content of *A. setaceus* was related to the DPPH radical and superoxide anion scavenging activities. Guillen et al. (2008) suggested that *A. officinalis* can be considered an excellent source of natural antioxidants because high among of phenolic compounds. The antioxidant properties of *A. officinalis* may implicate for different human diseases. Previously, no reports have dealt with total phenolic contents and antioxidant activities of *A. setaceus*. Our studies suggest that *A. setaceus* contains higher levels of total phenolic contents, as compared to *A. officinalis*. The higher contents of total phenolic in *A. setaceus* probably explain why it has greater radical scavenging capacity. Sun and Ho (2005) reported that antioxidant activity in many plant materials primarily originate from their phenolic content, indicating a significant positive relationship between total phenolic contents and antioxidant capacity. The phenolic groups in polyphenols can accept an

electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components (Scalbert *et al*., 2005)

In general, plant flavonoids are highly effective free radical scavengers and antioxidants. In this study, the total flavonoid content of the *Asparagus* species extracts was calculated as rutin equivalents (RE) in milligrams per gram sample. Apparently, the total flavonoid contents of *A. densiflorus* cv. Myers (163.26 mg/g) was highest than other species (Table 4.3). Makris et al. (2001) suggested that flavonoid content related with antioxidant activity of *A. officinalis*. Our finding suggested that the antioxidant activity of *A. densiflorus* cv. Myers may relate with total flavonoid contents. *A. densiflorus* cv. Myers should be evaluated further for the possible isolation of active antioxidant compounds.

Table 4.3 Reducing power ability, total phenolic and total flavonoid contents of methanol extracts from roots of *Asparagus* species.

Asparagus species	Reducing power ability	Total phenolic contents	Total flavonoid contents
	TE (mM)/g DW[g]	CE (mg)/g DW[h]	RE (mg)/g DW[i]
A. officinalis	21.44 ± 0.36[b]	19.99 ± 0.42[d]	37.02 ± 2.48[d]
A. densiflorus cv. Myers	35.42 ± 0.51[a]	130.81 ± 0.58[b]	163.27 ± 5.81[a]
A. racemosus	2.85 ± 0.54[d]	5.49 ± 0.42[e]	13.03 ± 0.22[f]
A. setaceus	34.53 ± 0.53[a]	152.09 ± 0.49[a]	49.62 ± 1.18[c]
A. aethiopicus	13.51 ± 0.59[c]	22.54 ± 0.42[d]	20.14 ± 0.44[e]
A. umbellatus	19.23 ± 0.41[b]	61.39 ± 0.43[c]	59.57 ± 1.01[b]
A. filicinus	11.42 ± 0.45[c]	8.91 ± 0.42[e]	16.96 ± 5.59[e]
A. densiflorus	14.18 ± 0.46[c]	10.19 ± 0.43[e]	9.36 ± 1.08[f]

The different letters (a-f) within the same column are significant difference at the level of $p<0.05$ according to the Duncan test.

[g] Trolox equivalent in millimolar per gram dry weight.

[h] Catechin equivalent in milligram per gram dry weight.

[i] Rutin equivalent in milligram per gram dry weight.

4.3.2 Cytotoxicity activity

The cytotoxicity effects of compounds and plant extracts in *in vitro* models are widely used (Songsiang *et al*., 2011; Ngamrojanavanich, *et al*. 2007). Cell culture systems can be more sensitive and more reproducible than tests involving intact animals (Cetin and Bullerman, 2005). In the cytotoxicity studies, methanol extracts of *Asparagus* species were tested against human epidermoid carcinoma of oral cavity (KB), breast adenocarcinoma (MCF-7), human small cell lung cancer (NCI-H187) cell lines and normal cells (Vero cells). Interestingly, the extract of *A. setaceus* showed cytotoxicity against KB cell and NCI-H187 cell with an IC_{50} values of 19.59 and 32.60 µg/ml, respectively, while it showed moderate cytotoxicity against Vero cells (IC_{50} = 46.55 µg/ml) (Table 4.4). The results indicate that the extract of *A. setaceus* may have anti-cancer properties. Bioactive constituents from *A. setaceus* should be further evaluated.

Table 4.4 Cytotoxicity of methanol extracts from roots of *Asparagus* species.

Asparagus species	Cell line[a], IC_{50} (µg/ml)			
	Vero cell	KB	NCI-H187	MCF-7
A. officinalis	>50[b]	>50[b]	>50[b]	>50[b]
A. densiflorus cv. Myers	>50[b]	>50[b]	>50[b]	>50[b]
A. racemosus	>50[b]	>50[b]	>50[b]	>50[b]
A. setaceus	46.55	32.60	19.59	>50[b]
A. aethiopicus	>50[b]	>50[b]	>50[b]	>50[b]
A. umbellatus	>50[b]	>50[b]	>50[b]	>50[b]
A. filicinus	>50[b]	>50[b]	>50[b]	>50[b]
A. densiflorus	>50[b]	>50[b]	>50[b]	>50[b]
Ellipticine[c]	1.18	0.769	0.333	-
Doxorubicin[c]	-	0.779	0.115	8.34
Tamoxifen[c]	-	-	-	8.99

[a] Results are mean of three replications.

[b] Considered inactive.

[c] Cytotoxic reference compound.

- = Test not determined

4.4 Conclusions

This is the first report of the antioxidant and cytotoxic activities from root extracts of *Asparagus* species. Methanol extracts of *A. densiflorus* cv. Myers and *A. setaceus* showed moderate antioxidant activity Interestingly, *A. setaceus* exhibited significant cytotoxic activity against KB cells and NCI-H187 cells, with weak activity against normal cells (Vero cells). However, *A. setaceus* should be evaluated further for the possible isolation of active anti-cancer compounds.

CHAPTER V

CONCLUSION

In the present study, nucleotide sequences of the *mat*K gene of eight *Asparagus* spp., *A. racemosus, A. setaceus*, *A. aethiopicus*, *A. officinalis*, *A. filicinus*, *A. densiflorus*, *A. umbellatus* and *A. densiflorus* were analyzed. The entire *mat*K sequences of the eight taxa were found to be 1,557 bp in length. Only ten nucleotide-substitutions were observed among the eight taxa. The *mat*K gene sequences provided valuable information for identification of these *Asparagus* species.

The molecular phylogeny potentially divided the eight *Asparagus* species into two clades, the subgenus *Asparagus* clade of Eurasian species (*A. setaceus*, *A. officinalis* and *A. filicinus*), and the subgenus *Protasparagus* of Southern African species (*A. racemosus*, *A. densiflorus*, *A. umbellatus* and *A. densiflorus* cv. Myers). *A. aethiopicus* was the only taxon not grouped specifically with any other species. To investigate the genetic diversity among populations as well as the relatedness to their chemical compositions, further study such as with AFLP and RAPD markers should be used to survey the whole-genome information of different *Asparagus* germplasm.

Asparagus racemosus, a medicinally phytoestrogenic species, was separated from other *Asparagus* spp. by a molecular technique. We successfully demonstrated PCR-RFLP authentication of *A. racemosus*. The *BssK*I restriction enzyme was shown to specifically digest such species-specific polymorphic sites found in the *mat*K region. The PCR-RFLP method was applied to commercial crude drugs claimed to be derived from *A. racemosus*. From our results, there is the presence of adulterants of, or substitutes for, *A. racemosus* in the Thai traditional drug markets. The PCR-RFLP methods described in this study could be applied for high levels of quality control of *A. racemosus* raw materials. Subsequently research could focus on a quantitative analysis of major steroidal saponin productions, shatavarins. Tissue culture technique could be utilized for increasing the secondary products.

The antioxidant and cytotoxic activities of the root extracts of *Asparagus* species were evaluated. Interestingly, *A. setaceus* exhibited significant cytotoxicity against KB cells and NCI-H187 cells, but showed weak cytotoxic activity against normal cells (Vero cells). Bioassay-guided isolation of roots of *A. setaceus* for active principles should be further investigated.

Due to the fact that therapeutic methods used in cancer treatment are often ineffective, it is necessary to look for new solutions. Plants have been a prime source of new drugs for the treatment of cancer and will likely be the basis of novel anticancer agents. The genus *Asparagus* exhibited highly valuable plant species having therapeutic and nutraceutical importance. Saponins and their antitumor activity are especially interesting. Plants in the genus *Asparagus* could be one of the interests as a new source of natural medicine.

REFERENCES

A Consortium for the Barcode of Life (CBOL) Plant Working Group. 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America 106: 12794-12797.

Ahmad, S., Ahmad, S., Jain, P.C. 1991. Chemical examination of Satavari (*Asparagus racemosus*). Bulletin of Medico Ethnobotanical Research 12:157-160.

Aranishi, F., Okimoto, T., Izumi, S., 2005. Identification of gadoid species (Pisces, Gadidae) by PCR-RFLP analysis. Journal of Applied Genetics 46: 69-73.

Asahina, H., Shinozaki, J., Masuda, K., Morimitsu, Y., Satake, M. 2010. Identification of medicinal *Dendrobium* species by phylogenetic analyses using *mat*K and *rbc*L sequences. Journal of Natural Medicine 64: 133-138.

Belhouchet, Z., *et al*. 2008. Steroidal saponins from roots of *Smilax aspera* subsp. mauritanica. Chemical and Pharmaceutical Bulletin 56: 1324-1327.

Benzie, I. F. F., Strain, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. Analytical Biochemistry 239: 70-76.

Bhattacharya, S.K., Bhattacharya, A., Chakrabarti, A. 2000. Adaptogenic activity of Siotone, a polyherbal formulation of Ayurvedic rasayanas. Indian Journal of Experiment Biology 38: 119-128.

Bhutani, K.K., Gohil, V.M. 2010. Natural products drug discovery research in India: Status and appraisal. Indian Journal of Experimental Biology 48: 199-207.

Bopana, N., Saxena, S. 2007. *Asparagus racemosus*-Ethnopharmacological evaluation and conservation needs. Journal of Ethnopharmacology 110: 1-15.

Brien, J.O., Wilson, I., Orton, T., Pognan, F. 2000. Investigation of the alamar blue (resazulin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry 267: 5421-5426.

Cerutti, P. A. 1991. Oxidant stress and carcinogenesis. European Journal of Clinical Investigation 21: 1-11.

Cetin, Y., Bullerman, L.B. 2005. Cytotoxicity of Fusarium mycotoxins tomammalian cell cultures as determined by the MTT bioassay. Food and Chemical Toxicology 43: 755-764.

Chang, L.W., Yen, W. J., Huang, S. C., Duh, P. D. 2002. Antioxidant activity of sesame coat. Food Chemistry 78: 347-354.

Charles, G., Jean, L., Marianne, P., Serge, L., Samuel, T., Andre, P. 2009. Synthesis, cytotoxicity and hemolytic activity of chacotrioside lupane-type neosaponins and their germanicane-type rearrangement products. Bioorganic and Medicinal Chemistry Letters 19: 2310-2314.

Chen, C.W., Ho, C.T. 1995. Antioxidant properties of polyphenols extracted from green and black teas. Journal Food Lipids 2: 35-46.

Chen, X., Tamanian, K.G. 1753. Asparagaceae Linnaeus, ed. Flora of China, pp. 208

Choi, Y.E., Ahn, C.H., Kim, B.B., Yoon, E.S. 2008. Development of species specific AFLP-derived SCAR marker for authentication of *Panax japonicus* C.A. Meyer. Biological and Pharmaceutical Bulletin 31: 135-138.

Clifford, H.T., Conran, J.G. 1987. Asparagaceae. In: George, A.S. ed. Flora of Australia, pp. 159-164. Australian: Government Publishing Service, Canberra, Australian.

Cook, N.C., Samman, S. 1996. Flavonoids-chemistry, metabolism cardioprotective effects and dietary sources. Nutrition Biochemistry 7: 66-76.

Cui, X.M., Lo, C.K., Yip, K.L., Dong, T.T., Tsim, K.W. 2003. Authentication of *Panax notoginseng* by 5S-rRNA spacer domain and random amplified polymorphic DNA (RAPD) analysis. Planta Medica 69: 584-586.

Dasgupta, N., De, B. 2004. Antioxidant activity of *Piper betle* L. leaf extract *in vitro*. Food Chemistry 88: 219-224.

Deli, J., Matus, Z., Toth, G. 2000. Carotenoid composition in the fruits of *Asparagus officinalis*. Journal of Agricultural and Food Chemistry 48:2793-2796.

Ding, Y., Yang, C.R. 1990. Steroidal saponins from *Asparagus filicinus*. Yao Xue Xue Bao 25:509-514.

Dong, X.Y., Wang, Z.T., Zhou, K.Y., Xu, L.S., Xu, H., Wang, Y.Q. 2003. Allele-specific primers for diagnostic PCR authentication of *Dendrobium officinale*. Planta Medica 69: 587-588.

El-Sayed, S. A. 2009. Total phenolic contents and free radical scavenging activity of certain Egyptian *Ficus* species leaf samples. Food Chemistry 114: 1271-1277.

Fukuda, T., *et al*. 2005. Molecular phylogeny of the genus *Asparagus* (Asparagaceae) inferred from plastic *pet*B intron and *pet*D-*rpo*A intergenic spacer sequences. Plant Species Biology 20: 121-32.

Gautam, M., Diwanay, S., Gairola, S., Shinde, Y., Patki, P., Patwardhan, B. 2004. Immunoadjuvant potential of *Asparagus racemosus* aqueous extract in experimental system. Journal of Ethnopharmacology 91: 251-255.

Gautam, M., *et al*. 2009. Immunomodulatory activity of *Asparagus racemosus* on systemic Th1/Th2 immunity: Implications for immunoadjuvant potential. Journal of Ethnopharmacology 121:241-247.

Gopumadhavan, S., Venkataranganna, M.V., Rafiq, M. 2005. Evaluation of estrogenic effect of Menosan using the rat models of uterotrophic assay. Medicine Update 13: 37-41.

Gordon, M. H. 1990. The mechanism of antioxidant action *in vitro*, In: Hudson, B.J.F. ed. Food antioxidants, pp. 1-18. London: Elsevier Applied Science.

Grady, D., Gebretsadik, T., Kerlikowske, K., Ernster, V., Pettiti, D. 1995. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. Obstetrics and Gynecology 85: 304-313.

Guillén, R., *et al*. 2008. Antioxidants from asparagus spears: phenolics. Acta Horticulturae 776: 247-253.

Gupta, V.K., Sharma, S.K. 2006. Plants as natural antioxidants. Natural Product Radiance 5: 326-334.

Gyamfi, M.A., Yonamine, M., Aniya, Y. 1999. Free-radical scavenging action of medicinal herbs from Ghana *Thonningia sanguinea* on experimentally-induced liver injuries. General Pharmacology 32: 661-667.

Halliwell, B. 1997. Antioxidants and human diseases: A general introduction. Nutrition Reviews 55: 44-52.

Hayes, P.Y., Jahidin, A.H., Lehmann, R., Penman, K., Kitching, W., De Voss, J.J. 2006. Structural revision of shatavarins I and IV, the major components from the roots of *Asparagus racemosus*. Tetrahedron Letters 47: 6965-6969.

Hayes, P.Y., Jahidin, A.H., Lehmann, R., Penman, K., Kitching, W., De Voss, J.J. 2008. Steroidal saponins from the roots of *Asparagus racemosus*. Phytochemistry 69: 796-804.

Hiroshi, M., Hiromichi, N., Makoto, C., Mitsuharu, T. 1998. Antioxidant activity of capsanthin and the fatty acid esters in Paprika (*Capsicum annuum*). Journal of Agricultural and Food Chemistry 46: 3468-3472.

Hinneburg, I., Damien Dorman, H.J., Hiltunen, R. 2006. Antioxidant activities of extracts from selected culinary herbs and species. Food Chemistry 97: 122-129.

Huang, K.C. 1993. In The Pharmacology of Chinese Herbs, p. 361. CRC Press, Boca Raton, FL, USA.

Huang, X.F., Lin, Y.Y., Kong, L.Y. 2008. Steroids from the roots of *Asparagus officinalis* and their cytotoxic activity. Journal of Integrative Plant Biology 50:717-722.

Hutchinson, J., Dalziel, J.M. 1968. Flora of West Tropical Africa Part 1 (2nd ed.), vol. 3, p. 93. Crown Agents for Overseas Governments and Administration, London.

Jia-Jun, W., *et al*. 2010. Steroidal saponins and ecdysterone from *Asparagus filicinus* and their cytotoxic activities. Steroids 75: 734-739.

Joshi, J., Dev, S. 1988. Chemistry of ayurvedic crude drugs: Part VIII: Shatavari: 2. Structure elucidation of bioactive shatavarin-I and other glycosides. Indian Journal of Chemistry B 27: 12-16.

Ke, H., Xinsheng, Y. 2003. The cytotoxicity of methyl protodioscin against human cancer cell line *in vitro*. Cancer Investigation 21: 389-393.

Khaliq-Uz-Zaman, S. M., Shameel, S., Ahmad, V. U. 1998. Saponins from the genus *Asparagus*. Journal of the Chemical Society of Pakistan 20: 299-306.

Koo, H. N., *et al*. 2000. Inhibition of tumor necrosis factor α-induced apoptosis by *Asparagus cochinchinensis* in Hep G2 cells. Journal of Ethnopharmacology 73: 137-143.

Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A., Janzen, D.H. 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences of the United States of America 102: 8369-8374.

Kubota, S., Konno, I., Kanno, A. 2012. Molecular phylogeny of the genus *Asparagus* (Asparagaceae) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* species. Journal of Applied Genetics 124: 345-354.

Lee, D.Y., *et al*. 2009. Anti-inflammatory effects of *Asparagus cochinchinensis* extract in acute and chronic cutaneous inflammation. Journal of Ethnopharmacology 121: 28-34.

Lee, Y.O., Kanno, A., Kameya, T. 1997. Phylogenetic relationships in the genus *Asparagus* based on the restriction enzyme analysis of the chloroplast DNA. Japanese Journal of Breeding 47: 375-378.

Li, Y.F., *et al*. 2003. Identification of bulb from *Fritillaria cirrhosa* by PCR with specific primers. Planta Medica 69: 184-186.

Liang, Z.Z., Aquino, R., De Simone, F., Dini, A., Schettino, O., Pizza, C. 1988. Oligofurostanosides from *Asparagus cochinchinensis*. Planta Medica 54:344-346.

Liu, H., Yan, G., Finnegan, P.M., Sedgley, R. 2007. Development of DNA markers for hybrid identification in *Leucadendron* (Proteaceae). Scientia Horticulturae 113: 376-382.

Liu, W., *et al*. 2009. Asparanin A induces G_2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma Hep G2 cells. Biochemical and Biophysical Research Communications 381: 700-705.

MacDonald-Wicks, L. K., Wood, L. G., Garg, M. L. 2006. Methodology for the determination of biological antioxidant capacity *in vitro*: A review. Journal of the Science of Food and Agriculture 86: 2046-2056.

Makris, D.P., Rossiter, J.T. 2001. Domestic processing of onion bulbs (*Allium cepa*) and asparagus spears (*Asparagus officinalis*): effect on flavonol content and antioxidant status. Journal of Agricultural and Food Chemistry 49: 3216-3222.

Mandal, D., Banerjee, S., Mandal, N.B., Chakravarty, A.K., Sahu NP. 2006. Steroidal saponins from the fruits of *Asparagus racemosus*. Phytochemistry 67: 1316-1321.

Mandal, S.C., *et al*. 2000. Antitussive effect of *Asparagus racemosus* root against sulfur dioxide-induced cough in mice. Fitoterapia 71: 686-689.

Mayo, J.L. 1998. Black cohosh and chastberry: harbs valued by woman for centuries. Clinical Nutrition Insight 6: 1-4.

Miguel, M.G. 2010. Antioxidant activity of medicinal and aromatic plants. A review. Flavour and Fragrance Journal 25: 291-293.

Moon, J. K., Shibamoto, T. Y. 2009. Antioxidant assays for plant and food components. Journal of Agriculture and Food Chemistry 57: 1655-1666.

Mortada, M. E. 1998. Molluscidal steroidal saponins from *Agave ferox*. Zagazig Journal of Pharmaceutical ciences 7: 73-79.

Muruganandan, S., Garg, H., Lal, J., Suresh, C., Dinesh, K. 2001. Studies on the immunostimulant and antihepatotoxic activities of *Asparagus racemosus* root extract. Journal of Medicinal and Aromatic Plant Sciences 22: 49-52.

Negi, J.S., Singh, P., Joshi, G.P., Rawat, M.S., Bisht, V.K. 2010. Chemical constituents of *Asparagus*. Pharmacognosy Reviews 4: 215-220.

Ngamrojanavanich, N., *et al*. 2007. Cytotoxic constituents from *Butea superba* Roxb. Journal of Ethnopharmacology 109: 354-358.

Nieri, P., Adinolfi, B., Morelli, I., Breschi, M.C., Simoni, G., Martinotti, E. 2003. Genetic characterization of the three medicinal *Echinacea* species using RAPD analysis. Planta Medica 69: 685-686.

Nwafor, P.A., Okwuasaba, F.K. 2003. Anti-nociceptive and anti-inflammatory effects of methanolic extract of *Asparagus pubescens* root in rodents. Journal of Ethnopharmacology 84: 125-129.

Nwafor, P.A., Okwuasaba, F.K., Onoruvwe, O.O. 1998. Contraceptive and non-estrogenic effects of methanolic extract of *Asparagus pubescens* root in experimental animals. Journal of Ethnopharmacology 62: 117-122.

Ohsako, T., Ohnishi, O. 2000. Intra- and interspecific phylogeny of wild *Fagopyrum* (Polygonaceae) species based on nucleotide sequences of noncoding regions in chloroplast DNA. American Journal of Botany 87: 573-582.

Oonsivilai, R., Ferruzzi, M.G., Ningsanond, S. 2008. Antioxidant activity and cytotoxicity of Rang Chuet (*Thunbergia laurifolia* Lindl.) extracts. Asian Journal of Food and Agro-Industry 1: 116-128.

Pant, G., Panwar, M.S., Negi, D.S., Rawat, M.S., Morris, G. A. 1988. Spirostanol glycoside from fruits of *Asparagus officinalis*. Phytochemistry 27: 3324-3325.

Pant, G., Panwar, M.S., Negi, D.S., Zagorski, M. 1988. Steroidal glycosides of the roots of *Asparagus plumosus* baker. Herba Polonica 34: 175-181.

Park, M.J., Kim, M.K., In, J.G., Yang, D.C. 2006. Molecular identification of Korean ginseng by amplification refractory mutation system-PCR. Food Research International 39: 568-574.

Pongboonrod, S. 1950. Mai Tet Muang Thai, Kasembunnakich Press, Bangkok.

Potduang, B., Meeploy, M., Giwanon, R., Benmart, Y., Kaewduang, M., Supatanakul, W. 2008. Biological activities of *Asparagus racemosus*. African Journal of Traditional, Complementary and Alternative Medicine 5: 230-237.

Rao, A. R. 1981. Inhibitory action of *Asparagus racemosus* on DMA-induced mammary carcinogenesis in rats. International Journal of Cancer 28: 607-610.

Rastogi, R.P., Mehrotra, B.N. 1969. Compendium of Indian Medicinal Plants.Vol. 1. Central Drug Research Institute, Lucknow and Publications & Information Directorate, New Delhi p. 49-50.

Rastogi, R.P., Mehrotra, B.N. 1979. Compendium of Indian Medicinal Plants. Vol. 2. Central Drug Research Institute, Lucknow and Publications & Information Directorate, New Delhi. p. 80-81.

Ravikumar, P.R., Soman, R., Chetty, G.L., Pandey, R.C., Dev, S. 1987. Chemistry of ayurvedic crude drugs: Part VI: Shatavari-1: Structure of shatavarin-IV. Indian Journal of Chemistry B 26: 1012-1017.

Riccardi, P., Casali, P.E., Mercati, F., Falavigna, A., Sunseri, F. 2011. Genetic characterization of asparagus doubled haploids collection and wild relatives. Scientia Horticulturae 130: 691-700.

Rodriguez, R., *et al*. 2005. Antioxidant activity of ethanolic extracts from several *Asparagus* cultivars. Journal of Agricultural and Food Chemistry 53: 5212-5217.

Sairam, K., Priyambada, S., Aryya, N.C., Goel, R.K. 2003. Gastroduodenal ulcer protective activity of *Asparagus racemosus*: an experimental, biochemical and histological study. Journal of Ethnopharmacology 86: 1-10.

Sanchez-Gonzalez, I., Jimenez-Escrig, A., Saura-Calixto, F. 2005. *In vitro* antioxidant activity of coffees brewed using different procedures (Italian, espresso and filter). Food Chemistry 90: 133-139.

Sasaki, Y., Fushimi, H., Komatsu, K., 2004. Application of single-nucleotide polymorphism analysis of the *trn*K gene to the identification of *Curcuma* plants. Biological and Pharmaceutical Bulletin 27: 144-146.

Sati, O. P., Pant, G. 1985. New furostanol glycosides from *Asparagus plumosus* Leaves. Journal of Natural Products 48: 390-394.

Sati, O. P., Pant, G., Hostettmann, K. 1984. Potent molluscicides from *Asparagus plumosus*. Pharmazie 39: 581.

Sati, O. P., Pant, G. 1985. Spirostanol glycosides from *Asparagus plumosus*. Phytochemistry 24: 123-126.

Saxena, V.K., Chourasia, S., 2001. A new isoflavone from the roots of *Asparagus racemosus*. Fitoterapia 72: 307-309.

Sautour, M., Miyamoto, T., Lacaille-Dubois, M. A. 2007. Steroidal saponins from *Asparagus acutifolius*. Phytochemistry 68: 2554-2562.

Scalbert, A., Manach, C., Morand, C., Remesy, C. 2005. Dietary polyphenols and the prevention of disease. Critical Reviews in Food and Nutrition 45: 287-306.

Sekine, T., Fukasawa, N., Kashiwagi, Y., Ruangrungsi, N., Murakoshi, I. 1994. Structure of asparagamine A: A novel polycyclic alkaloid from *Asparagus racemosus*. Chemical Pharmaceutical Bulletin 42: 1360-1362.

Sekine, T., Fukasawa, N., Murakoshi, I., Ruangrungsi, N. 1997. A 9,10-dihydrophenanthrene from *Asparagus racemosus*. Phytochemistry 44: 763-764.

Sharma, S.C., Chand, R., Sati, O.P. 1982a. Steroidal Saponins of *Asparagus adscendens*. Phytochemistry 21: 2075-2078.

Sharma, S.C., Chand, R., Bhatti, B.S., Sati, O.P. 1982b. New oligospirostanosides and oligofurostanosides from *Asparagus adscendens* roots. Planta Medica 46: 48-51.

Sharma, S.C., Sati, O.P., Chand. R. 1982c. Steroidal constituents of different parts of *Asparagus curillus* Buch. Ham Current Science 51: 280-281.

Sharma, S.C., Sati, O.P., Chand, R. 1982d. Steroidal saponins of *asparagus curillus.* Phytochemistry 21: 1711-1714.

Sharma, S.C., Sati, O.P., Chand, R. 1983. Steroidal saponins from *Asparagus curillus* fruits. Planta Medica 47: 117-120.

Sharma, S.C., Sharma, H.C. 1984. Oligofuro and spirostanosides of *Asparagus adscendens*. Phytochemistry 23: 645-648.

Sharma, S.C., Sharma, H.C. 1993. Oligofurostanosides from *Asparagus curillus* leaves. Phytochemistry 33: 683-686.

Sharma, S.C., Shrikant, M. 2000. Effect of shatavari on diabetic retionopathy. Proceedings of International Congress on Ayurveda-2000. Chennai, India, p. 85-86.

Sharma, S. C., Thakur, N. K. 1994. Furostanosides from *Asparagus filicinus* roots. Phytochemistry 36: 469-471.

Sharma, S. C., Thakur, N. K. 1996. Oligofuranosides and oligospiranosides from roots of *Asparagus filicinus*. Phytochemistry 42: 599-603.

Sharma, P. C., Yelne, M. B., Dennis, T.J. 2000. Database on medicinal plants used in Ayurveda, pp. 418-430. Delhi: Documentation & publication Division, Central Council for Research in Ayurveda & Siddha.

Shasnay, A. K., Darokar, M. P., Sakia, D., Rajkumar, S., Sundaresan, V., Khanuja, S. P. S. 2003. Genetic diversity and species relationship in *Asparagus* spp. using RAPD analysis. Journal of Medicinal and Aromatic Plant Sciences 25: 698-704.

Shimoyamada, K., Suzuki, M., Maruyama, M., Watanabe, K. 1990. An antifungal saponin from White asparagus (*Asparagus officinalis* L.) Bottoms. Journal of the Science of Food and Agriculture 72: 430-434.

Singleton, V. L., Orthofer, R., Lamuela-Raventós, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology 299: 152-178.

Smitinand, T. 2001. Thai Plant Names (botanical names-vernacular names). Revised edition. Bangkok: The Forest Herbarium, Royal Forest Department.

Songsiang, U., Hahnvajanawong, C., Yenjai, C. 2011. Cytotoxicity of chemical constituents from the stem of *Dalbergia parviflora*. Fitoterapia 82: 1169-1174.

Stajner, N., Bohanec, B., Javornik, B. 2002. Genetic variability of economically important *Asparagus* species as revealed by genome size analysis and rDNA ITS polymorphisms. Plant Sciences 162: 931-937.

Sui, X., Huang, Y., Tan, Y., Guo, Y., Long, C. 2011. Molecular authentication of the ethnomedicinal plant *Sabia parviflora* and its adulterants by DNA barcoding technique. Planta Medica 77: 492-496.

Sugita, M., Shinozaki, K., Sugiura, M. 1985. Tobacco chloroplast $tRNA^{LYS}$(UUU) gene contains a 2.5-kilobase-pair intron: an open reading frame and a conserved boundary sequence in the intron. Proceedings of the National Academy of Sciences of the United States of America 82: 3557-3561.

Sun, T., Ho, C. T. 2005. Antioxidant activities of buckwheat extracts. Food Chemistry 90: 743-749.

Sun, Y., Fung, K. P., Leung, P. C., Shi, D., Shaw, P. C. 2004. Characterization of medicinal *Epimedium* species by 5S-rRNA gene spacer sequencing. Planta Medica 70: 287-288.

Takashi, M., Yukiko, G., Kaori, I., Yasushiro, F., Keiji, H., Kooichi, M. 2001. Antioxidative activity of capsorubin and related compounds from Paprika (*Capsicum annuum*). Journal of Oleo Science 50: 663-665.

Tandon, M., Shukla, Y. N., Thakur, R. S. 1990. Constituents of *Asparagus adscendens*. Fitoterapia 61: 473.

Tandon, M., Shukla, Y. N., Thakur, R. S. 1990. Steroid glycosides from *Asparagus adscendens*. Phytochemistry 29: 2957-2959.

Tang, X. H., Gao, J. 2001. Inhibitory effects of juice from *Asparagus officinalis* L. on cyclophosphamide (CTX)-induced mutagenic activities in mice. Journal of Nanjing University 37: 569-573.

Tetsuro, K., Ming-Yu, C., Tatsuya, H., Fumihide, T., Tonihisa, O. 2011. Anti-inflammatory and anti-melanogenic steroidal saponin glycosides from fonugreek (*Trigonella foenum-graecum* L.) seeds. Planta Medica 77: 705-710.

Velioglu, Y. S., Mazza, G., Gao, L., Oomah, B. D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. Journal of Agricultural and Food Chemistry 46: 4113-4117.

Vijay, N., Sairkar, P., Silawat, N., Garg, R. K., Mehrotra, N. N. 2009. Genetic variability in *Asparagus racemosus* (Willd.) from Madhya Pradesh, India by random amplified polymorphic DNA. African Journal of Biotechnology 8: 3135-3140.

Vongsak, B., Kengtong, S., Vajrodaya, S., Sukrong, S. 2008. Sequencing analysis of the medicinal plant *Stemona tuberosa* and five related species existing in Thailand based on *trn*H-*psb*A chloroplast DNA. Planta Medica 74: 1764-1766.

Wang, C.Z., Li, P., Ding, J.Y., Peng, X., Yuan, C.S. 2007. Simultaneous identification of Bulbus *Fritillariae cirrhosae* using PCR-RFLP analysis. Phytomedicine 14: 628-632.

Weising, K., Nybom, H., Wolff, K., Kahl, G. 2005. DNA fingerprinting in plants: principles, methods, and application 2 nd ed. Florida: CRC Press.

Wiboonpun, N., Phuwapraisirisan, P., Tip-pyang, S. 2004. Identification of antioxidant compound from *Asparagus racemosus*. Phytotheraphy Research 18: 771-773.

Wolf, H.T., Zundorf, I., Winckler, T., Bauer, R., Dingermann, T. 1999. Characterization of *Echinnacea* species and detection of possible adulterations by RAPD analysis. Planta Medica 65: 773-774.

Wong, C. C., Li, H., Cheng, K., Chen, F. 2006. A systemic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chemistry 97: 705-711.

Wu, J. H., Tung, Y. T., Chyu, C. F., Chien, S. C., Wang, S. Y., Chang, S. T. 2008. Antioxidant activity and constituents of extracts from the root of *Garcinia multifora*. Journal of Wood Science 54: 383-389.

Xu, B. J., Chang, S. K. C. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. Journal of Food Science 72, 159-166.

Xu, D. H., Abe, J., Kanazawa, A., Gai, J. Y., Shimamoto, Y. 2001. Identification of sequence variations by PCR-RFLP and its application to the evaluation of cpDNA diversity in wild and cultivated soybeans. Theoritical and Applied Genetics 102: 683-688.

Yang, C. X., Huang, S. S., Yang, X. P., Jia, Z. J. 2004. Norlignans and steroid saponins from *Asparagus gobicus*. Planta Medica 70: 446-451.

Yang, D.Y., Fushimi, H., Cai, S.Q., Komatsu, K. 2004. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS) analyses of medicinally used *Rheum* species and their application for identification of Rhei Rhizoma. Biological and Pharmaceutical Bulletin 27: 661-669.

Yang, M., Zhang, D., Liu, J., Zheng, J. 2001. A molecular marker that is specific to medicinal Rhubarb based on chloroplast *trn*L/*trn*F sequences. Planta Medica 67: 784-786.

Yu, B. P. 1994. Cellular defenses against damage from reactive oxygen species. Physiological Reviews 74: 139-162.

Zhang, H. J., *et al*. 2004. Bioactive constituents from *Asparagus cochinchinensis*. Journal of Natural Products 67: 194-200.

Zhang, Y. B., Wang, J., Wang, Z. T., But, P. P. H., Shaw, P.C. 2003. DNA microarray for identification of the herb of *Dendrobium* species from Chinese medicinal formulations. Planta Medica 69: 1172-1174.

Zhao, K. J., Dong, T. T. X., Cui, X. M. 2003. Genetic distinction of Radix Adenophorae from its adulterants by the DNA sequence of 5S-rRNA spacer domains. The American Journal of Chinese Medicine 31: 919-926.

Zhong-Hua-Ben-Cao editorial board. 1999. Shanghai, p. 7147. Shanghai Science and Technology Press.

Zhu, S., Fushimi, H., Cai, S. Q., Komatsu, K. 2004. Species identification from Ginseng drugs by multiplex amplification refractory mutation system (MARMS). Planta Medica 70: 189-192.

Zuo, Y., Chen, Z., Kondo, K., Funamoto, T., Wen, J., Zhou, S. 2011. DNA Barcoding of *Panax* Species. Planta Medica 77: 182-187.

APPENDICES

APPENDIX A

Asparagus species used in this study

Figure A1 *A. aethiopicus* L. (Synonym *A. sprengeri* Regel) a) fresh roots b) dry roots and c) crude drug

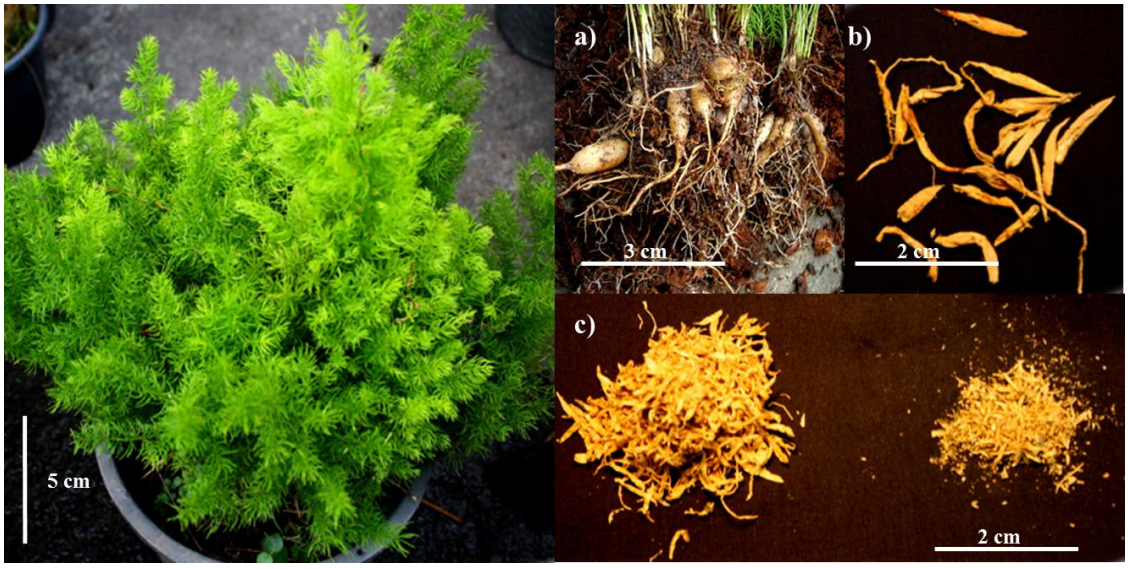


Figure A2 *A. densiflorus* (Kunth.) Jessop a) fresh roots b) dry roots and c) crude drug

Figure A3 *A. densiflorus* cv. Myers a) fresh roots b) dry roots and c) crude drug

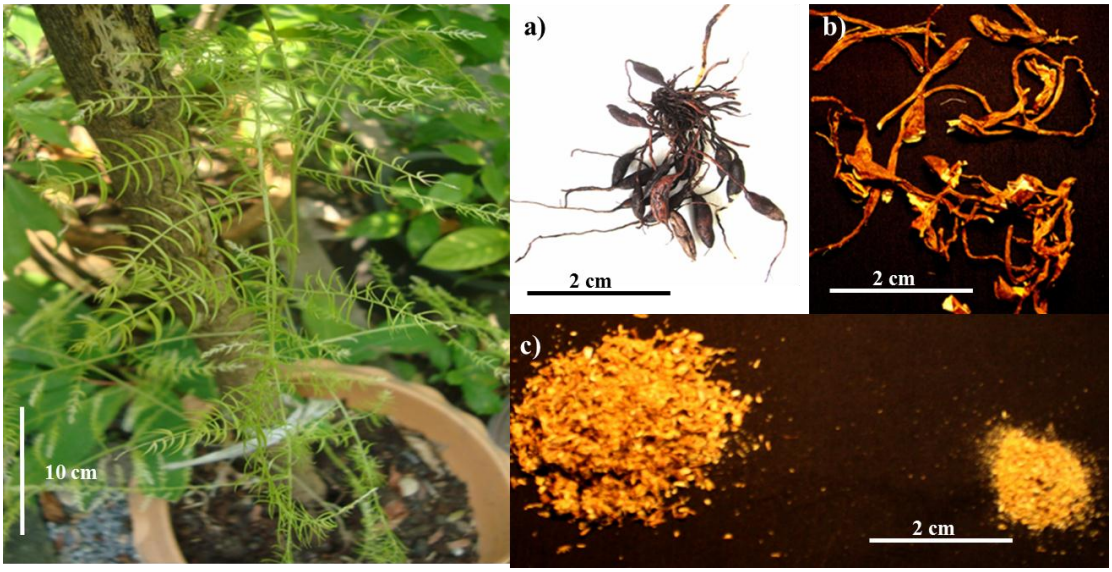


Figure A4 *A. filicinus* Buch.-Ham. a) fresh roots b) dry roots and c) crude drug

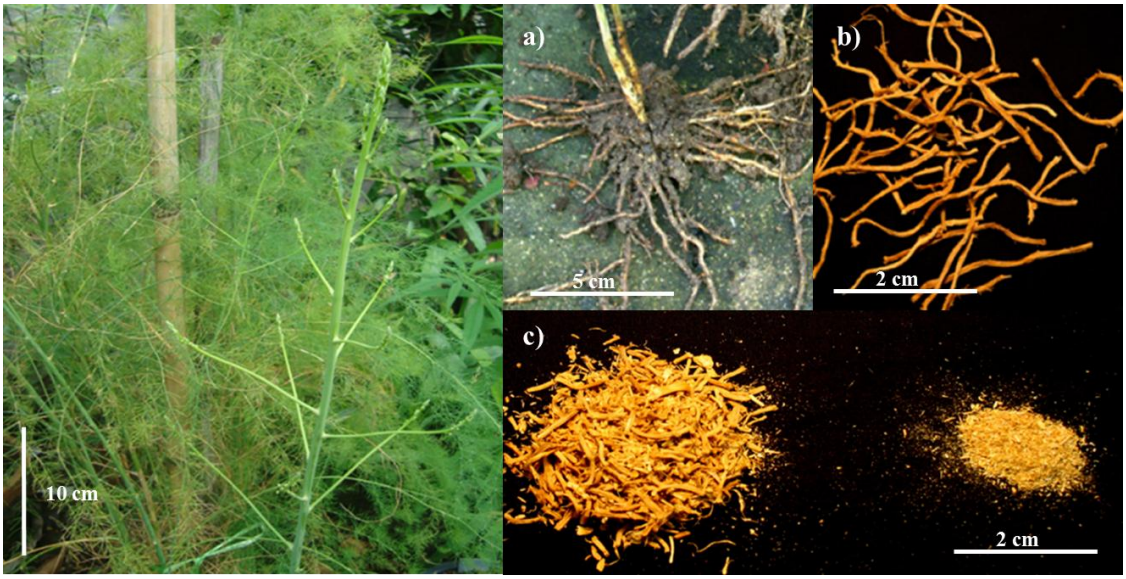


Figure A5 *A. officinalis* L. a) fresh roots b) dry roots and c) crude drug

Figure A6 *A. racemosus* Willd. a) fresh roots b) dry roots and c) crude drug

Figure A7 *A. setaceus* (Kunth.) Jessop a) fresh roots b) dry roots and c) crude drug

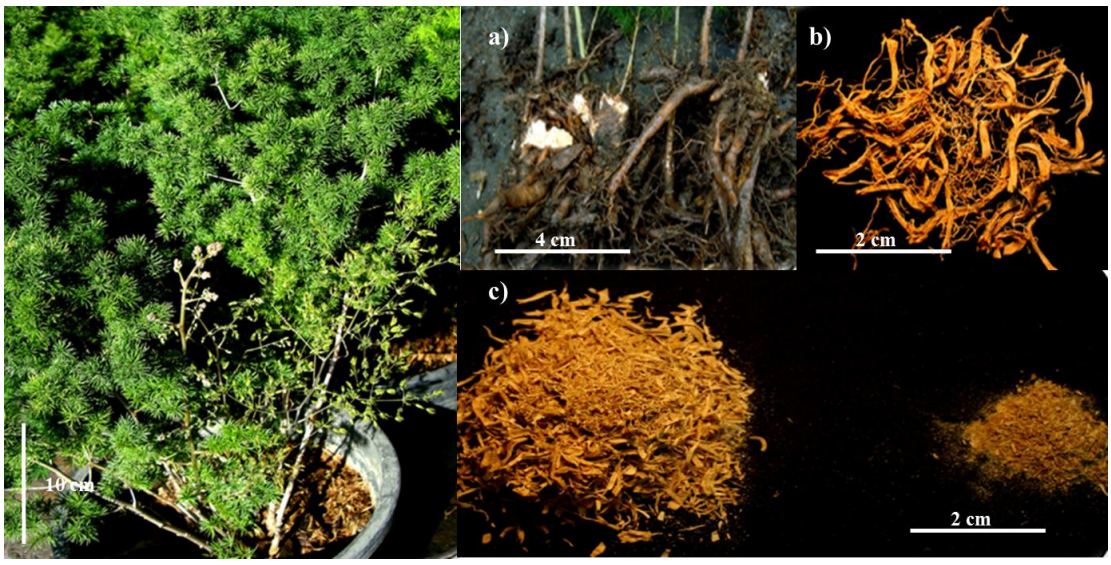
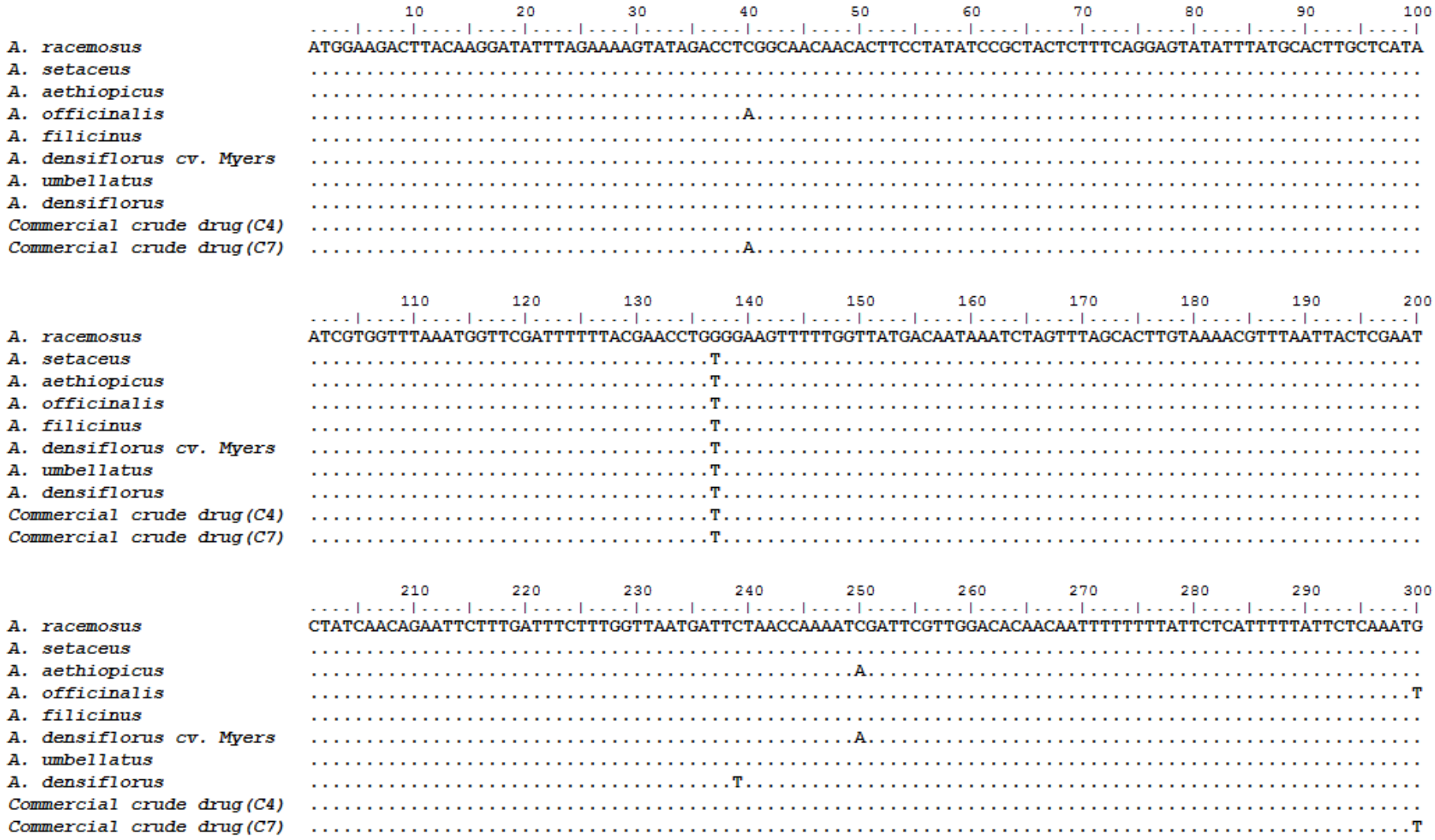
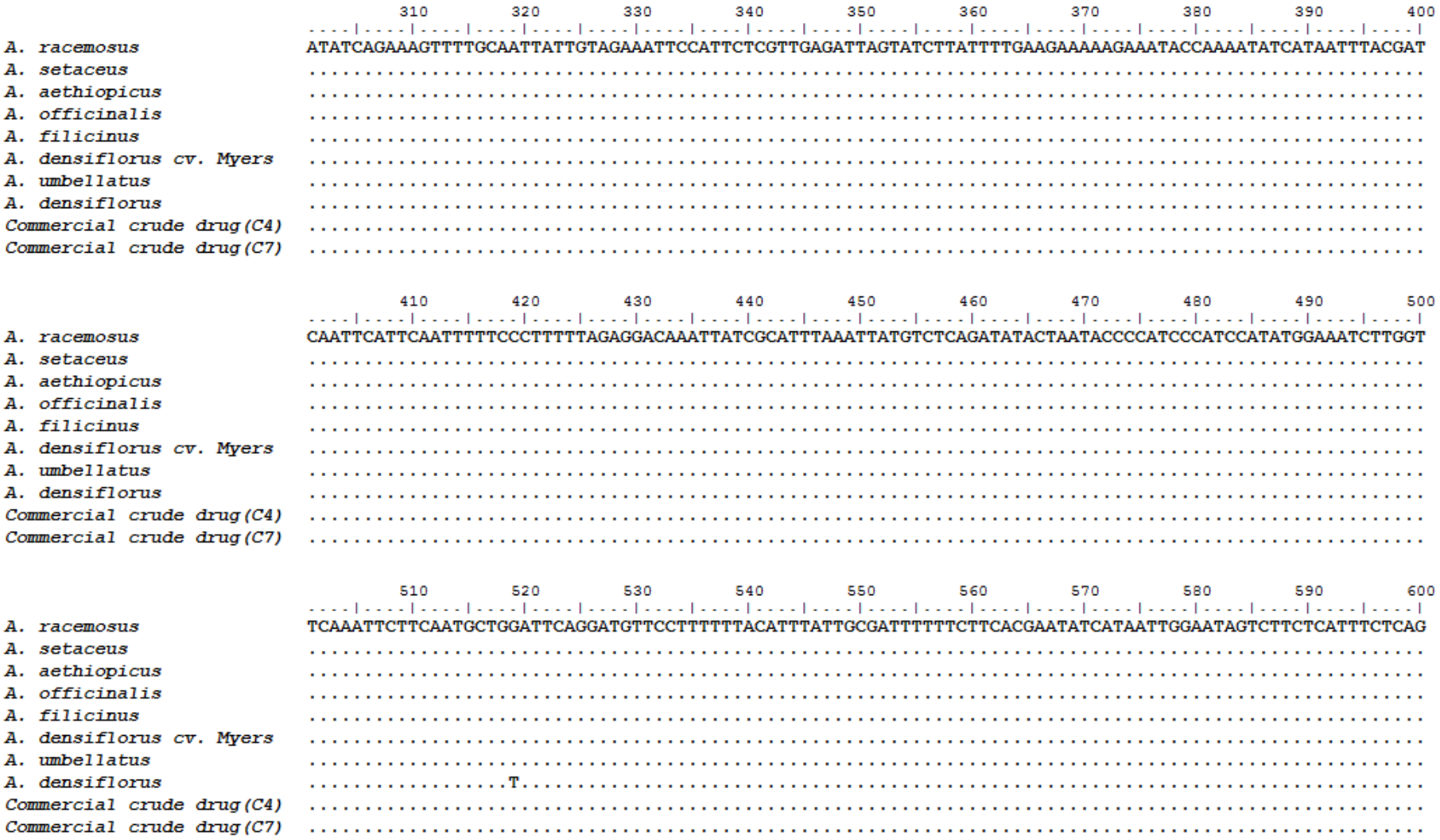


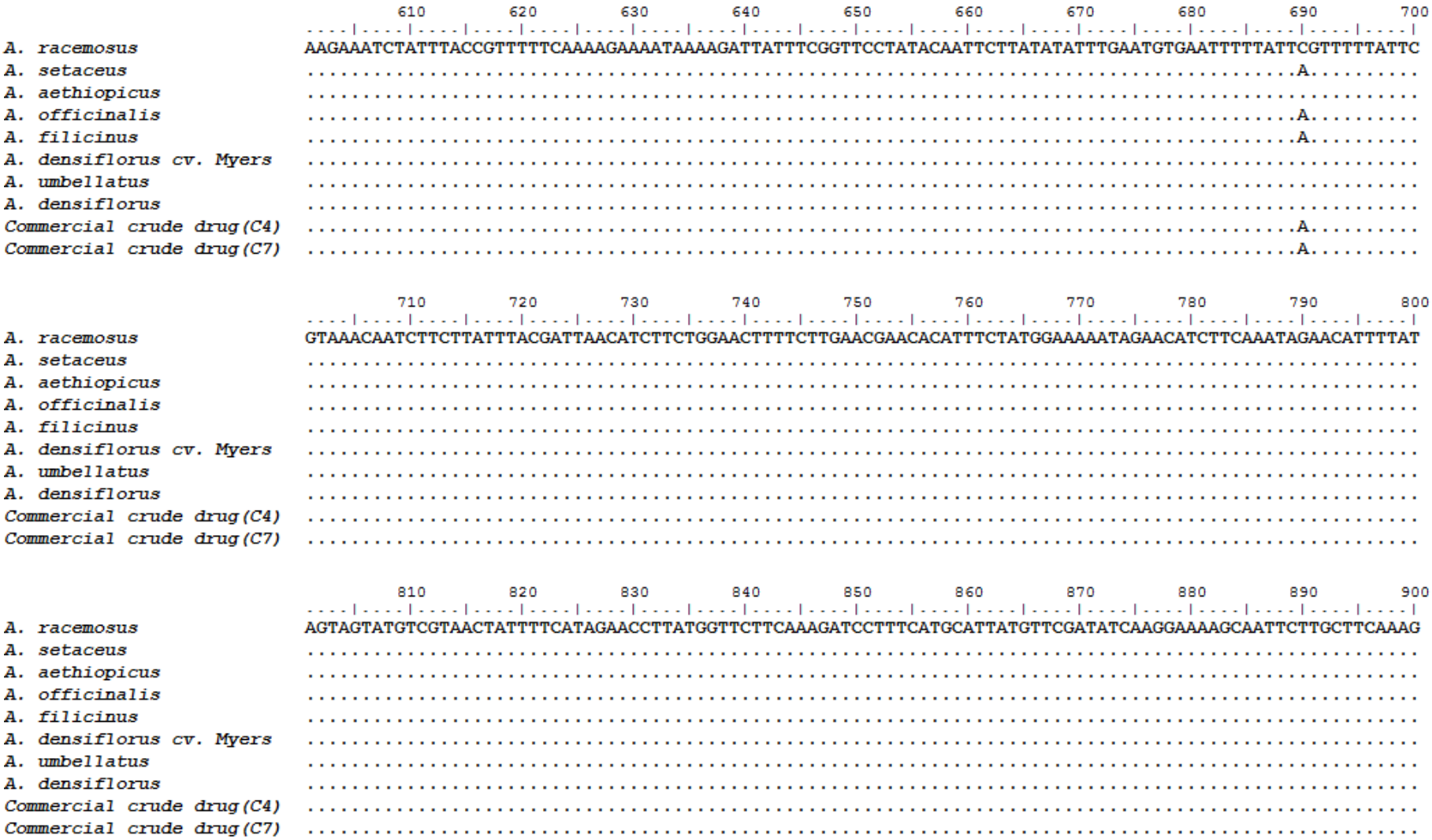
Figure A8 *A. umbellatus*. Royle a) fresh roots b) dry roots and c) crude drug

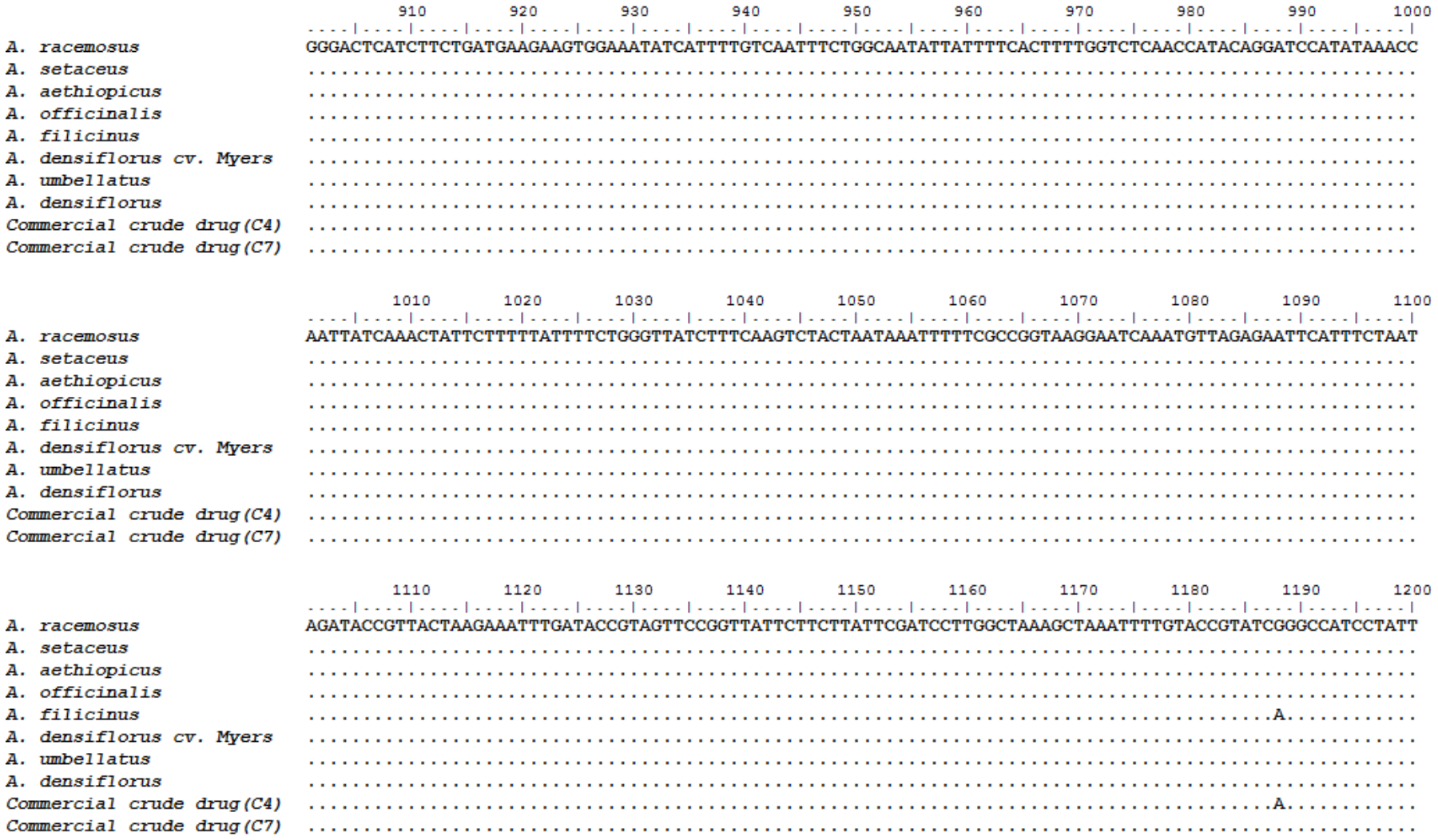
APPENDIX B

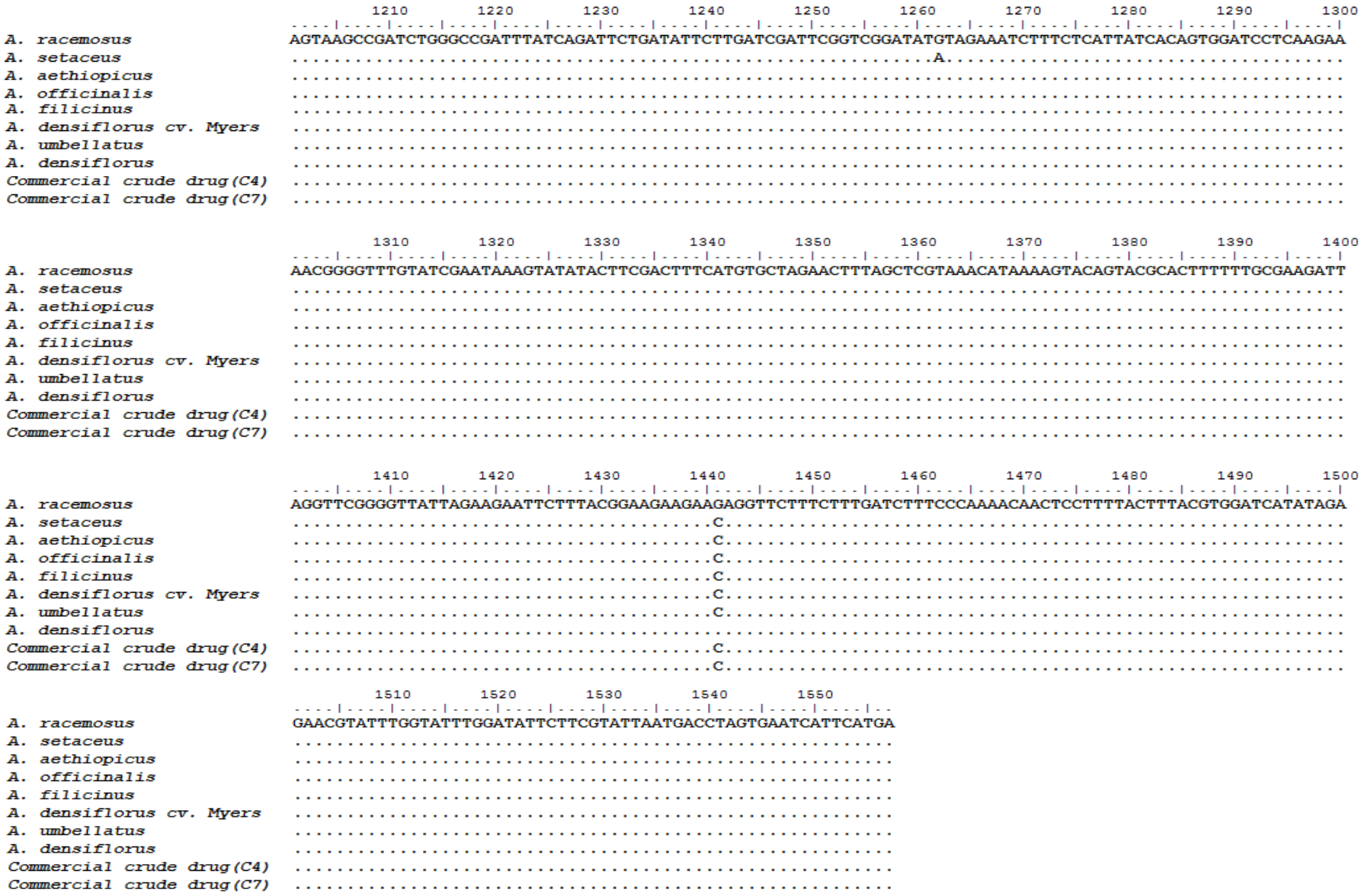
Figure B Comparison of the full *mat*K gene sequences among eight *Asparagus* species and commercial crude drugs (C4 and C7). Sequence alignment of complete *mat*K regions with the most variable sites generated by a pair of primers, **a** and **c**. Base substitutions are presented at the nucleotide positions 40, 137, 239, 250, 300, 519, 690, 1188, 1262, and 1441. Dot (.) indicates a nucleotide similar to *A. racemosus*.


10 20 30 40 50 60 70 80 90 100
A. racemosus ATGGAAGACTTACAAGGATATTTAGAAAAGTATAGACCTCGGCAACAACACTTCCTATATCCGCTACTCTTTCAGGAGTATATTTATGCACTTGCTCATA
A. setaceus
A. aethiopicus
A. officinalis .....A.....
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4)
Commercial crude drug(C7) .....A.....
110 120 130 140 150 160 170 180 190 200
A. racemosus ATCGTGGTTTAAATGGTTCGATTTTTTACGAACCTGGGGAAGTTTTTGGTTATGACAATAAATCTAGTTTAGCACTTGTAAAACGTTTAATTACTCGAAT
A. setaceus .....T.....
A. aethiopicus .....T.....
A. officinalis .....T.....
A. filicinus .....T.....
A. densiflorus cv. Myers .....T.....
A. umbellatus .....T.....
A. densiflorus .....T.....
Commercial crude drug(C4) .....T.....
Commercial crude drug(C7) .....T.....
210 220 230 240 250 260 270 280 290 300
A. racemosus CTATCAACAGAATTCTTTGATTTCTTTGGTTAATGATTCTAACCAAAATCGATTCGTTGGACACAACAATTTTTTTTATTCTCATTTTTATTCTCAAATG
A. setaceus
A. aethiopicus .....A.....
A. officinalis .....T
A. filicinus
A. densiflorus cv. Myers .....A.....
A. umbellatus
A. densiflorus .....T.....
Commercial crude drug(C4)
Commercial crude drug(C7) .....T


310 320 330 340 350 360 370 380 390 400
A. racemosus ATATCAGAAAGTTTTGCAATTATTGTAGAAATTCCATTCTCGTTGAGATTAGTATCTTATTTTGAAGAAAAAGAAATACCAAAATATCATAATTTACGAT
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4)
Commercial crude drug(C7)
410 420 430 440 450 460 470 480 490 500
A. racemosus CAATTCATTCAATTTTTCCCTTTTTAGAGGACAAATTATCGCATTTAAATTATGTCTCAGATATACTAATACCCCATCCCATCCATATGGAAATCTTGGT
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4)
Commercial crude drug(C7)
510 520 530 540 550 560 570 580 590 600
A. racemosus TCAAATTCTTCAATGCTGGATTCAGGATGTTCCTTTTTTACATTTATTGCGATTTTTTCTTCACGAATATCATAATTGGAATAGTCTTCTCATTTCTCAG
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus .....T.....
Commercial crude drug(C4)
Commercial crude drug(C7)


610 620 630 640 650 660 670 680 690 700
A. racemosus AAGAAATCTATTTACCGTTTTTCAAAAGAAAATAAAAGATTATTTCGGTTCCTATACAATTCTTATATATTTGAATGTGAATTTTTATTCGTTTTTATTC
A. setaceus .....A.....
A. aethiopicus .....
A. officinalis .....A.....
A. filicinus .....A.....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....A.....
Commercial crude drug(C7) .....A.....
710 720 730 740 750 760 770 780 790 800
A. racemosus GTAAACAATCTTCTTATTTACGATTAACATCTTCTGGAACTTTTCTTGAACGAACACATTTCTATGGAAAAATAGAACATCTTCAAATAGAACATTTTAT
A. setaceus .....
A. aethiopicus .....
A. officinalis .....
A. filicinus .....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....
Commercial crude drug(C7) .....
810 820 830 840 850 860 870 880 890 900
A. racemosus AGTAGTATGTCGTAACTATTTTCATAGAACCTTATGGTTCTTCAAAGATCCTTTCATGCATTATGTTCGATATCAAGGAAAAGCAATTCTTGCTTCAAAG
A. setaceus .....
A. aethiopicus .....
A. officinalis .....
A. filicinus .....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....
Commercial crude drug(C7) .....


910 920 930 940 950 960 970 980 990 1000
A. racemosus GGGACTCATCTTCTGATGAAGAAGTGGAAATATCATTTTGTCAATTTCTGGCAATATTATTTTCACTTTTGGTCTCAACCATACAGGATCCATATAAACC
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4)
Commercial crude drug(C7)
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
A. racemosus AATTATCAAACTATTCTTTTTATTTTCTGGGTTATCTTTCAAGTCTACTAATAAATTTTTCGCCGGTAAGGAATCAAATGTTAGAGAATTCATTTCTAAT
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4)
Commercial crude drug(C7)
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
A. racemosus AGATACCGTTACTAAGAAATTTGATACCGTAGTTCCGGTTATTCTTCTTATTCGATCCTTGGCTAAAGCTAAATTTTGTACCGTATCGGGCCATCCTATT
A. setaceus
A. aethiopicus
A. officinalis
A. filicinus A
A. densiflorus cv. Myers
A. umbellatus
A. densiflorus
Commercial crude drug(C4) A
Commercial crude drug(C7)


1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
A. racemosus AGTAAGCCGATCTGGGCCGATTTATCAGATTCTGATATTCTTGATCGATTCGGTCGGATATGTAGAAATCTTTCTCATTATCACAGTGGATCCTCAAGAA
A. setaceus .....A.....
A. aethiopicus .....
A. officinalis .....
A. filicinus .....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....
Commercial crude drug(C7) .....
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
A. racemosus AACGGGGTTTGTATCGAATAAAGTATATACTTCGACTTTCATGTGCTAGAACTTTAGCTCGTAAACATAAAAGTACAGTACGCACTTTTTTGCGAAGATT
A. setaceus .....
A. aethiopicus .....
A. officinalis .....
A. filicinus .....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....
Commercial crude drug(C7) .....
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
A. racemosus AGGTTCGGGGTTATTAGAAGAATTCTTTACGGAAGAAGAAGAGGTTCTTTCTTTGATCTTTCCCAAAACAACTCCTTTTACTTTACGTGGATCATATAGA
A. setaceus .....C.....
A. aethiopicus .....C.....
A. officinalis .....C.....
A. filicinus .....C.....
A. densiflorus cv. Myers .....C.....
A. umbellatus .....C.....
A. densiflorus .....
Commercial crude drug(C4) .....C.....
Commercial crude drug(C7) .....C.....
1510 1520 1530 1540 1550
A. racemosus GAACGTATTTGGTATTTGGATATTCTTCGTATTAATGACCTAGTGAATCATTCATGA
A. setaceus .....
A. aethiopicus .....
A. officinalis .....
A. filicinus .....
A. densiflorus cv. Myers .....
A. umbellatus .....
A. densiflorus .....
Commercial crude drug(C4) .....
Commercial crude drug(C7) .....

VITA

Mr. Teerawat Boonsom was born on May 13, 1986 in Khon Kaen, Thailand. He received Bachelor's degree of Science in Pharmacy in 2010 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Poster presentation

Teerawat Boonsom, Thatree Phadungcharoen and Suchada Sukrong. Nucleotide analysis of *mat*K gene in the genus *Asparagus*. Proceedings of the 5th RSPG Researchers Club conference "Thai Resources: Reach out to the world", 3rd-5th November 2011, Nongrawiang Training Centre, Muang, Nakhon Ratchasima, Thailand. p. 86-87.

Publication

Boonsom, T., Waranuch, N., Ingkaninan, K., Denduangboripant, J., Sukrong, S. 2012. Molecular analysis of the genus *Asparagus* based on *mat*K sequences and its application to identify *A. racemosus*, a medicinally phytoestrogenic species. Fitoterapia 83: 947-953.