Degradation Profiling of Chemical Constituents in Andrographis Herb

Abdulaziz Wadeng

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2017

Degradation Profiling of Chemical Constituents in Andrographis Herb

Abdulaziz Wadeng

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2017

Thesis Title	Degradation Profiling of Chemical Constituents in Andrographis Herb
Author	Mr. Abdulaziz Wadeng
Major Program	Pharmaceutical Sciences

Major Advisor

……..
(Assoc. Prof. Dr. Anuchit Plubrukarn)

Co-Advisor

……..
(Asst. Prof. Dr. Chatchai Wattanapiromsakul)

Examining Committee :

……...Chairperson
(Assoc. Prof. Dr. Kornkanok Ingkaninan)

……...… .Committee
(Assoc. Prof. Dr. Anuchit Plubrukarn)

…….Committee
(Asst. Prof. Dr. Chatchai Wattanapiromsakul)

…….Committee
(Asst. Prof. Dr. Luelak Lomlim)

The graduate school, Prince of Songkla University, has approved this thesis as partial fulfillment of the Master of Pharmacy Degree in Pharmaceutical Sciences

……..
(Assoc. Prof. Dr. Teerapol Srichana)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……Signature

(Assoc. Prof. Dr. Anuchit Plubrukarn)

Major Advisor

……Signature

(Mr. Abdulaziz Wadeng)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……Signature

(Mr. Abdulaziz Wadeng)

Candidate

ชื่อวิทยานิพนธ์ ระเบียนการสลายตัวขององค์ประกอบทางเคมีในสมุนไพรฟ้าทะลายโจร

ผู้เขียน นายอับดุลอาซิ วาเด็ง

สาขาวิชา เภสัชศาสตร์

ปีการศึกษา 2559

บทคัดย่อ

ศึกษาระเบียนการสลายตัวของสมุนไพรฟ้าทะลายโจร ภายใต้สภาวะการเก็บรักษาจริง ในกรอบระยะเวลา 6 เดือน โดยใช้เทคนิค ^{1}H NMR-based metabolomics และวิเคราะห์ผลด้วยวิธีการวิเคราะห์ตัวแปรพหุคูณ โดยตรวจสอบสารสกัดชั้นคลอโรฟอร์มและชั้นเมทานอล-น้ำ ของสมุนไพรจาก 4 แหล่งผลิต พบว่าองค์ประกอบเมตาโบโลมในสารสกัดมีการเปลี่ยนแปลงที่สัมพันธ์กับระยะเวลาการเก็บรักษา อย่างไรก็ตาม อนุพันธ์ไดเทอร์ปีนแลคโตน ซึ่งเป็นองค์ประกอบหลักที่ออกฤทธิ์ในฟ้าทะลายโจร สามารถละลายได้เฉพาะในชั้นคลอโรฟอร์ม ทั้งนี้อนุพันธ์ไดเทอร์ปีนแลคโตนที่ใช้ในการติดตามและสามารถตรวจวัดได้ในการวิจัยนี้ ได้แก่ andrographolide, 14-deoxyandrographolide และ 14-deoxy-11,12-didehydroandrographolide โดยพบว่า 14-deoxy-11,12-didehydroandrographolide มีปริมาณเพิ่มขึ้นอย่างสม่ำเสมอ ซึ่งชี้ให้เห็นว่า 14-deoxy-11,12-didehydroandrographolide เป็นผลิตภัณฑ์สลายตัวที่เกิดขึ้น และสามารถใช้ในการพิจารณาอายุชั้นการเก็บรักษาของสมุนไพรได้ ผลการติดตามการสลายตัวโดยการวิเคราะห์ปริมาณแลคโตนรวม พบว่าปริมาณแลคโตนรวมลดลง โดยอัตราการสลายตัวเป็นปฏิกิริยาอันดับศูนย์ (k = -0.2545 - -0.9002 mo^{-1}) จากผลการวิจัยนี้ มีข้อเสนอแนะว่า ในกรณีของวัตถุดิบฟ้าทะลายโจรนั้น เพื่อให้สามารถเก็บสมุนไพรได้นานพอสมควร ควรคัดเลือกวัตถุดิบสมุนไพรที่มีปริมาณแลคโตนรวมสูงสุดเท่าที่เป็นไปได้ ส่วนในกรณีของผลิตภัณฑ์ฟ้าทะลายโจร ควรกำหนดปริมาณแลคโตนรวมที่ระบุในฉลากยาสูงกว่าข้อกำหนดต่ำสุดในวัตถุดิบ ซึ่งจะทำให้สามารถกำหนดวันหมดอายุที่สัมพันธ์กับปริมาณแลคโตนรวมที่คงเหลือตลอดช่วงการเก็บรักษา ทั้งนี้ จากผลการวิจัย ปริมาณแลคโตนรวมที่ระบุบนฉลากผลิตภัณฑ์ ควรอยู่ในช่วง 9 – 11% เพื่อให้สามารถเก็บรักษาสมุนไพรได้เป็นระยะเวลา 12 เดือน

Thesis Title	Degradation profiling of chemical constituents in Andrographis Herb
Author	Mr. Abdulaziz Wadeng
Major program	Pharmaceutical Sciences
Academic Year	2016

ABSTRACT

A degradation profile of the Andrographis Herb under standard storage conditions (6 months), using ^{1}H NMR-based metabolomics approach and multivariate analysis, was performed on the $CHCl_3$- and aq MeOH-extracts from four suppliers. The metabolomics profiles in both extracts changed according to the storage time. Focusing on the active diterpene lactones, however, only $CHCl_3$ can extract the main ingredients, among which; andrographolide, 14-deoxyandrographolide, and 14-deoxy-11,12-didehydroan-drographolide, were the indicative markers. Specifically, 14-deoxy-11,12-didehydro-andrographolide, was the only lactone that increased consistently throughout the storage period and was identified as the major decomposition product; therefore it could be proposed as an indicator for the storage age. A stability profile on the total lactone content was performed. Total lactone contents decreased consistently, revealing a zeroth order kinetics (k = -0.2545 - -0.9002 mo^{-1}). Based on the results, crop selection for the highest lactone contents would be the most important for longer storage time of the raw materials. As for the herbal products, the results suggested that raising the labeled amount to a higher content will allow the lactone contents to remain in an acceptable range throughout the storage period. This can be in a range of 9 – 11%; therefore the products can be stored as long as 12 month after the manufacturing dates.

ACKNOWLEDGMENTS

First I would like to express my sincere thanks to my thesis advisor, Assoc. Prof. Dr. Anuchit Plubrukarn for his invaluable help and constant encouragement throughout the course of this research. I am most grateful for his 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 him.

My sincere thanks are expressed to my thesis co-advisor, Asst. Prof. Dr. Chatchai Wattanapiromsakul, for his valuable advice.

I would like to express my thank to Mrs. Pranee Rattanasuwan, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, for her identification and support in the Andrographis Herb samples.

I would like to thank all staff of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, and none of this would have been possible without love and encouragement of my friends. I thank them all for their understanding and support me.

I would like to thank the Discipline of Excellence in Pharmacy, phase 2, Faculty of Pharmaceutical sciences, and the Graduate School Research Support Funding for Thesis, for the financial support.

Finally, I would like to express my grateful thank to my family, for all of their encouragement, understanding, suggestion, and support throughout my study period.

Abdulaziz Wadeng

CONTENTS

CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF SCHEME

CHAPTER 1

INTRODUCTION

Andrographis paniculata (Burm. f.) Wall. ex Nees ([ฟ้าทะลายโจร], "Fa-Ta-Lai Chon" [Thai]; 穿心蓮, "Chuan Xin Lian" [Mandarin]; कीरायत, "Kirayat" [Hindi]; কালমেঘ, "Kālmegh" [Bengali]; Hempedu Bumi, [Bahasa]), is an annual herbaceous plant belonging to the family Acanthaceae. The plant is found widely spread throughout East and Southeast Asia, and Indian subcontinent, and is well known as one of the most widely used medicinal plants in several Asian countries, including Thailand, for the treatment of common cold.

Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998) has described *A. paniculata* as followed

…*A. paniculata* is an annual herb, up to 1 m high, erect, stem acutely quadrangular. Leaves simple, opposite, lanceolate, acute, glabrous, entire-slightly undulate, 2 to 12 cm long and 1 to 3 com wide, upper once often bracteiform; petiole short. Inflorescence plant, in panicle, 10 to 30 cm long; terminal and axillary, bract small, pedicel short. Calyx 5-partite, small, linear. Corolla tube narrow about 6 mm long; limb not shorter than the tube, bilabiate; upper lip oblong, white with a yellowish top; lower lip broadly cuneate, trifid white with violet marking. Stamens 2, inserted in the throat and far exserted, anthers basally bearded. Superior ovary, 2-celled, style far exserted. Capsule erect, linear-oblong, 1 to 2 cm long and 2 to 5 mm wide, compressed, longitudinally furrowed on the broad faces, thinly glandular-hairy. Seed small, subquadrate...

Andrographis Herb (Herba Andrographitis), which is the term used as a common herbal drug name of *A. paniculata* throughout this thesis, is described as the aerial parts of *A. paniculata* (both fresh and dried) (Ministry of Public Health, 1998). The traditional use of the Andrographis Herb has been well documented throughout several Asian countries. It is an important and widely used medicinal plant particularly among East and Southeast Asian countries, as well as in Indian subcontinent. For examples, in Ayurvedic Medicine, Andrographis Herb can be found as an ingredient in as many as 26 Ayurvedic recipes (Sudhakaran et al., 2012), prescribed for the treatments of diabetes, dysentery, helminth infections, peptic ulcer, skin infections, and snake bite (Jarukamjorn et al., 2008). In Chinese traditional medicines, the herb is described for the treatment of fever, common cold, laryngitis, pharyngitis, and respiratory infections (Jarukamjorn et al., 2008). In Thailand, *A. paniculata* has been recognized and selected as one of the medicinal plants for the treatments of fever, common cold, non-infectious diarrhea, diabetes, and hypertension (National Drug Committee, 2016, Jarukamjorn et al., 2008). It has been listed in Thailand's National

Figure 1. *Andrographis paniculata* (Burm. f.) Wall. ex Nees (family Acanthaceae), whole plant (a), flower (b), and capsules (c)

Drug Lists (2016) as drug used in digestive and respiratory system, and is recommended for the relief of the symptoms of the common cold and non-infectious diarrhea (National Drug Committee, 2016). This effective use of the Andrographis Herb, particularly for the treatment of common cold, has led to the recognition of the herb by WHO to be used for symptomatic treatment of the upper respiratory infections (such as common cold, uncomplicated sinusitis, bronchitis, and pharyngotonsillitis), acute diarrhea, lower urinary tract infections (World Health Organization, 2004). The recommended regimens for common cold and non-infectious bronchitis are 500 mg – 2 g of powdered Andrographis Herb capsules four time daily for three days, and for infectious diarrhea are 1.5 – 3 g of powdered Andrographis Herb capsules four time daily for three days. The herb is considered fairly safe to be used as frequently as needed, with one major caution that it should not be used continuously for longer than two weeks.

Being clinically proven and acceptably effective as an alternative approach for the treatment of common cold and non-infectious diarrhea, and officially recommended both nationally and internationally, it is not surprising that the Andrographis Herb is among the most cultivated medicinal plants. In Thailand, the plants can be grown widely in almost every region throughout the country. And with such a short cultivating period of three months, it can be cultivated multiple times per year. The first ten production areas in Thailand are Nakhon Pathom, Ratchaburi, Prachin Buri, Khon Kaen, Chiang Rai, Phayao, Udon Thani, Surin, Chaiyaphum, Sa Kaeo, and Suphan Buri (Ministry of Agriculture and Cooperatives, 2014). The annual gross production of the Andrographis Herbs is approximately 25-27 tons per hectare (4.23 tons per rai) of fresh harvest, which leads to an annual income of about 16,250

Baht per hectare (2,600 Baht per rai) (Ministry of Agriculture and Cooperatives, 2014). As for the herbal products, the major dosage form of the Andrographis Herb that is available in market is Andrographis Capsules, containing either 250 or 500 mg of dried powdered Andrographis Herb. To date, up to seven brands of Andrographis Herb products can be found in drugstores and health care product shops.

Despite being used worldwide as a clinically proved herbal medicine with only few minor safety issues, and despite being one of the most widely marketed medicinal herbs, there is one major problem that has hampered the extensive development of the Andrographis Herb or any of its active constituents toward a modern medicine. The diterpene lactones, which are the major components and primary active ingredients in the herb, are relatively unstable, and have been reported to have as a short shelf-life as 6-12 months. Under the standard storage conditions (25 °C, 51% relative humidity) the Thai Herbal Pharmacopoeia recommended that the herb should be used within one year, and a frequent – up to every three months – quantitative determination for its active diterpene lactones is recommended throughout the storage period. An extensive review on the stability of the Andrographis Herb and its active diterpene lactones will be visited later in section 1.2 of this chapter.

1.1 Chemical constituents and biological activities of *A. paniculata*

A. paniculata is one of the most extensively investigated medicinal plants. To date, there have been more than 30 reports documenting as many as 90 chemical constituents isolated from the plant. Among these, the major chemical components fall into the class of labdane-type diterpene lactones, among which andrographolide (Table 1) is the major compound. Other classes of compounds that

have been isolated from *A. paniculata* include flavonoids, xanthones, iridoids, and quinic acids (Fujita et al., 1984; Rao et al., 2004; Dua et al., 2004; Xu et al., 2012; Hossain et al., 2014).

As for the diterpene lactones, up to 60 compounds have been isolated and reported from *A. paniculata*. Andrographolide, which is the major component, can be isolated in a range of 1 - 4% of dry herb (Cheung et al., 2001; Sharma et al., 2012). The Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998) describes the assay for the Andrographis Herb based either on the total lactone content, using a back titration method, or on the amount of andrographolide, using an HPLC-based assay**,** specifying that the total lactone contents in the Andrographis Herb must not be less than 6% of the dried herb weight, calculating on the basis of andrographolide, or the amount of andrographolide must not be less than 1% of the dried herb weight.

The diterpene lactones that have been isolated from *A. paniculata* are present either in a form of a glycoside or an aglycone, and there are some derivatives that have also been found in both species. Apart from andrographolide, three additional diterpene lactones, 14-deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide (Table 1), all of which are structurally related to andrographolide, have been isolated in high proportion from *A. paniculata* (7.17, 11.98, and 7.16 $mg \cdot g^{-1}$ of *A. paniculata* respectively) (Yang et al., 2012), and can also be considered major chemical constituents. All the diterpene lactones that have been reported from *A. paniculata* to date are summarized in Table 1. The atomic numbering system described throughout this thesis is referred that originally assigned to andrographolide (Fujita et al., 1984).

Table 1. Chemical constituents in *A. paniculata*

I. Diterpenoids

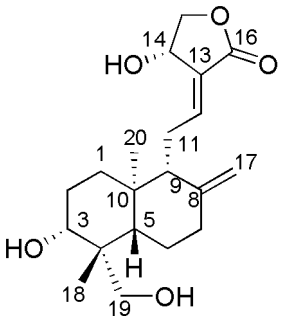
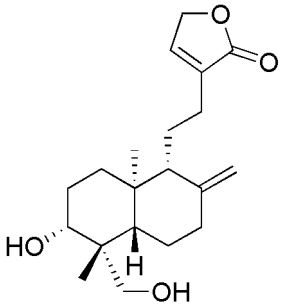
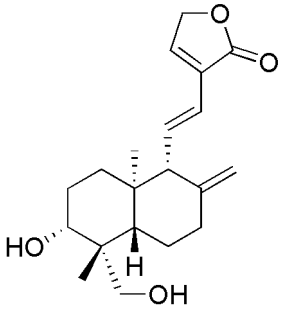
Name	Structures	References
andrographolide		Fujita et al., 1984; Matsuda et al., 1994; Rao et al., 2004; Chen et al., 2006; Shen et al., 2006; Chen et al., 2008; Kulyal et al., 2010; Zou et al., 2010
14-deoxyandrographolide		Fujita et al., 1984; Rao et al., 2004; Chen et al., 2006; Chen et al., 2008; Kulyal et al., 2010
14-deoxy 11,12-didehydroandrographolide		Fujita et al., 1984; Matsuda et al., 1994; Rao et al., 2004; Chen et al., 2006; Shen et al., 2006; Chen et al., 2008

Table 1. (cont.)

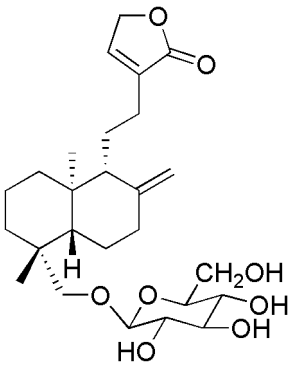
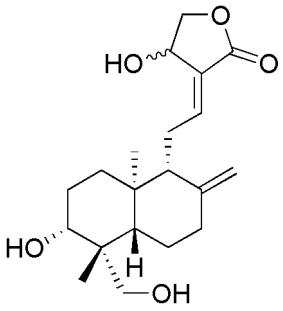
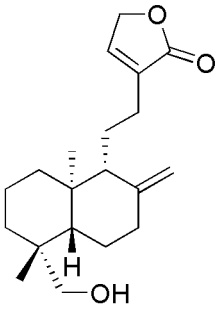
neoandrographolide		Fujita et al., 1984; Matsuda et al., 1994; Rao et al., 2004; Chen et al., 2006; Shen et al., 2006; Chen et al., 2008; Kulyal et al., 2010; Zou et al., 2010
isoandrographolide		Matsuda et al., 1994; Chen et al., 2006; Shen et al., 2006
andrograpanin		Fujita et al., 1984; Chen et al., 2006

Table 1. (cont.)

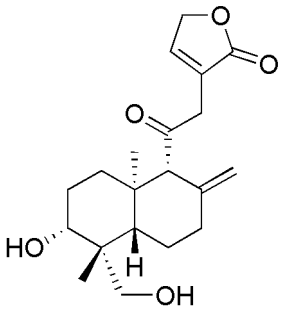
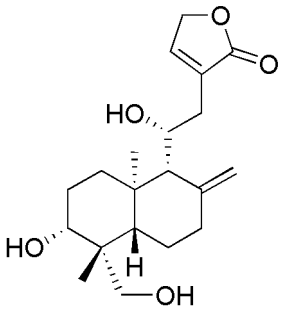
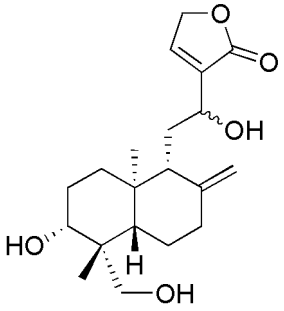
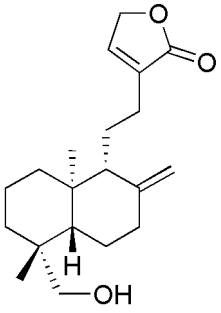
Name	Structures	References
14-deoxy-11-oxo-andrographolide		Fujita et al., 1984; Kulyal et al., 2010
14-deoxy-11-hydroxyandrographolide		Matsuda et al., 1994; Rao et al., 2004
14-deoxy-12-hydroxyandrographolide		Matsuda et al., 1994; Kulyal et al., 2010
3,14-dideoxyandrographolide		Chen et al., 2006; Chen et al., 2008; Kulyal et al., 2010

Table 1. (cont.)

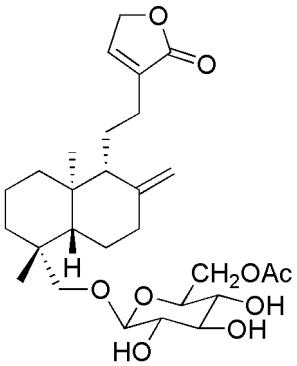
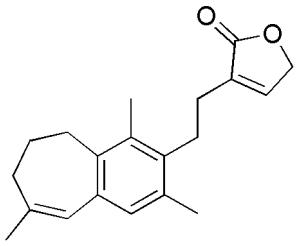
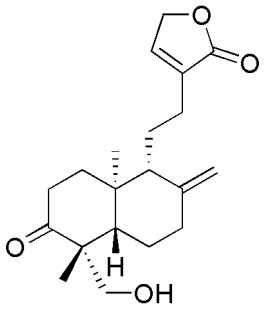
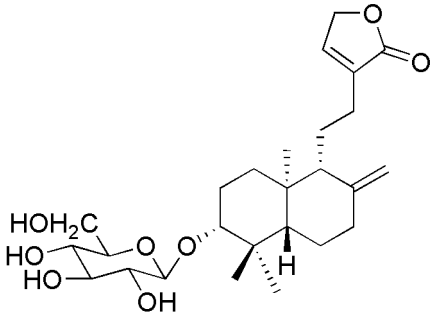
Name	Structures	References
6'-acetylneoandrographo-lide		Matsuda et al.,1994
andrographolactone		Wang et al., 2009
3-oxo-14-deoxyandrogra-pholide		Chen et al., 2008
3-*O*-*β*-D-glucopyranosyl-14,19-dideoxyandrogra-pholide		Shen et al., 2006

Table 1. (cont.)

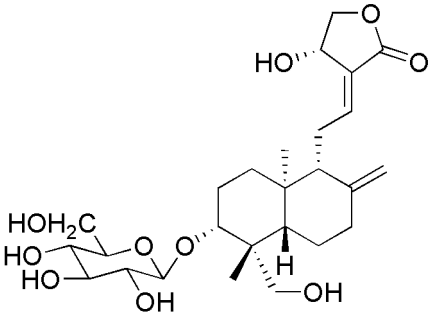
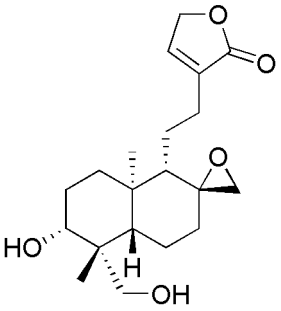
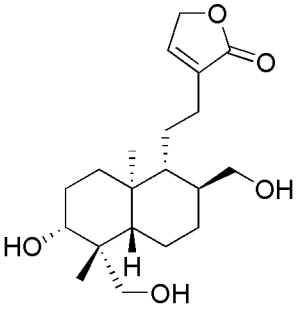
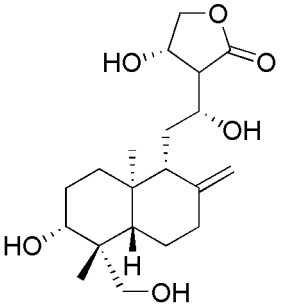
Name	Structures	References
3-*O*-*β*-D-glucopyranosylan-drographolide		Shen et al., 2006
8,17-epoxy-14-deoxyandro-grapholide		Shen et al., 2006
14-deoxy-17*β*-hydroxyan-drographolide		Shen et al., 2006
12*S*-hydroxyandrographolide		Shen et al., 2006

Table 1. (cont.)

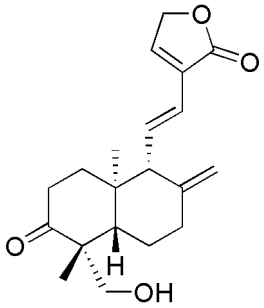
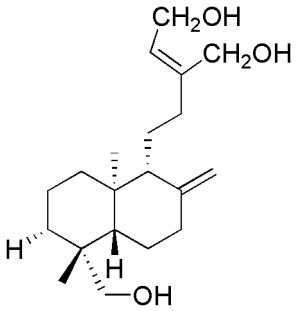
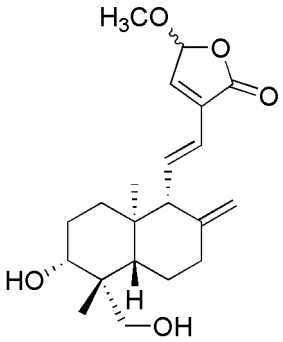
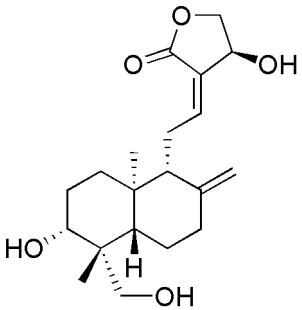
Name	Structures	References
3-oxo-14-deoxy-11,12-dide-hydroandrographolide		Chen et al., 2008
8(17),13-*ent*-labdadiene-15,16,19-triol		Chen et al., 2008
15-methoxy-3,19-dihydroxy-8(17)11,13-*ent*-labda-trien-16,15-olide		Chen et al., 2008
andropanolide		Prammanick et al., 2006

Table 1. (cont.)

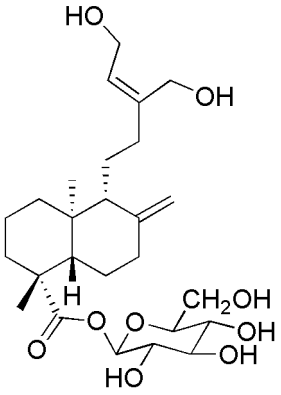
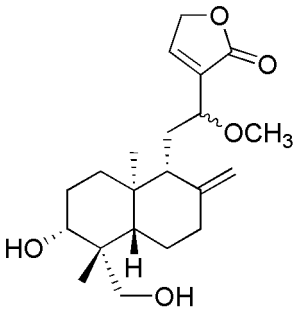
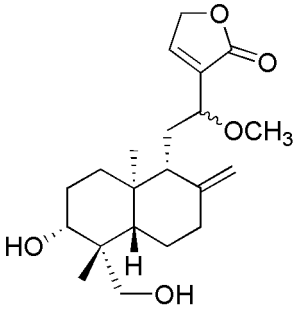
Name	Structures	References
andrographatoside		Shen et al., 2006
14-deoxy-12-methoxyandro-grapholide		Fujita et al., 1984; Matsuda et al., 1994
12-*epi*-14-deoxy-12-methoxyandrographolide		Matsuda et al., 1994

Table 1. (cont.)

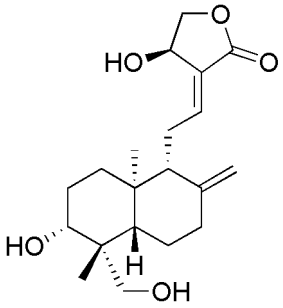
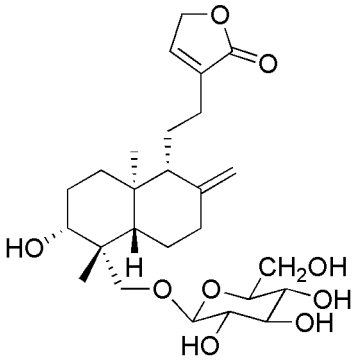
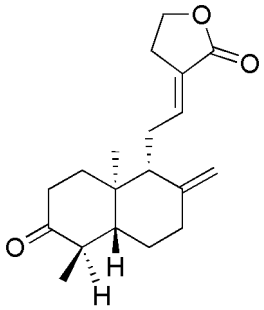
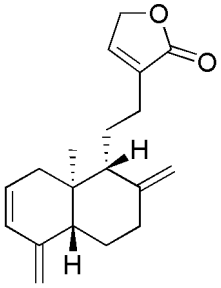
Name	Structures	References
14-*epi*-andrographolide		Matsuda et al., 1994
deoxyandrographiside		Chen et al., 2006; Zou et al., 2010
3-dehydro-14-deoxy-19-nor-andrographolide		Zhang et al., 2006
2,4(18)-diene-14-deoxy-19-norandrographolide		Zhang et al., 2006

Table 1. (cont.)

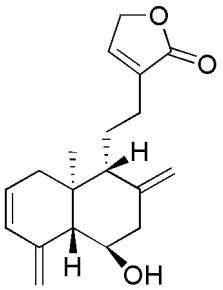
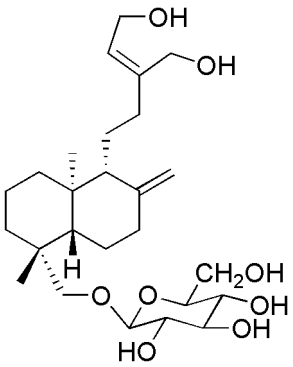
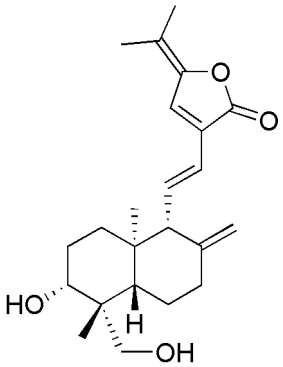
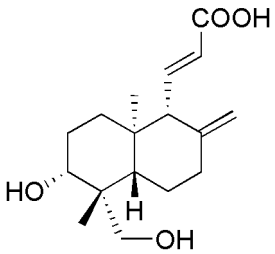
Name	Structures	References
6*β*-hydroxy-2,4(18)diene-14-deoxy-19-norandrographolide		Zhang et al., 2006
19-*O*-*β*-D-glucopyranosyl-*ent*-labda-8(17),13-dien-15,16,19-triol		Zou et al., 2010
14-deoxy-15-isopropylidene-11,12-didehydroandrographolide		Reddy et al., 2003; Rao et al., 2004
3,19-dihydroxy-14,15,16-trinor-*ent*-labda-8(17),11-dien-13-oic acid		Chen et al., 2006

Table 1. (cont.)

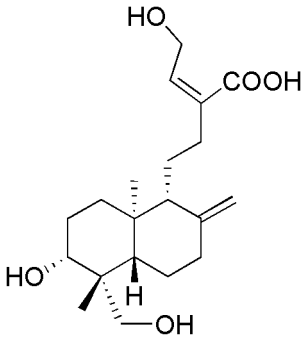
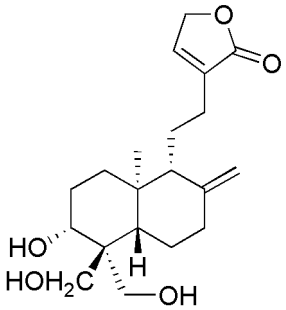
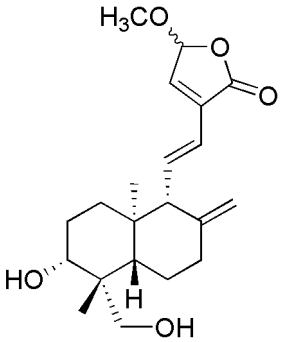
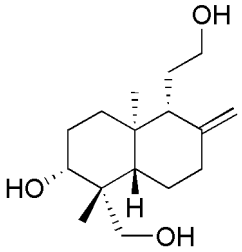
Name	Structures	References
3,15,19-trihydroxy-*ent*-labda-8(17),13-dien-16-oic acid		Chen et al., 2006; Chen et al., 2008
3,18,19-trihydroxy-*ent*-labda-8(17),13-dien-16,15-olide		Chen et al., 2006
3,19-dihydroxy-15-methoxy-*ent*-labda-8(17),11,13-trien-16,15-olide		Chen et al., 2006
13,14,15,16-tetranor-*ent*-labda-8(17)-ene-3,12,19-triol		Chen et al., 2006; Chen et al., 2008

Table 1. (cont.)

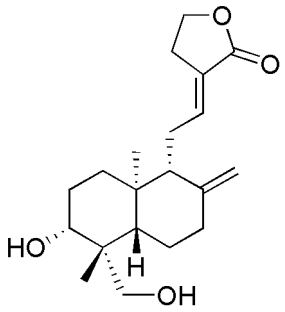
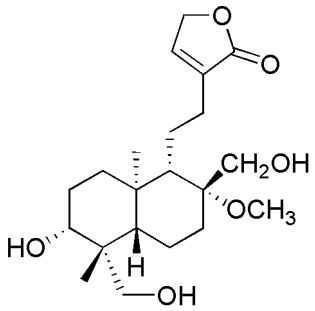
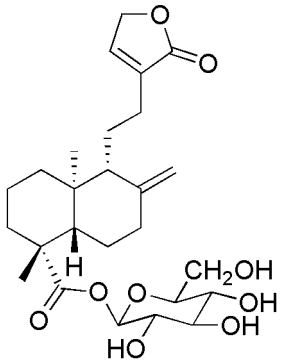
Name	Structures	References
3,19-dihydroxy-*ent*-labda-8(17),12-dien-16,15-olide		Chen et al., 2006
8*α*-methoxyl-14-deoxy-17*β*-hydroxyandrographolide		Ma et al., 2010
19-[(*β*-D-glucopyranosyl)-oxy]-19-oxo-*ent*-labda-8(17),13-dien-16,15-olide		Chen et al., 2006

Table 1. (cont.)

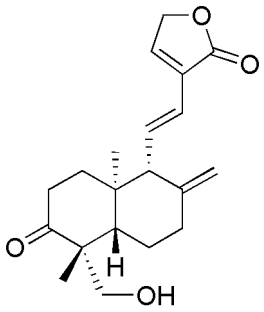
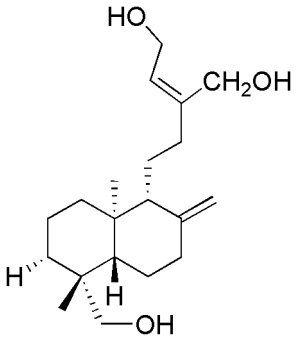
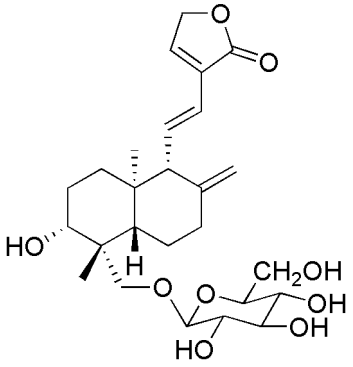
Name	Structures	References
19-hydroxy-3-oxo-*ent*-labda-8(17),11,13-trien-16,15-olide		Chen et al., 2006
ent-labda-8(17),13-dien-15,16,19-triol		Chen et al., 2006
14-deoxy-11,12-didehydroandrographiside		Matsuda et al., 1995; Chen et al., 2006

Table 1. (cont.)

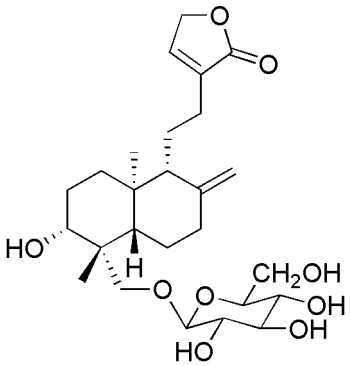
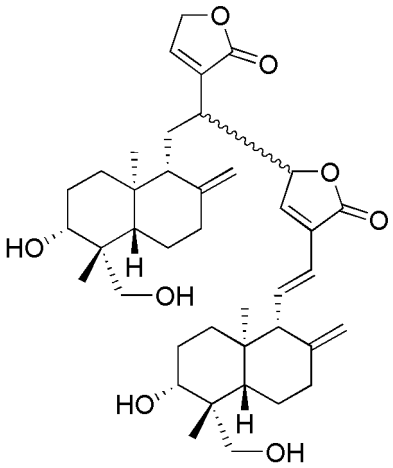
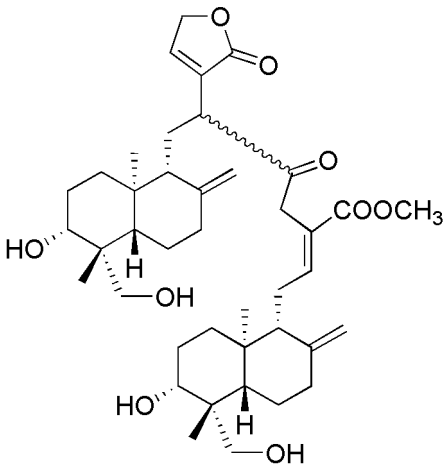
Name	Structures	References
andropanoside		Fujita et al., 1984
bisandrographolide A,B,C		Matsuda et al., 1994
bisandrographolide D		Matsuda et al., 1994

Table 1. (cont.)

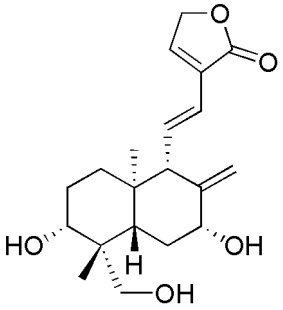
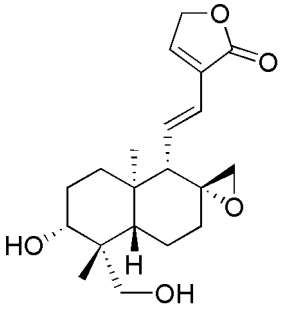
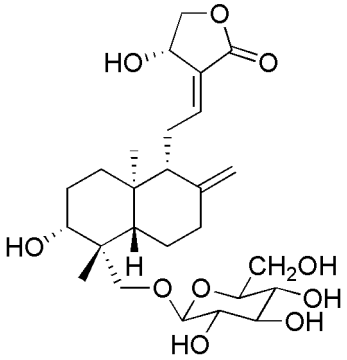
Name	Structures	References
3,7,19-trihydroxy-8,11,13-*ent*-labdatrien-15,16-olide		Ma et al., 2009
8*α*,17*β*-epoxy-3,19-dihydroxy-11,13-*ent*-labdatrien-15,16-olide		Ma et al., 2009
andrographiside		Matsuda et al., 1994; Chen et al., 2006; Shen et al., 2006; Chen et al., 2008; Kulyal et al 2010; Zou et al.., 2010

Table 1. (cont.)

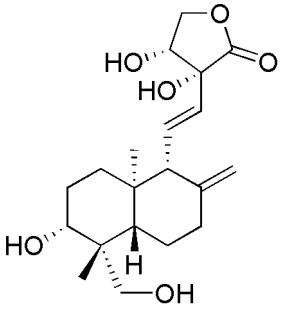
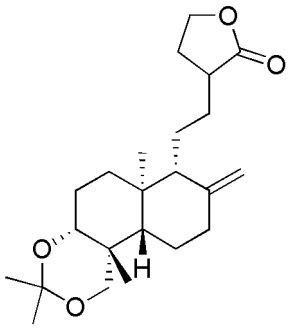
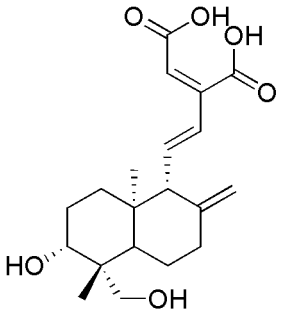
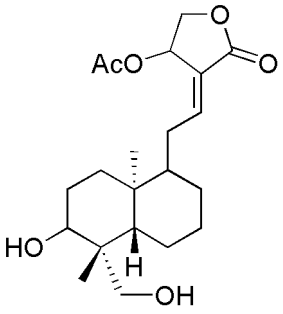
Name	Structures	References
(13*R*,14*R*)3,13,14,19-tetra-hydroxy-*ent*-labda-8(17),11-dien-16,15-olide		Xu et al., 2010
3,19-isopropylidene-14-deoxy-*ent*-labda-8(17),13-dien-16,15-olide		Xu et al., 2010
andrographic acid		Li et al., 2007
14-acetylandrographolide		Chao et al., 2010

Table 1. (cont.)

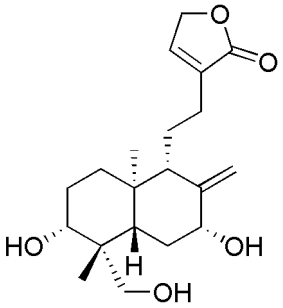
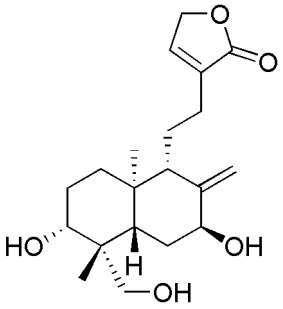
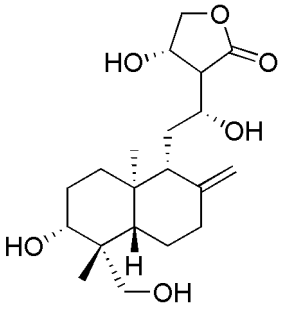
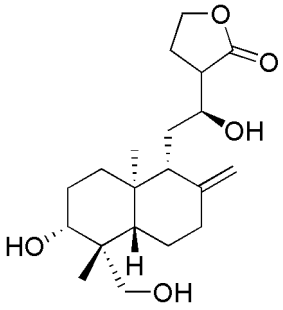
Name	Structures	References
7*R*-hydroxy-14-deoxyandrographolide		Chen et al., 2008
7*S*-hydroxy-14-deoxyandrographolide		Chen et al., 2008
12*S*,13*S*-hydroxyandrographolide		Chen et al., 2008
12*R*,13*R*-hydroxyandrographolide		Chen et al., 2008

Table 1. (cont.)

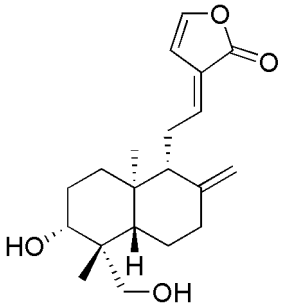
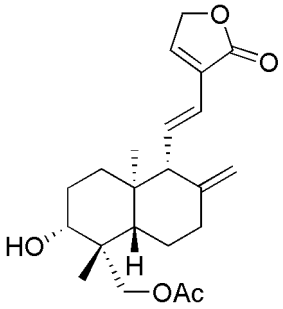
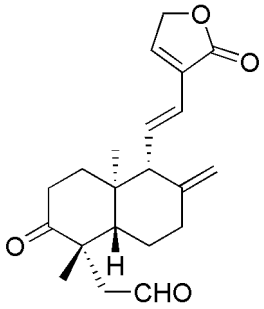
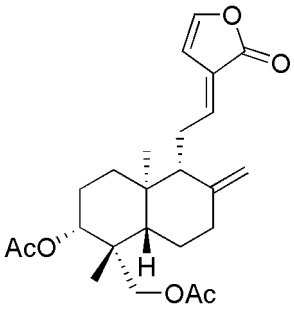
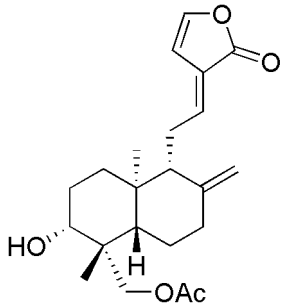
Name	Structures	References
14-deoxy-14,15-dehydroandrographolide		Chao et al., 2010
19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide		Chao et al., 2010
3,19-dioxolabda-8(17),11*E*,13-trien-16,15-olide		Chao et al., 2010
3,19-*O*-diacetylanhydroandrographolide		Chao et al., 2010

Table 1. (cont.)

Name	Structures	References
19-*O*-acetylanhydroandro-grapholide		Chao et al., 2010

II. Flavonoids

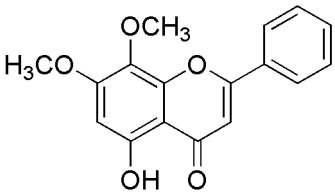
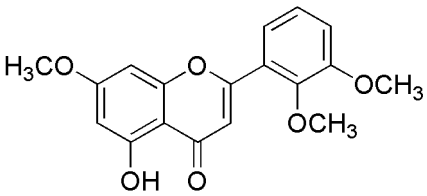
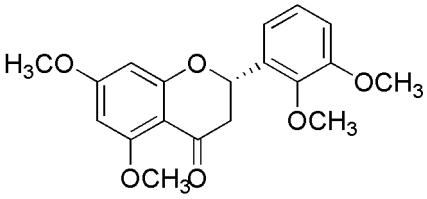
Name	Structures	References
7-*O*-methylwogonin		Rao et al., 2004
5-hydroxy-7,2',3'-trimethoxyflavone		Rao et al., 2004
5,7,2',3'-tetramethoxy-flavanone		Rao et al., 2004

Table 1. (cont.)

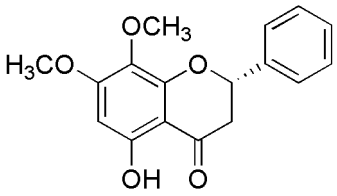
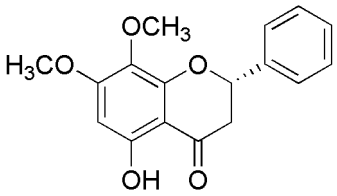
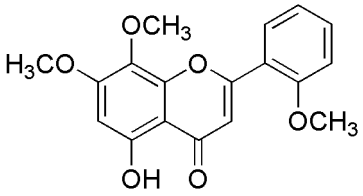
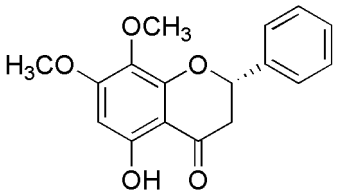
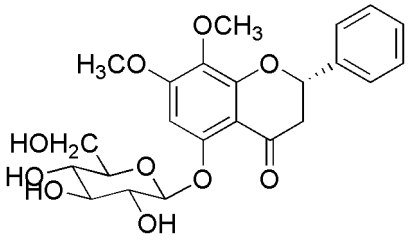
Name	Structures	References
5-hydroxy-7,8-dimethoxyflavanone		Chao et al., 2010
7-*O*-methyldihydrowo-gonin		Rao et al., 2004
skullcapflavone-12'-methylether		Rao et al., 2004
5-hydroxy-7,8-dimethoxyflavone		Chao et al., 2010
andrographidine A		Li et al., 2007

Table 1. (cont.)

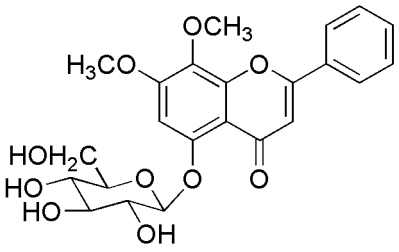
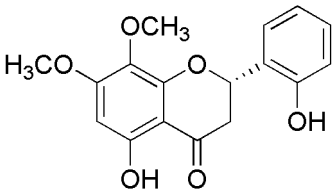
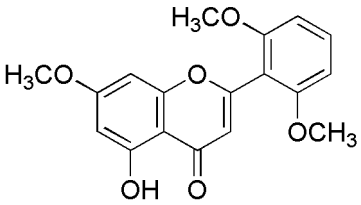
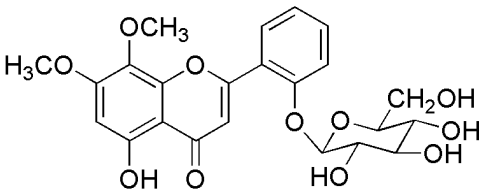
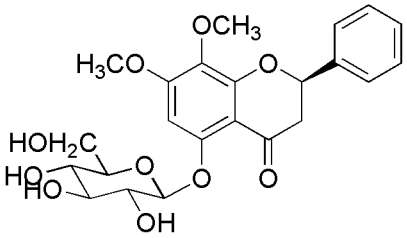
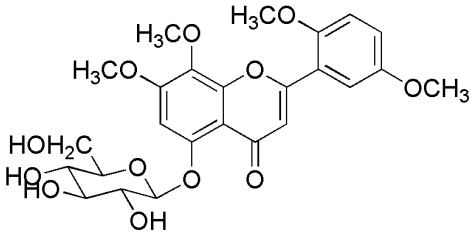
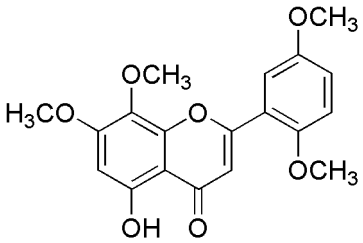
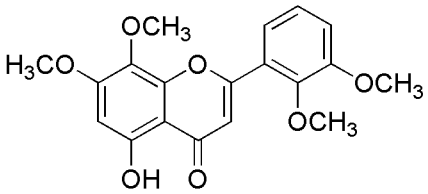
Name	Structures	References
7-*O*-methylwogonin-5-glucoside		Rao et al., 2004
dihydroskullcapflavone I		Rao et al., 2004
5-hydroxy-7,2',6'-tri-methoxyflavone		Reddy et al., 2003; Rao et al., 2004
skullcapflavone I 2'-gluco-side		Rao et al., 2004
5-hydroxy-7,8-dimethoxy (2*R*)-flavanone-5-*O*-*β*-D-glucopyranoside		Li et al., 2007

Table 1. (cont.)

Name	Structures	References
5-hydroxy-7,8,2',5'-tetramethoxy-flavone-5-*O*-β-D-glucopyranoside		Li et al., 2007
5-hydroxy-7,8,2',5'-tetramethoxyflavone		Rao et al., 2004
5-hydroxy-7,8,2',3'-tetramethoxyflavone		Rao et al., 2004

III. Xanthones

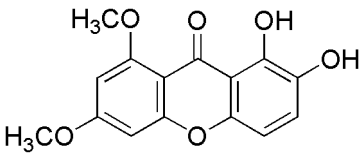
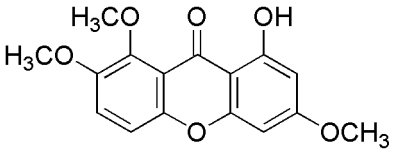
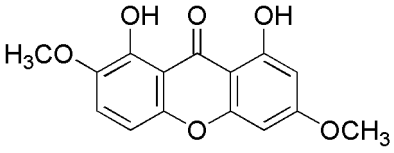
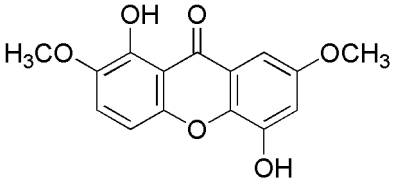
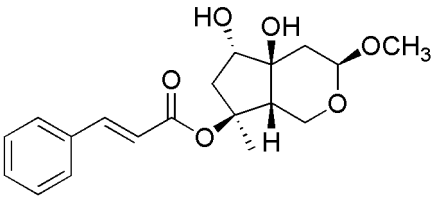
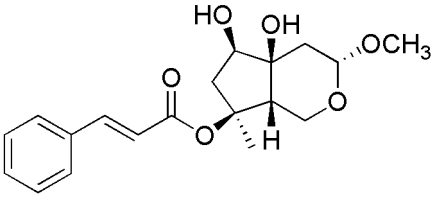
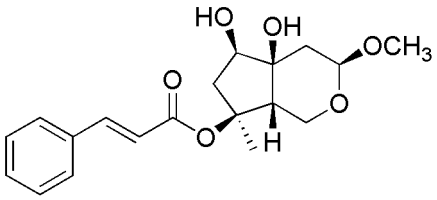
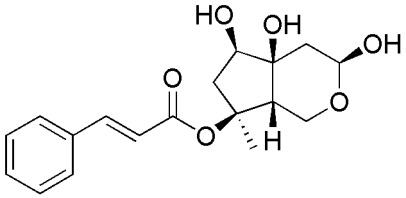
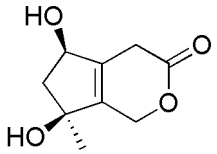
Name	Structures	References
1,2-dihydroxy-6,8-dimethoxyxanthone		Dua et al., 2004

Table 1. (cont.)

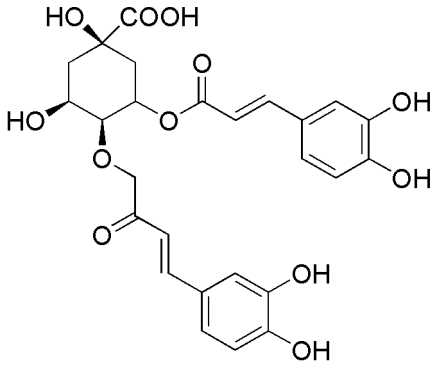
3,7,8-trimethoxy-1-hydroxyxanthone		Dua et al., 2004
1,8-dihydroxy-3,7-dimethoxyxanthone		Dua et al., 2004
4,8-dihydroxy-2,7-dimethoxyxanthone		Dua et al., 2004

IV. Noriridoids

Name	**Structures**	**References**
andrographidoid A		Xu et al., 2012
andrographidoid B		Xu et al., 2012

andrographidoid C		Xu et al., 2012
andrographidoid D		Xu et al., 2012
andrographidoid E		Xu et al., 2012

V. Quinic acid

Name	**Structures**	**References**
3,4-dicaffeoylquinic acid		Hossain et al., 2014

Given the long history of *A. paniculata* as a medicinal plant and one of the ingredient in herbal drugs used widely throughout several Asian countries, the plant,

either alone or as an ingredient in the herbal drug formulae, has also been investigated extensively for its biological activities. As pure compounds, andrographolide, as well as several other major diterpenes, are also the subjects of interest in the pharmacological investigations. The pharmacological and/or biological activities that have been focused on include the effects on common cold, anti-inflammatory, cytotoxic, antimicrobial, and anti-hyperglycemic activities, most of which are closely related to the recommendation and the uses of the plant as described above. All the biological activities of *A. paniculata* that have been investigated and reported to date are summarized in Table 2.

Table 2. Biological activities of chemical constituents in *A. paniculata*

Names	Activities	References
andrographolide	- Analgesic (300 $mg \cdot kg^{-1}$ dose)	Madav et al., 1995
	- Antihyperglycemic effect (effective dose 1.5 $mg \cdot kg^{-1}$)	Yu et al., 2003
	- Cytotoxic (IC_{50} 14.01 $\mu g \cdot mL^{-1}$ against HL-60 cells)	Cheung et al., 2005
	- Antiviral (IC_{50} 8.28 $\mu g \cdot mL^{-1}$ against herpes simplex virus 1 (HSV-1)	Wiart et al., 2005
	- Anti-influenza (IC_{50} 1.2 ± 0.4 $\mu g \cdot mL^{-1}$)	Ko et al., 2006
	- Cytotoxic (ED_{50} 6.5 $\mu g \cdot mL^{-1}$ against KB cells)	Li et al., 2007

Table 2. (cont.)

Names	Activities	References
andrographolide	- Antiproliferative effect (GI_{50} 9.33 ± 0.92 μM against HL-60 cells)	Chen et al., 2008
14-deoxyandrogra-pholide	- Antimalarial (80.9 ± 3.7 % inhibition against *Plasmodium berghei* NK 65 at 2.5 $mg \cdot kg^{-1}$)	Misra et al., 1992
	- Cytotoxic (EC_{50} 2.8 $\mu g \cdot mL^{-1}$ against T-47D cells)	Tan et al., 2005
	- Antibacterial (MIC 15.6 $\mu g \cdot mL^{-1}$ against *Staphylococcus aureus*)	Sule et al., 2011
	- Cardiovascular activity (reduced coronary perfusion pressure by up to −19.5 ± 7.0 mmHg)	Awang et al., 2012
	- Antifungal (MIC and MFC 50 $\mu g \cdot mL^{-1}$ against *Microsporum canis*)	Sule et al., 2012
14-deoxyan-11,12-didehydrodrographolide	- Cytotoxic (EC_{50} 1.5 $\mu g \cdot mL^{-1}$ against T-47D cells)	Tan et al., 2005
	- Antiviral (IC_{50} 11.1 $\mu g \cdot mL^{-1}$ against herpes	Wiart et al., 2005

Table 2. (cont.)

Names	Activities	References
14-deoxyan-11,12-didehydrodrographolide	- Antifungal (MIC and MFC 100 and 150 $\mu g \cdot mL^{-1}$ against *Aspergillus niger* and *Candida tropicalis*)	Sule et al., 2012
neoandrographolide	- Antimalarial (84.4 ± 6.4 % inhibition against *Plasmodium berghei* NK 65 at 2.5 $mg \cdot kg^{-1}$)	Misra et al., 1992
	- Antiviral (IC_{50} 7.97 $\mu g \cdot mL^{-1}$ against herpes simplex virus 1 (HSV-1)	Wiart et al., 2005
	- Anti-proliferative effect (GI_{50} 26.67 ± 0.81 μM against HL-60 cells)	Chen et al., 2008
isoandrographolide	- Anti-proliferative effect (GI_{50} 6.30 ± 0.65 μM against HL-60 cells)	Chen et al., 2008
3,14-dideoxyandrogra-pholide	- Anti-proliferative effect (GI_{50} 20.01 ± 1.22 μM against HL-60 cells)	Chen et al., 2008

Table 2. (cont.)

Names	Activities	References
andrographolactone	- Cytotoxic effect (IC_{50} 0.05 and 0.06 mM against LoVo and NCI-H460 cells respectively)	Wang et al., 2009
3-oxo-14-deoxyandrogra-pholide	- Anti-proliferative effect (GI_{50} 22.80 ± 1.55 μM against HL-60 cells)	Chen et al., 2008
3-oxo-14-deoxy-11,12-didehydroandrogra-pholide	- Anti-proliferative effect (GI_{50} 19.17 ± 2.09 μM against HL-60 cells)	Chen et al., 2008
8(17),13-*ent*-labdadiene-15,16,19-triol	- Anti-proliferative effect (GI_{50} 20.41 ± 0.73 μM against HL-60 cells)	Chen et al., 2008
15-methoxy-3,19-dihy-droxy-8(17)11,13-*ent*-labda-trien-16,15-olide	- Anti-proliferative effect (GI_{50} 26.36 ± 1.89 μM against HL-60 cells)	Chen et al., 2008
3,19-dihydroxy-14,15,16-trinor-8(17),11-*ent*-labda-dien-13-oic acid	- Anti-proliferative effect (GI_{50} 28.81 ± 1.39 μM against HL-60 cells)	Chen et al., 2008

Table 2. (cont.)

Names	Activities	References
13,14,15,16-tetranor-*ent*-labda-8(17)-ene-3,12,19-triol	- Anti-proliferative effect (GI_{50} 24.95 ± 2.10 μM against HL-60 cells	Chen et al., 2008
andrographiside	- Anti-proliferative effect (GI_{50} 28.83 ± 2.18 μM against HL-60 cells	Chen et al., 2008
7*R*-hydroxy-14-deoxy-andrographolide	- Anti-proliferative effect (GI_{50} 22.48 ± 1.83 μM against HL-60 cells	Chen et al., 2008
7*S*-hydroxy-14-deoxy-andrographolide	- Anti-proliferative effect (GI_{50} 25.18 ± 1.47 μM against HL-60 cells	Chen et al., 2008
12*S*,13*S*-hydroxyandro-grapholide	- Anti-proliferative effect (GI_{50} 24.43 ± 2.19 μM against HL-60 cells	Chen et al., 2008
12*R*,13*R*-hydroxyandro-grapholide	- Anti-proliferative effect (GI_{50} 26.56 ± 1.79 μM against HL-60 cells	Chen et al., 2008
1,2-dihydroxy-6,8-di-methoxyxanthone	- Anti-plasmodial activity (IC_{50} 4 $\mu g \cdot mL^{-1}$ against *Plasmodium falciparum*)	Dua et al., 2004

Table 2. (cont.)

Names	Activities	References
3,15,19-trihydroxy-8(17),13-*ent*-labdadien-16-oic acid	- Anti-proliferative effect (GI_{50} 22.42 ± 1.88 μM against HL-60 cells	Chen et al., 2008

1.2 The stability of the Andrographis Herb and its primary diterpene lactones

Whereas being used as an effective alternative approach for the treatment of common cold and non-infectious diarrhea, the Andrographis Herb has such a short shelf-life that hampers its extensive development both as a herbal medicine and as a modern drug. As described previously, the Thai Herbal Pharmacopoeia states that the herb should be stored no longer one year, during which time a quantitation should be frequently conducted. The monograph of the Andrographis Herb, however, did not provide an extensive detail on the precised shelf-life or kinetics of the decomposition, nor was the primary decomposition product(s) resulted from such a degradation mentioned. A few publications have been reporting the degradation/stability of either the Andrographis Herb or its active components, namely andrographolide, or both.

As a purely isolated, single component, andrographolide in its crystalline form is highly stable, and showed no traceable decomposition after being exposed to a temperature as high as 70 °C (75% relative humidity) for a period of 3 months. However, in an amorphous form, a solid dispersion of andrographolide in Poly Vinyl Pyrrolidone decomposed promptly within two months under the accelerated conditions. The degradation underwent a second-order kinetics ($k = 3.8 \times 10^{-6}$ d^{-1}) with

a half-life, calculated for an ambient temperature, as short as 7.83 years. At 70 °C, the primary decomposition product of andrographolide was 14-deoxy-11,12-didehydroandrographolide (Lomlim et al., 2003). The decomposition of andrographolide toward 14-deoxy-11,12-didehydroandrographolide agreed well with that reported by Pholphana et al (2004). The stability of the ground herb under the accelerated conditions has also been investigated. The kinetics of the degradation was also in the second order ($k = 6.58 \times 10^{-6}$ d^{-1}), with a comparable half-life of 4.2 years (Plubrukarn et al., 2006)

On the other hand, a study on the whole plant of *A. paniculata*, stored at a refrigerated (5 ± 2° C) and ambient (25 ± 2° C, 60% ± 5% RH; and 30 ± 2° C, 60% ± 5% RH) conditions, showed no significant reduction of andrographolide over a period of three months (Ibrahim et al., 2008). The discrepancy among the latter and the previous reports may lie on the forms of the materials used (ground *vs.* whole plants) and the length of the investigation, which may lead to the different impacts on the decomposition and transformation of the primary diterpene lactones presented in the herb.

1.3 Metabolomics and metabolomics workflow

Metabolomics is a term used to describe the science of the simultaneous measurement and analysis of thousands all of the small molecules, both primary and secondary metabolites. The metabolome, the term that was coined to reflect the connection with other omic research subject represents the collection of all the metabolites present in a biological system, i.e., the collective end products of the cellular metabolism(s). Relying on the advantages of the advanced separation

technology, particularly in the areas of LC- and GC-MS, and highly sensitive spectroscopic measurements of high-field NMR and FT-IR, combined with the development of statistic software for the multivariate analyses, the approach on metabolomics therefore has become a powerful tools that can be used extensively in the biological and chemical research areas, ranging from the quality control, drug toxicity, microbiology, chemotaxonomy (Liu et al., 2010).

In general, the most straightforward workflow for the metabolomics studies involves sample or specimen treatments and preparation, data acquisition, and data mining. Depending on the purposes of the investigation, the sample treatment may involve a harvesting or collecting of the treated plants or specimens to be investigated in a systematically organized manner. For the plant metabolomics studies, the specimen preparation may include size reducing, drying, and extracting in such a way that all the metabolites of interest are retrieved in a comparable and proportionate fashion. Specifically for the extraction, universal solvents such as chloroform, methanol, or ethanol may be employed, either independently or as a two-phase extraction (Kim et al., 2010). When necessary, a quick, partial purification may be applied. This is of particular importance in a workflow that focuses on the minor components whose signals could be overwhelmed by the larger proportion of the major primary metabolites. Also, in a metabonomics research where the primary foci are the cellular metabolites in the tested animals or human subjects, in which the prior pharmacological or physiological experiments have been performed, treatment of the biological samples to remove cellular metabolic backgrounds is among the primary concerns.

Owing to the need of simultaneous analyses of all the metabolites, the data acquisition for the metabolomics workflow therefore generally employs the

analytical methods that allow the determination of all the present analytes within one single run. For such purposes, the reliable analytical tools are high-performance chromatography, both GC and LC. Equipped with the universal and highly sensitive detectors such as a photodiode array detector for HPLC, or a mass spectrometer for both chromatographic methods, GC and LC are among the most powerful analytical methods for the metabolomics studies.

The disadvantages of the chromatographic separations are the low reproducibility and the inability to identify any given compounds without an aid of reliable standards or extensive databases (Lisec et al., 2006; De Vos et al., 2007). This can be overcome by the uses of spectroscopic and spectrometric approaches, particularly IR and NMR spectrometry. The advances in Fourier transformation technology, and, particularly for the NMR, the development of the superconducting magnet, have made up and compensated the low sensitivity of both methods, and have made the IR and NMR spectrometry to become the powerful tools for the metabolomics research (Kim et al., 2010). The specific signals identified for any relevant markers can be selected and used to determine the presence, or even the amount, of the selected compounds. In addition, whereas the spectral complexity could add the difficulty to the data analysis, signal bucketing or binning, hence simplifying the spectra and data handling, can be performed without compromising the integrity of the data (Kim et al., 2010).

Once acquired, all the data from all the chemical analyses will be compared and analyzed by way of data mining, generally, with multivariate analyses, either by means of principal component analysis and/or partial least square method. The principal component analysis (PCA) primarily indicates the similarity (or dissimilarity)

within the dataset and suggests the most possible variables – whether the chemical shifts, wave numbers, or retention times – that may govern such a similarity (Kim et al., 2010; Alonso et al., 2015).

As mentioned earlier, the metabolomics research has become one of the powerful research approaches that allow the total metabolites in any given living systems to be studied simultaneously. The approach has been employed in a wide range of research field. For examples –Van der Kooy et al (2010) used a rapid NMR targeted metabolomics approach combined with principle component analysis (PCA) to examine that the capsules are indeed *A. afra* and not *A. annua,* and showed that NMR-based metabolomics with the multivariate analysis (PCA), the concentration of artemisinin in the plant material was determined. It can be a rapid and valuable tool in the quality control of herbal supplements. Farag et al (2010) investigated the metabolic fingerprint and chemical profile of commercial cultivars of *Humulus lupulus* L. The results showed that metabolomics approach provided new insights for the supplementary and concurrence for the different technology platform applications in similar plant metabolomics projects. In addition, Yilmaz et al (2010) studied the ^{1}H NMR metabolic fingerprinting for discrimination of authenticity of saffron using principal component analysis (PCA) modeling, and demonstrated that the metabolic fingerprinting technique can provide immediate means of unsupervised classification of the saffron samples.

1.4 Research aims and objectives

As described earlier, the Andrographis Herb is one of the most commonly used herbal medicines for the primary health care as an alternative for the

treatment of common cold and non-infectious diarrhea. The effectiveness of the Andrographis Herb is highly appreciated even among the health care personnel. However, the stability of the diterpene lactones, which leads to such a short storage period, is the major course that limits the extensive development of the Andrographis Herb as a modern medicine. Also, the short storage period can be problematic for the manufacturers and crop producers to implement any reasonable storage and shipping plans.

Whereas the shelf-life of the diterpene lactones, namely andrographolide, either as a purely isolated compound or as a component in the herbal matrix, has already been reported, and the storage time for the herb itself is officially recommended to be only one year (Ministry of Public Health, 1998), the degradation paths of andrographolide, particularly with a spontaneous transformation in the plant matrix over the real-time storage, have never been specifically reported. In addition, the dynamics of the transformation among the diterpene lactones is practically unknown. It is of important to align such transformation with the shelf-life of the herb, preferably in a real-time manner, to determine the fate of the diterpene lactones, and to set up a strong foundation that could allow a reasonably implementable storage plans and shelving periods.

In this investigation, an NMR-based metabolomics approach is opted to determine the composition changes in the Andrographis Herb over a period of six months under standard storage conditions similar to that be perceivably applied by general manufacturers. The objectives of this investigation are

1. To study the degradation profile of chemical constituents in the Andrographis Herb;

2. To determine the chemical marker(s) for the chemical degradation in the Andrographis Herb; and

3. To suggest a possible and reasonable storage conditions and shelf life of the Andrographis Herb.

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and instrumentations

All the chemicals and organic solvents for the general purposes are laboratory grade and were all used as purchase. Chromatographic solvents were commercial grade and were re-distilled prior to use. Thin layer chromatography was performed using SiO_2 60 F_{254} (0.20 mm thickness) on the aluminum support (Merck®), visualized (1) under 254-nm light, and (2) after sprayed with Kedde reagent (3,5-dinitrobenzoic acid in MeOH, followed by 5% methanolic KOH) then heated (purple spots on white background). Chromatographic separations were performed on SiO_2 60 (mesh size 0.04 - 0.06 mm; Salicycle®). Ultraviolet spectra were record on a UV-spectrophotometer (Genesis-6). The principle bands (λ_{max}) were recorded in nm with log ε, using MeOH as a solvent. Infrared spectra were recorded on an FT-IR spectrophotometer (Bruker; VERTEX 70), and the major bands (ν) were recorded in cm^{-1} (KBr pellets). The ^{1}H and ^{13}C NMR spectra used for the chemical identification were recorded on 500 MHz FT-NMR (Varian Unity Inova 500) spectrometer. The NMR chemical shifts are recorded in part per million (δ) referencing either $CDCl_3$ (7.24 ppm of residual $CHCl_3$ for ^{1}H NMR, and 77.0 ppm for ^{13}C NMR), or DMSO-d_6 (2.50 ppm of residual CD_3SOCD_3 for ^{1}H NMR, and 39.5 ppm for ^{13}C NMR). The ESI-MS were performed using a LC mass spectrometer (Waters, Alliance 2690, and Micromass, LCT)

2.2 Plant materials

All the plant materials of *A. paniculata* used throughout this investigation were identified by Mrs. Pranee Rattanasuwan of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. A voucher specimen is deposited at the herbarium of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University (SKP 001 01 16 01).

The plant materials of *A. paniculata* used for preparing the standard diterpene lactones was purchased as unprocessed dried herbs from Suphan Buri Province. The materials were used in the further extraction without additional drying.

The Andrographis Herbs from four suppliers were used in the metabolomics and stability profiling experiments. Three of which were bought as whole, dried aerial parts from local farmers in Nakhon Pathom, Khon Kaen, and Suphan Buri Provinces. The other one was obtained in-house from the nursery of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The sources for the herbs used in this study were selected primarily to demonstrate the variations due to the different and unspecified crop productions with no intention to reflect the geographical impacts that may influent the chemical accumulation or productivity.

2.3 Isolation and purification of standard diterpene lactones

The dried aerial parts of *A. paniculata* (300 g) was ground and refluxed in EtOH (3 L, 2 hr), yielding a crude extract (11.02 g) upon solvent removal. An aliquot of the crude extract (1.63 g) was chromatographed on a column of SiO_2, eluted with

gradient eluting solvents, starting from 80% EtOAc in CH_2Cl_2, through 100% EtOAc and 5% MeOH in EtOAc, to 20% MeOH in EtOAc. Seven major fractions were collected after the fractional pool. Recrystallization of fraction 2 ($CHCl_3$/MeOH = 2:1) yielded andrographolide (297 mg). Fraction 1 was chromatographed on a $AgNO_3$-impregnated SiO_2 (EtOAc/$CHCl_3$/CH_3CN = 70:20:10) to afford 14-deoxyandrographolide (35 mg) and 14-deoxy-11,12-didehydroandrographolide (75 mg). The more polar fraction 5, which was another major fraction, was subjected to the recrystallization (MeOH), and neoandrographolide (38 mg) was obtained.

Andrographolide. Tabular crystal, UV (MeOH) λ_{max} (log ε) 225 (6.53) nm; IR (KBr) ν 3399, 1727, 1674, 909 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 6.95 (1H, td, J = 7.0, 1.7 Hz, H-12), 5.01 (1H, t, J = 6.5 Hz, H-14), 4.88 (1H, s, H-17), 4.56 (1H, s, H-17), 4.44 (1H, dd, J = 10.5, 6.1 Hz, H-15), 4.24 (1H, dd, J = 10.5, 2.0 Hz, H-15), 4.17 (1H, dd, J = 11.2, 2.3 Hz, H-19), 3.47 (1H, dt, J = 11.2, 9.5 Hz, H-3), 3.32 (1H, t, J = 10.1 Hz, H-19), 2.70 (2H, dd, J = 8.7, 2.5 Hz, H-11), 1.96 (1H, m, H-9), 1.83 (1H, m, H-7), 1.80 (2H, m, H-1 and H-6), 1.76 (1H, m, H-2), 1.23 (3H, s, H-18), 0.68 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.0 (C, C-16), 148.6 (CH, C-12), 146.0 (C, C-8), 127.0 (C, C-13), 108.6 (CH_2, C-17), 80.5 (CH, C-3), 74.0 (CH_2, C-15), 66.0 (CH, C-14), 64.0 (CH_2, C-19), 55.5 (CH, C-9), 55.0 (CH, C-5), 48.8 (C, C-4), 38.0 (C, C-10), 37.5 (CH_2, C-1, and C-17), 27.8 (CH_2, C-2), 24.6 (CH_2, C-11), 23.6 (CH_2, C-6, and CH_3, C-18), 15.1 (CH_3, C-20); HRESIMS m/z 373.1997 $[M+Na]^+$ (cald for $C_{20}H_{30}O_5Na$, 373.1991).

14-Deoxyandrographolide. White solid, UV (MeOH) λ_{max} (log ε) 215 (6.49) nm; IR (KBr) ν 3362, 3079, 2935, 2850, 1749, 1645, 1448, 1347 and 893 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.07 (1H, t, J = 1.6 Hz, H-14), 4.85 (1H, s, H-17), 4.75 (2H, dd, J = 4.2, 2.0 Hz, H-15), 4.57 (1H, s, H-17), 4.15 (1H, d, J = 11.1 Hz, H-19), 3.45 (1H, t, J

= 5.0 Hz, H-3), 3.30 (1H, d, *J* = 11.3 Hz, H-19), 2.43 (1H, m, H-12), 2.39 (1H, m, H-7), 2.10 (1H, dt, *J* = 15.8, 8.0 Hz, H-12), 1.92 (1H, m, H-7), 1.80 (overlapped, H-1, H-2, and H-6), 1.72 (2H, m, H-11), 1.57 (1H, m, H-9), 1.30 (1H, m, H-6), 1.23 (3H, s, H-18), 1.18 (1H, dd, *J* = 12.8, 4.2 Hz, H-5), 1.16 (1H, m, H-1), 0.62 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.3 (C, C-16), 146.7 (C, C-8), 144.0 (CH, C-14), 134.6 (C, C-13), 107.3 (CH_2, C-17), 80.6 (CH, C-3), 70.1 (CH_2, C-15), 64.2 (CH_2, C-19), 56.0 (CH, C-9), 55.2 (CH, C-5), 42.8 (C, C-4), 39.0 (C, C-10), 38.1 (CH_2, C-7), 36.8 (CH_2, C-1), 28.2 (CH_2, C-2), 24.5 (CH_2, C-12), 23.9 (CH_2, C-6), 22.7 (CH_3, C-18), 21.9 (CH_2, C-11), 15.2 (CH_3, C-20); HRESIMS *m/z* 357.2041 $[M+Na]^+$ (cald for $C_{20}H_{30}O_4Na$, 357.2042).

14-Deoxy-11,12-didehydroandrographolide. White solid, UV (MeOH) λ_{max} (log ε) 210 (6.65), 249 (6.72) nm; IR (KBr) *v* 3437, 2936, 2873, 1748, 1640, and 891 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.15 (1H, t, *J* = 2.0 Hz, H-14), 6.82 (1H, dd, *J* = 15.8, 10.1 Hz, H-11), 6.08 (1H, d, *J* = 15.8 Hz, H-12), 4.79 (2H, d, *J* = 2.0 Hz, H-15), 4.74 (1H, dd, *J* = 2.0, 1.6 Hz, H-17), 4.49 (1H, dd, *J* = 1.9, 1.6 Hz, H-17), 4.18 (1H, d, *J* = 11.0 Hz, H-19), 3.44 (1H, ddd, *J* = 11.4, 4.8, 1.1 Hz, H-3), 3.32 (1H, d, *J* = 12.0 Hz, H-19), 2.41 (1H, ddd, *J* = 13.8, 4.2, H-7), 2.28 (1H, d, *J* = 10.0 Hz, H-9), 2.01 (1H, ddd, *J* = 13.0, 10.0, 4.8 Hz, H-7), 1.75 (2H, m, H-6), 1.70 (1H, m, H-2), 1.47 (1H, dt, *J* = 13.0, 3.4 Hz, H-1), 1.30 (1H, m, H-2), 1.22 (3H, s, H-18), 1.17 (1H, dd, *J* = 12.7, 2.5 Hz, H-5), 1.09 (1H, td, *J* = 14.0, 13.3, 4.1 Hz, H-1), 0.77 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.4 (C, C-16), 148.1 (C, C-8), 143.1 (CH, C-14), 136.0 (CH, C-11), 129.2 (C, C-13), 121.1 (CH, C-12), 109.2 (CH_2, C-17), 80.8 (CH, C-3), 69.7 (CH_2, C-15), 64.2 (CH2, C-19), 61.6 (CH, C-9), 54.6 (CH, C-5), 42.8 (C, C-4), 38.5 (C, C-10), 38.2

(CH_2, C-1), 36.5 (CH_2, C-7), 28.0 (CH_2, C-6), 22.9 (CH_2, C-2), 22.7 (CH_3, C-18), 15.9 (CH_3, C-20); HRESIMS *m/z* 355.1895 $[M+Na]^+$ (cald for $C_{20}H_{28}O_4Na$, 355.1885).

Neoandrographolide. Needles, UV (MeOH) λ_{max} (log ε) 225 (6.83) nm; IR (KBr) ν 3447, 2929, 2851, 1747, 1644, 1444, 1353, and 909 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 7.45 (1H, dd, *J* = 2.6, 1.4 Hz, H-14), 4.80 (2H, m, H-17, and H-15), 4.60 (1H, m, H-17), 3.87 (1H, d, *J* = 9.6 Hz, H-19), 2.30 (1H, m, H-7), 2.20 (1H, m, H-12), 2.00 (1H, m, H-12),1.90 (1H, m, H-7), 1.83 (1H, d, *J* = 13.1 Hz, H-3), 1.77 (1H, m, H-6), 1.71 (1H, m, H-1), 1.67 (1H, m, H-11),1.60 (1H, m, H-9), 1.53 (1H, m, H-2), 1.47 (1H, m, H-11), 1.38 (1H, m, H-2), 1.29 (1H, qd, *J* = 12.8, 4.1 Hz, H-6), 1.21 (1H, dd, *J* = 12.9, 2.0 Hz, H-5), 1.00 (1H, td, *J* = 13.0, 3.6 Hz, H-1), 0.95 (3H, s, H-18), 0.86 (1H, td, *J* = 13.3, 3.6 Hz, H-3), 0.60 (3H, s, H-20); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 174.0 (C, C-16), 147.8 (C, C-8), 147.0 (CH, C-14), 132.0 (C, C-13), 106.0 (CH_2, C-17), 71.0 (CH_3, C-19), 70.5 (CH_2, C-15), 55.9 (CH, C-9), 55.5 (CH, C-5), 39.0 (C, C-10), 38.5 (CH_2, C-1), 38.1 (CH_2, C-7), 38.0 (C, C-4), 35.8 (CH_2, C-3), 27.0 (CH_3, C-18), 24.2 (CH_2, C-12), 24.1 (CH_2, C-6), 21.0 (CH_2, C-11), 18.0 (CH_2, C-2), 15.0 CH_3, C-20); HRESIMS *m/z* 503.2631 $[M+Na]^+$ (cald for $C_{26}H_{40}O_8Na$, 503.2621).

2.4 Stability and metabolomics profiling

2.4.1 Specimen preparation and extraction

All the herbs purchased from three crop producers in Nakhon Pathom, Khon Kaen, and Suphan Buri were specified as fully grown crops, harvested at flower blossom but before fruiting. All were subjected to the stability experiments with no further drying process. The Songkhla specimens were also harvested as indicated above. The herbs were rinsed thoroughly, air-dried, and oven-dried (40 – 60 °C) for 24

- 48 hr. The specimens from each source were processed separately in an individual-source manner. The herbs were cut into small pieces (3 - 5 cm) (Figure 2), and packed in a series of light-protected paper sachets (approximately 900 mg per sachet). All the specimens were stored in the storage unit of the Traditional Medicine Manufacturing Facility, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The storage conditions were controlled at 25 ± 5 °C and 51 ± 5% relative humidity as recommended by the Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998).

Starting on the day of arrival at the laboratory as day 0, therefore mimicking the storage conditions that may be practiced by any manufacturers, the herb specimens from each source were collected in a random manner for the NMR-based (triplicate) and titrimetric-based (duplicate) stability profiling experiments. The collecting intervals for the NMR-based experiments were weekly during months 1 and 2, once every two weeks during months 3 and 4, and monthly from month 5 onward. Hence, the sampling schedules for the samples from each source were on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 98, 112, 140, and 168. The collecting intervals for the titrimetric-based experiments were monthly, and were carried out for six months; hence

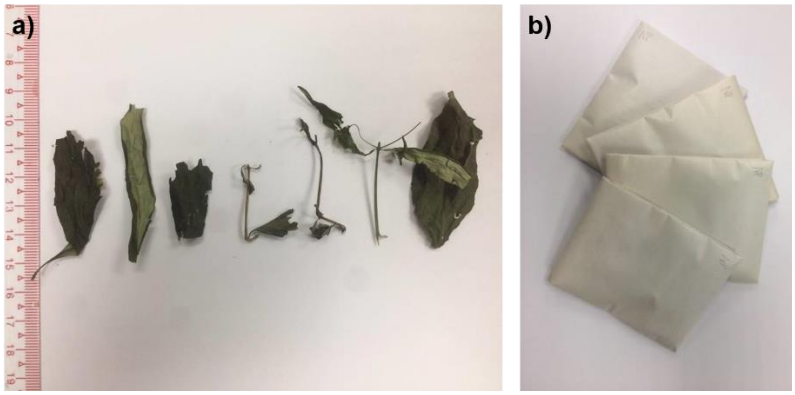


Figure 2. Andrographis Herb cut into 3 – 5 cm (a) and packed in a series of light-protected paper sachets (b)

the sampling times were scheduled on days 0, 28, 56, 84, 112, 140, and 168. Once collected, all the samples were preserved separately at -20 °C until the time of extraction.

The samples to be subjected to the titrimetric method of determination were assayed for the total lactone contents (Ministry of Public Health, 1998). The assay protocol is described in section 2.5. As for the NMR-based experiments, each collected sample was separately ground with the aid of liquid nitrogen. About 100 mg of the resulting fine powder was accurately weighed and extracted with a mixture of $CHCl_3$/MeOH/water (4:2:2 mL). This was vortexed for 10 s, and ultra-sonicated for 20 min at room temperature. The mixture was set aside at an ambient temperature for 10 min to allow a clear separation into two fractions. A 3-mL aliquot of each fraction was brought to dryness under a reduced pressure. The resulting dried extracts were stored at -20 °C until the dates scheduled for the NMR experiments.

2.4.2 NMR acquisition and bucketing

The NMR acquisition for the metabolomics experiments were performed at 25° C on a 500 MHz Varian Unity Inova 500 NMR spectrometer, operating at a ^1H NMR frequency of 499.7 MHz. A 128-scan for the FID was acquired and recorded with the following parameters: pulse width 30°; acquisition time 2.7 s; and relaxation delay 1.5 s. An exponential window function lb 0.3 was used prior to the Fourier transformation. For the non-polar $CHCl_3$-extracts, each sample was dissolved in a quantitatively 0.8 mL of $CDCl_3$ (99.9% D, containing 0.05% v/v, tetramethylsilane [TMS]). Similarly, each polar aq MeOH-extract was dissolved in a 0.8 mL of D_2O (99.9% D, containing 0.1% v/v 3-[trimethylsilyl]propionic-2,2,3,3-d_4 acid sodium salt

[TMSP])**,** buffered with KH_2PO_4 (pH 6.0 ± 0.1). TMS and TMSP were referred to as the internal standards for the chemical shifts (both at 0 ppm in their respective solvents) and for the quantitative resonance integration.

The recorded FID of each sample was Fourier transformed on MestReNova 9.0.1 (Mestrelab Research S.L). Each spectrum was phased and baseline-corrected manually. The peak integration was normalized according to the reference signal as mentioned above (integral range 0.2 - -0.2 ppm, equivalent to 1 integral unit). Each spectrum was then bucketed to an equal width of 0.04 ppm per bucket, corresponding to a spectral range of 8.0 - 0.2 ppm (a total of 196 buckets). The spectral regions between 1.64 - 1.50 ppm (residual water) and between 7.32 - 7.20 ppm (residual $CHCl_3$) were excluded from all the spectra of the non-polar extracts, and that between 4.82 - 4.68 ppm (residual HOD) were excluded from the spectra of the polar extracts.

2.4.3. Principal component analysis

The principal component analysis (PCA) was performed on a SIMCA 13.0.3.0 software (Umetrics AB, Umea). The bucketed spectral data from each sample, once normalized (an equivalent of 1 integration unit for the reference signal in each spectra, see 2.4.2), were imported into an Excel format (Microsoft Excel 2010). The resulting normalized buckets were recalculated on a per-weight of dried herb basis. The processed data were imported to SIMCA for the multivariate analysis using pareto scaling approach.

2.5 Total lactone content

The sampled herbs set aside for the titrimetric method of quantitative determination as described in section 2.4.1 was subjected to the assay for the total

lactone contents according to the protocol in the Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998). In brief, the assay protocol is as follow;

An accurately about 100 g of finely ground Andrographis Herb was reflux (2 hr) in 85% EtOH (50 mL). Once cool, the mixture was filtered, and the marc was washed with 85% of EtOH to a clear, colorless filtrate. To the combined filtrate and washing was added basic lead acetate (1 mL). The solution was set aside for 15 min, then filtered and washed with EtOH to a clear solution. 25% Na_2SO_4 (1 mL) was then added. After setting aside for 1 hr, decolorizing charcoal (500 mg) was added and the mixture was refluxed for 10 min. The hot solution was filtered through a pad of charcoal (500 mg), and washed with hot EtOH (2 mL $\times$ 3). Once cool, 20 mL of water was added. This was titrated to an end-point (phenolphthalein) with 0.1 N NaOH. An additional 0.1 N NaOH (5 mL) was added, and the solution was brought to reflux (30 min). The solution was cooled and titrated with 0.05 N HCl, using phenolphthalein as an indicator.

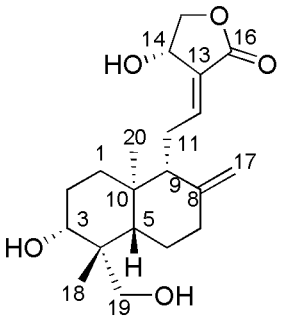
CHAPTER 3

RESULTS AND DISCUSSION

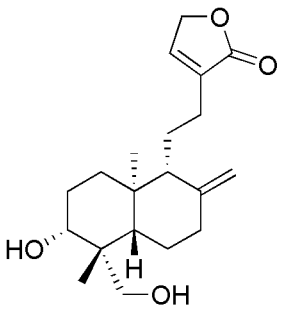
In this study, an NMR-based metabolomics approach is adopted for the investigation of the changes in metabolomics profiles of the Andrographis Herb over a period of six months. The primary aim is to determine the most relevant chemical marker(s), whether primary or secondary metabolites, that may reflect the stability of the herb. Specifically for the diterpene lactones, the investigation is focusing on the dynamics in transformation among the major diterpene lactones, namely andrographolide; 14-deoxyandrographolide; and 14-deoxy-11,12-didehydroandrographolide, during the course of storage in the recommended, standard conditions. The alignment among the changes in the metabolomics profiles and the transformation among the major diterpene lactones that were determined using the NMR-based approach and the stability that was profiled on the basis of total lactone contents will be attempted.

3.1. ^{1}H NMR spectra of the standard diterpene lactones and the crude extracts

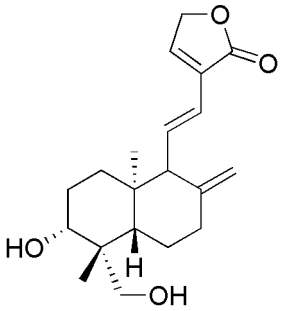
The four major diterpene lactones; andrographolide; 14-deoxyandrographolide; 14-deoxy-11,12-didehydroandrographolide; and neoandrographolide, which are used as the reference standards in this investigation, were prepared in-house. All were identified on the basis of the complete spectroscopic analyses, and by the spectral comparison with the published data (Fujita et al., 1984; Chen et al., 2006). Their purity was referred to their ^{1}H NMR spectra, in which no detectable impurities were observed.



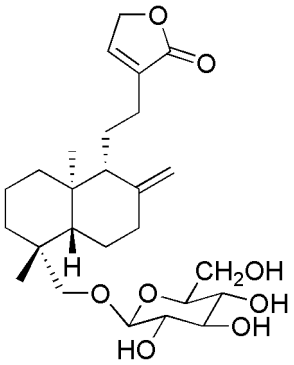
andrographolide



14-deoxyandrographolide



14-deoxy-11,12-didehydro andrographolide



neoandrographolide

Figure 3. The chemical structures of andrographolide, 14-deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide

The ^{1}H NMR spectra (500 MHz) of the three less polar compounds; i.e., andrographolide; 14-deoxyandrographolide; and 14-deoxy-11,12-didehydroandrographolide (Figures 4a-c, respectively), were performed in $CDCl_3$, which was also the solvent that was used to obtain the spectra of the $CHCl_3$-extracts (vide infra). Their spectra showed the close resemblance among each other in the high-field regions (3.5 – 0.8 ppm), belonging to most protons in the *trans*-decalin moiety. In the low-field regions (7.5 – 4.0 ppm), the differences in the oxygenated functionalities and the saturation, particularly those surrounding the lactone moiety, provide the distinctive

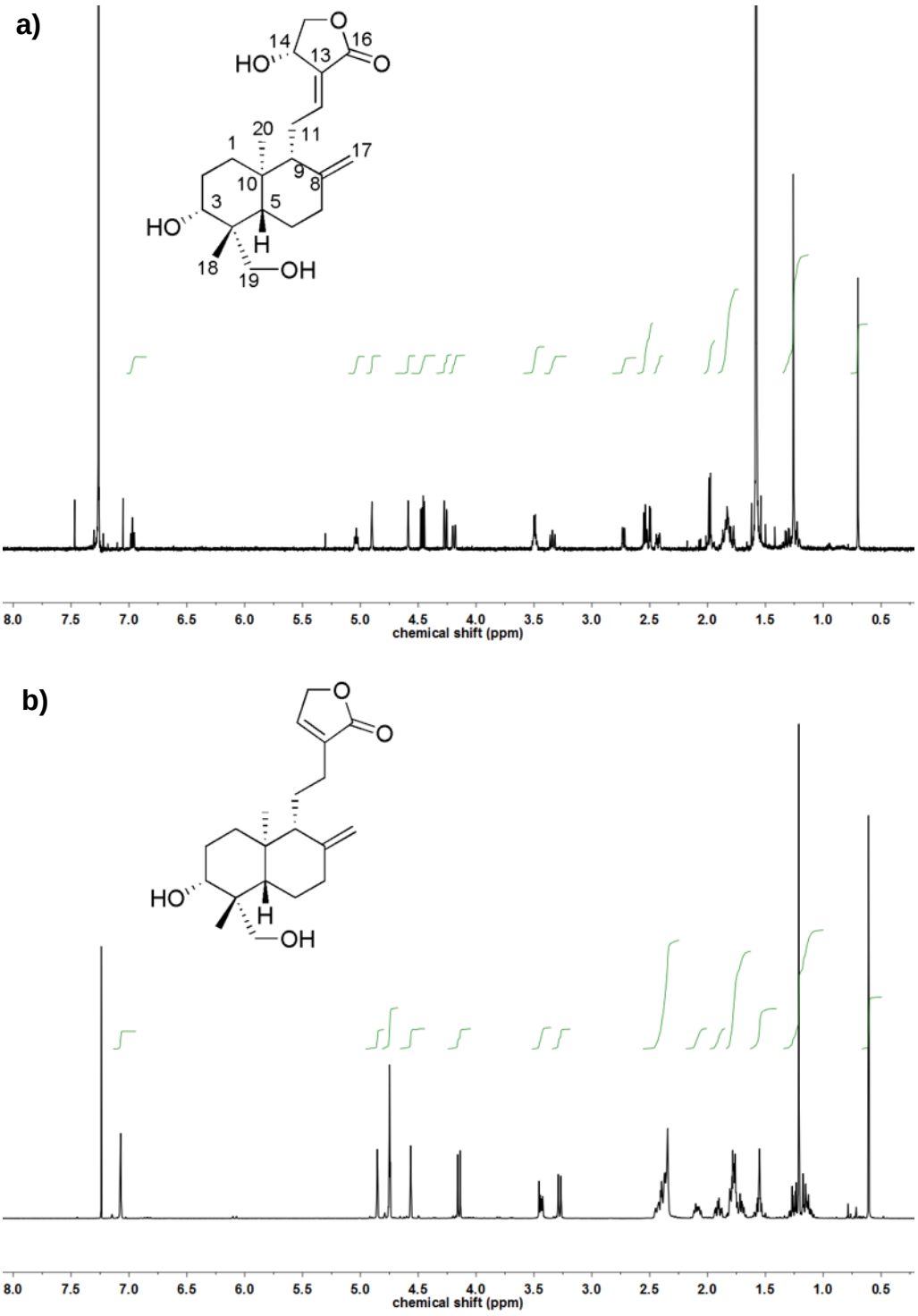


Figure 4. The ^{1}H NMR spectra of andrographolide (a), 14-deoxyandrographolide (b) (this page), 14-deoxy-11,12-didehydroandrographolide (c) (all in $CDCl_3$, 500 MHz), and and neoandrographolide (d) (in DMSO-d_6, 500 MHz) (next page)

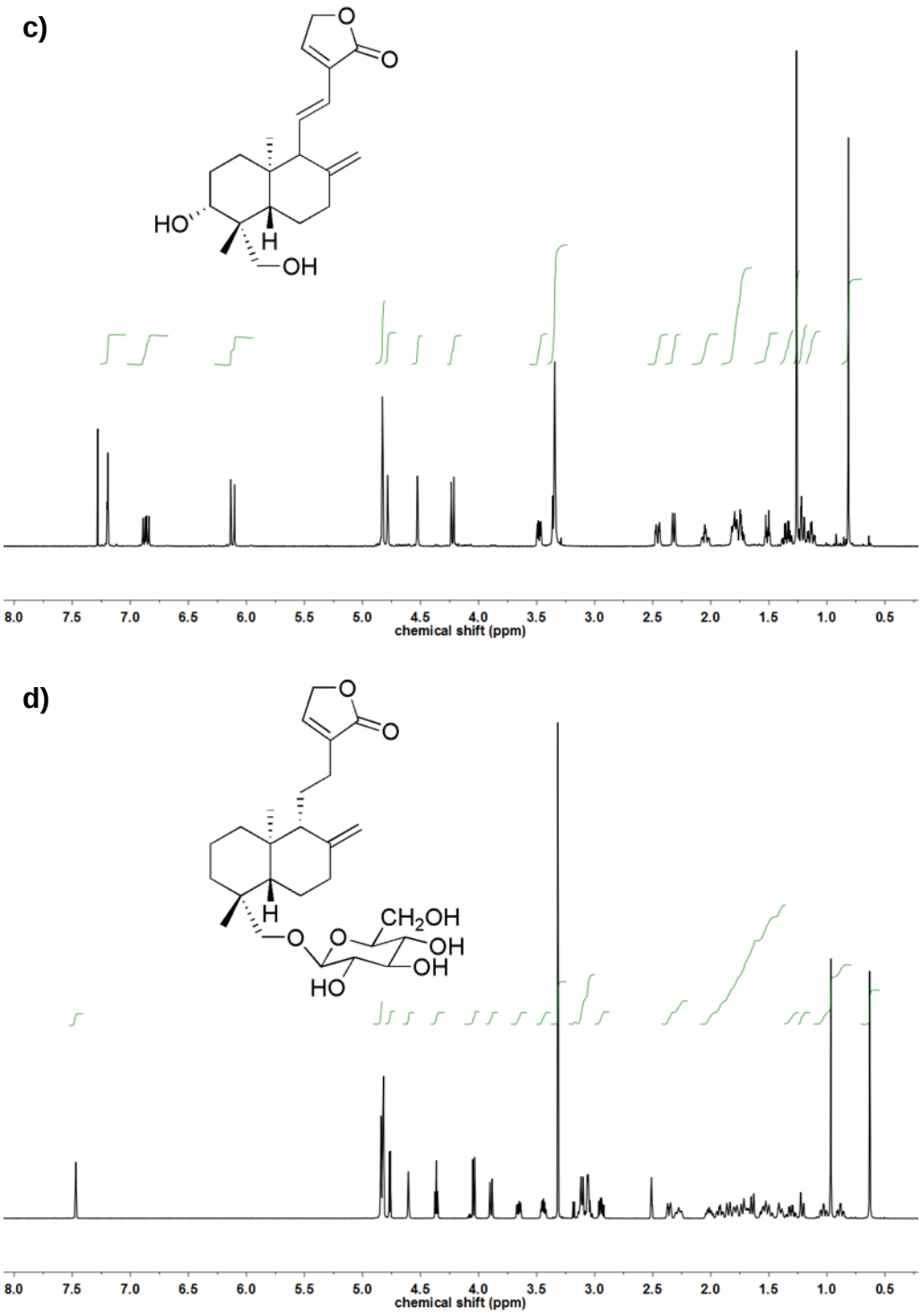


Figure 4. (cont)

and indicative resonances of each compounds. Of particular interest were the resonances at δ 6.95 (td, J = 7.0, 1.7 Hz), belonging to H-12 of andrographolide; δ 7.07 (t, J = 1.6 Hz) belonging to H-14 of 14-dideoxyandrographolide; and δ 7.15 (t, J = 2.0 Hz), 6.82 (dd, J = 15.8, 10.1 Hz), and 6.08 (d, J = 15.8 Hz), respectively belonging to H-14, H-11, and H-12 of 14-deoxy-1,12-didehydroandrographolide. These are the indicative resonances that are used to identify and determine the presence of each compound in the spectra of the extracts (vide infra).

Being a glycosidic derivative of 14-deoxyandrographolide, neoandro-grapholide is more polar than the other three compounds, and its ^{1}H NMR spectrum (500 MHz; Figure 4d) was obtained in DMSO-d_6. Apart from the resonances of the glucose residue, most of its NMR signals are comparable to that of 14-deoxyandrographolide. The relevant signal indicative to neoandrographolide is at δ 7.45 (dd, J = 2.6, 1.4 Hz, H-14). Note here that despite being more hydrophilic, the compound is not soluble in water (or D_2O), which is the NMR-operating solvent used for the aq MeOH-extracts.

Shown in Figures 5 and 6 are the ^{1}H NMR spectra of the $CHCl_3$- and aq MeOH-extracts of the Andrographis Herb from the four locations (500 MHz, $CDCl_3$ and D_2O), representatively selected from the day-0 samples of each location. In the spectra of the $CHCl_3$-extracts, all the three referencing compounds, andrographolide, 14-deoxyandro-grapholide, and 14,deoxy-11,12-didehydroandrographolide, are present in all four samples, with all the indicative resonances as described above clearly separated and easily identifiable (Figure 5). All the three compounds will therefore be used as the indicative markers for the further discussion. The other classes of compounds that are observable in these spectra are fat and steroids, the signal of which

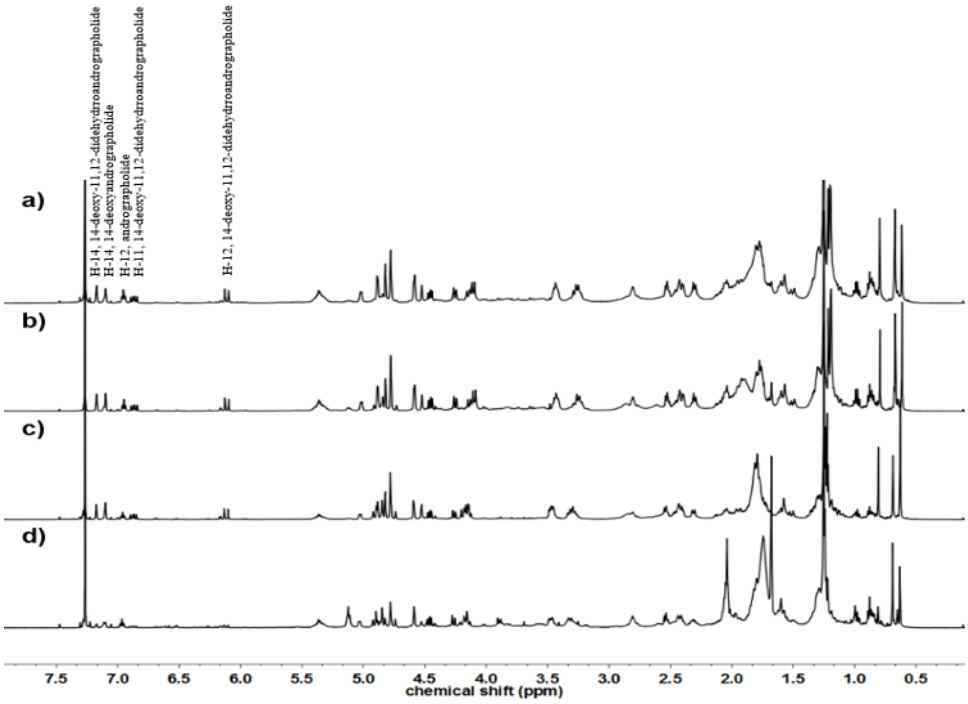


Figure 5. ^{1}H NMR spectra (500 MHz; $CDCl_3$) of $CHCl_3$-extracts of the Andrographis Herbs from (a) Suphan Buri; (b) Nakhon Pathom; (c) Khon Kaen; and (d) Songkhla; all from day-0 of the samples from each supplier

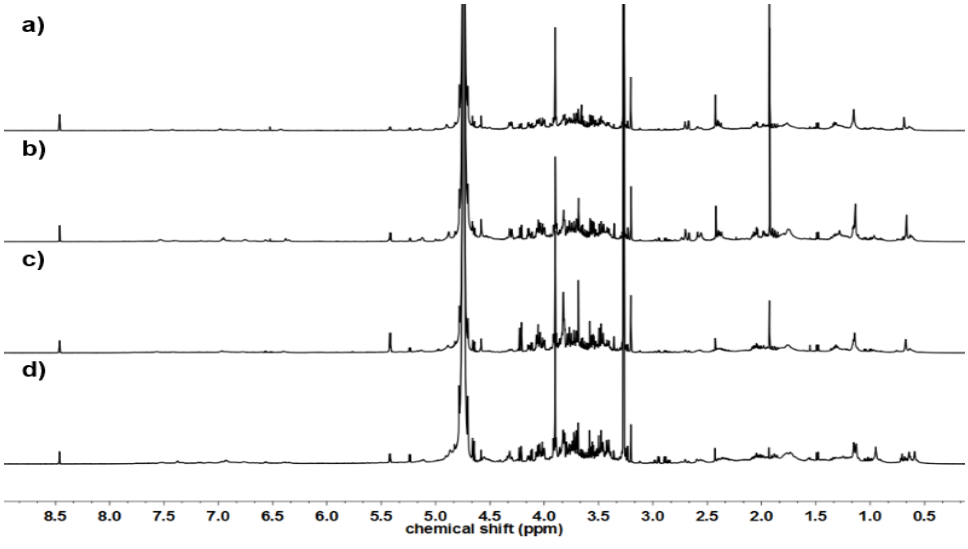


Figure 6. ^{1}H NMR spectra (500 MHz; D_2O) of aq MeOH-extracts of the Andrographis Herbs from (a) Suphan Buri; (b) Nakhon Pathom; (c) Khon Kaen; and (d) Songkhla; all from day-0 of the samples from each supplier

are unidentifiable and overlapped in the high-field regions (2.2 – 1.2 ppm).

The ^{1}H NMR spectra of the aq MeOH-extracts (Figure 6), on the other hand, are void of any identifiable diterpene lactones. Even the polar neoandrographolide, which was isolated in a good yield, was absent in these extracts. The characteristic signals at 4.5 – 3.0 ppm, although not fully identified here, can be deduced to be primarily sugars and amino acids (see Appendix B).

3.2 Metabolomics profiles of the Andrographis Herb from different locations

As stated earlier, this investigation does not intend to identify certain geographical locations that may facilitate the best crop production for the Andrographis Herb, nor does it intend to examine any geographical impacts on the crop productivity or the production yield of the herbs. However, comparing the metabolomics profiles in the herbs from different sources may help examining the possibility of using the selected references standards as reliable chemical markers. Also, knowing that the samples from different crop locations certainly accumulate different initial amounts of diterpene lactones, comparison across different crops may show whether their stability profiles are consistent and parallel to each other.

For each location, the samples from day 0 were selected as representative. This is to avoid the complication due to the degradation and transformations among the diterpene lactones over the storing period. The ^{1}H NMR spectrum of each sample was acquired and processed. Once normalized and bucketed, the multivariate analysis was performed. For the $CHCl_3$-extracts, PCA yielded a total of eight principal components (PC), with a final cumulative R^2 of 0.99. The first two principal components, however, readily yielded an acceptable ccumulative R^2 of 0.79.

The score plots and loading plots from the other principal components were readily examined to show a similar clustering patterns to those obtained from the analysis based on the first two components (data not shown). The following discussion is therefore focusing on only the first two components.

The score plot between PC1 and PC2 (Figure 7a) shows four clearly discriminating clusters corresponding to the herbs from the four provinces. Among these, the samples from Songkhla set themselves apart toward the positive quadrants of PC1. Those from the other three locations aggregate densely on the negative PC1, but spread out more widely and clearly on PC2 scales. The samples from Nakhon Pathom lean toward the positive PC2, whereas those from Songkhla and Suphan Buri do toward the negative scale, and those from Khon Kaen fall in the middle.

The loading plots indicate the influences of the three reference diterpene lactones in discriminating the herbs of each province from the others. Specifically focusing on the indicative signals of the three lactones (δ 6.95, H-12 of andrographolide; 7.07, H-14 of 14-deoxyandrographolide; and 7.15, 6.82, and 6.08, H-14, H-11, and H-12 of 14-deoxy-11,12-didehydroandrographolide; see section 3.1). The PC1 loading plot (54.2% contribution; Figure 7b), showed that all fell towards the negative PC scales. This indicates that the three compounds inversely discriminate the Songkhla samples from those of other three provinces. In other words, the Andrographis Herbs from Suphan Buri, Khon Kaen, and Nakhon Pathom have been characterized by the higher amount of the three diterpene lactones – and possibly also most of other minor related lactone derivatives – than that in the herbs from Songkhla. The positive scales of the three compounds shown in the PC2 loading plot (24.8% contribution; Figure 7c) indicate that the samples from Nakhon Pathom were set apart

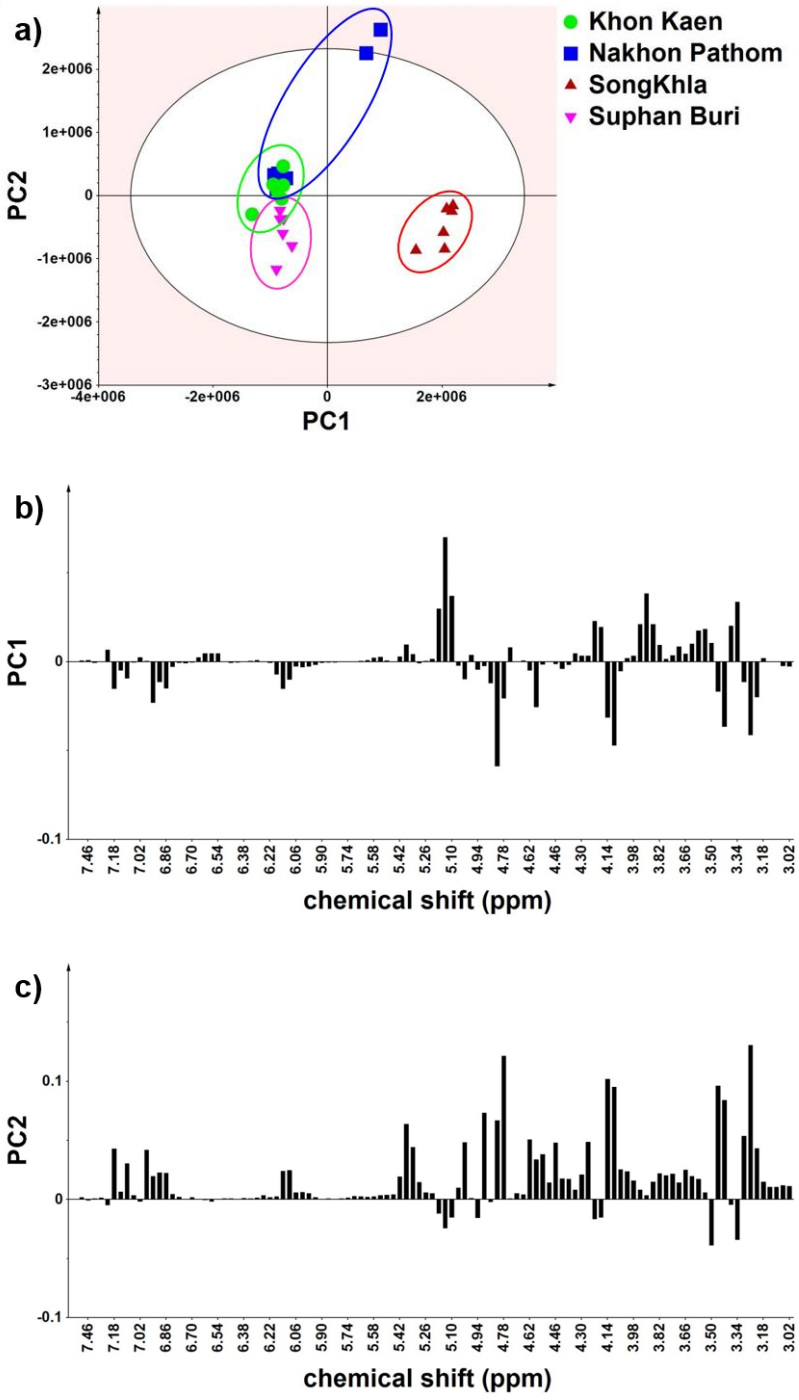


Figure 7. The score plot of PC1 and PC2 score of $CHCl_3$-extracts of the Andrographis Herbs (a), the loading plots; focusing between δ 7.5 – 3.0 ppm, for PC1 (b), and PC2 (c) of $CHCl_3$-extracts of the Andrographis Herbs, from the day-0 specimens from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla provinces

from others by their higher amount of the diterpene lactones.

For the aq MeOH-extracts, a total of three principal components were obtained, contributing to 93% of cumulative R^2 after the last PC. Shown in Figure 8a is the score plot of the aq MeOH-extracts based on the contribution of the first two PC (cumulative R^2 0.80). Four distinctive clusters corresponding to the four provinces can also be clearly observed. However, with very little, if any, diterpene lactones present in the aq MeOH-extracts, the contribution from the diterpene lactones, which are the major secondary metabolites in the Andrographis Herb, were considered negligible. Focusing on the loading plots based on PC1 and PC2 (61 and 19% contribution respectively) (Figure 8b-c), the most prominent signals were those resonating in the range of 3 - 5 ppm, belonging to the protons on the oxygenated carbons of sugars, and the α-protons of the amino acids. (see Appendix B).

Specifically focusing on the diterpene lactones, which are the major secondary metabolites and the active components in the Andrographis Herb, the results from the $CHCl_3$-extracts confirmed that the three major lactones, i.e., andrographolide, 14-deoxyandrographolide, and 14-deoxy-11,12-didehydroandrographolide, can be used as the markers for the metabolomics study in the following sections. On the other hand, the aq MeOH-extracts, which contain mainly the primary metabolites, and in which no diterpene lactones were observed, carry less conclusive information regarding the stability of the diterpene lactones in the herb. As for the effects of geographic variation, the Andrographis Herb from different crops naturally and expectedly yielded different metabolomics profiles both in the $CHCl_3$- and aq MeOH-extracts, i.e., the non-polar and polar compartments of the plants. Whereas this thesis does not attempt to

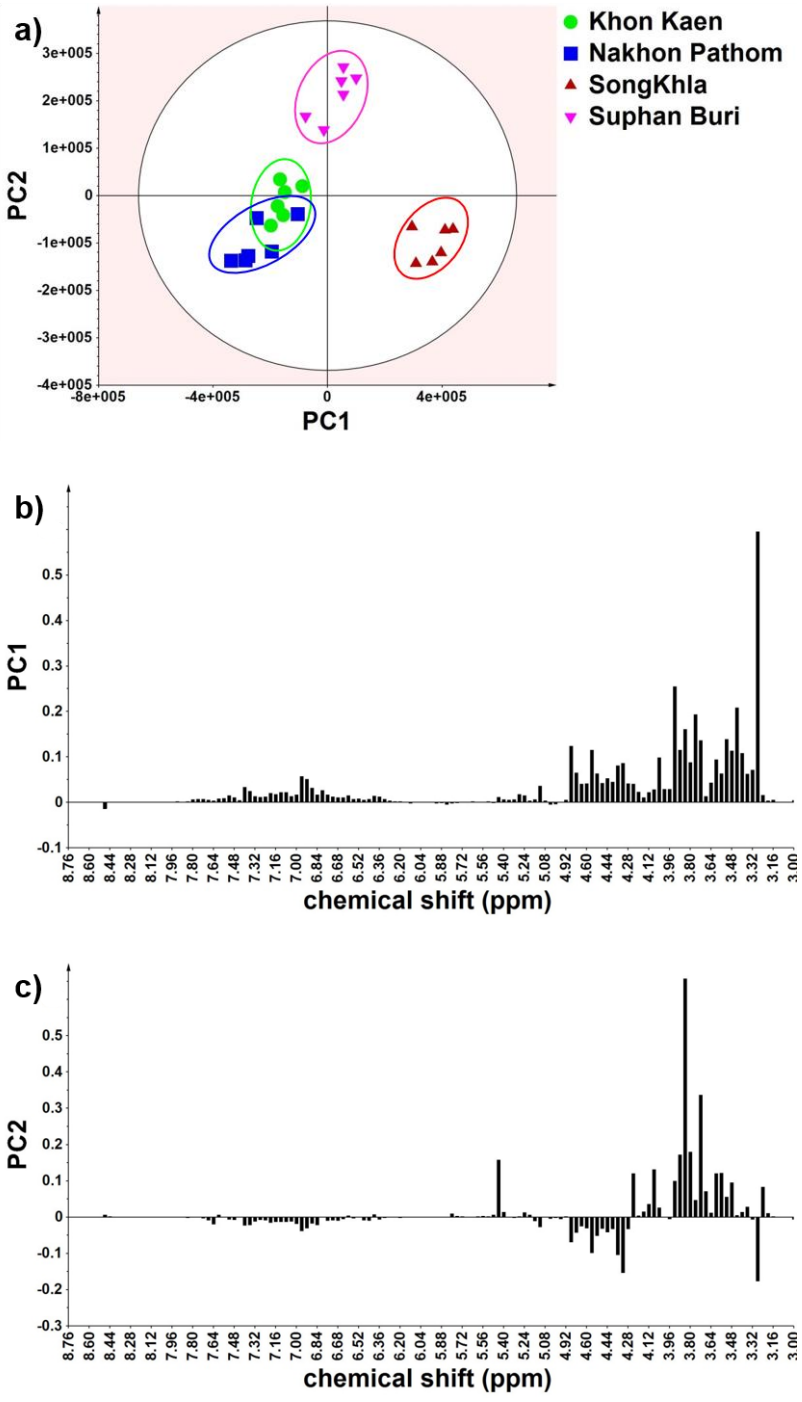


Figure 8. The score plot of PC1 and PC2 score of aq MeOH-extracts of the Andrographis Herbs (a), the loading plots; focusing between δ 8.8 – 3.0 ppm, for PC1 (b), and PC2 (c) of aq MeOH-extracts of the Andrographis Herbs, from the day-0 specimens from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla provinces

investigate either the genetic or environmental impacts on such differences, the results support the generalization that different crops from different locations and agricultural practices lead to the herbs of different quality.

3.3. Stability profile of the Andrographis Herb

3.3.1 Stability profiles based on the ^{1}H NMR-based metabolomics approach

As stated earlier, the primary objectives of this investigation is to examine the stability profiles of the Andrographis Herb using the NMR-based metabolomics approach in a real-time manner. That is, to examine if there are any systematic and consistent changes in the metabolomics profiles of the herb upon an extended period of conventional storage, and if there is any, which compounds could be the best indicative markers for such changes. The herbs from each supplier, including the one that was grown in-house, were stored separately in the storage conditions similar to those conventionally practiced by any manufacturers; i.e., storing of the whole, dried, and unprocessed plants as purchased in a series of light-protected packages. The storage periods started on the day that each purchased plant materials arrived at the lab as day 0. This is to mimic the real storage conditions as most manufacturers may have no knowledge on the precise harvesting dates and drying periods conducted by each supplier.

The stability profiles of the Andrographis Herb were performed on both the $CHCl_3$- and aq MeOH-extracts of the herbal specimens from each supplier as described. Using PCA for the statistical analysis, the results from each supplier were consistent and parallel well to the others (vide infra). Here, the results from Suphan Buri Province are selected as a representative for the discussion and as an introduction

for baseline. The extended discussion and comparison with the other three suppliers will be addressed as an individual supplier basis.

PCA of the $CHCl_3$-extracts of the Andrographis Herb from Suphan Buri Province after six-month storage yielded a total of six principal components, with cumulative R^2 of 0.99 and q^2 of 0.97 from the first three PCs. The score plots based on the combination of the first three PCs (Figure 9; (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3) were all parallel, and showed that the dataset clustered according to the storage time. For example, the PC2-PC3 plot (Figure 9c), which is the clearest representative, shows that the data from the first two months cluster on the negative scale of PC2 axis, and the plot gradually shifts toward the positive end as time progresses (see the scatterplot for PC2, Figure 10b).

Note that, for this dataset ($CHCl_3$-extracts of samples from Suphan Buri), PC2 showed a gradual shift in a good accordance to time whereas the other PCs, despite allowing the observable clusters, did not conform to a time-wise manner (Figure 9; also see the scatterplots for each PC, Figure 10). This can be attributed to the variables that fluctuate over the storage period; for examples, compound(s) of which the transformation is in equilibria with other related derivative(s) or undergoes multi-step transformations/degradations. Nevertheless, in this study, the discussion is primarily focusing on the compounds that either incline or decline consistently (i.e., in this case, the PC2 of $CHCl_3$-extracts of Suphan Buri samples), hence allowing the direct prediction for the stability of the herb itself.

The loading plot based on PC1 from the $CHCl_3$-extracts of Suphan Buri samples (Figure 11a) showed the significant positive scores for all the indicative signals

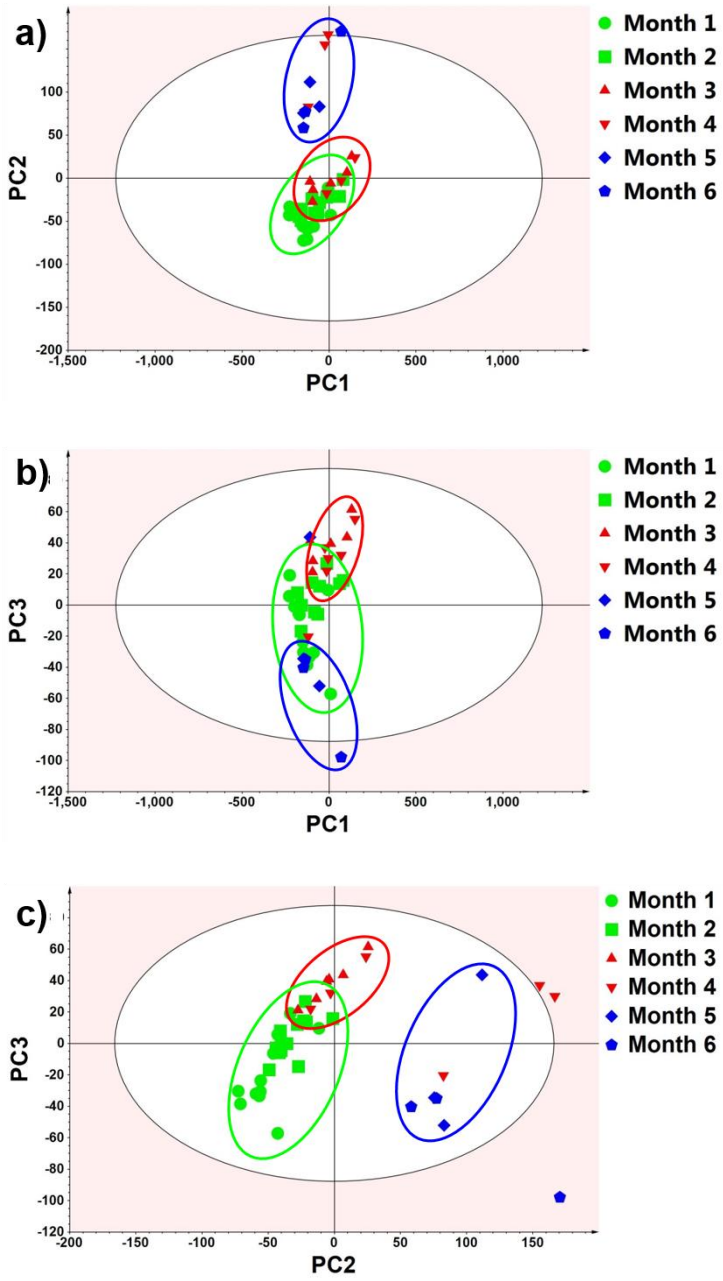


Figure 9. The score plots of $CHCl_3$-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative $R^2 = 0.99$ and $q^2 = 0.97$, after first three PCs)

Note; For clarify, the outlier were removed from the plot between PC1/PC2 and PC1/PC3

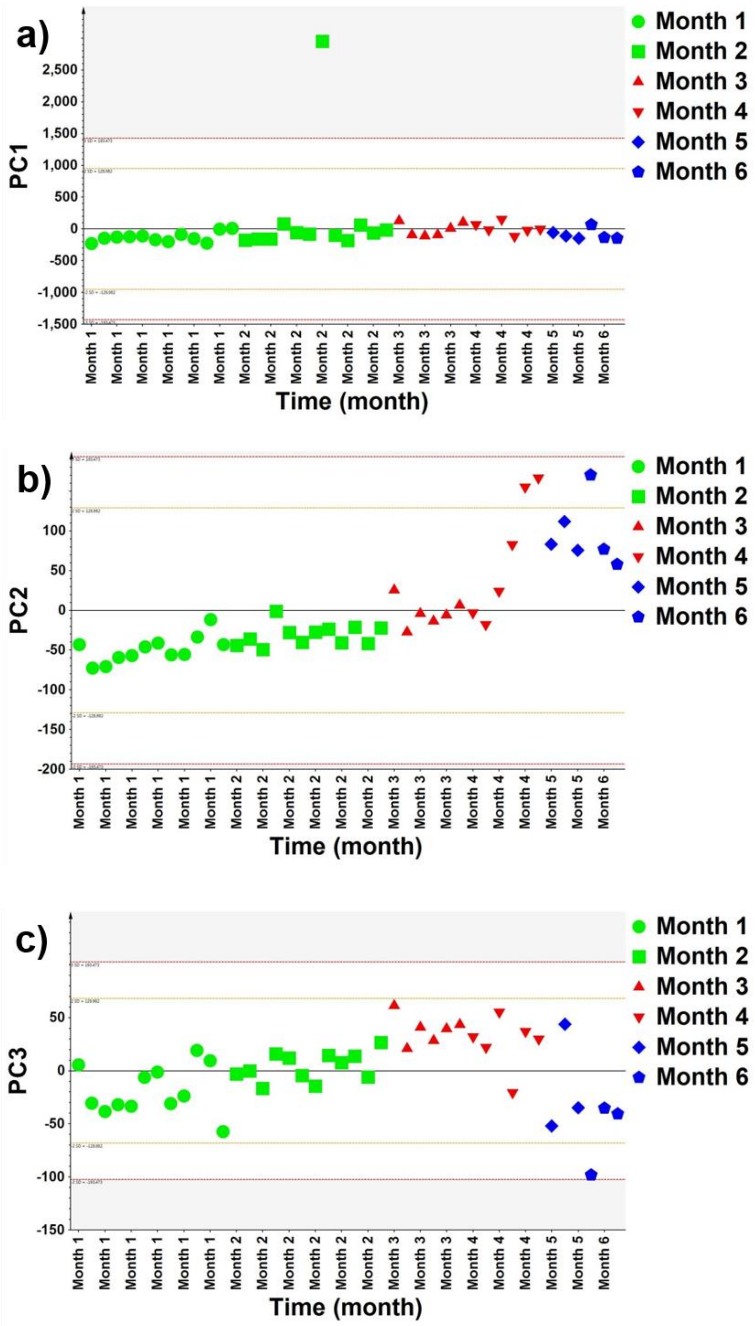
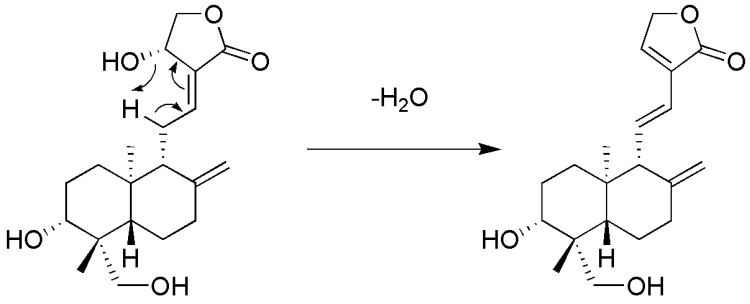


Figure 10. The scatterplots of $CHCl_3$-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

of the three diterpene lactone markers (δ 6.95, H-12 of andrographolide; 7.07, H-14 of 14-deoxyandrographolide; and 7.15, H-14; 6.82, H-11; and 6.08, H-12 of 14-deoxy-11,12-didehydroandrographolide). This signifies the importance of the three compounds to each sample in this dataset. Focusing on the PC2 loading plot (Figure 11b), however, only the indicative signals of 14-deoxy-11,12-didehydroandrographolide (δ 7.15, 6.82, and 6.08) stood out and yielded their distinguishably positive scores. Referred to the aforementioned scatterplot based on PC2 (Figure 10b), these positive scores indicated that 14-deoxy-11,12-didehydroandrographolide was the compound that influenced the time-wise clustering as seen in PC2/PC3 score plot (Figure 9c). That is, throughout the storage period of the Andrographis Herb, the amount of 14-deoxy-11,12-didehydroandrographolide had gradually increased, thus suggesting that 14-deoxy-11,12-didehydroandrographolide is one of the primary decomposition products. This agrees well with the previous report by Lomlim et al (2003), in which 14-deoxy-11,12-didehydroandrographolide was identified as the major decomposition product of andrographolide upon being exposed to the heat-accelerated conditions. The mechanism of this decomposition toward 14-deoxy-11,12-didehydroandrographolide may be proposed as a cascade of elimination/dehydration on C-11 – C-14 of andrographolide (Scheme 1).



Scheme 1. Degradation of andrographolide toward 14-deoxy-11,12-didehydroandrographolide

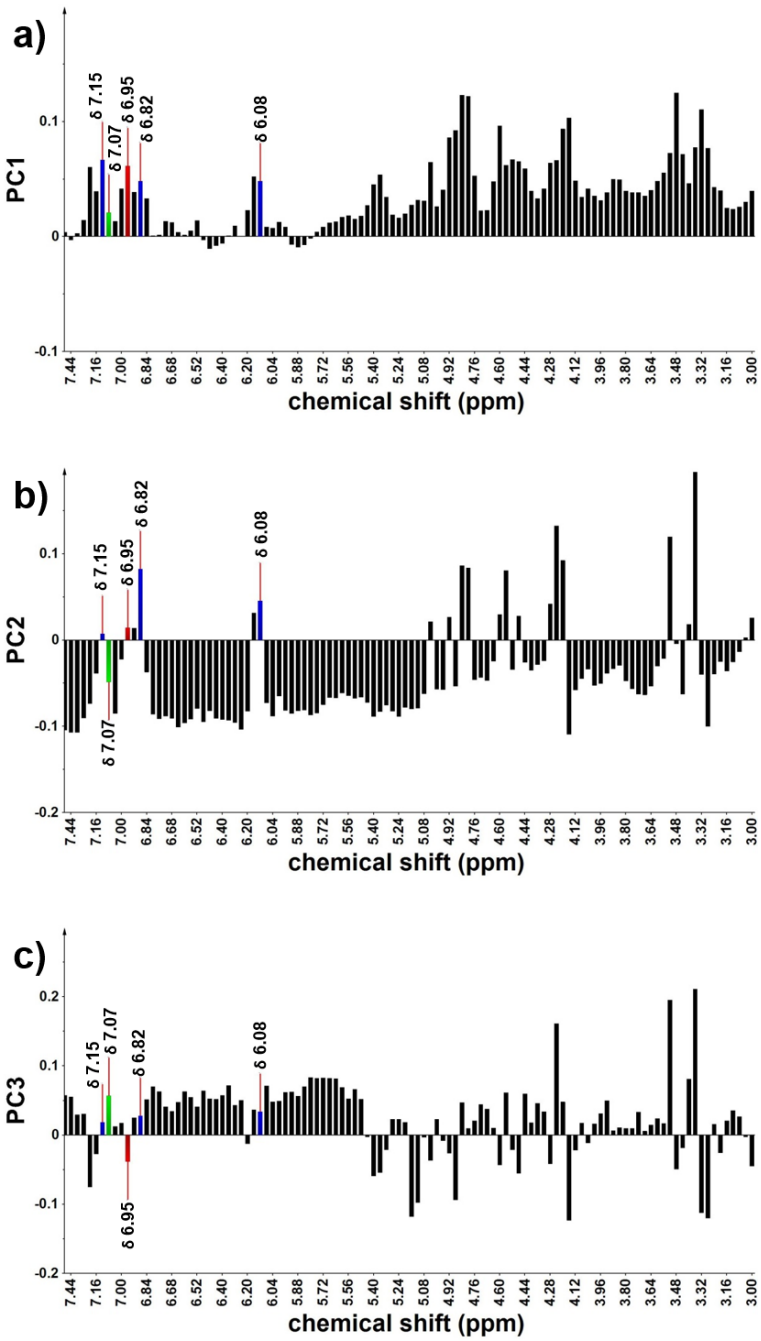


Figure 11. The loading plots of $CHCl_3$-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

The gradual increase of 14-deoxy-11,12-didehydroandrographolide can also be seen directly in the NMR spectra of the $CHCl_3$-extracts of the stored herb (Figure 12). The three indicative signals (δ 7.15, 6.82, and 6.08) had gradually increased over the storage period. On the other hand, although unable to be accounted for their changes, the integrations for the signals of andrographolide and 14-deoxyandrographolide (δ 6.95 and 7.07, respectively) were inconsistent and did not allow a precise extrapolation.

Similarly, the score plots based on the samples from the other three suppliers, i.e., from Khon Kaen, Nakhon Pathom, and Songkhla (Figures 13, 16, and 19, respectively), were examined in the same manner as that for the samples from Suphan Buri. Whereas the plots seem not allowing a direct comparison due to different

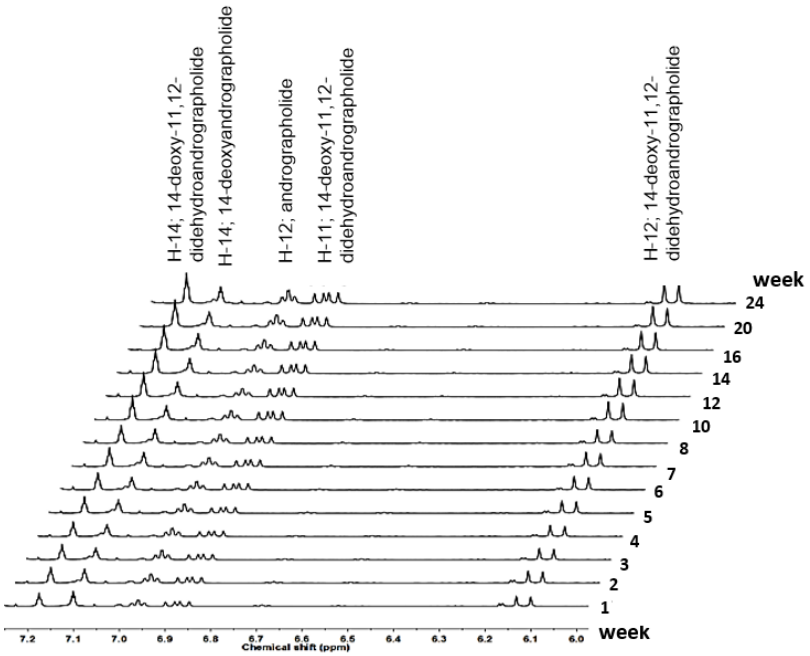


Figure 12. Overlaid ^1H NMR spectra (500 MHz; $CDCl_3$) of the $CHCl_3$-extracts of the Andrographis Herb from Suphan Buri Province (δ 7.3 – 6.0 ppm, weeks 0-24)

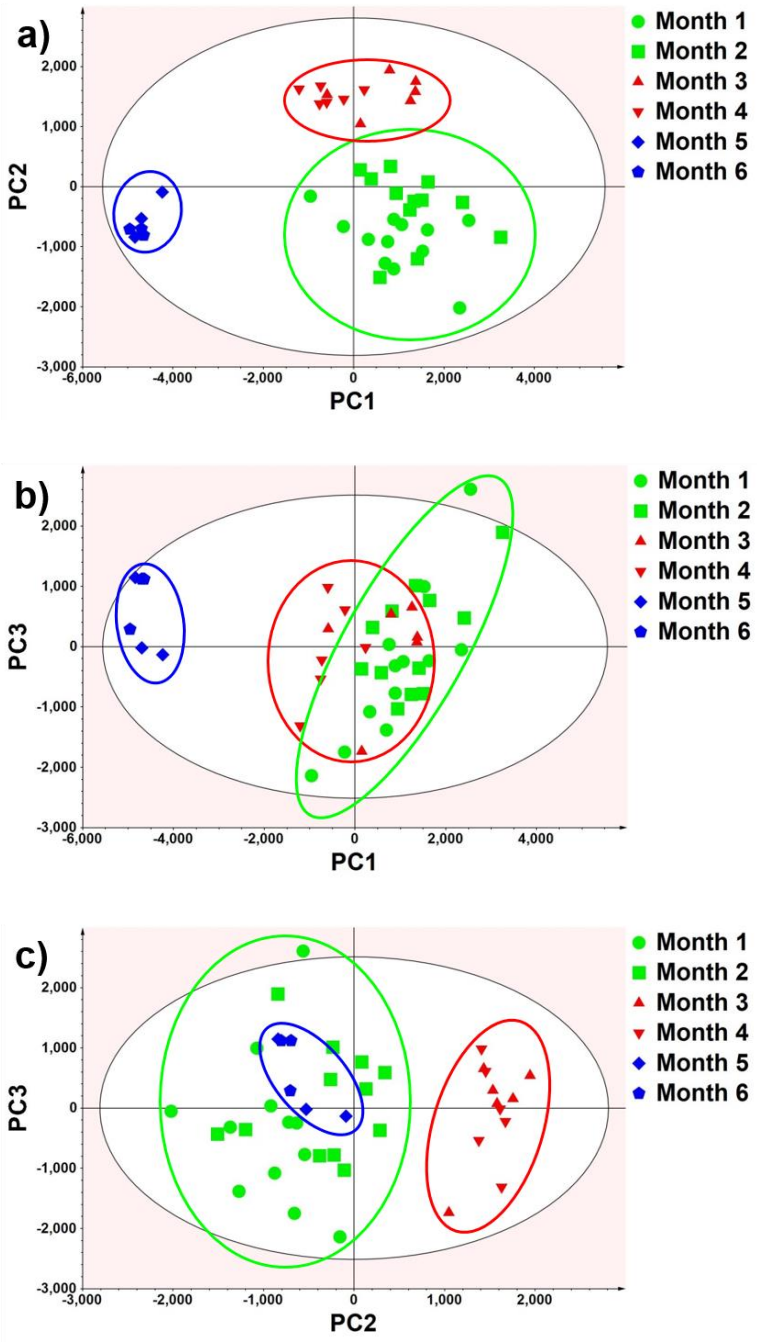


Figure 13. The score plots of $CHCl_3$-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative $R^2 = 0.84$ and $q^2 = 0.76$, after first three PCs)

metabolomics baselines (see section 3.2), they are all in fact comparable. In the samples from each supplier, the dataset clustered according to the storage time, with a gradual shift in a time-wise manner that can be observed from at least one PC (see the scatterplots of PC1 for Khon Kaen, PC3,for Nakhon Pathom, and PC2 for Songkhla, Figures 14a, 17c, and 20b, respectively). The loading plots based on the according PCs showed the relevance between the indicative signals of 14-deoxy-11,12-didehydroandrographolide (δ 7.15, 6.82, and 6.08) and the time-wise shifts discussed above (Figures 15b, 18c, and 21b, for the samples from Khon Kaen, Nakhon Pathom, and Songkhla, respectively). The results agreed well those observed in the samples from Suphan Buri, and confirmed that among the three diterpene lactones, 14-deoxy-11,12-didehydroandrographolide, as a decomposition product, is the best indicator for tracing the stability of the Andrographis Herb. In other words, the amount (or the ratio to other diterpene lactones) of 14-deoxy-11,12-didehydroandrographolide may be used as a suggestive indicator of how old the Andrographis Herb raw material is and how long the herb has been storage. This is also supported by the increasing intensity in the ^{1}H-NMR signals of 14-deoxy-11,12-didehydroandrographolide as seen in the overlaid spectra of the extracts from each supplier according to the storage times (Figures 22a-c).

PCA on the aq MeOH-extracts from each supplier yielded the score plots in which the dataset formed a series of time-related clusters in a comparable fashion to that of the $CHCl_3$-extracts (Figures 23,26, 29, and 32, for the samples from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla, respectively). From the score plots from each suppliers, the relevant loading plots (Figures 25, 28, 31, and 34, for the samples from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla, respectively),

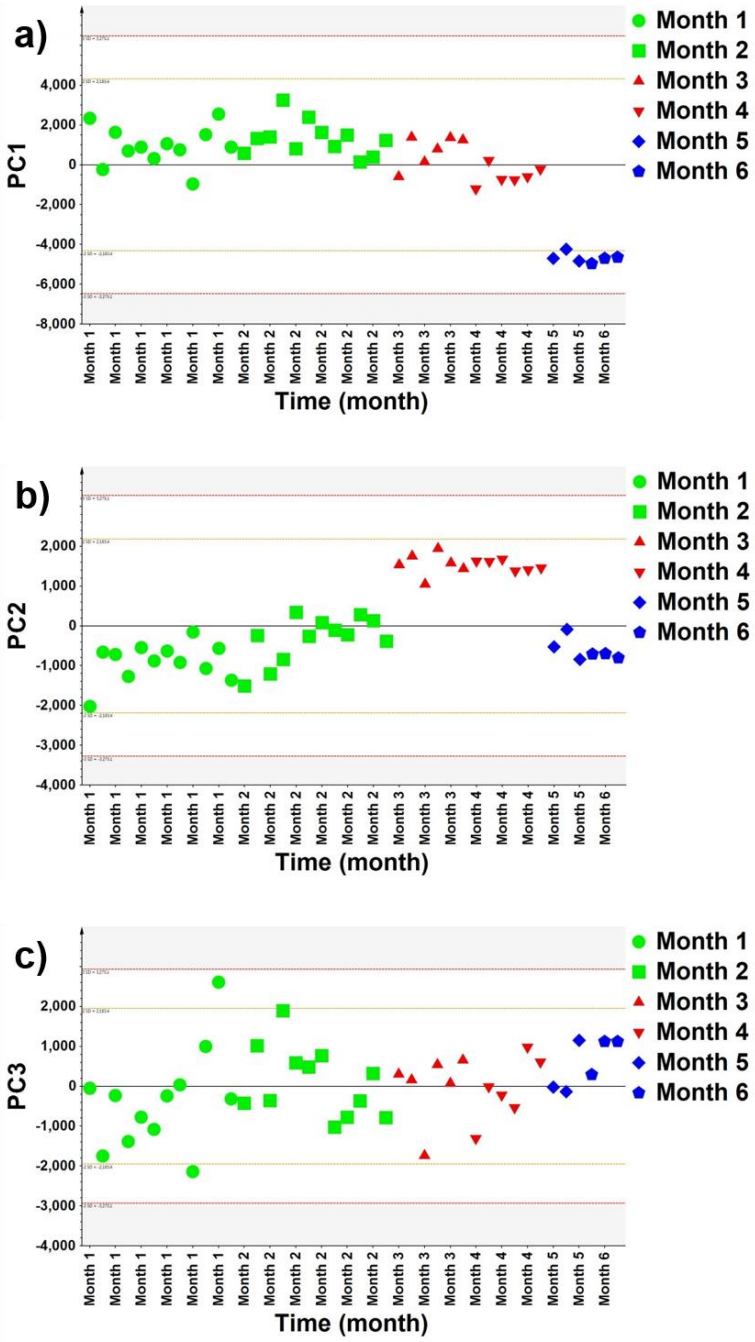


Figure 14. The scatterplots of $CHCl_3$-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

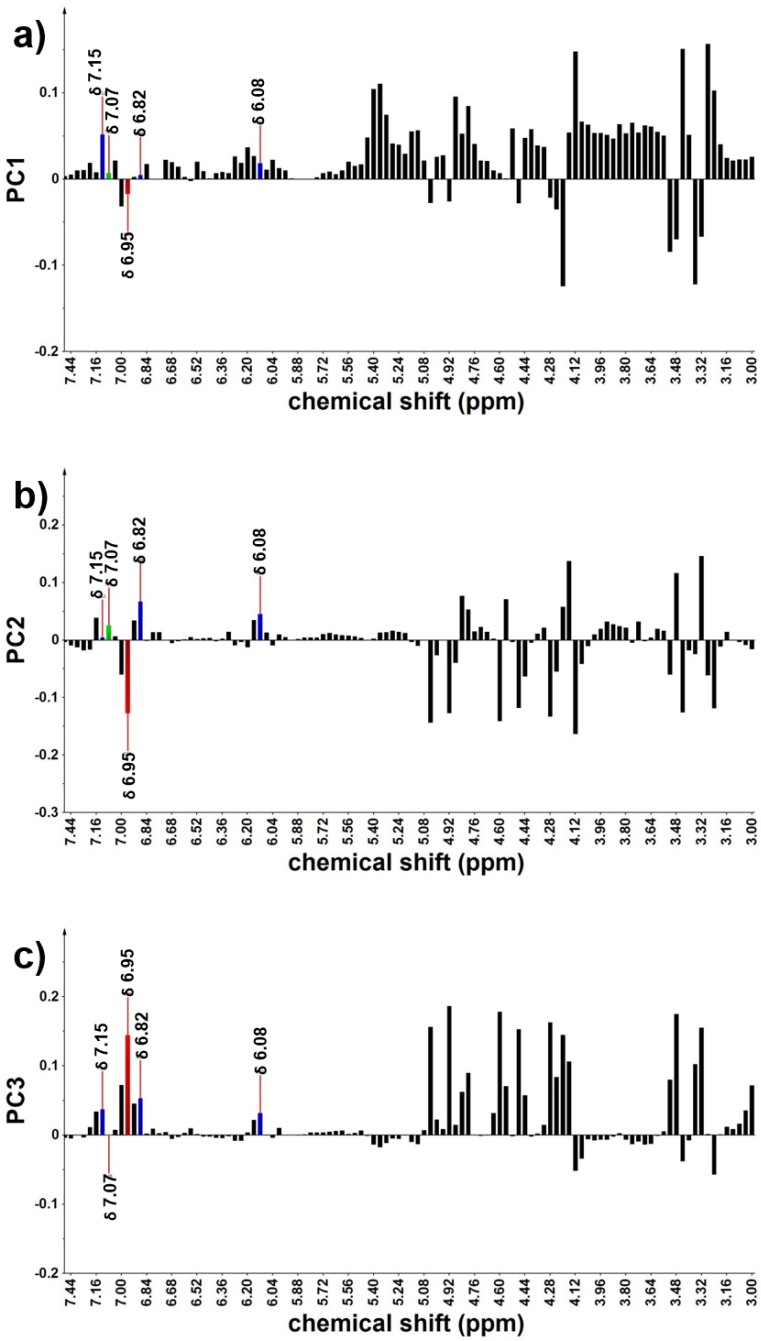


Figure 15. The loading plots of $CHCl_3$-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

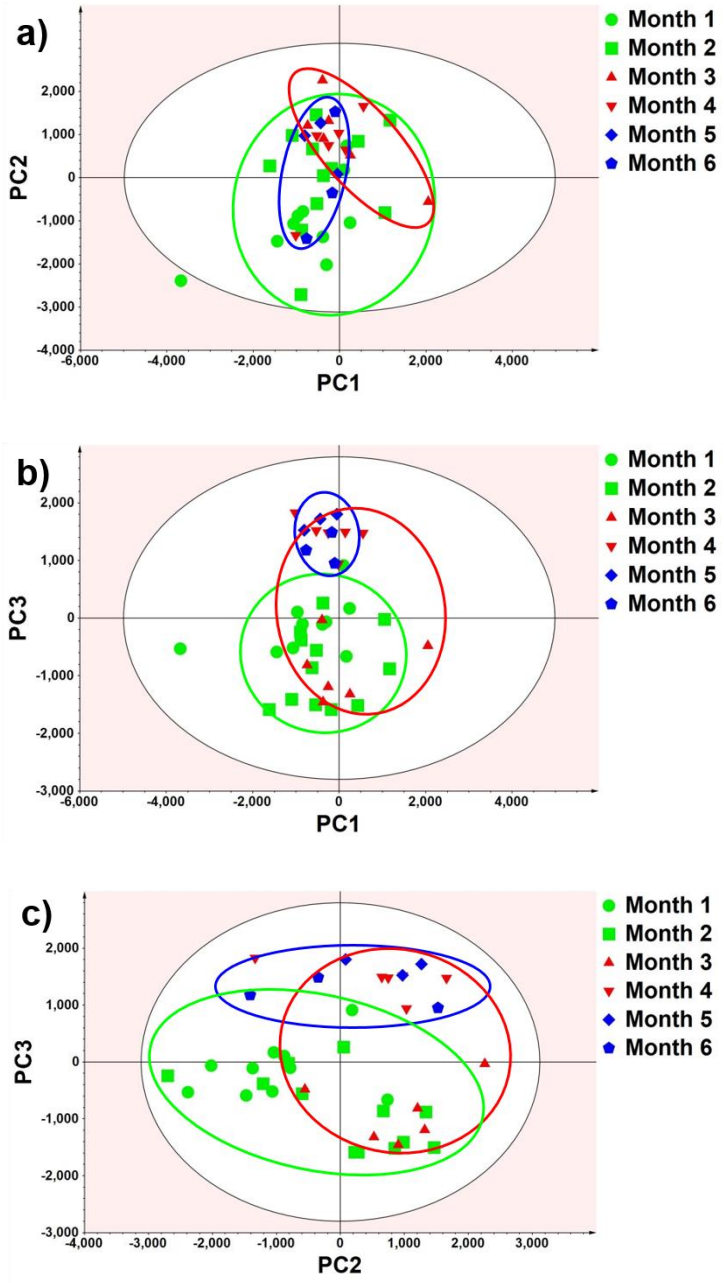


Figure 16. The score plots of $CHCl_3$-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative $R^2 = 0.84$ and $q^2 = 0.76$, after first three PCs)

Note; For clarify, the outlier were removed from the plot between PC1/PC2 and PC1/PC3

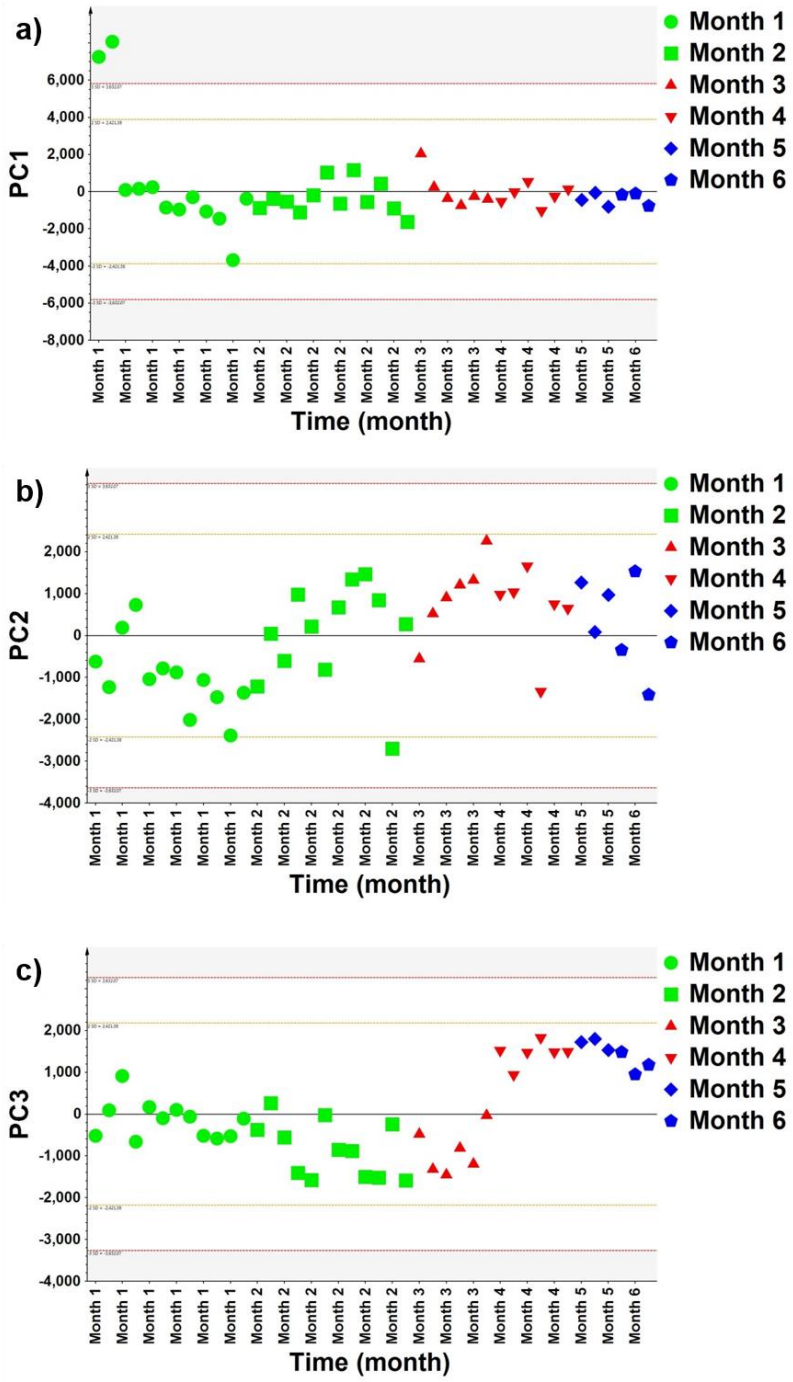


Figure 17. The scatterplots of $CHCl_3$-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

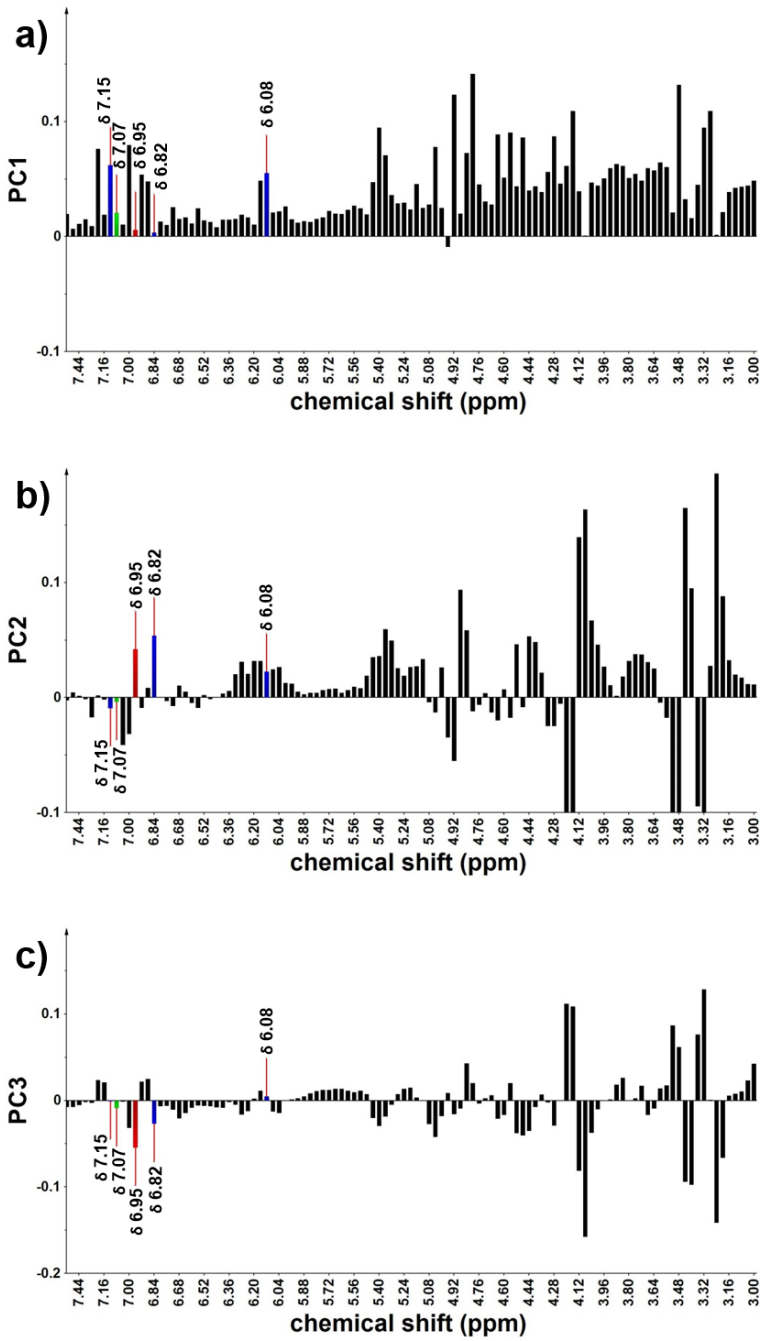


Figure 18. The loading plots of $CHCl_3$-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

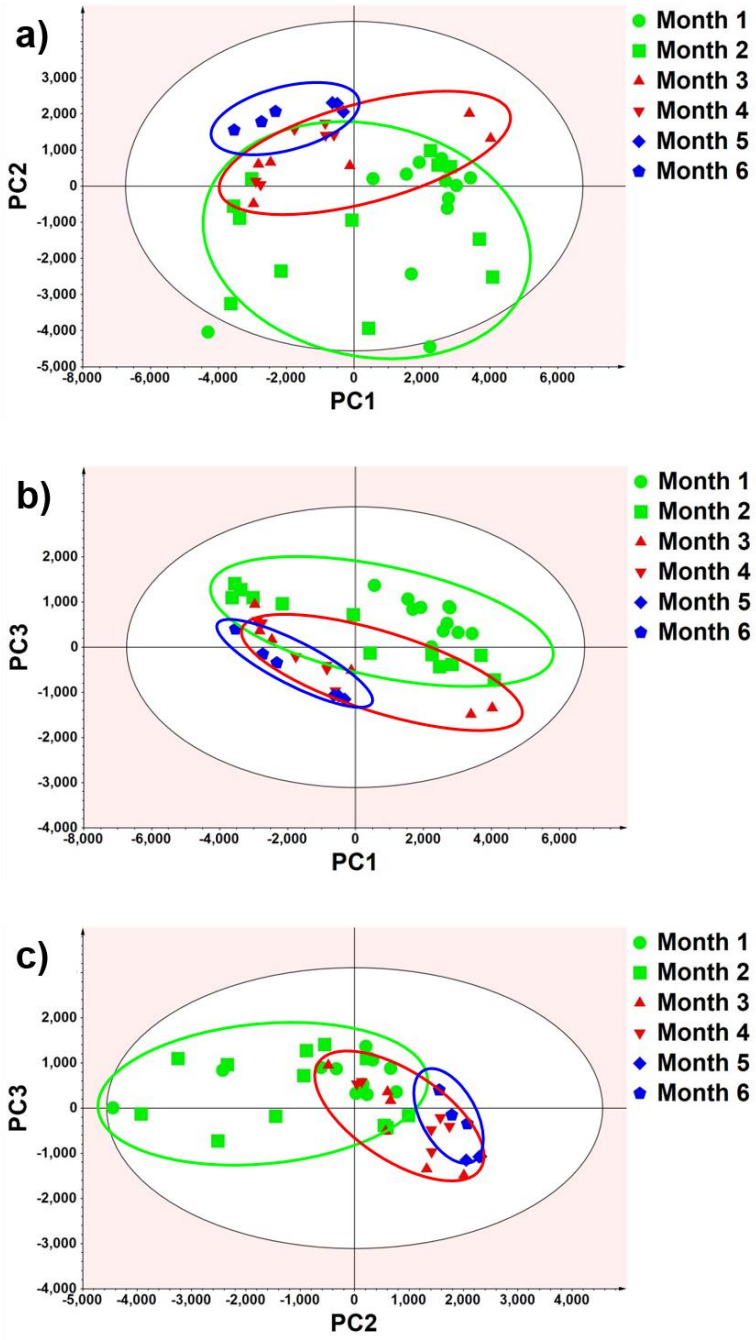


Figure 19. The score plots of $CHCl_3$-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative $R^2 = 0.75$ and $q^2 = 0.51$, after first three PCs) Note; For clarify, the outlier were removed from the plot between PC1/PC3 and PC2/PC3

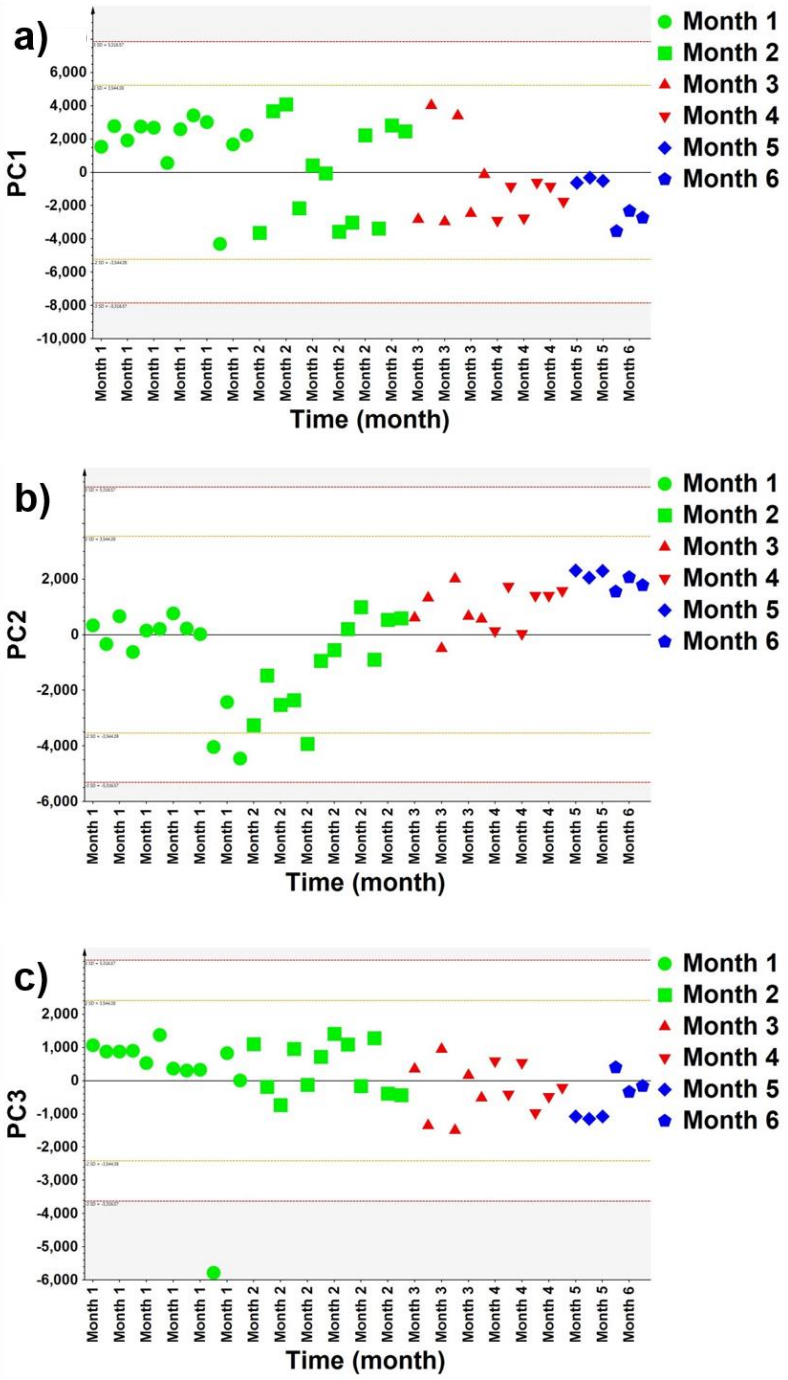


Figure 20. The scatterplots of $CHCl_3$-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

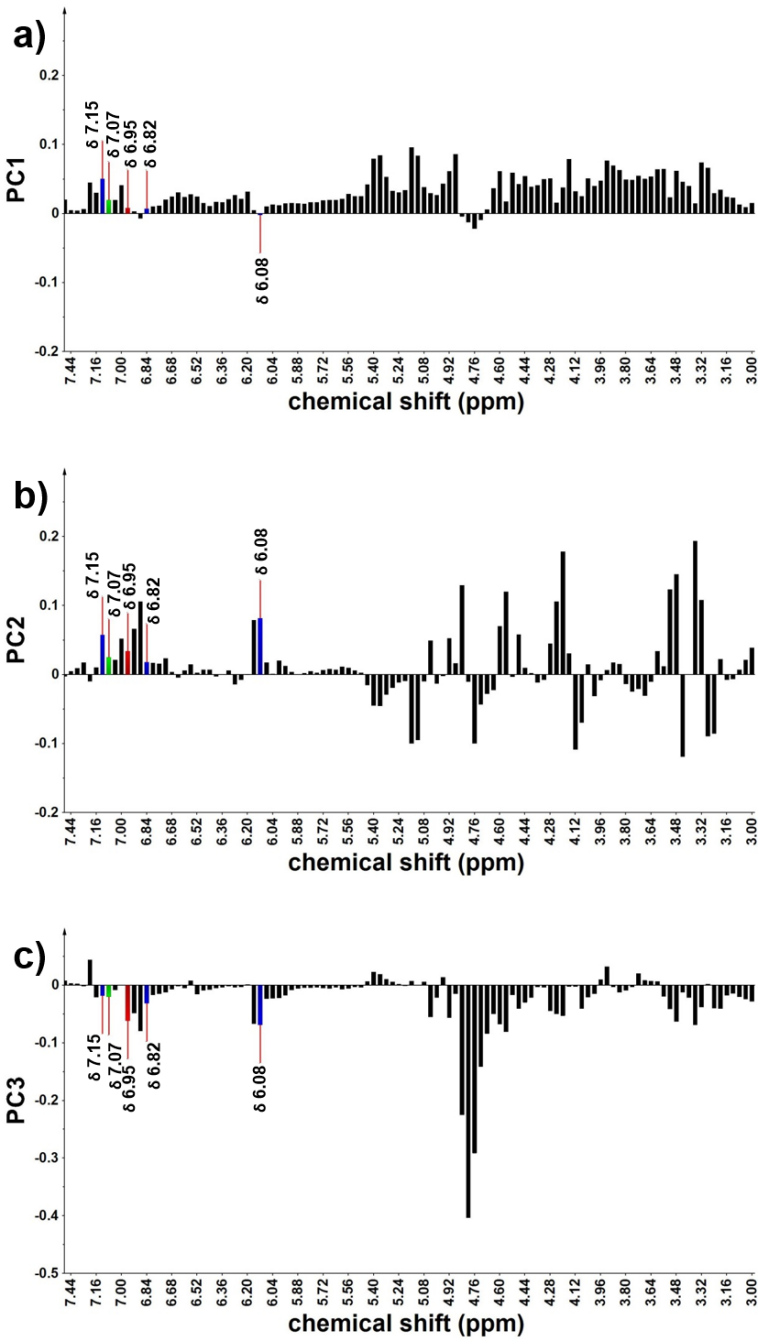


Figure 21. The loading plots of $CHCl_3$-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

showed that the NMR signals corresponding to the clusters described above fell into the range of 3 - 4 ppm, mainly belonging to sugars (see section 3.2). In most cases, the sugar contents in the herbs gradually decreased over the period of the storage. This spontaneous loss of sugar and related primary metabolites is not surprising. However, with no significant presence of the diterpene lactones in the aq MeOH-extracts from the herbs from any suppliers, the aq MeOH-extracts have less implication on tracing the stability of the Andrographis Herb than do the $CHCl_3$-extracts.

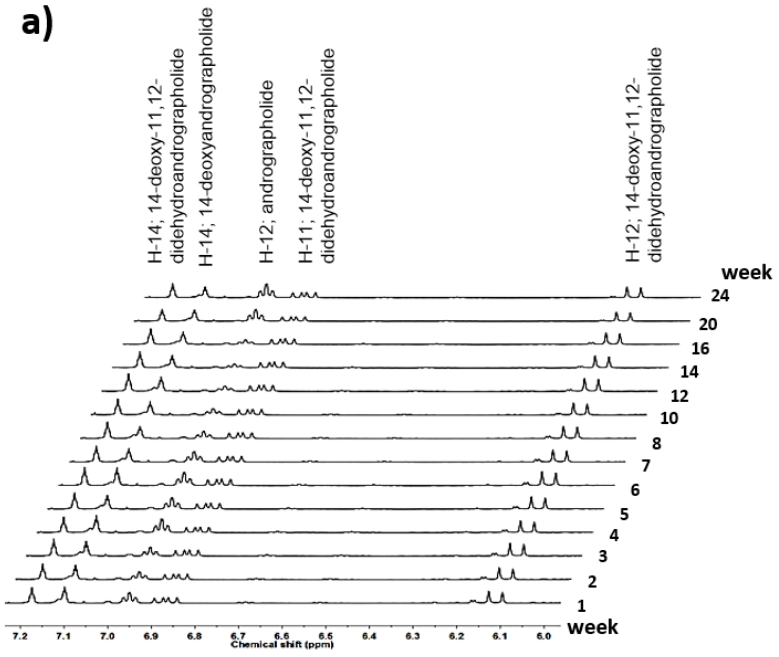


Figure 22. Overlaid ^{1}H NMR spectra (δ 7.3 – 6.0 ppm, 500 MHz; $CDCl_3$) of the $CHCl_3$-extracts of the Andrographis Herb from Khon Kaen (a) (this page), Nakhon Pathom (b), and Songkhla (c) (next page)

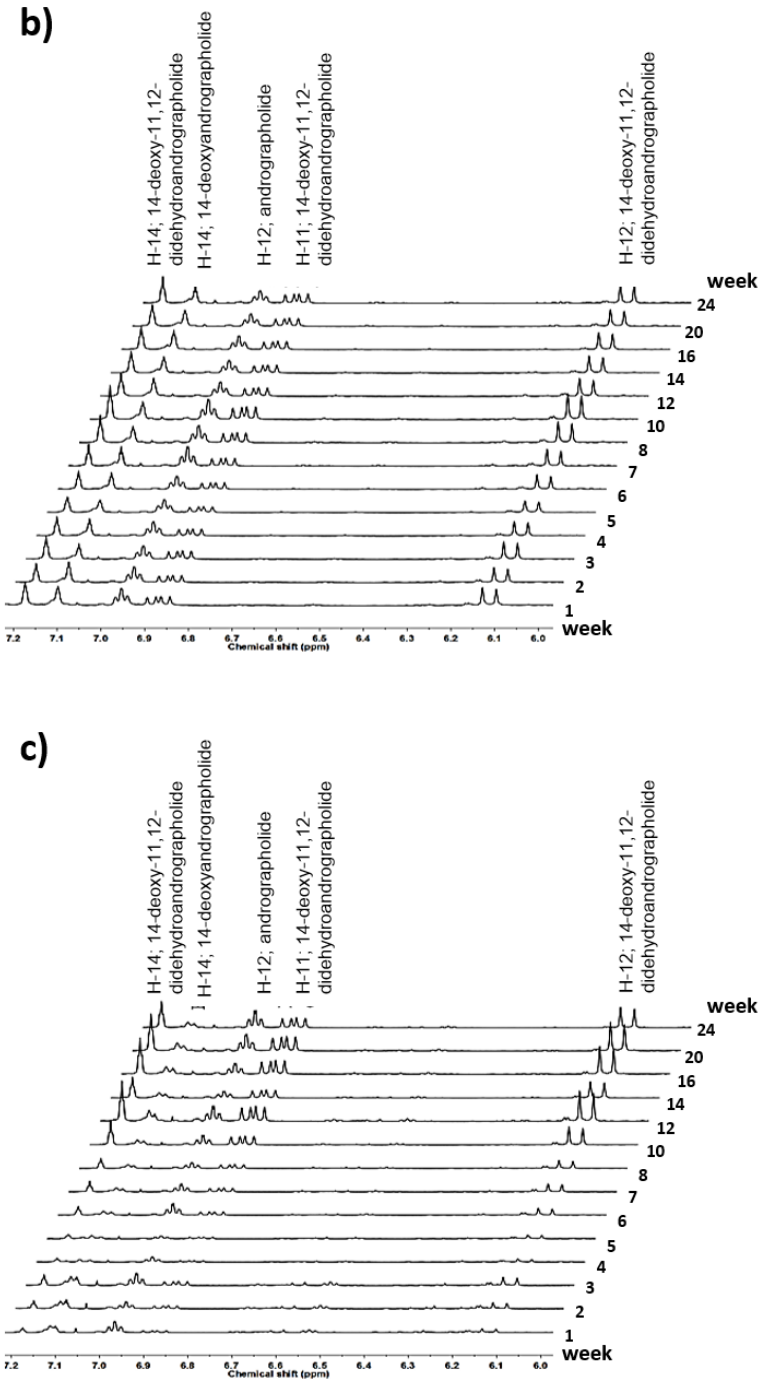


Figure 22. (cont).

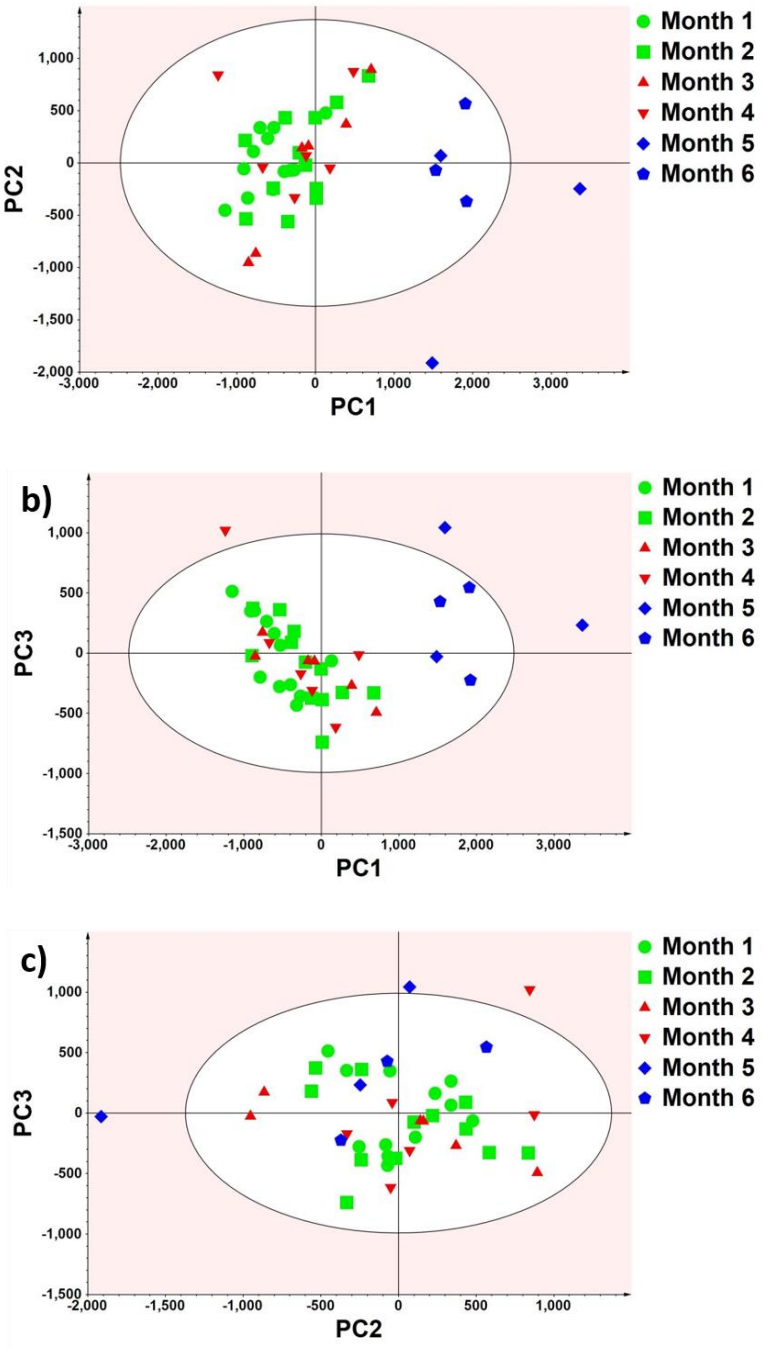


Figure 23. The score plots of aq MeOH-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative R^2 = 0.86 and q^2 = 0.64, after first three PCs)

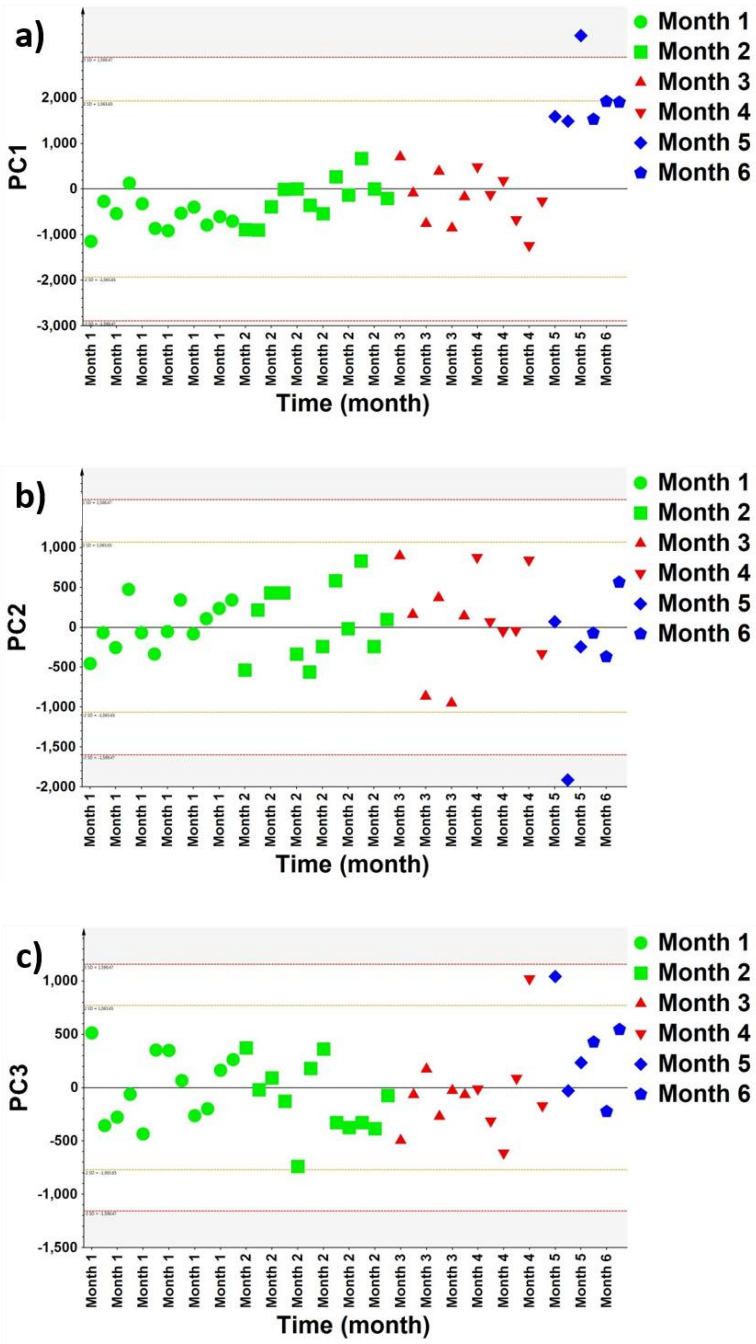


Figure 24. The scatterplots of aq MeOH-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

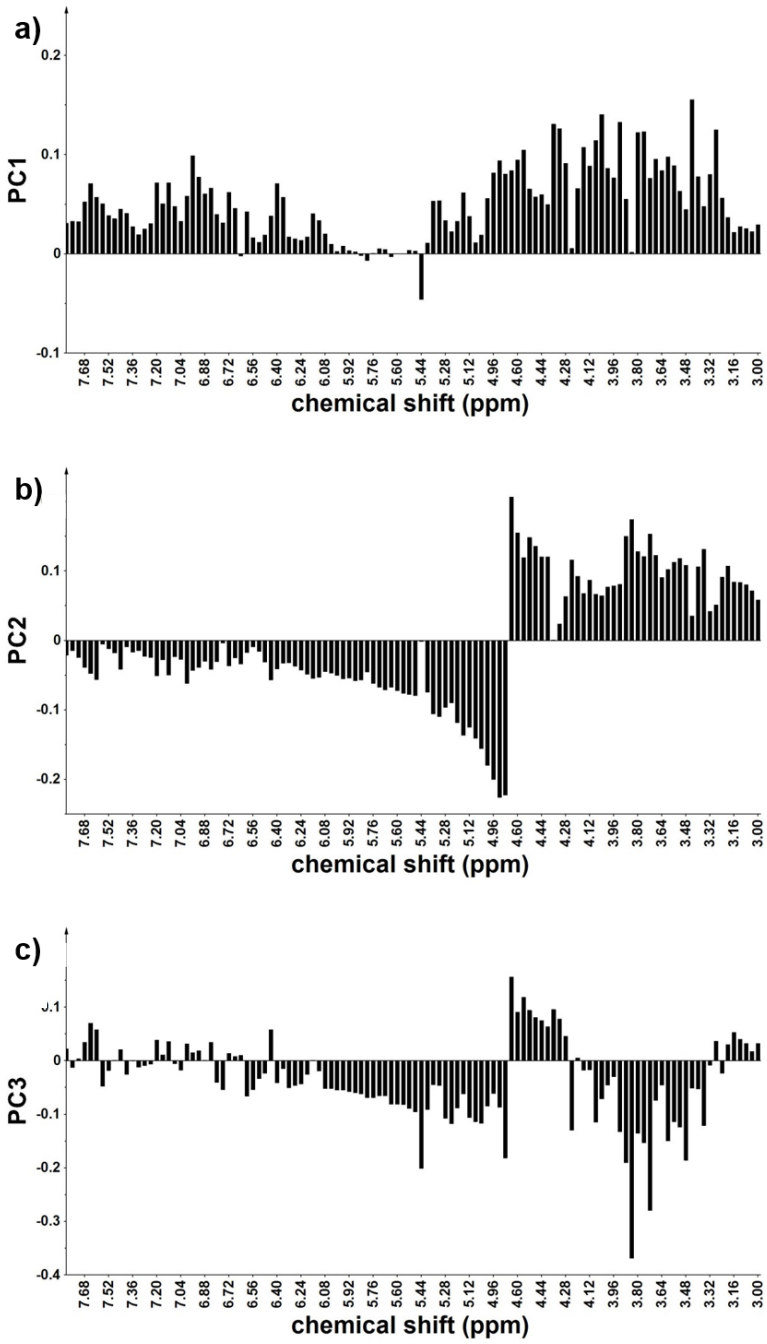


Figure 25. The loading plots of aq MeOH-extracts of the Andrographis Herb from Suphan Buri Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

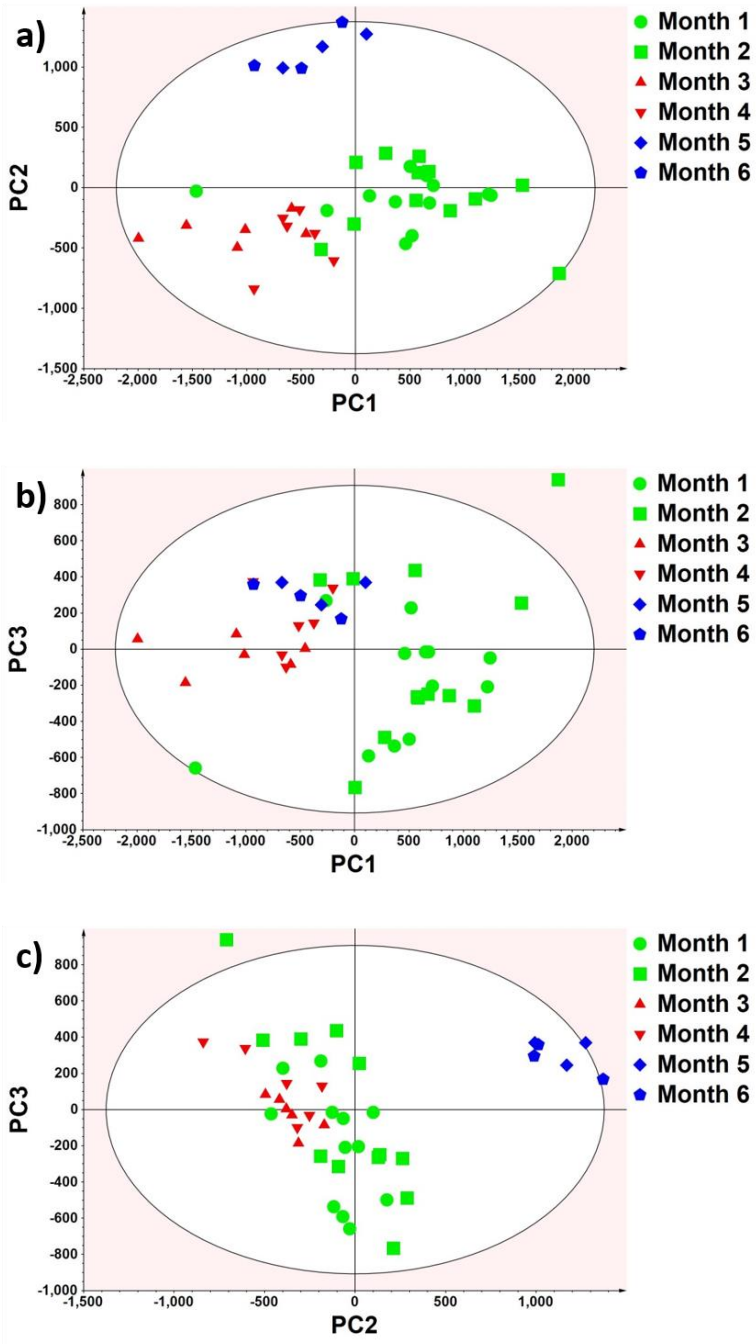


Figure 26. The score plots of aq MeOH-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative R^2 = 0.77 and q^2 = 0.68, after first three PCs)

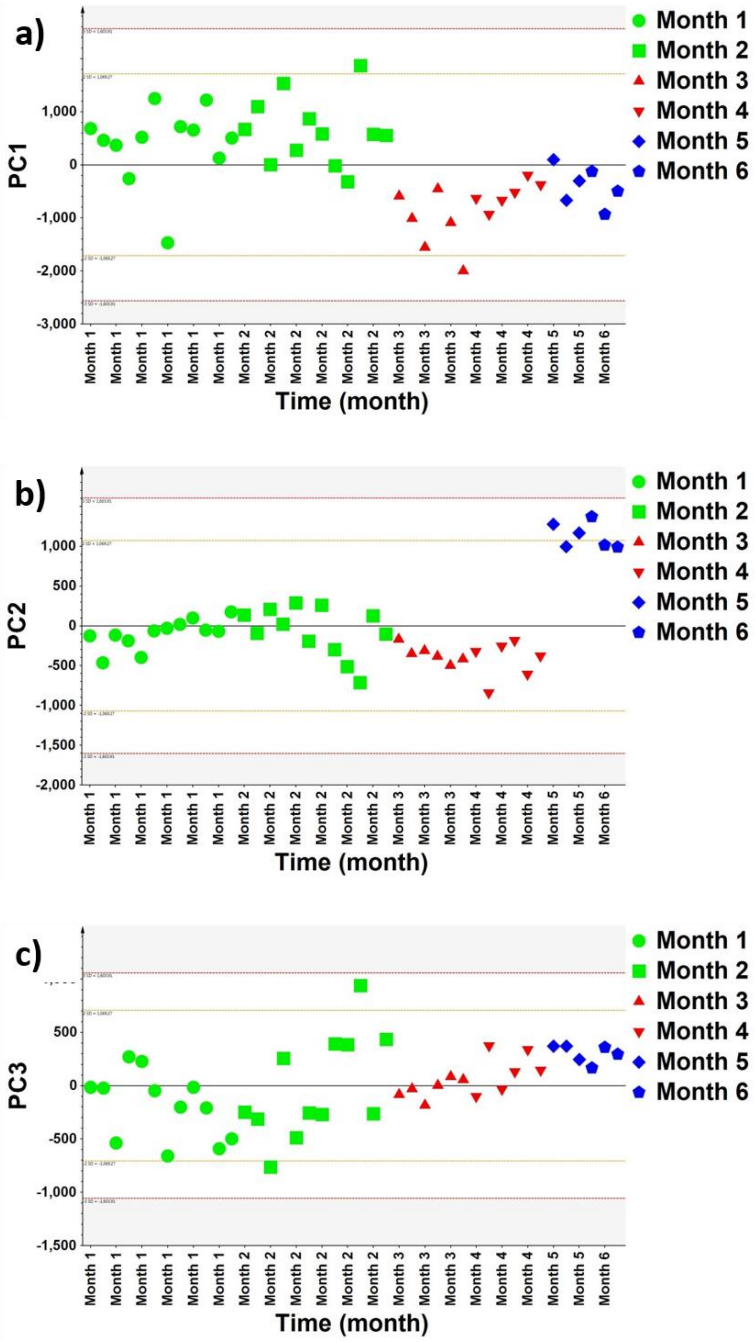


Figure 27. The scatterplots of aq MeOH-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

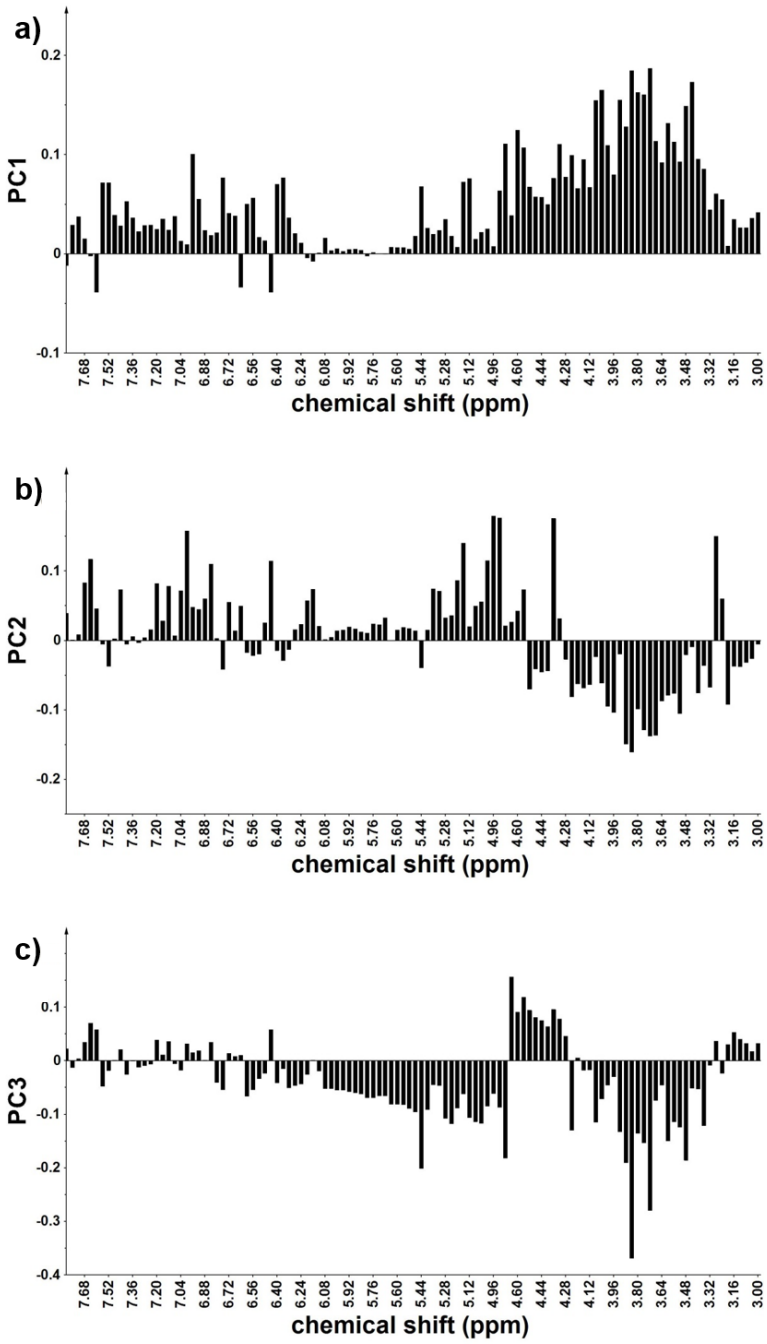


Figure 28. The loading plots of aq MeOH-extracts of the Andrographis Herb from Khon Kaen Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

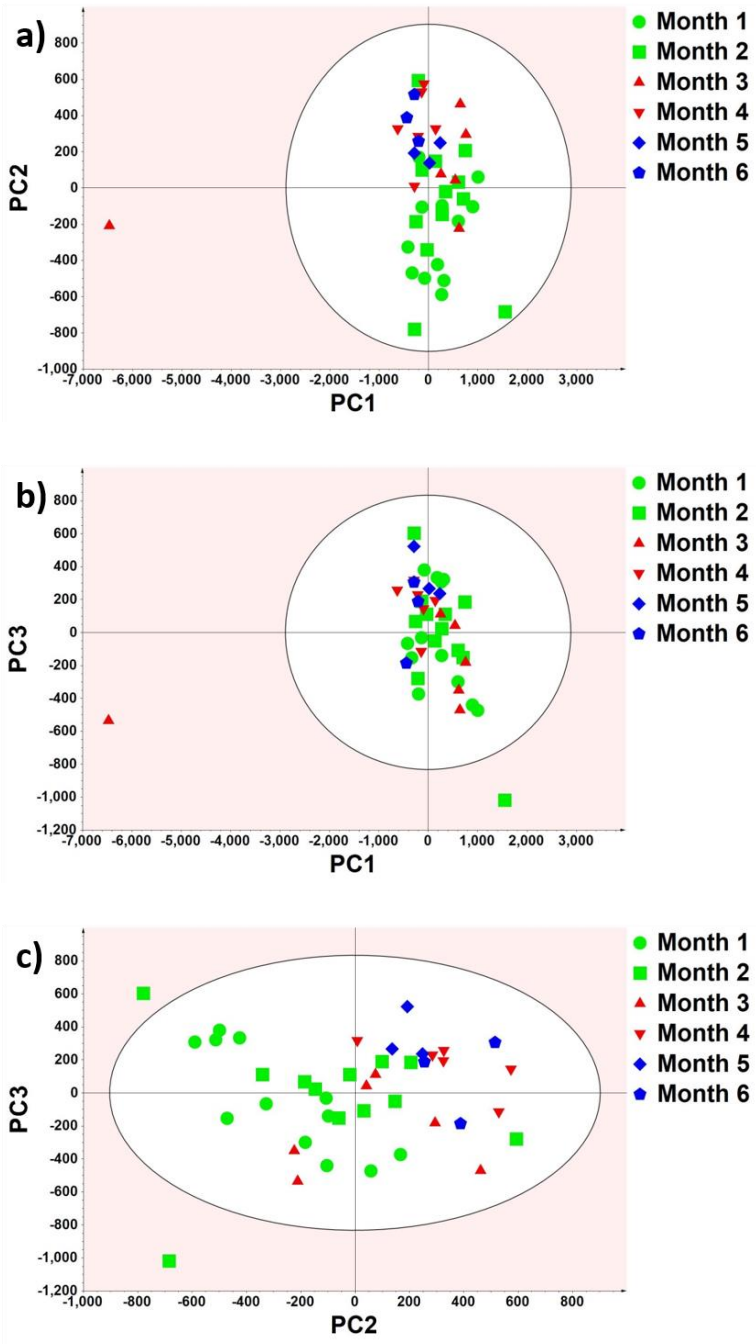


Figure 29. The score plots of aq MeOH-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative R^2 = 0.86 and q^2 = 0.64, after first three PCs)

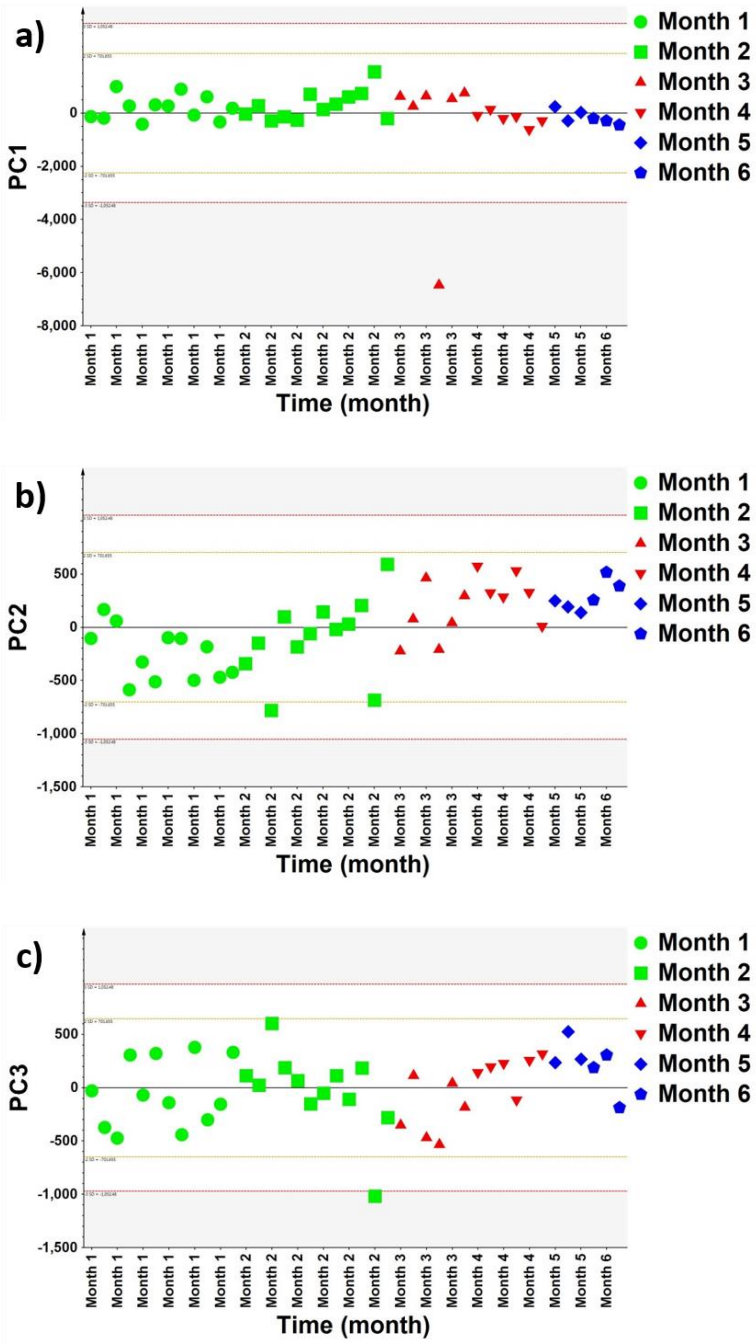


Figure 30. The scatterplots of aq MeOH-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

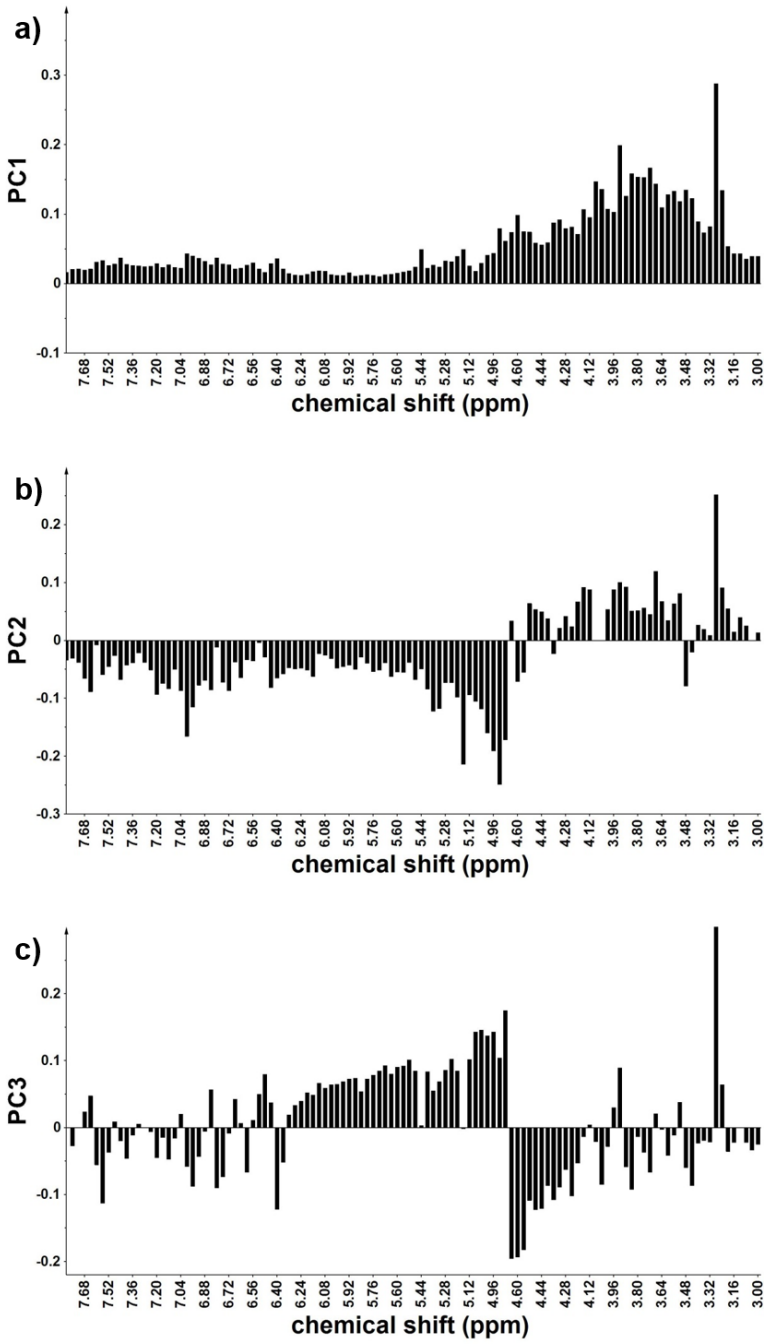


Figure 31. The loading plots of aq MeOH-extracts of the Andrographis Herb from Nakhon Pathom Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

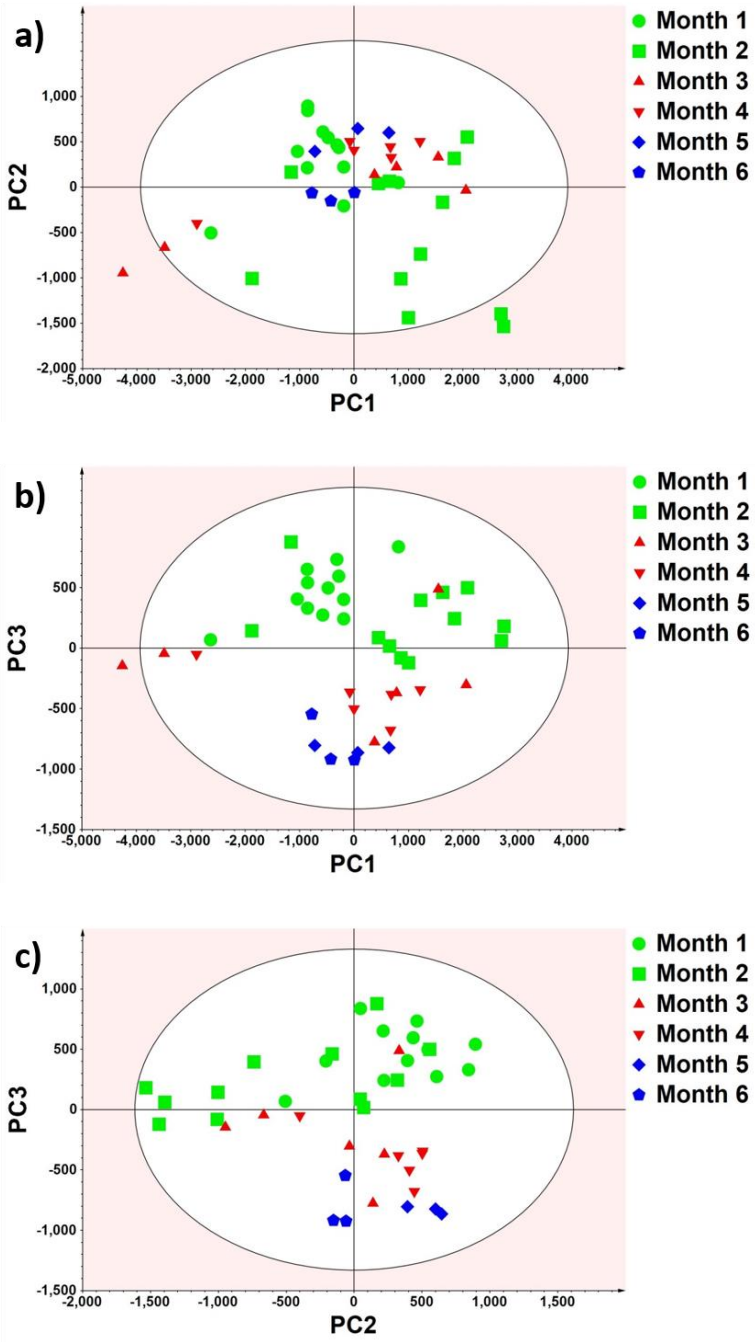


Figure 32. The score plots of aq MeOH-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1 vs. PC2, (b) PC1 vs. PC3, and (c) PC2 vs. PC3 respectively (cumulative R^2 = 0.86 and q^2 = 0.64, after first three PCs)

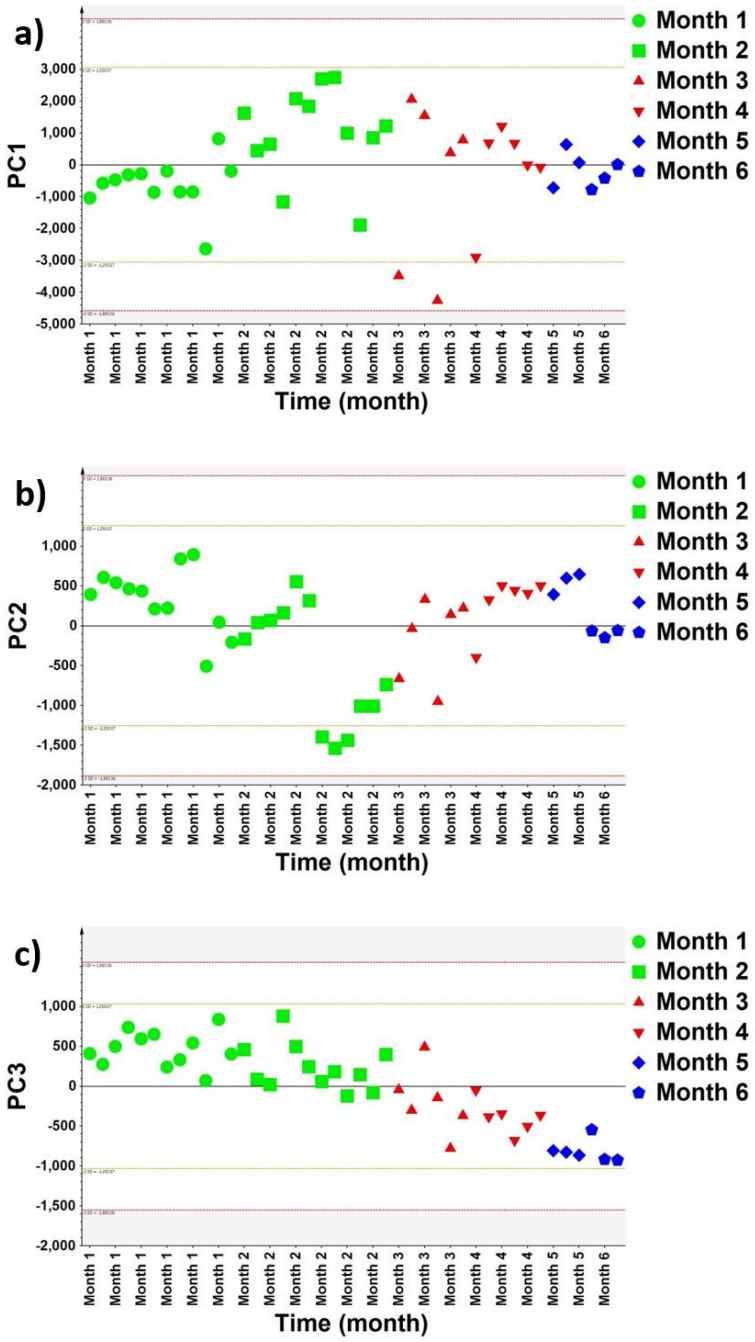


Figure 33. The scatterplots of aq MeOH-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

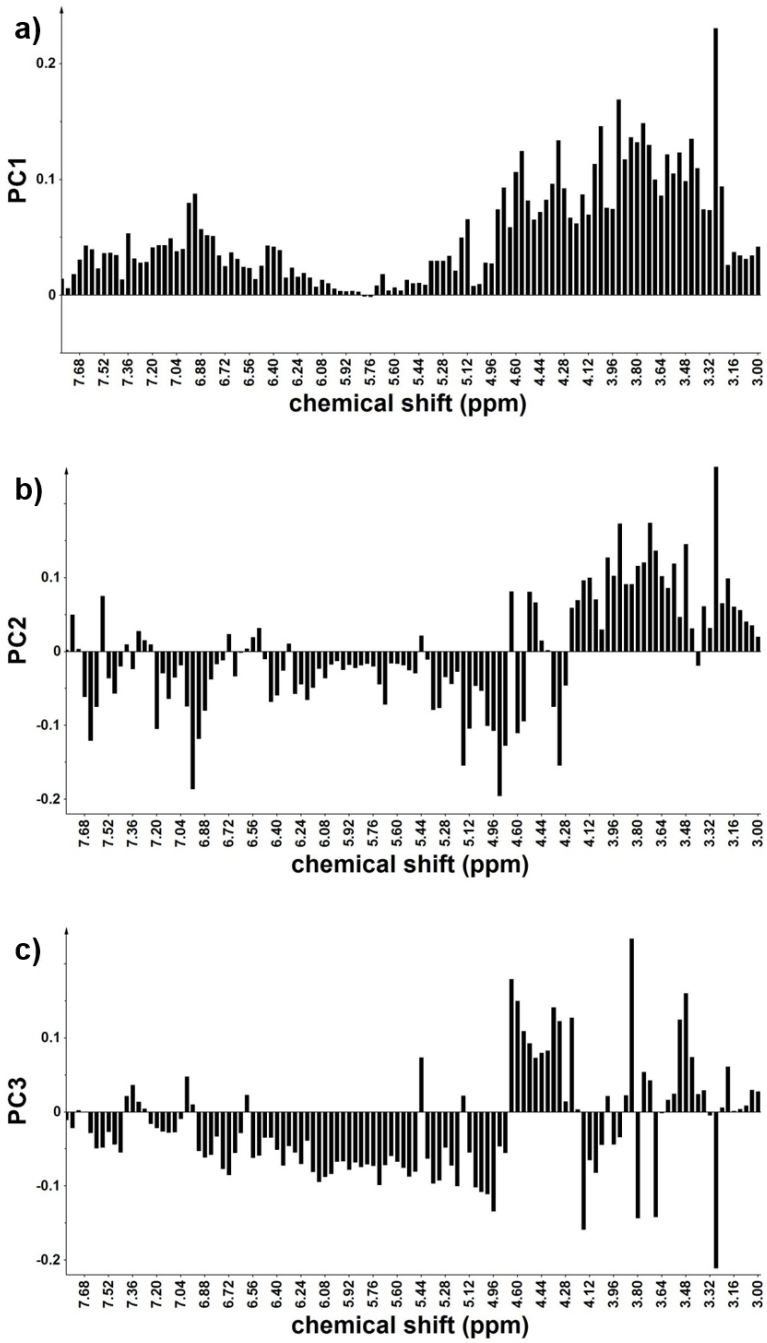


Figure 34. The loading plots of aq MeOH-extracts of the Andrographis Herb from Songkhla Province corresponding to the storage period, (a) PC1, (b) PC2, and (c) PC3 respectively

3.3.2 Stability profiles based on the total lactone contents

The samples of the Andrographis Herb that had been stored over the same period as that described in section 3.3.1 were also subjected to the assay for total lactone contents. This is to vouch and compare the NMR-based stability profiles with the official method. The Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998) recommends either the volumetric method, based on the assay for the total lactone content, or the reverse phase HPLC-based method for the andrographolide content (Ministry of Public Health, 2016;–Appendix 3.5), as the standard assay methods for the Andrographis Herbs. Here, the assay for the total lactone contents is chosen on the assumption that it is easier and requires less investment than the HPLC-based one; hence, it is adopted and officially registered by most local manufacturers. In addition, the method, which analyzes all the saponifiable lactones simultaneously, may reflect the integrated results and agree well with the metabolomics concepts in this study.

The Andrographis Herbs from the four suppliers similar to that of the NMR-based experiments were sampled on a monthly basis, and the lactone contents in the herbs were determined in a duplicate manner (Table 3). Linear correlation between the storage times and the lactone contents was met (Figure 35). Except for the Songkhla samples (slope = -0.9002), the plot from the other three suppliers was comparably parallel (slope of the plots from Suphan Buri, Khon Kaen, and Nakhon Pathom samples -0.3991, -0.2545, and -0.3052 respectively). The resulting correlation indicated that the lactone contents in the Andrographis Herb degraded with the zeroth-order kinetics, and the rate equations can be expressed as,

$c = -0.3991t + 16.144$ (1)

$c = -0.2545t + 12.634$ (2)

$c = -0.3052t + 10.851$ (3)

$c = -0.9002t + 17.353$ (4)

where c = concentration

t = time

equations (1) - (4) represent the kinetic equations for the herbs from Suphan Buri (1), Khon Kaen (2), Nakhon Pathom (3), and Songkhla (4), respectively.

The rate constants (*k*) deduced from the rate equations were -0.3991, -0.2545, -0.3052, and -0.9902 mo^{-1} for the samples from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla Provinces, respectively. Although not the same values, all *k*s were in a comparable magnitude of 10^{-1} mo^{-1}.

Whereas the samples from Suphan Buri, Khon Kaen, and Nakhon Pathom degraded parallelly, the results from Songkhla samples did not follow suit. At the moment, no account can be given for such an incoherence. However, a close observation suggested that from month 3 onward, the degradation plot of Songkhla specimens had become comparably parallel to the others. The major difference among Songkhla samples and others is that the samples from Songkhla had a real day 0 after harvesting, whereas others were virtual ones (see 3.3.1). It is reasonable to consider that the decomposition during the first months after harvesting might undergo different routes or rates. The transformation among the lactones may arrive to another equilibrium after certain period of storage.

Table 3. The total lactone contents of the Andrographis Herb from Suphan Buri, Khon Kaen, Nakhon Pathom and Songkhla provinces

Time	Total lactone	Suphan Buri			Khon Kaen			Nakhon Pathom			Songkhla		
		1	2	average	1	2	average	1	2	average	1	2	average
Month 0	(mg)	162.54	166.30	164.42	124.08	128.09	126.08	111.29	110.47	110.88	172.45	171.22	171.84
	(%)	16.20	16.60	16.40	12.40	12.61	12.50	11.06	10.96	11.01	17.07	16.93	17.00
Month 1	(mg)	162.28	159.65	160.96	121.63	125.53	123.58	105.65	104.05	104.85	166.37	166.73	166.55
	(%)	16.13	15.88	16.06	12.10	12.50	12.30	10.55	10.36	10.46	16.52	16.64	16.58
Month 2	(mg)	154.30	151.81	153.06	123.70	117.61	120.66	104.00	100.60	102.30	165.94	165.40	165.67
	(%)	15.36	15.15	15.26	12.30	11.70	12.00	10.32	10.03	10.18	16.46	16.50	16.48
Month 3	(mg)	141.26	139.49	140.38	127.10	120.20	123.65	98.95	99.10	99.02	140.20	138.96	139.58
	(%)	14.10	13.91	14.00	12.64	11.96	12.30	9.88	9.86	9.87	14.01	13.87	13.94
Month 4	(mg)	147.81	146.22	147.02	119.49	117.19	118.34	95.59	96.65	96.12	137.54	135.42	136.48
	(%)	14.76	14.61	14.68	11.94	11.68	11.81	9.54	9.65	9.60	13.72	13.53	13.62
Month 5	(mg)	143.39	141.62	142.50	114.89	112.76	113.82	92.93	94.17	93.55	130.64	129.93	130.28
	(%)	14.33	14.14	14.24	11.47	11.25	11.36	9.28	9.41	9.34	13.04	12.98	13.01
Month 6	(mg)	141.79	139.49	140.64	108.66	108.30	108.48	89.68	92.90	91.29	120.73	118.25	119.49
	(%)	14.17	13.92	14.04	10.85	10.79	10.82	8.93	9.26	9.10	12.06	11.80	11.93

The zeroth-order kinetics of total lactone degradation contradicted the results reported previously for the degradation of andrographolide in the heat-accelerated conditions, in which andrographolide in the PVP-solid dispersion and in the powdered Andrographis Herb degraded with the second-order kinetics (*k*s = 3.8×10^{-6} and 6.6×10^{-6} d^{-1}, respectively) (Lomlim et al., 2003; Plubrukarn et al., 2006). The correlations based on the first- and second-order kinetics between the storage time and the total lactone contents, i.e., reciprocals of the lactone contents for first-order, and the contents in logarithmic scales for second-order, had been attempted; however, the direct correlation between storage time and lactone contents, hence zeroth-order kinetics, yielded the best R^2s regardless of the herbs suppliers. The differences between the results reported here and those previously reported are unable to be accounted for; however, it could be speculated that this discrepancy is resulted from the integrated

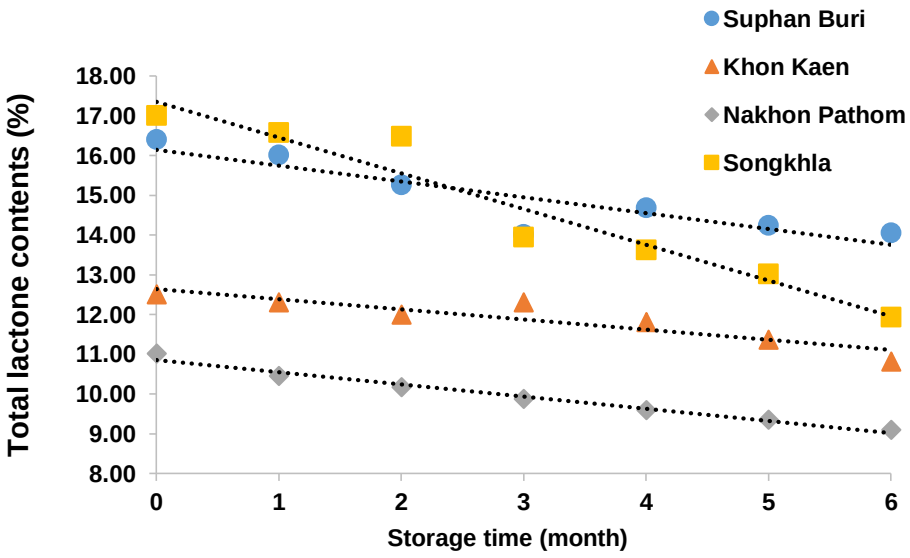


Figure 35. Zeroth-Order Plots for the total lactone content in the Andrographis Herb of the sample from each supplier (R^2 = 0.7961, 0.8409, 0.9814, and 0.9361 for Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla Provinces, respectively)

transformation among the diterpene lactones presented in the herbs. And such kinetics as described by the above kinetics equations is the appearing kinetics but not an actual one.

The fluctuation in the amounts of andrographolide and 14-deoxyandrographolide in the samples from each suppliers as seen in the NMR spectra (see the overlaid spectra, Figures 12, and 22a-c, from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla respectively), may reflected the discrepancy in the total lactone contents encountered above. The amount of both compounds dropped after the first two month but increased to double the original concentrations after six months. On the other hand, although consistently increase, the increasing rates of 14-deoxy-11,12-didehydroandrographolide did not match those of total lactones either. The results indicate that 14-deoxy-11,12-didehydroandrographolide might be a final product for total lactone decomposition, whereas the degradation of andrographolide and/or 14-deoxyandrographolide might be a multi-step transformation, and may involve an equilibrium that shifted the concentrations of either lactones in a non-linear manner.

Regardless of the disagreeable results, all equations showed the comparably short half-lives ($t_{½}$). The calculated $t_{½}$s based on the assay for the total lactone contents are 20.5, 24.6, 18.0, and 9.4 months, for the samples from Suphan Buri, Khon Kaen, Nakhon Pathom, and Songkhla, respectively. With the accelerated conditions, $t_{½}$s reported for andrographolide in solid dispersion and in powdered herbs were 7.83 and 4.2 years, respectively (Lomlim et al., 2003; Plubrukarn et al., 2006).

3.4 Half-life, shelf-life, and the storage of the Andrographis Herb

The total lactone contents in the Andrographis Herb from the four suppliers used in this investigation were in the range of 11-18% on day 0, and decreased to 9-15% after a six-month storage. With the Thai Herbal Pharmacopoeia stating the standard for the total lactone contents in the Andrographis Herb not less than 6% w/w, the total lactone contents in all the samples passed well above the standard throughout the storage period.

However, the rate equations established on the assay for the total lactone contents suggested that the herbs may have the estimated ($t_{½}$s) in a range of 9-20 months, and shelf-lives (t_{90}s) of 2-6 months. These are slightly shorter than $t_{½}$s and t_{90}s suggested in the previous reports ($t_{½}$s and t_{90}s = 7.83 and 0.87 years, Lomlim et al., 2003; 4.2 and 0.46 years, Plubrukarn et al., 2006), but could be considered in a comparable range. Having a zeroth-order degradation kinetics, i.e., a non-exponential decay, $t_{½}$s and t_{90}s of total lactone contents in the Andrographis Herb therefore rely on the initial lactone contents. That is, as long as the raw materials contain a high contents of total lactones, they are usable throughout the estimated period or even long after the recommended $t_{½}$s and t_{90}s. For examples, a hypothetical herb that originally contained as high as 10.8% of the total lactones would last and be usable as a raw materials for 12 months, calculated based on the equation established from Suphan Buri samples, equation (1).

As a product with a limited shelf-life, the Thai Herbal Pharmacopoeia (Ministry of Public Health, 1998) specifically recommends that the Andrographis Herb should be kept for no longer than one year and air-dried every 2 – 3 months. Given that

the other diterpene lactones presenting in *A. paniculata* are also active with a comparable potency, and no pronounced adverse effects have been reported, a lengthy storage periods may be allowed as long as the lactone contents remain in an acceptable quantity (Ministry of Public Health, 1998). With crop selection and good agricultural and harvesting practices to assure that the raw materials is of high lactone contents, the manufacturers may manage the storage of the raw materials more practically and even longer than the recommended ones. Additional recommendation is that the raw materials could be regularly quantitated for example, every three to six months over the storage period.

Different consideration is needed when the attention is turned from the raw materials to the marketed products. The most common herbal preparation of the Andrographis Herb is the powdered Andrographis Herb capsules (250-500 mg/capsule). For the preparations in which the herb is used in an unprocessed form, i.e., as a merely dried, powdered herb, but not extracted and compounded into other dosage forms, The Thai Herbal Pharmacopoeia uses the same limit as that of the raw materials. This leads to a few questions concerning the quality assurance of the products. On one hand, the use of herbal products from the very high lactone contents may assure a longer storage period similar to that for the raw materials. The materials of higher lactone contents could be economically preferable in the consumers' viewpoints. On the other hand, from the practitioners' aspects, the products with higher lactone contents may add the risk of overdosing in the patients. Despite being comparatively safe, the Andrographis Herb has a main precaution of causing numbness on the extremity after a long-term use.

To compensate such a short shelf-life of the Andrographis Herb, it is reasonable to consider whether it is possible to raise the lower limit of the total lactone contents specifically for the herbal preparations containing the Andrographis Herb. This is based on the assumptions that (1) all the diterpene lactones possess a comparable potency, and (2) the herb has no severe adverse effects other than the well-documented numbness. Adopting *ks* in a similar range to those obtained in equations (1) - (3), the recommended label amount of total lactone contents in the products containing Andrographis Herb could be instead in a higher range of 9 – 11%. This will allow the products of Andrographis Herb to stand on the shelf for 12 months before the expiration dates, at which time the lactone contents would fall below the standard limit of 6%.

CHAPTER 4

CONCLUSION

The Andrographis Herb, the aerial part of *Andrographis paniculata*, is one of medicinal plants that have been widely used and officially recognized for its potential and effectiveness as an alternative medicine. The Thailand National Drug Lists for Herbal Medicines and recommends the Andrographis Herb for the relief of the symptoms of common cold and non-infectious diarrhea (National Drug Committee, 2016), and WHO recognizes the use of the Andrographis Herb for the symptomatic treatment of upper respiratory infections, such as the common cold, uncomplicated sinusitis, bronchitis, pharyngotonsillitis, acute diarrhea, lower urinary tract infections (World Health Organization, 2004). However, with the limited shelf-life, storage of the raw materials and processed products have been the major managerial concern among the manufacturers, and hampered the development process of this herbal medicine.

Employing the ^{1}H NMR-based metabolomics approach, this investigation has shown that the diterpene lactones in the dried herbs changed significantly over the storage period of six months under the standard storage conditions recommended by the Thai Herbal Pharmacopoeia. Focusing on four main diterpene lactones, andrographolide, 14-deoxyandrographolide, 14-deoxy-11,12-didehydro-andrographolide, and neoandrographolide, the amount of 14-deoxy-11,12-didehydroandrographolide increases consistently over the storage periods. This agrees well with the previous report (Lomlim et al, 2003), in which 14-deoxy-11,12-didehydroandrographolide was reported to be the primary degradation product. The amounts of andrographolide and 14-deoxyandrographolide, on the other hand, despite

falling and rising significantly over the storage periods parallelly among different product suppliers, did not allow a predictable trend. The two compounds hence may be the transformation intermediates. Otherwise, reverse or multi-step transformations may involve.

On the other hand, in order to trace the age of the Andrographis Herb raw materials and products, 14-deoxy-11,12-didehydroandrographolide is proposed here as a degradation marker. That is, the amount (or ratio) of 14-deoxy-11,12-didehydroandrographolide might be used as an indicator for the storage age of the raw materials and products. An extensive survey on raw materials from multiple sources and suppliers will allow the upper limit and acceptable level of 14-deoxy-11,12-didehydroandrographolide to be established.

The stability of the Andrographis Herb based on the assay for the total lactone contents indicated that the lactone contents in the herbs degraded rapidly with a zeroth-order kinetics. Among the samples from four supplying sources, except for Songkhla samples, the herbs from the other three suppliers yielded comparably paralleled kinetics plots, with *ks* in a range of -0.2545 - -0.9002 mo^{-1}. These resulted in $t_{½}$s in a range of 18 – 25 month, and t_{90}s of 3 – 6 month. This decomposition kinetics did not align well with the previous reports nor with the results from the NMR-based approach mentioned above. This in fact is not surprising, considering that the results from this assay method reflect the integrated decomposition from several lactones in a complex mixture; therefore, the decomposition in each of which might not proceed in the similar or comparable fashion. Regardless of the misalignment, the resulting $t_{½}$ and t_{90} were on the same range to those estimated when using other methods. This supports the short storage period as recommended by the Thai Herbal Pharmacopoeia.

The Thai Herbal Pharmacopoeia states only the lowest limit of the total lactone contents (not less than 6%) for the Andrographis Herb. For the raw materials, the short storage period can be overcome by crop selection and good agricultural and harvesting practices to assure that the good-quality herbs with high lactone contents are purchased and used. Also, a frequent quantitation should be performed throughout the storage period. However, additional concerns may be needed for the herbal products, i.e; the herbs that are processed and prepared into a ready-to-use dosage forms. The bottom line is that, once in a preparation, the contents of the active ingredients should present in a triturated dose with a certain and specific range. Based on the estimated degradation kinetics, the recommended dose per unit (i.e., dose per capsule of the Andrographis Herb) may be triturated into a range of 9 - 11% of total lactones. This will allow a storage time of 12 months, until which time the contents of the diterpene lactones fall below the standard range of 6% as regulated by the Thai Herbal Pharmacopoeia.

REFERENCES

Ahmad MS, Ahmad S, Arshad M, Afzal M. *Andrographia paniculata* a miracle herbs for cancer treatment: *In vivo* and *in vitro* studies against aflatoxin B1 toxicity. *Egypt J Med Hum Genet.* **2014**; 15: 163–171.

Alonso A, Marsal S, Julia A. Analytical methods in untargeted metabolomics: state of the art in 2015. *Front Bioeng Biotechnol*. **2015**; 3: 1-20.

Ameh SJ, Obodozie OO, Inyang US, Abubakar MS, Garba M. Quality control tests on *Andrographis paniculata* Nees (Family: Acanthaceae) – an Indian 'Wonder' plant grown in Nigeria. *Trop J Pharm Res.* **2010**; 9(4): 387-394.

Awang K, Abdullah NH, Abd. Hadi AH, Fong YS. Cardiovascular activity of labdane diterpenes from *Andrographis paniculata* in isolated rat hearts. *J Biomed Biotechnol*. **2012**; 1-5.

Chao WW, Lin BF. Isolation and identification of bioactive compounds in *Andrographis paniculata* (*Chuanxinlian*). *Chin Med*. **2010**; 5(17): 1-15.

Chen L, Zhu H, Wang R, Zhou K, Jing Y, Qiu F. *ent*-Labdane diterpenoid lactone stereoisomers from *Andrographis paniculata. J Nat Prod.* **2008**; 71: 852–855.

Chen LX, Qiu F, Wei H, Qu GX, Yao XS. Nine new *ent*-labdane diterpenoids from the aerial parts of *Andrographis paniculata. Helv Chim Acta*. **2006**; 89: 2660-2664.

Cheung HY, Cheung CS, Kong CK. Determination of bioactive diterpenoids from *Andrographis paniculata* by micellar electrokinetic chromatography. *J Chromatogr A.* **2001**; 930: 171– 176.

De Vos RC, Moco S, Lommen A, Keurentjes JJB, Bino RJ, Hall RD. Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat Protoc*. **2007**; 2(4): 778-791.

Dong HJ, Zhang ZJ, Yu J, Liu Y, Xu FG. Chemical fingerprinting of *Andrographis paniculata* (Burm. f.) Nees by HPLC and hierarchical clustering analysis. *J Chromatogr Sci*. **2009**; 47: 931-935.

Du Q, Jerz G, Winterhalter P. Separation of andrographolide and neoandrographolide from the leaves of *Andrographis paniculata* using high-speed counter-current chromatography. *J Chromatogr A*. **2003**; 984: 147–151.

Dua VK, Ojha VP, Roy R, Joshi BC, Valecha C, Devi CU, Bhatnagar MC, Sharma VP, Subbarao SK. Anti-malarial activity of some xanthones isolated from the roots of *Andrographis paniculata. J Ethnopharmacol*. **2004**; 95: 247–251.

Farag M, Porzel A, Schmidt J, Wessjohann AL. Metabolite profiling and fingerprinting of commercial cultivars of *Humulus lupulus* L. (hop): a comparison of MS and NMR methods in metabolomics. *Metabolomics*. **2012**; 8: 492-507.

Fujita T, Fujitani R, Tekada Y, Takaishi Y, Yamada T, Kido M, Miura I. On the diterpenoids of *Andrographis paniculata*: x-ray crystallographic analysis of andrographolide and structure determination of new minor diterpenoids. *Chem Pharm Bull.* **1984**; 32(67): 2117-2125.

Hossain MS, Urbi Z, Sule A, Rahman MH. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: A review of ethnobotany, phytochemistry, and pharmacology. *Scientific World J*. **2014**; 2014: 1-28.

Hu YM, Wang GC, Wang Y, Sung HHY, Williams ID, Ye WC. Diastereoisomeric *ent*-labdane diterpenoids from *Andrographis paniculata*. *Helv Chim Acta*. **2012**; 95: 120-126.

Ibrahim MN, Chong GH. Stability of andrographolide in *Andrographis paniculata* under selected storage conditions. *Int J Eng Technol*. **2008**; 5(1): 69-73.

Jarukamjorn K, Nemoto N. Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. *J Health Sci*. **2008**; 54(4): 370-381.

Jewvachdamrongkul Y, Jirawattanapong W, Dechatiwongse Na Ayudhya T. Modified method for determination of total lactones in Andrographis Herb. *Bull Dept Med Sci*. **1990**; 32(2): 53-61.

Joselin J, Jeeva S. *Andrographis paniculata*: A Review of its Traditional Uses, Phytochemistry and Pharmacology. *Med Aromat Plants*. **2014**; 3(4): 1-15.

Kim HK, Choi YH, Verpoorte R. NMR-based metabolomics analysis of plants. ***Nat Protoc*. 2010; 5(3): 536-549.**

Ko HC, Wei BL, Chiou WF. The effect of medicinal plants used in Chinese folk medicine on RANTES secretion by virus-infected human epithelial cells. *J Ethnopharmacol*. 2006; 107: 205-210.

Kongyingyoes B, Priengprom T, Pientong C, Aromdee C, Suebsasana S, Ekalaksananan T. 3,19-isopropylideneandrographolide suppresses early gene expression of drug-resistant and wild type herpes simplex viruses. *Antivir Res*. **2006**; 132: 281-286.

Kulyal P, Tiwari UK, Shukla A, Guar AK, Chemical constituents isolated from *Andrographis paniculata*. *Indian J Chem*. **2010**; 49: 356-359.

Kumar V, Thakur AK, Chatterjee SS. Perspective of *Andrographis paniculata* in neurological disorders. *Clinic Pharmacol Biopharmaceut*. **2014**; 1-4.

Li W, Xu X, Zhang H, Ma C, Fong H. Secondary metabolites from *Andrographis paniculata. Chem Pharm Bull.* **2007**; 55(3): 455-458.

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR. Gas chromatography mass spectrometry–based metabolite profiling in plants. *Nat Protoc*. **2006**; 1(1): 387-396.

Liu NQ, Cao M, Frederich M, Choi YH, Verpoorte R. Metabolomic investigation of the ethnopharmacological use of *Artemisia afra* with NMR spectroscopy and multivariate data analysis. *J Ethnopharmacol.* **2010**; 128: 230-235.

Lomlim L, Jirayupong N, Plubrukarn A. Heat-accelerated degradation of solid-state andrographolide. *Chem Pharm Bull.* **2003**; 51: 24-26.

Ma XC, Gou ZP, Wang CY, Yao JH, Xin XL, Lin Y, Liu KX. A new *ent*-labdane diterpenoid lactone from *Andrographis paniculata. Chinese Chem Lett*. **2010**; 21: 587–589.

Ma XC, Zhang BJ, Deng S, Huang SS, Liu KX, Jia JM. A new *ent*-labdane diterpenoid lactone from *Andrographis paniculata. Chinese Chem Lett*. **2009**; 20: 317–319.

Madav S, Tripathi HC, Mishra SK, Tandan SK. Analgesic, antipyretic and antiulcerogenic effects of andrographolide. *Indian J Pharm Sci*. **1995**; 57(3): 121-125.

Matsuda T, Kuroyanagi M, Sugiyama S, Umehara K, Ueno A, Nishi K. Cell differentiation-inducing diterpenoids from *Andrographis paniculata* Nees. *Chem Pharm Bull.* **1994**; 42(6): 1216-1225.

Ministry of Agriculture and Cooperatives; **2014** [cited 15 may 2017]. Available from: http://ssnet.doae.go.th/wp-content/uploads/2015/04/028_bunch-robber1.pdf

Ministry of Public Health. Fa Tha Lai. In Thai Herbal Pharmacopoeia, Vol. 1. Nonthaburi, Thailand, **1998**, 24–31.

Misra P, Pal NL, Guru PY, Katiyar JC, Srivastava V, Tandon JS. Antimalarial activity of *Andrographis paniculata* (Kalmegh) against *plasmodium berghei* NK 65 in *Mastomys natalensis. Int J Pharmacog.* **1992**; 30(4): 263-274.

Mulukuri NVL, Mondal NB, Prasad MR, Renuka S, Ramakrishna K. Isolation of diterpenoid lactones from the leaves of *Andrographis paniculata* and its anticancer activity. *Int J Pharm Pharm Res.* **2010; 3(3): 39-42.**

National Drug Committee, National Essential Drug List Committee, Ministry of Public Health, Bangkok, 2016.

Pholphana N, Rangkadilok N, Thongnest S, Ruchirawat S, Ruchirawat M, Satayavivad J. Determination and variation of three active diterpenoids in *Andrographis paniculata* (Burm.f.) Nees. *Phytochem Anal*. **2004**; 15: 365–371.

Plubrukarn A, Pinsuwan S, Ingkatawornwong S, Supavita T. Stability of andrographolide in powdered Andrographis Herb under accelerated conditions. *Planta Med*. **2006**; 72: 954-956.

Pramanick S, Banerjee S, Achari B, Das B, Sen AK, Mukhopadhyay S, Neuman A, Prang T. Andropanolide and isoandrographolide, minor diterpenoids from *Andrographis paniculata*: structure and x-ray crystallographic analysis. *J Nat Prod.* **2006**; 69: 403-405.

Quansah E, Karikari TK. Potential role of metabolomics in the improvement of research on traditional African medicine. *Phytochem Lett*. **2016**; 17: 270-277.

Rao YK, Vimalamma G, Rao CV, Tzeng YM. Flavonoids and andrographolides from *Andrographis paniculata. Phytochemistry*. **2004**; 65: 2317–2321.

Reddy MK, Reddy MVB, Gunasekari D, Murthy MM, Caux C, Bodo B. A flavone and an unusual 23-carbon terpenoid from *Andrographis paniculata. Phytochemistry*. **2003**; 62: 1271–1275.

Shahjahan M, Sollaiman AHM, Sultana N, Kabir K. Effect of organic fertilizers and spacing on growth and yield of Kalmegh (*Andrographis paniculata* Nees). *Intl J Agri Crop Sci*. **2013**; 6(11): 769-775.

Sharma M, Sharma A, Tyagi S. Quantitative HPLC analysis of andrographolide in *Andrographis paniculata* at two different stage of life cycle of plant. *Acta Chim Pharm Indica.* **2012**; 2(1): 1-7.

Shen YH, Li RT, Xiao WL, Xu G, Lin ZW, Zhao QS, Sun HD. *ent*-labdane diterpenoids from *Andrographis paniculata. J Nat Prod.* **2006**; 69: 319-322.

Sithara NV, Komathi S, Rajallakshmi G, Queen J, Bharathi D. Phytochemical analysis of *Andrographis paniculata* using different solvents. *European J. Biotechnol. Biosci.* **2016**; 4(8): 28-30.

Song YX, Liu SP, Jin Z, Qin JF, Jiang ZY. Qualitative and quantitative analysis of *Andrographis paniculata* by rapid resolution liquid chromatography/time-of-flight mass spectrometry. *Molecules.* **2013**; 18: 12192-12207.

Srivastav A, Misra H, Verma RK, Gupta MM. Chemical fingerprinting of *Andrographis paniculata* using HPLC, HPTLC and densitometry. *Phytochem Anal*. **2004**; 15: 280–285.

Subramanian R, Asmawi MZ, Sadikum A. A bitter plant with a sweet future? A comprehensive review of an oriental medicinal plant: *Andrographis paniculata. Phytochem Rev*. **2012**; 11: 39–75.

Sudhakaran MV. Botanical pharmacognosy of *Andrographis paniculata* (Burm. F.) Wall. Ex. Nees. *Phcog J*. **2012**; 4(32): 1-10.

Sule A, Ahmed QU, Abd.Samah O, Omar MN. Bacteriostatic and bactericidal activities of *Andrographis paniculata* extracts on skin disease causing pathogenic bacteria. *J Med Plant Res*. **2011**; 5(1): 7-14.

Sule A, Ahmed QU, Abd.Samah O, Omar MN, Hassan NM, Kamal LZ, Yarmo MA. Bioassay guided isolation of antibacterial compounds from *Andrographis paniculata. Am J Applied Sci.* **2011**; 8(6): 525-534.

Sule A, Ahmed QU, Latip J, Abd.Samah O, Omar MN, Umar A, Dogarai BBS. Antifungal activity of *Andrographis paniculata* extracts and active principles against skin pathogenic fungal strains *in vitro. Pharm Biol*. **2012**; 50(7): 850-856.

Tan MCS, Oyong GG, Shen CC, Consolacion Y. Secondary metabolites from *Andrographis paniculata* (Burm.f.) Nees. *Der Pharmacia Lettre*, **2016**; 8(13): 157-160.

Tan ML, Kuroyanagi M, Sulaiman SF, Najimudin N, and Muhammad TS. Cytotoxic activities of major diterpenoid constituents of *Andrographis paniculata* in a panel of human tumor cell lines. *Pharm Biol*. **2005**; 43(6): 501-508.

Van der Kooy F, Verpoorte R, Mayer MJJ. Metabolomic quality control of claimed anti-malarial *Artemisia afra* herbal remedy and *A. afra* and *A. annua* plant extracts. *S Afr J Bot*. **2008**; 74(2): 186-189.

Wang CH, Li W, Qin RX, Jiang MM, Li GQ. A new diterpenoid from the aerial parts of *Andrographis paniculata. Nat Prod Commun.* **2014**; 9(1): 13-14.

Wang GC, Wang Y, Williams LD, Sung HYH, Zhang XQ, Zhang DM, Jiang RW, Yao XS, Ye WC. Andrographolactone, a unique diterpene from *Andrographis paniculata. Tetrahedron Lett.* **2009**; 50: 4824-4826.

Wiart C, Kumar K, Yusof MY, Hamimah H, Fauzi ZM, Sulaiman M. Antiviral properties of *ent*-labdene diterpenes of *Andrographis paniculata* Nees, inhibitors of herpes simplex virus type 1. *Phytother Res.* **2005**; 19: 1069-1070.

World Health Organization. Herba Andrographidis. In WHO monographs on selected medicinal plant; WHO library cataloguing-in-publication data, Vol. 2. Geneva, **2004**. 12-24.

Wu TS, Chern HJ, Damu AG, Kuo PC, Su CR, Lee EJ, Teng CM. Flavonoids and *ent*-labdane diterpenoids from *Andrographis paniculata* and their antiplatelet

aggregatory and vasorelaxing effects. *J Asian Nat Prod Res*. **2008**; 10(1): 17-24.

Xu C, Chou GX, Wang ZT. A new diterpene from the leaves of *Andrographis paniculata* Nees. *Fitoterapia*. **2010**; 81: 610-613.

Xu C, Chou GX, Wang CH, Wang ZT. Rare noriridoids from the roots of *Andrographis paniculata. Phytochemistry*. **2012**; 77: 275-279.

Xu HW, Dai GF, Liu GZ, Wang JF, Liu HM. Synthesis of andrographolide derivatives: A new family of a-glucosidase inhibitors. *Bioorganic Med Chem*. **2007**; 15: 4247-4255.

Yang M, Wang J, Kong L. Quantitative analysis of four major diterpenoids in *Andrographis paniculata* by ^{1}H NMR and its application for quality control of commercial preparations. *J Pharmaceut Biomed.* **2012**; 70: 87-93.

Yang T, Yao S, Zhang X, Guo Y. Andrographolide inhibits growth of human T-cell acute lymphoblastic leukemia Jurkat cells by downregulation of PI3K/AKT and upregulation of p38 MAPK pathways. *Drug Des Dev*. **2016**; 10: 1389-1397.

Yilmaz A, Nyberg NT, Molgard P, Asili J, Jaroszewski JW. ^{1}H NMR metabolic fingerprinting of saffron extracts. *Metabolomics*. **2010**; 6(4): 511-517.

Yu BC, Hung CR, Chen WC, Cheng JT. Antihyperglycemic effect of andrographolide in Streptozotocin-induced diabetes rats. *Planta Med*. **2003**; 69: 1075-1079.

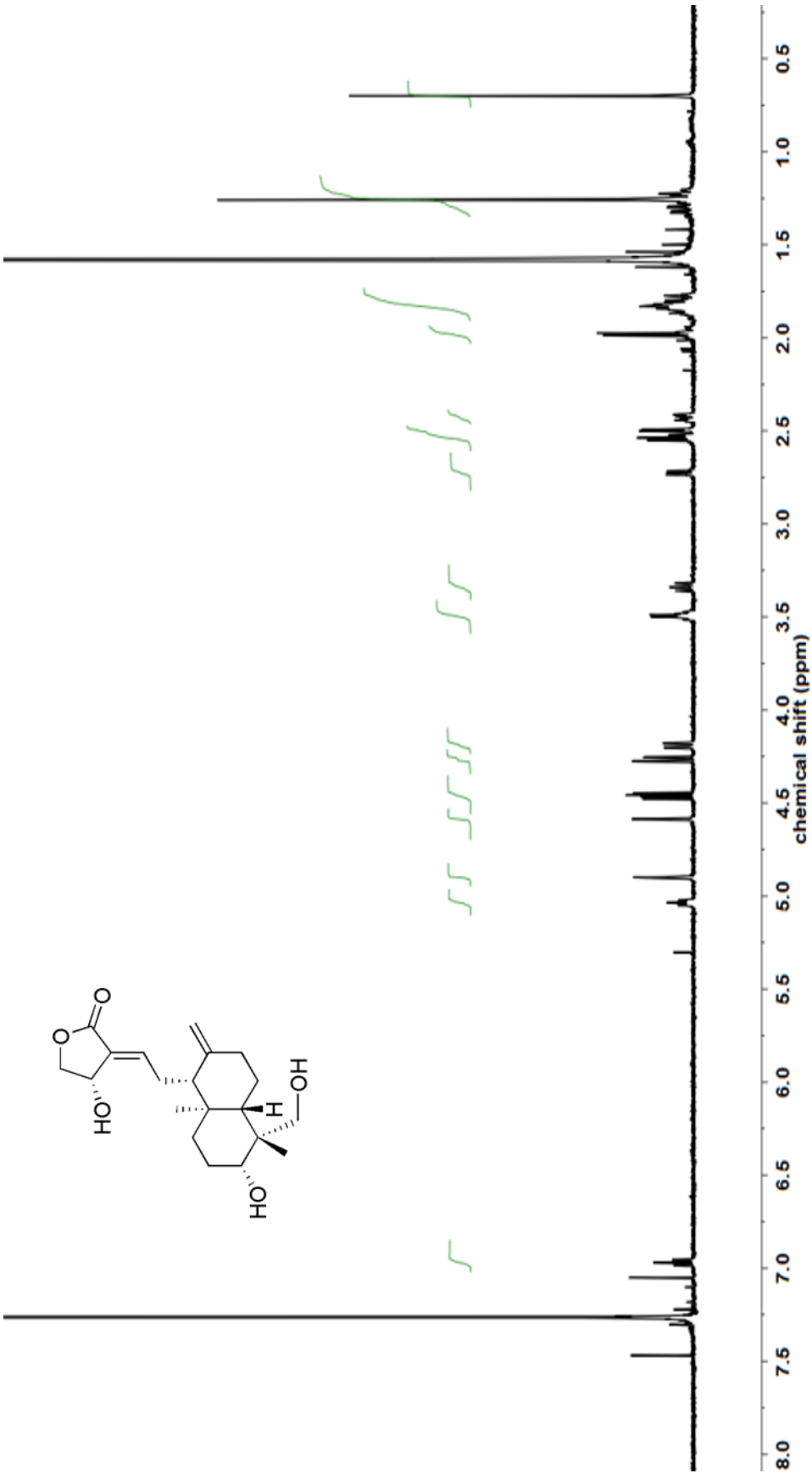
Yuan Y, Ji L, Luo L, Lu J, Ma X, Ma Z, Chen Z. Quinone reductase (QR) inducers from *Andrographis paniculata* and identification of molecular target of andrographolide. *Fitoterapi*a. **2012**; 83: 1506–1513.

Zhang XQ, Wang GC, Ye WC, Li Q, Zhou GX, Yao XS. New diterpenoids from *Andrographis paniculata* (Burm. f.) Nees. *J Integr Plant Biol*. **2006**; 48(9): 1122−1125.

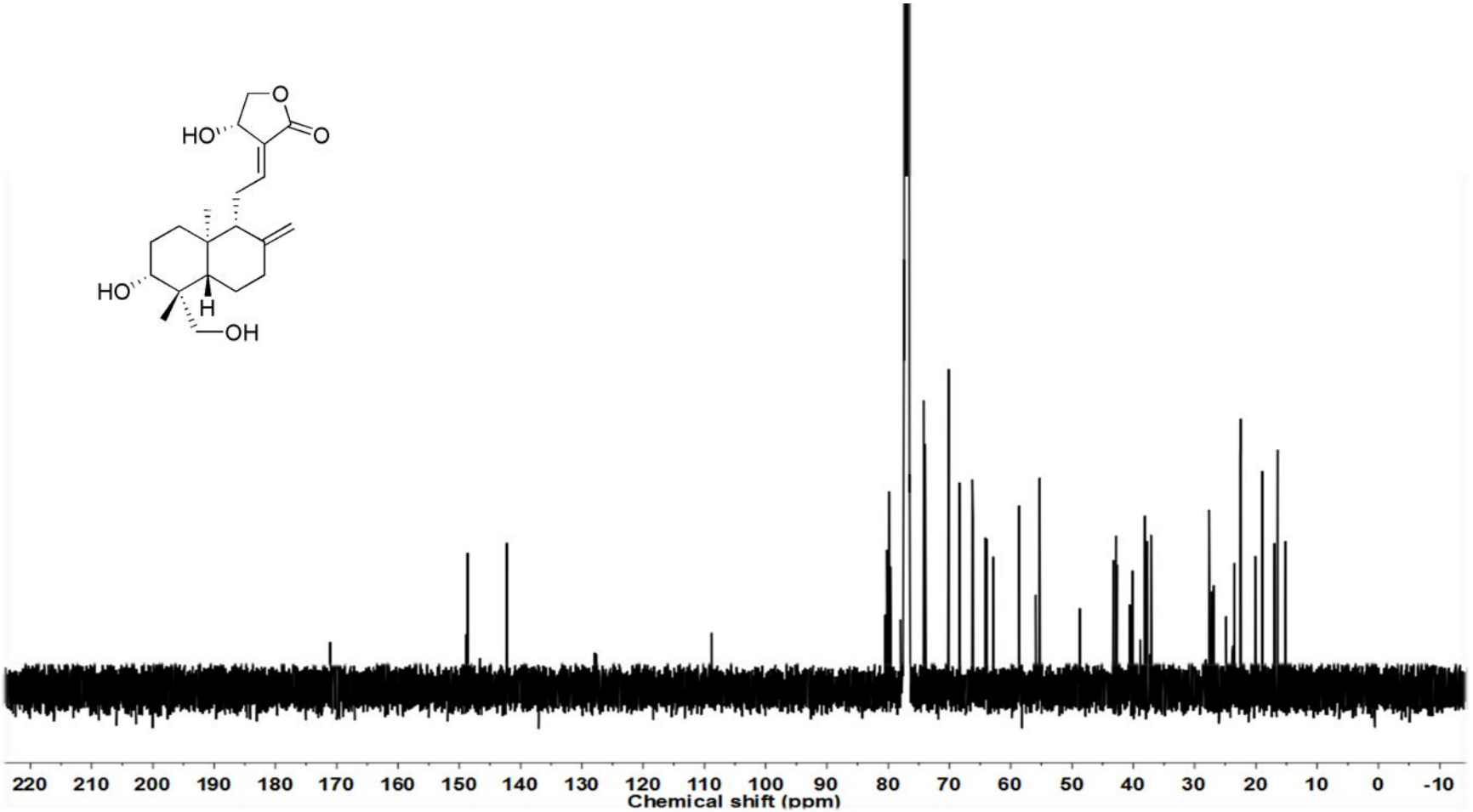
Zou QY, Li N, Dan C, Deng WL, Peng SL, Ding LS. A new *ent*-labdane diterpenoid from *Andrographis paniculata*. *Chinese Chem Lett*. **2010**; 21: 1091–1093.

APPENDICES

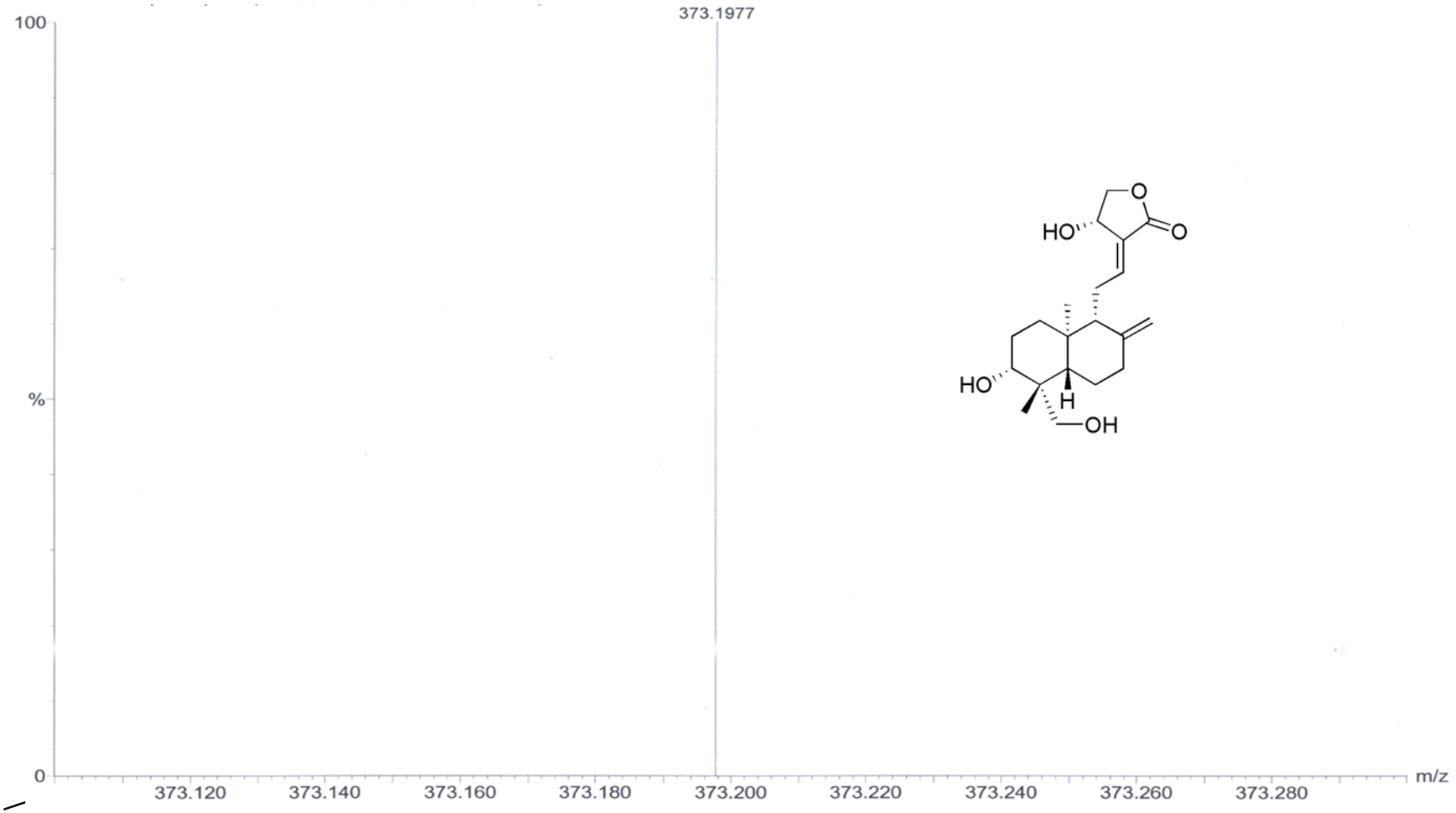
APPENDIX A



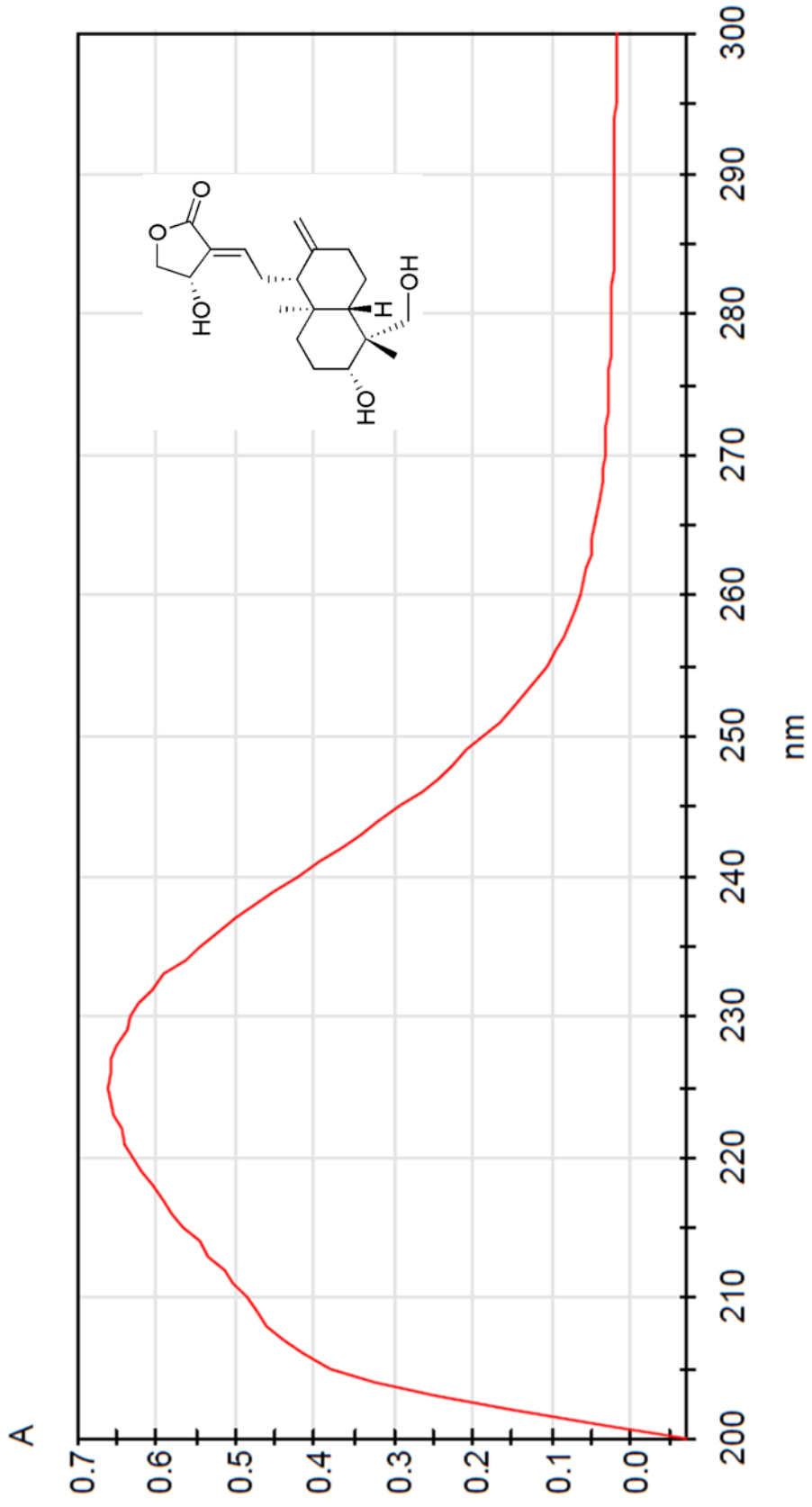
^{1}H NMR spectrum of andrographolide (500 MHz, $CDCl_3$)



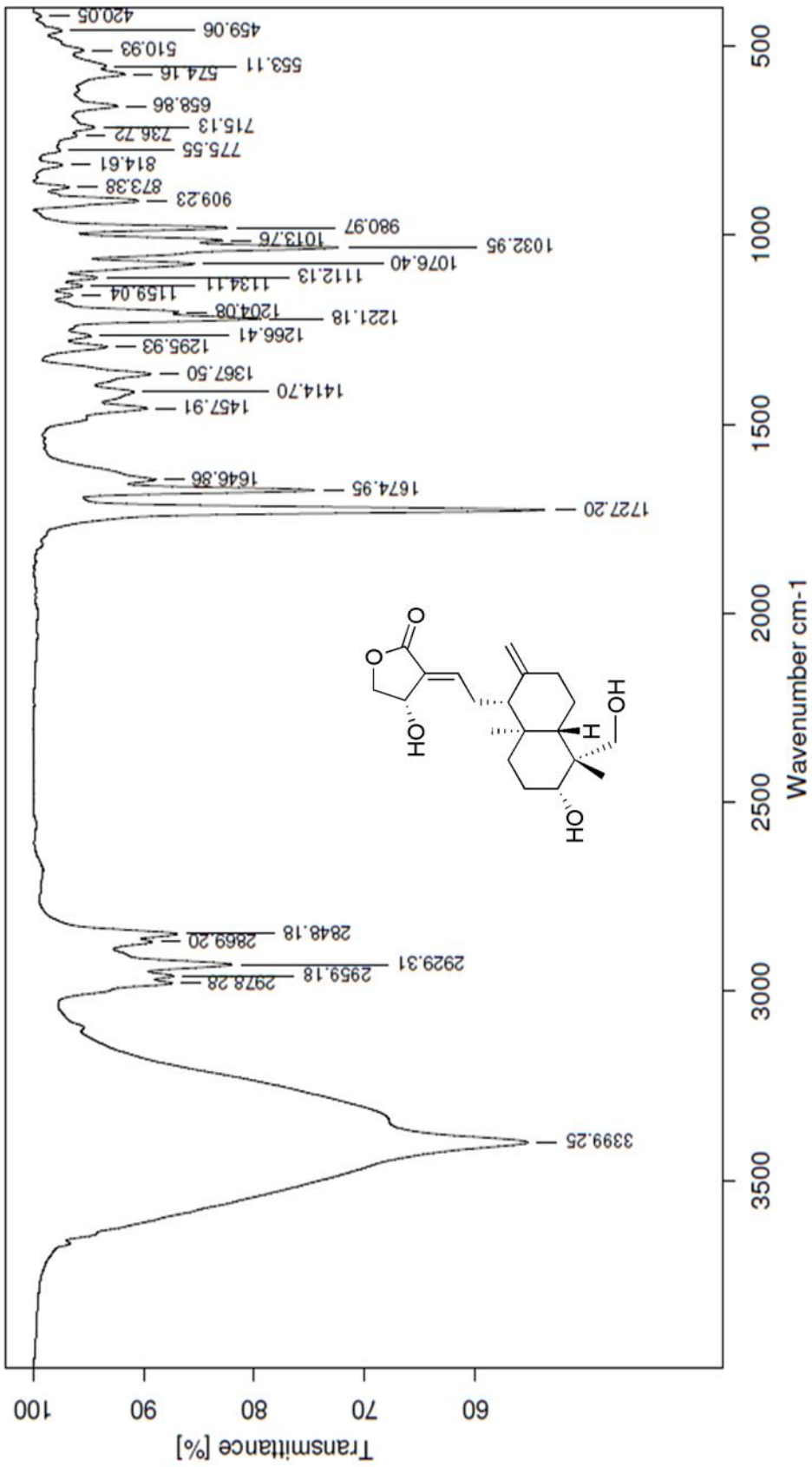
^{13}C NMR spectrum of andrographolide (125 MHz, $CDCl_3$)



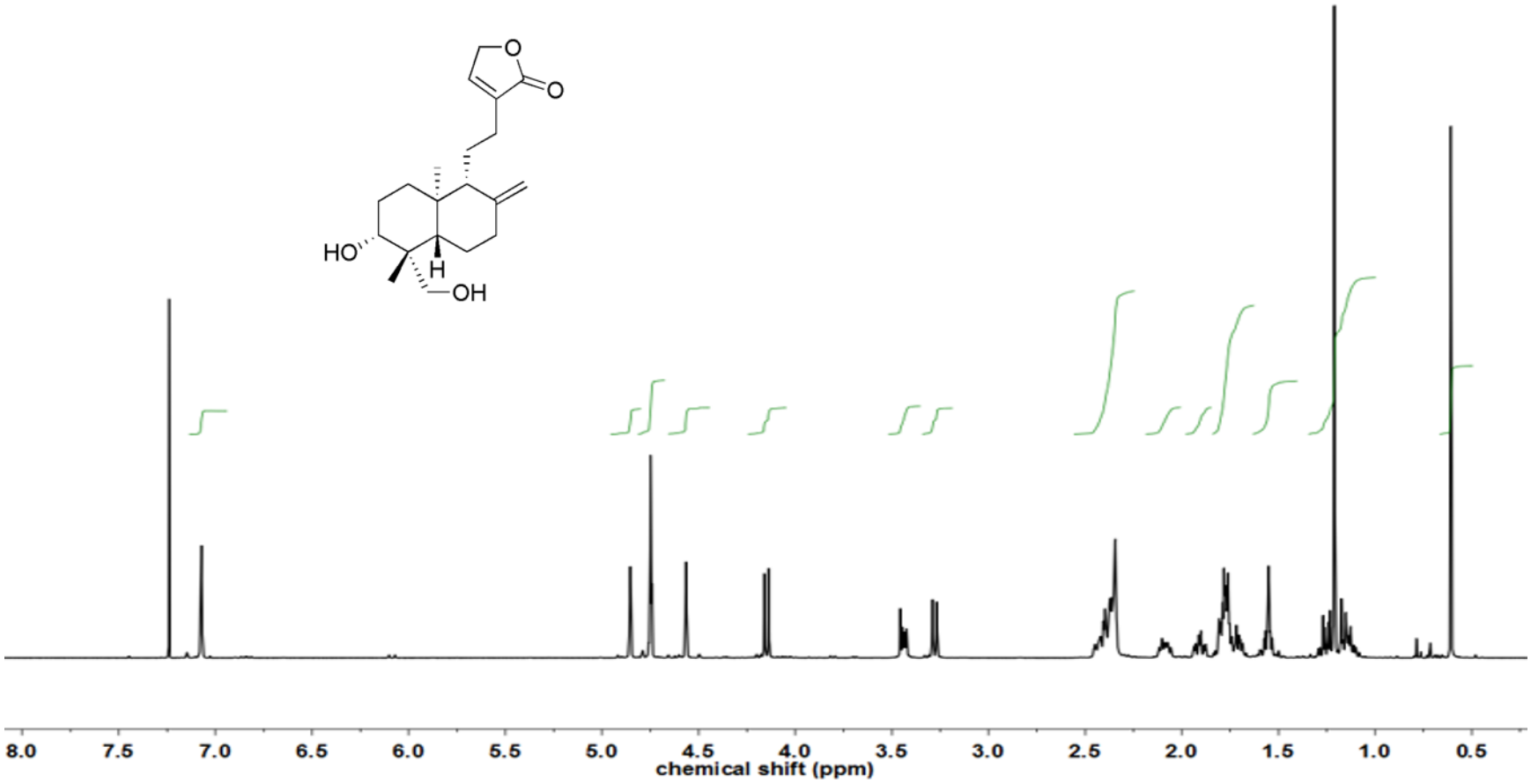
ESI mass spectrum of andrographolide



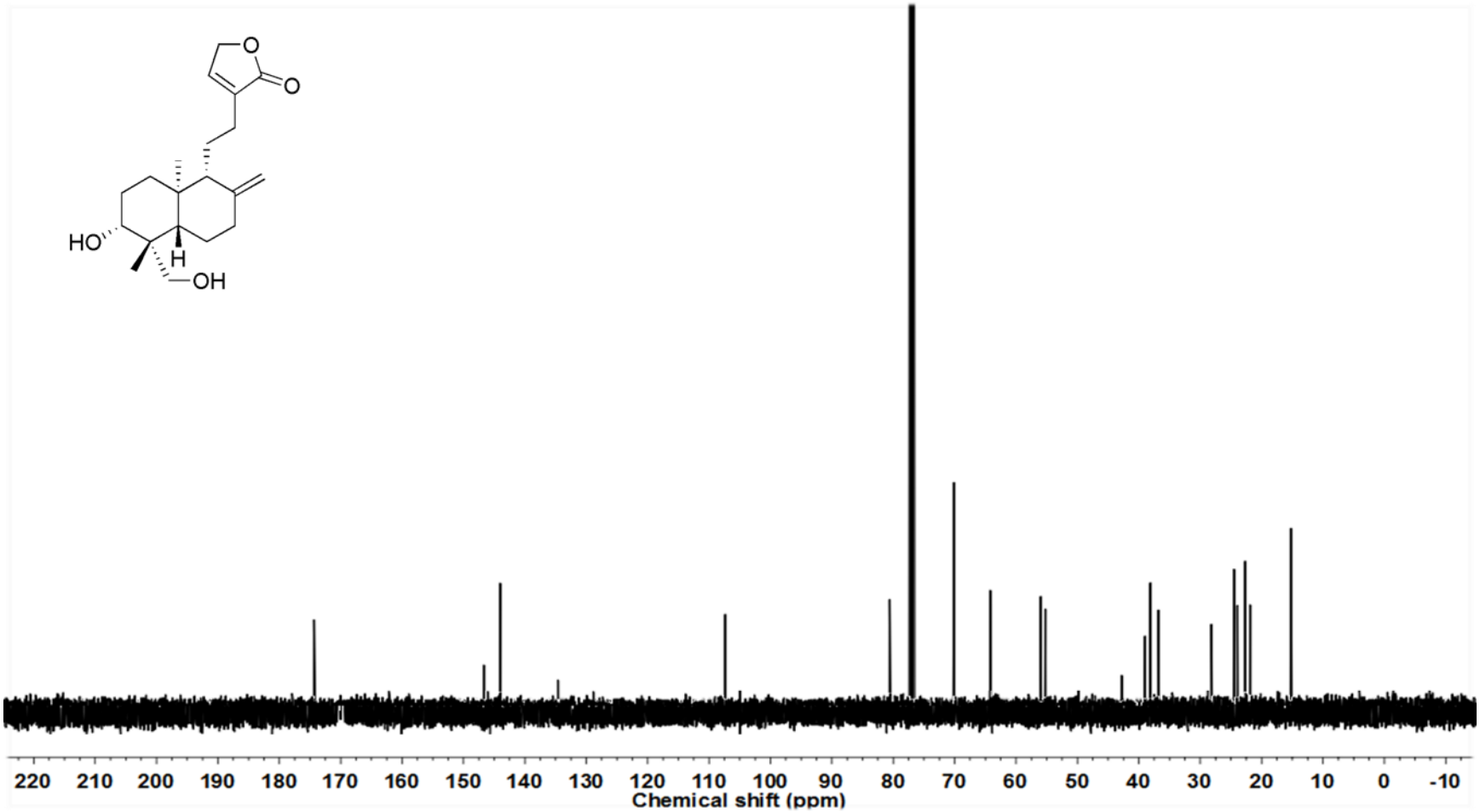
UV spectrum of andrographolide (MeOH)



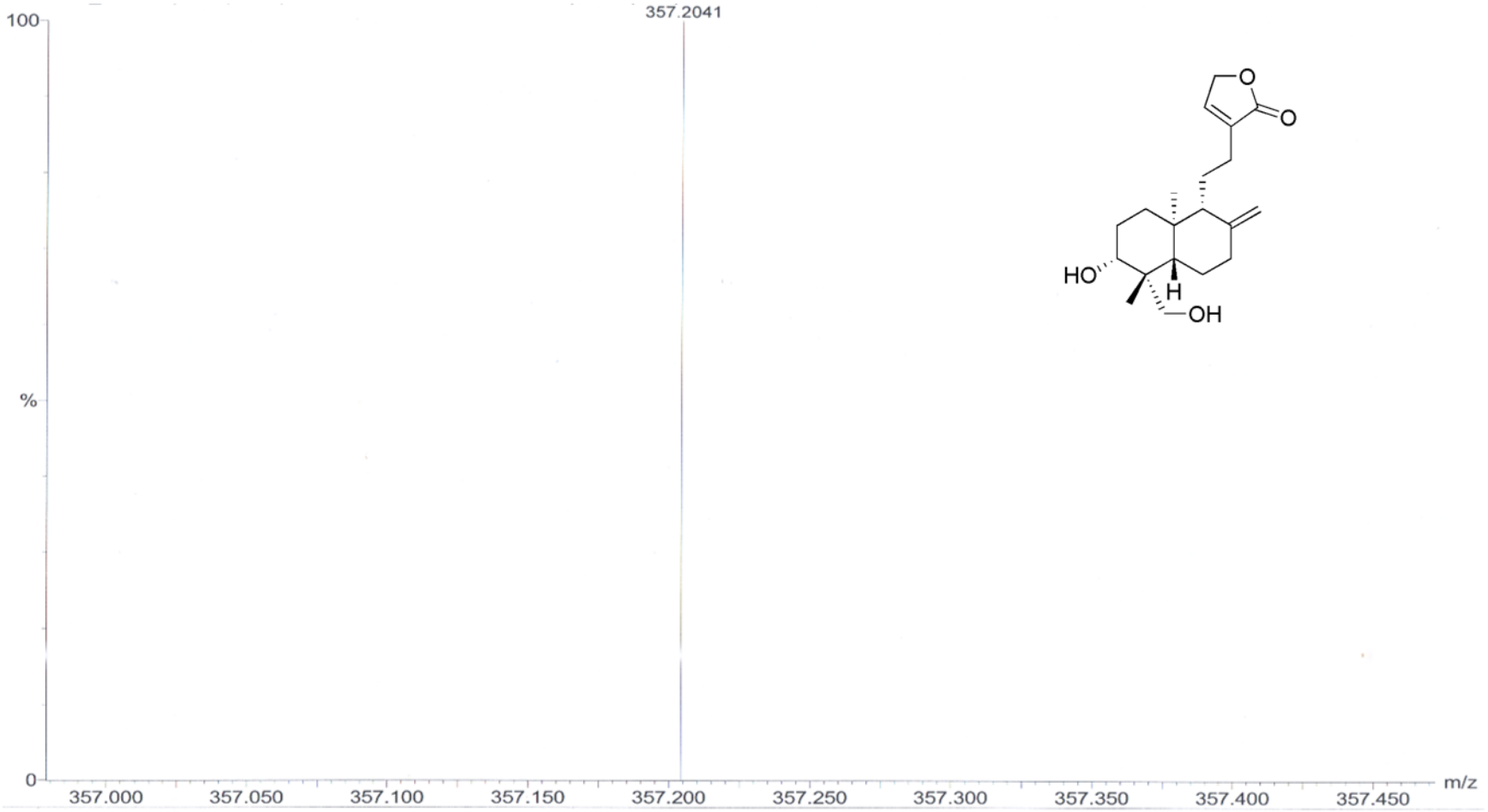
IR spectrum of andrographolide (KBr)



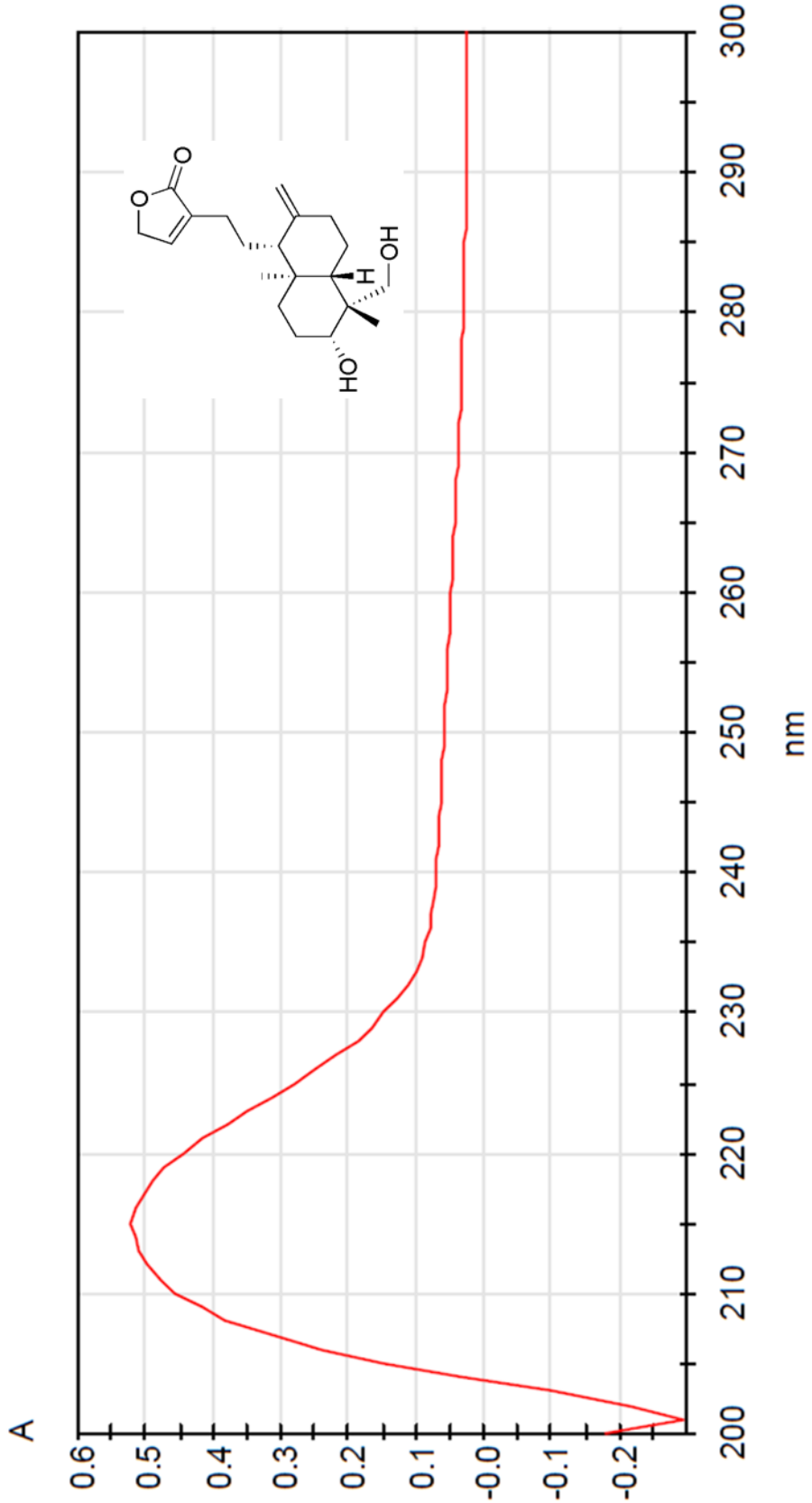
^{1}H NMR spectrum of 14-deoxyandrographolide (500 MHz, $CDCl_3$)



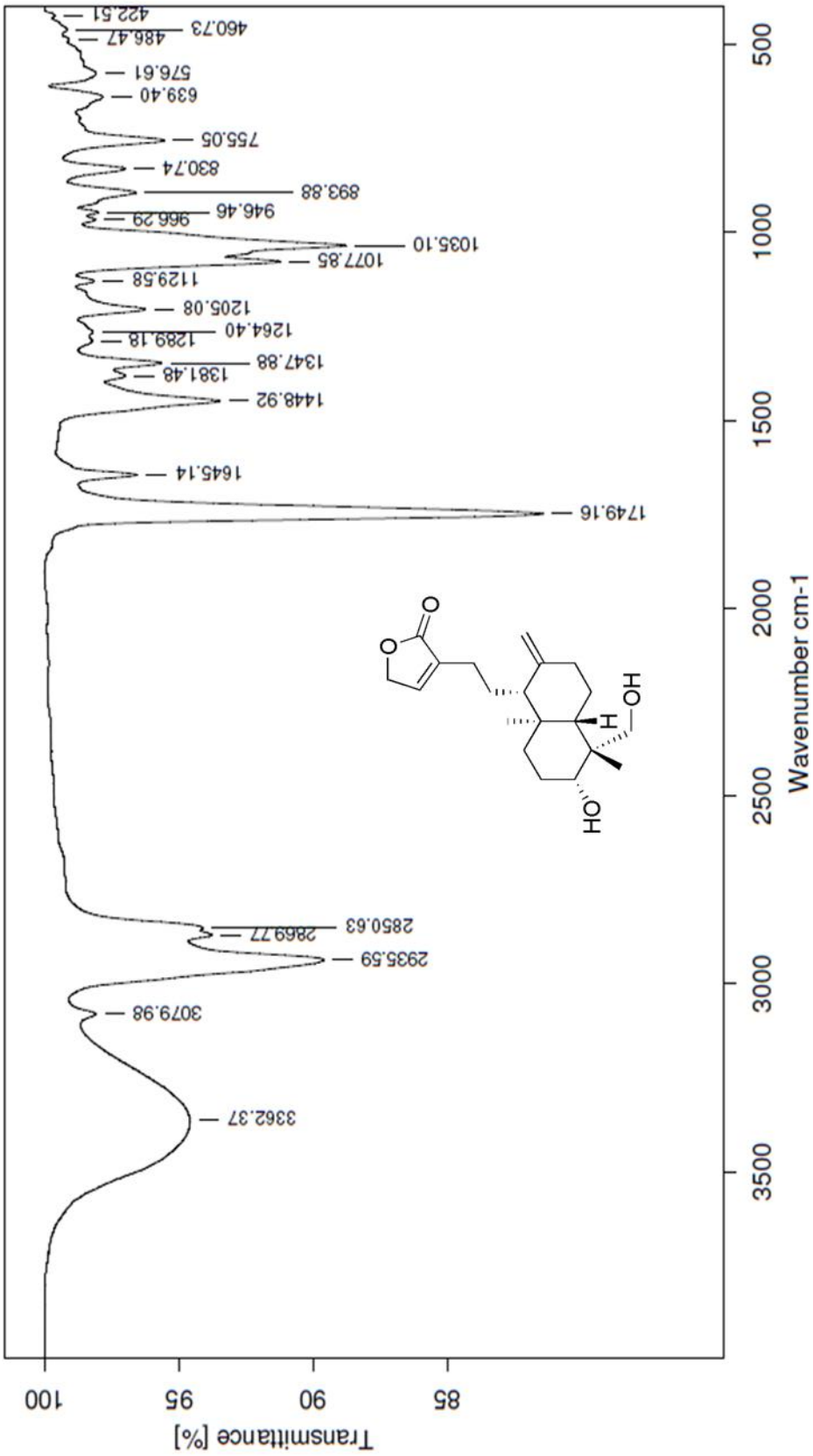
^{13}C NMR spectrum of 14-deoxyandrographolide (125 MHz, $CDCl_3$)



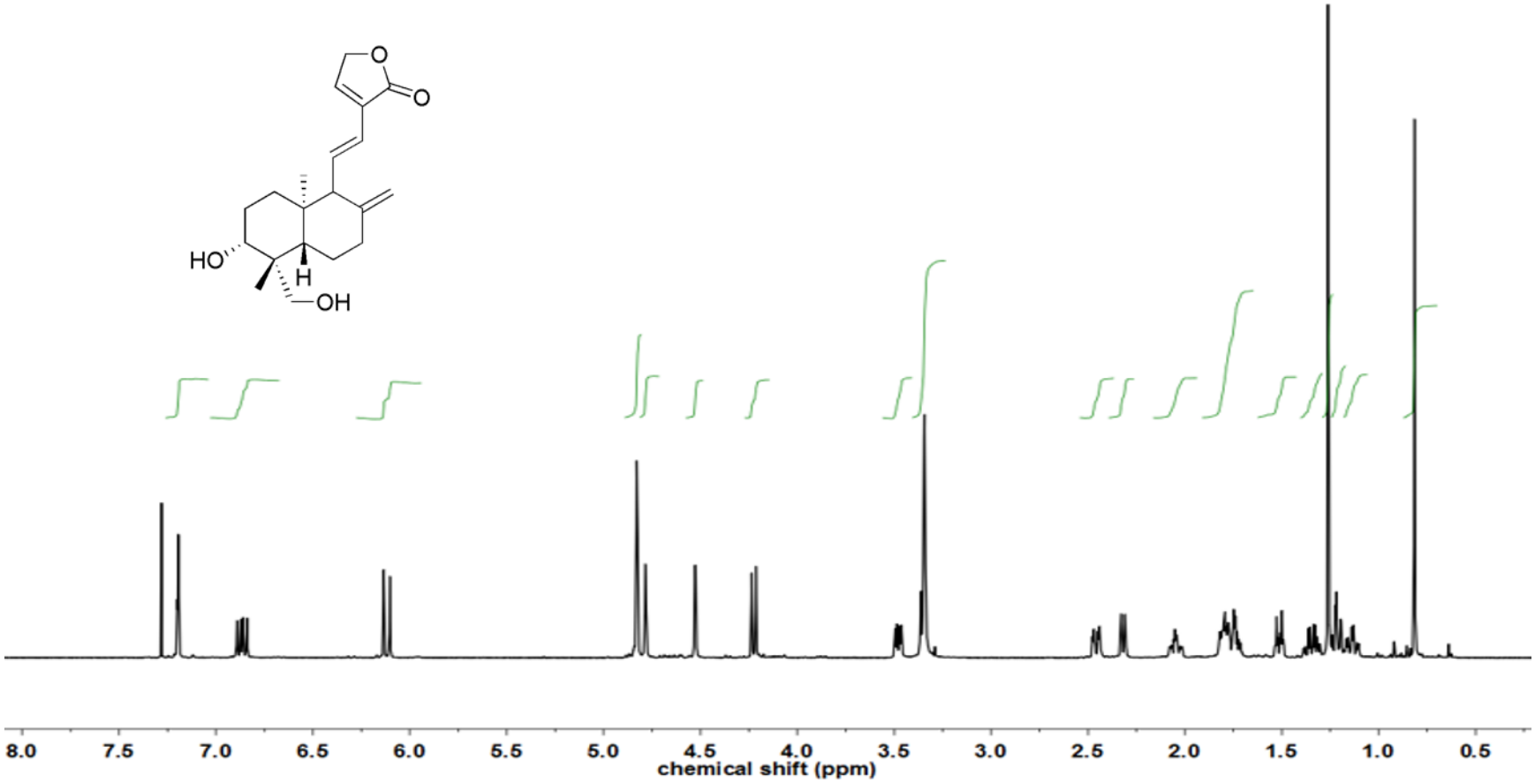
ESI mass spectrum of 14-deoxyandrographolide



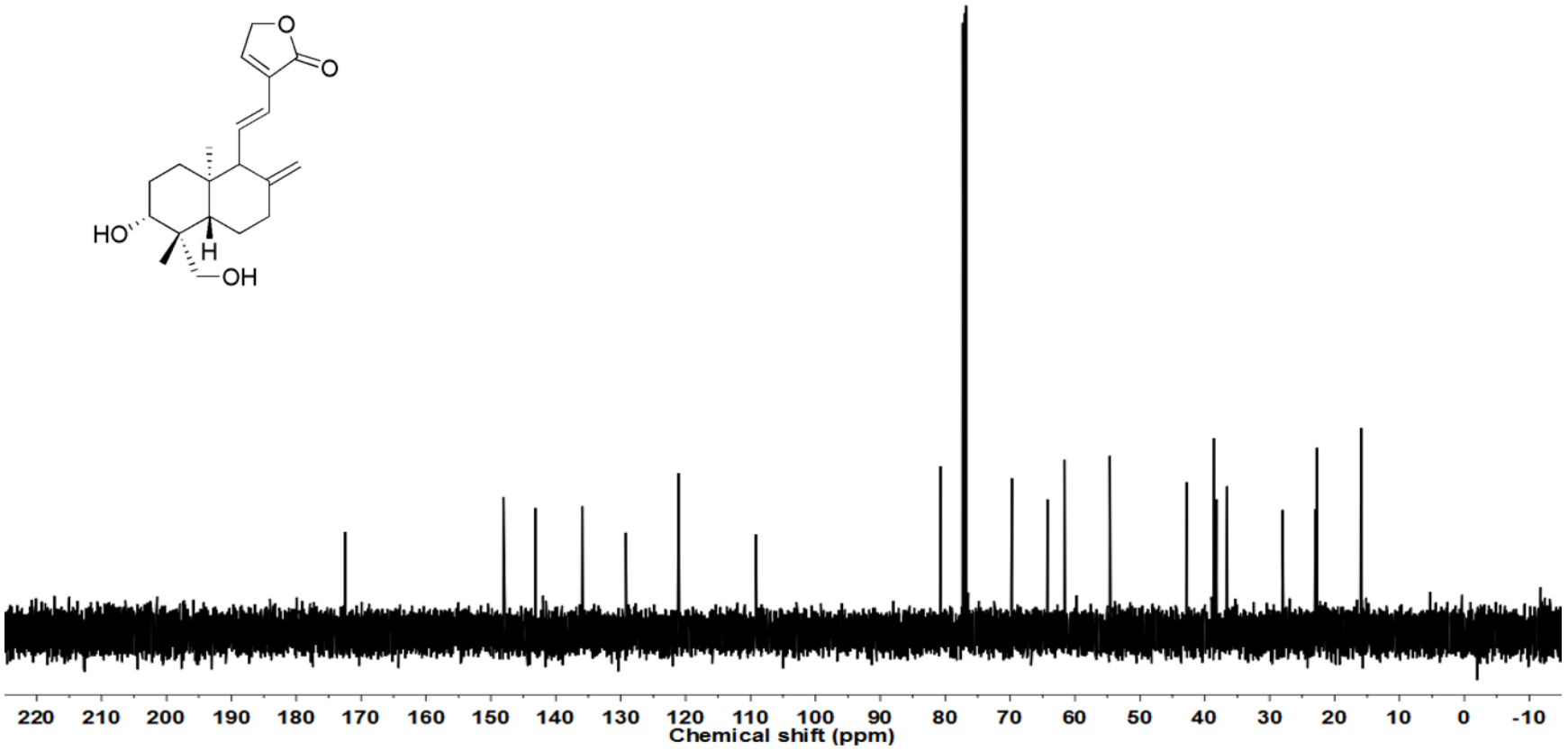
UV spectrum of 14-deoxvandrographolide (MeOH)



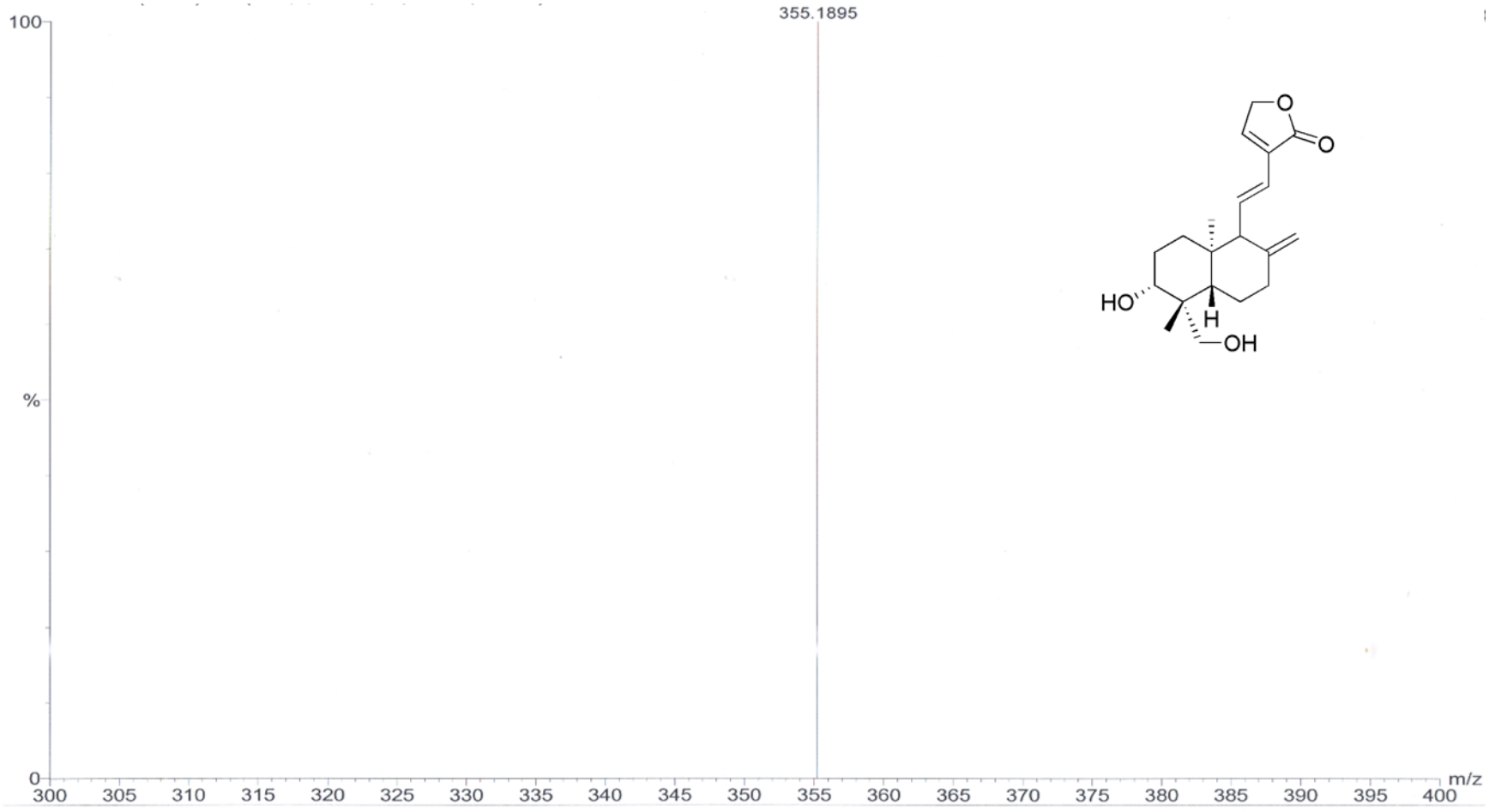
IR spectrum of 14-deoxyandrographolide (KBr)



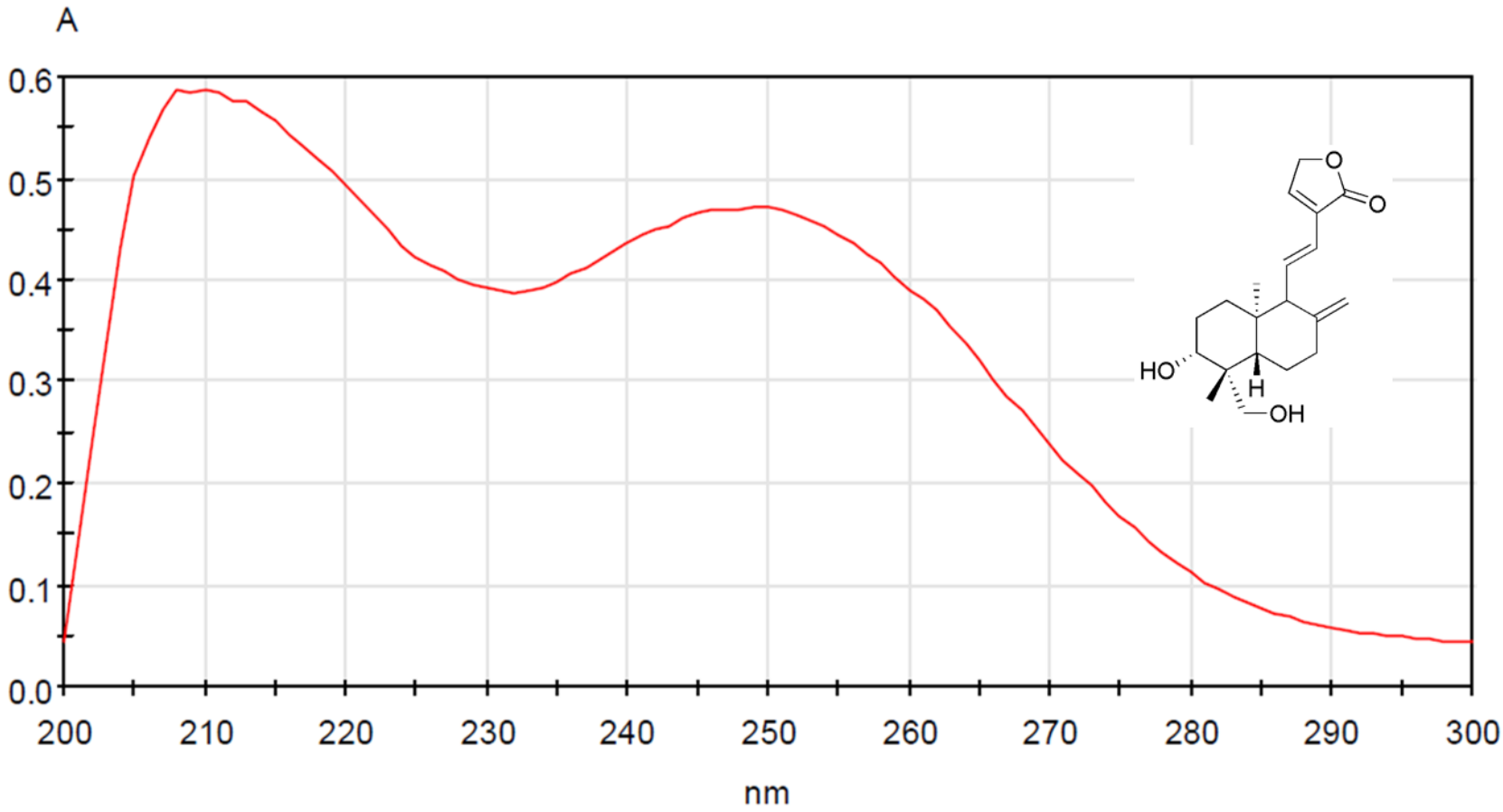
^{1}H NMR spectrum of 14-deoxy-11,12-didehydroandrographolide (500 MHz, $CDCl_3$)



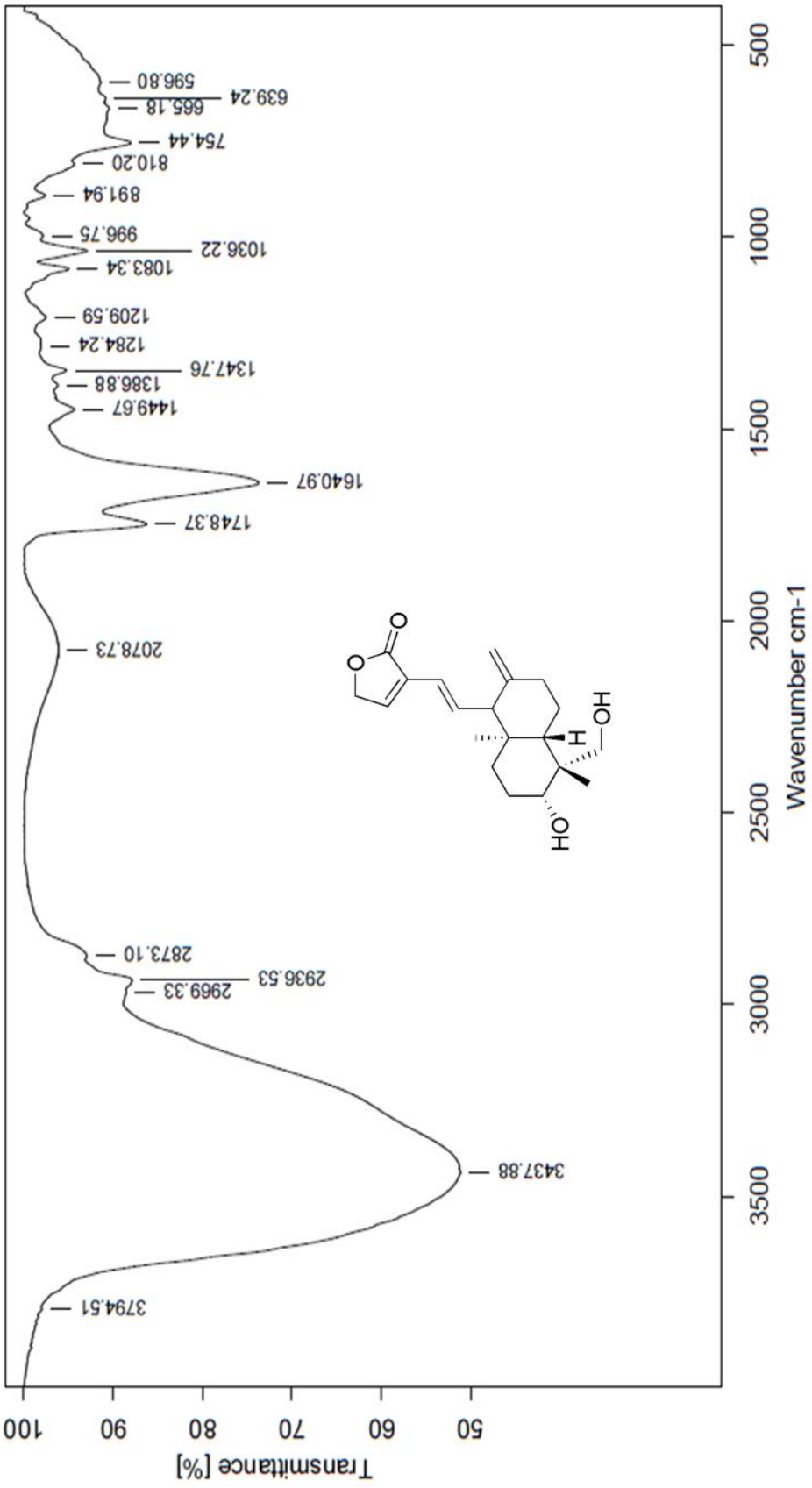
^{13}C NMR spectrum of 14-deoxy-11,12-didehydroandrographolide (125 MHz, $CDCl_3$)



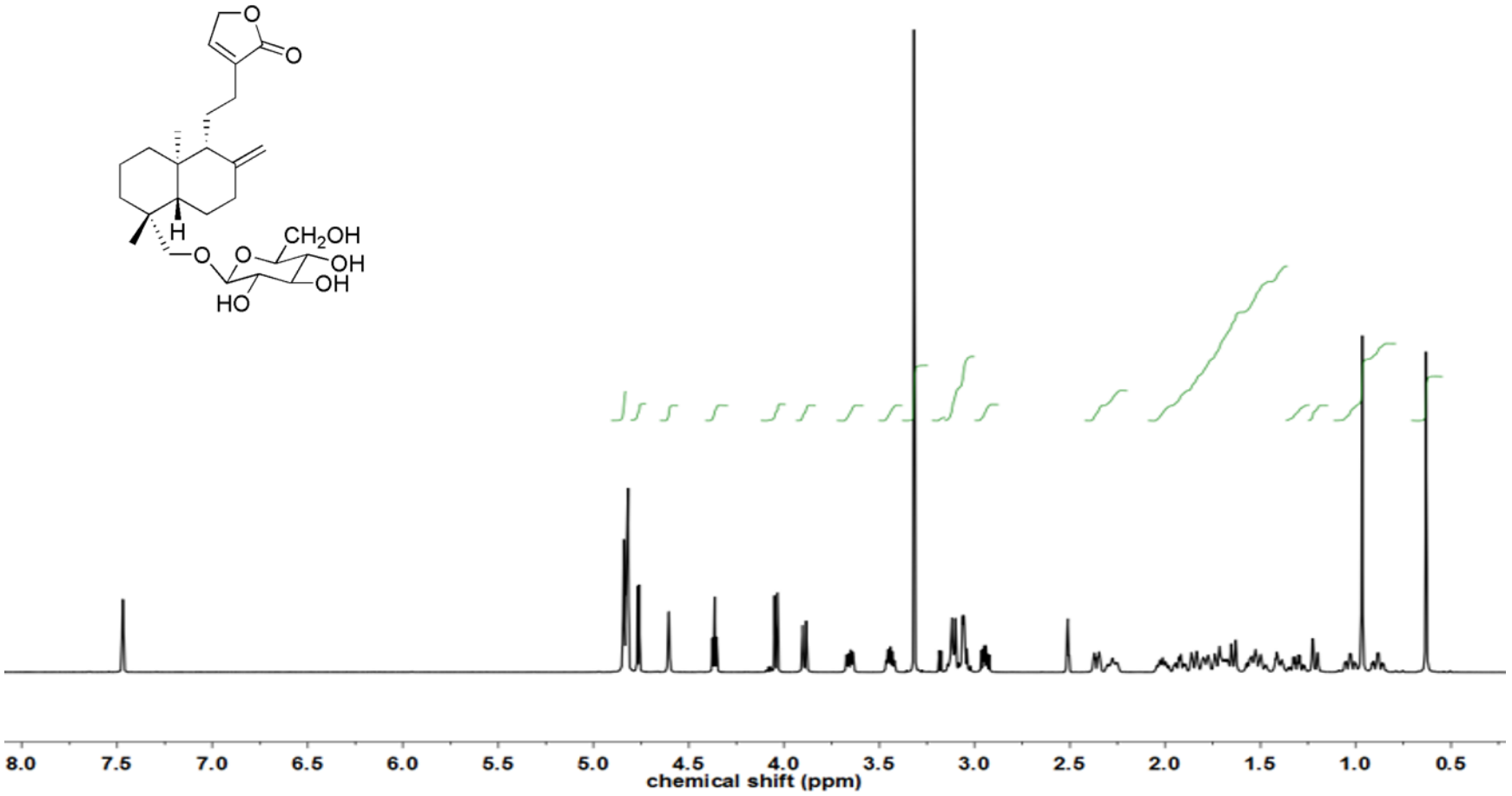
ESI mass spectrum of 14-deoxy-11,12-didehydroandrographolide



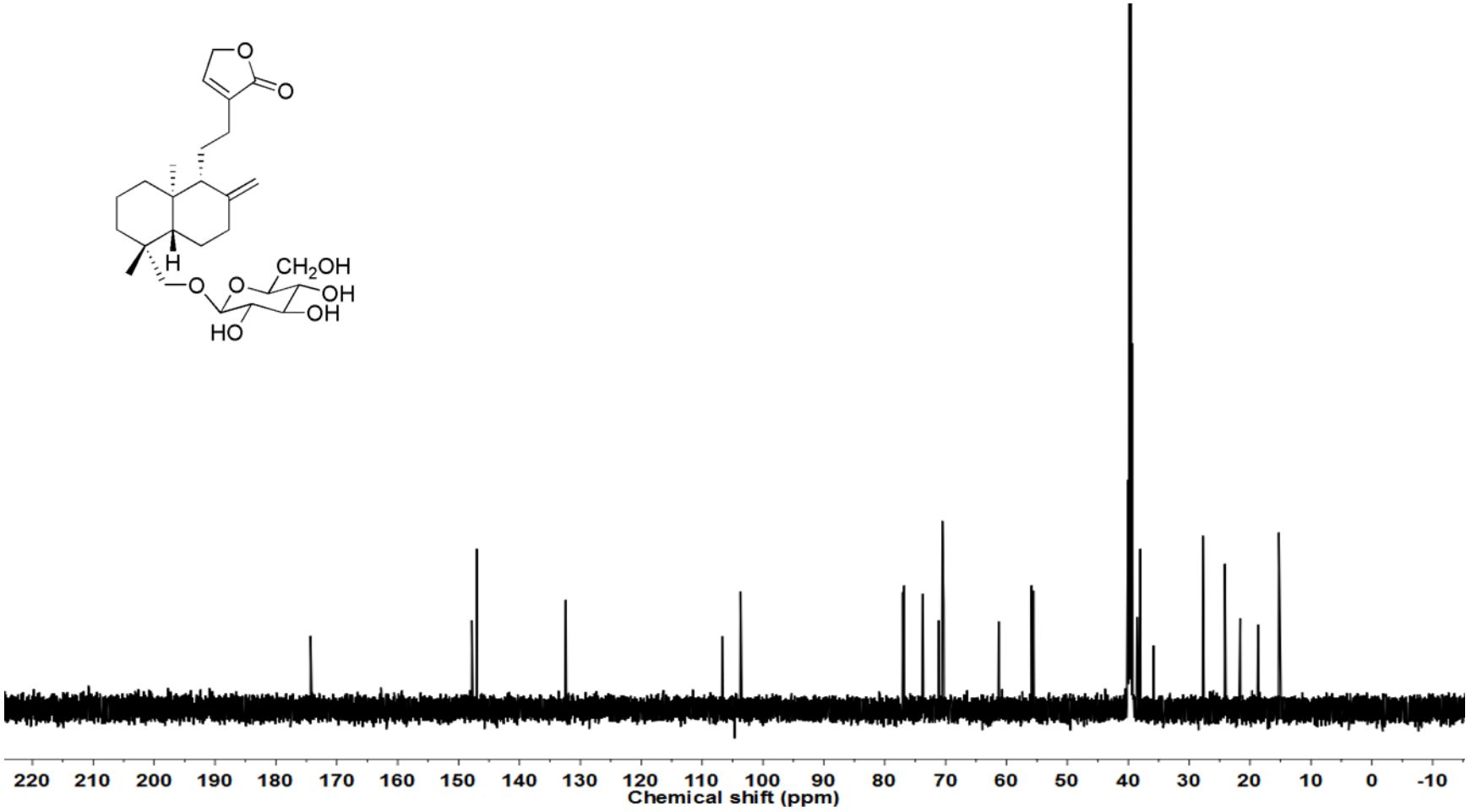
UV spectrum of 14-deoxy-11,12-didehydroandrographolide (MeOH)



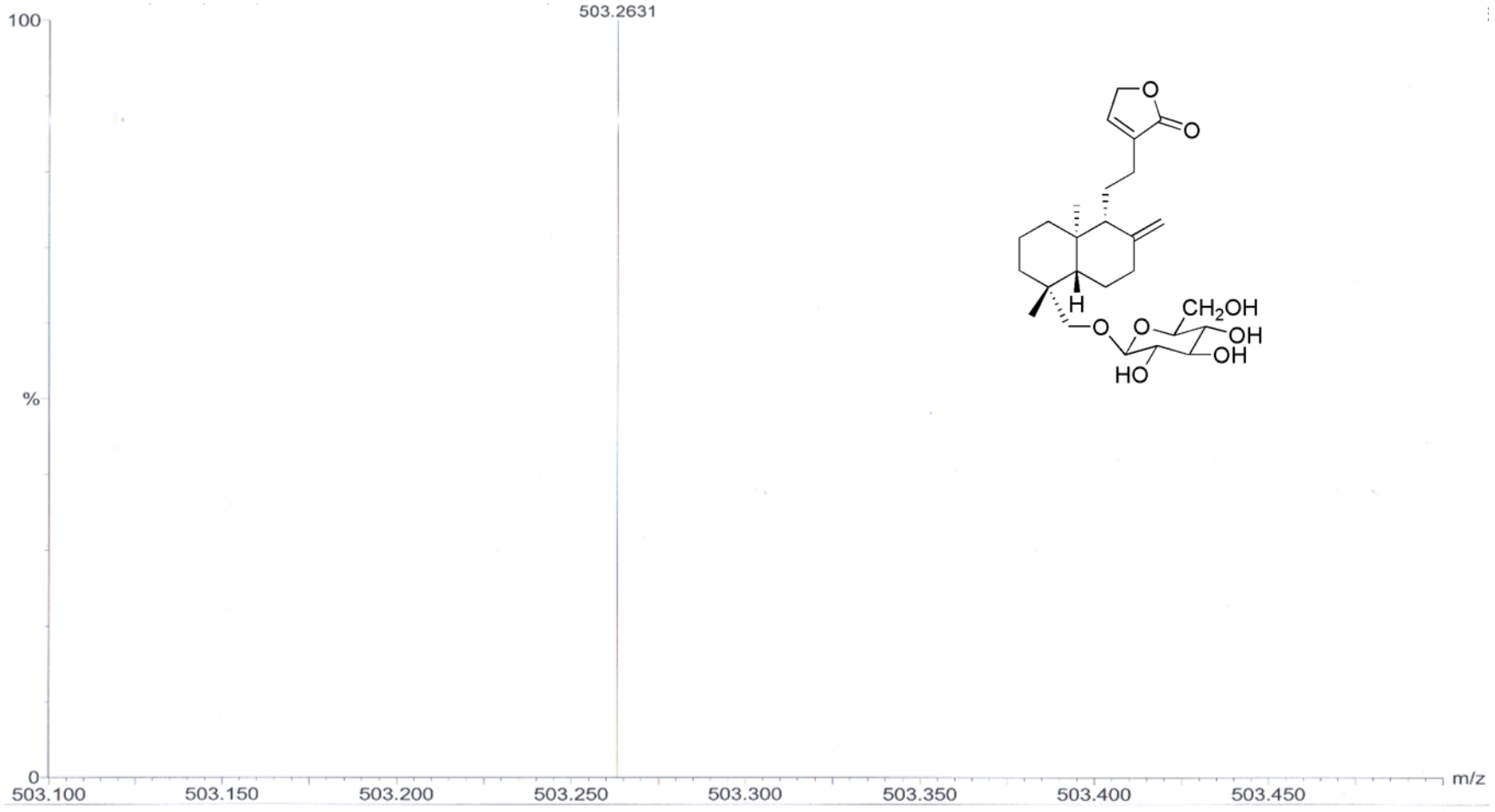
IR spectrum of 14-deoxy-11,12-didehydroandrographolide (KBr)



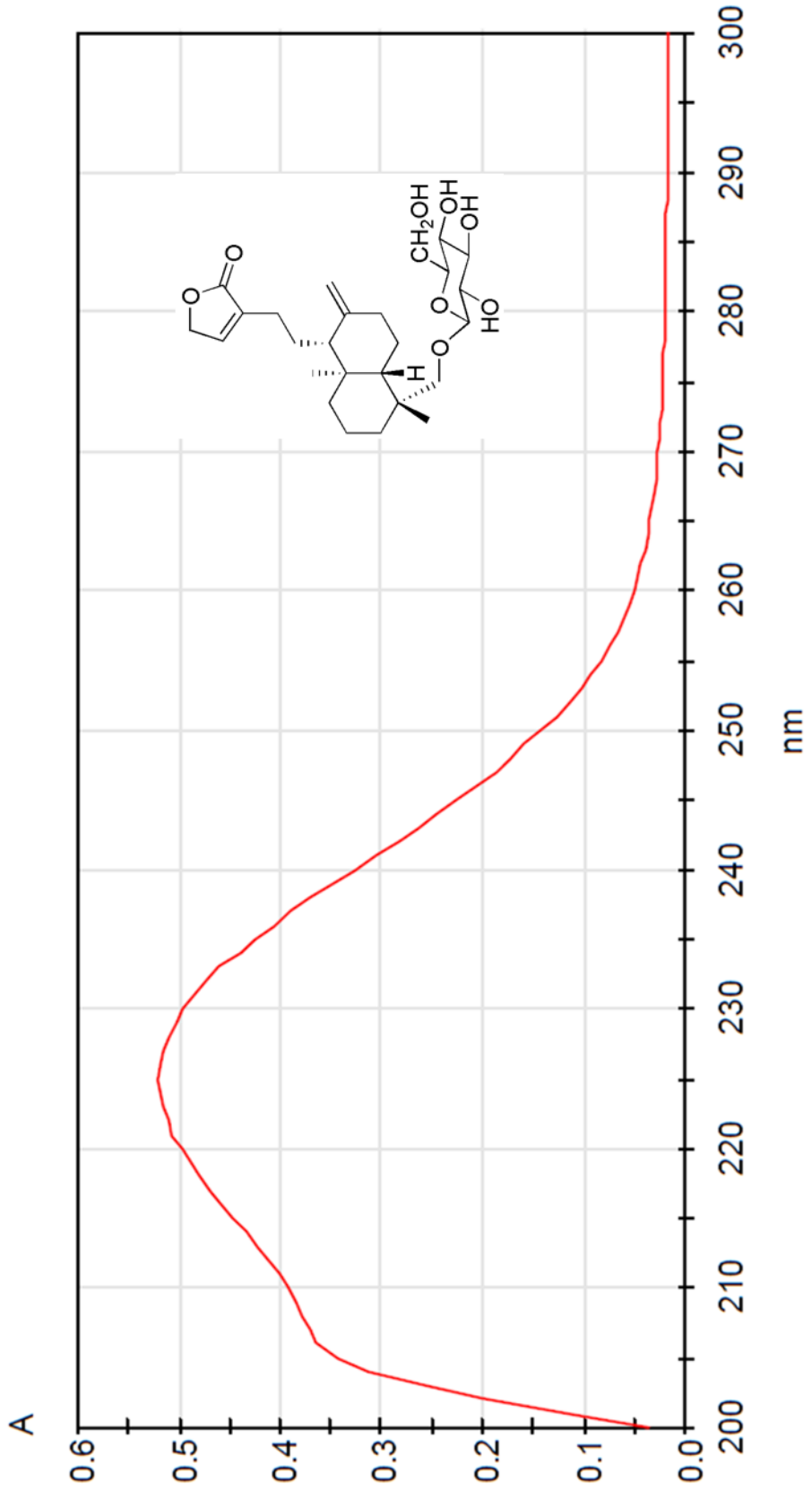
^{1}H NMR spectrum of neoandrographolide (500 MHz, DMSO-d_6)



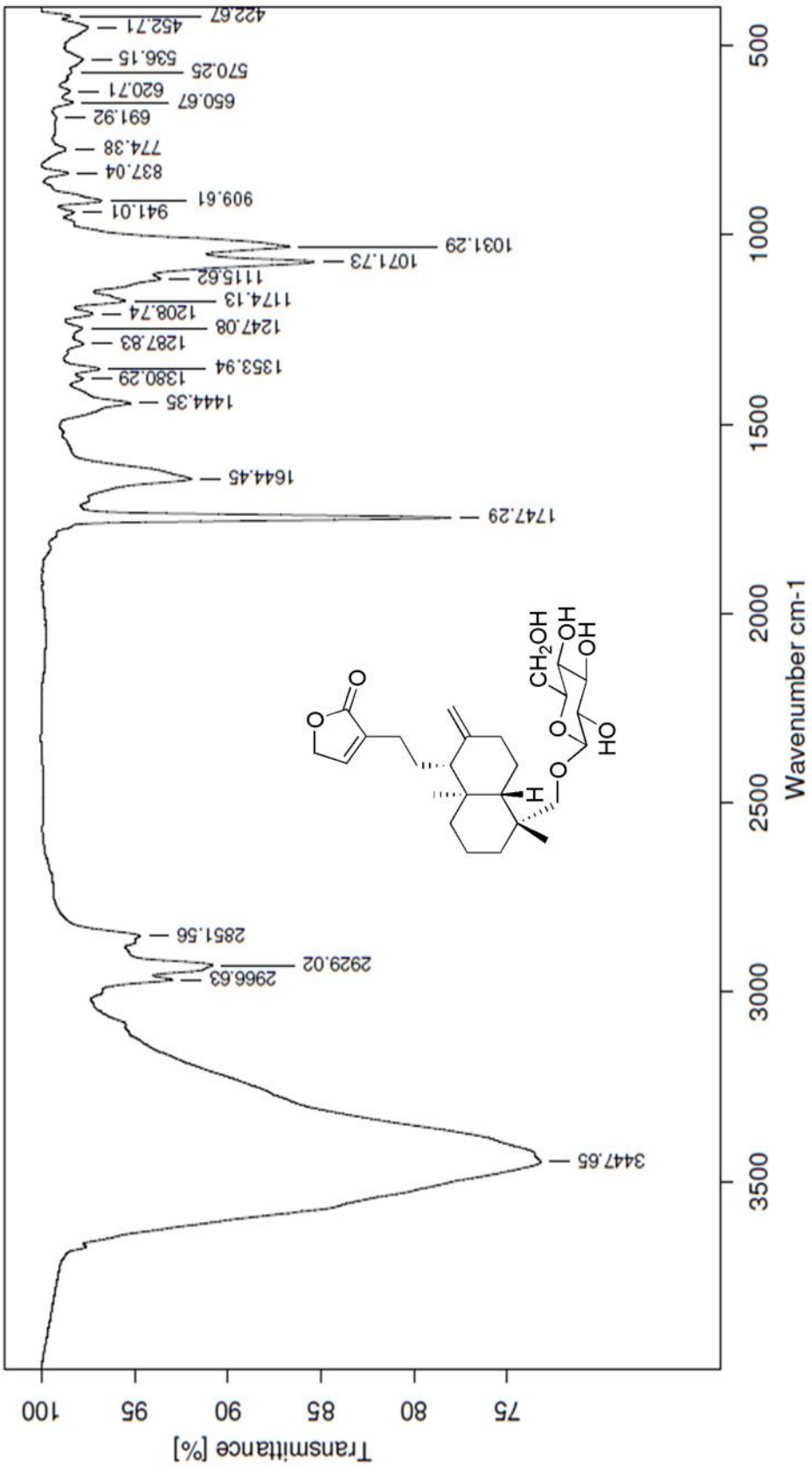
^{13}C NMR spectrum of neoandrographolide (125 MHz, DMSO-d_6)



ESI mass spectrum of neoandrographolide

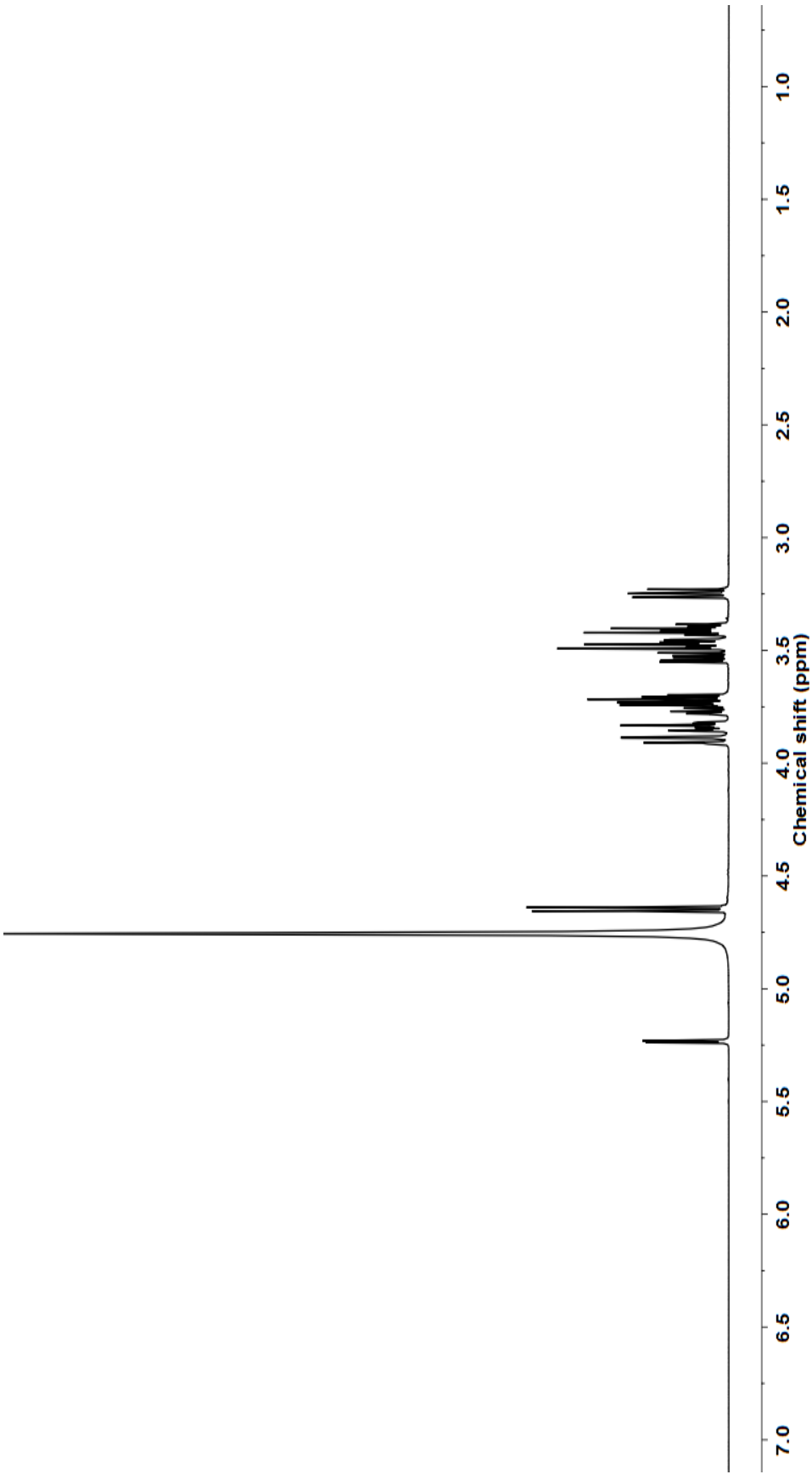


UV spectrum of neoandrographolide (MeOH)

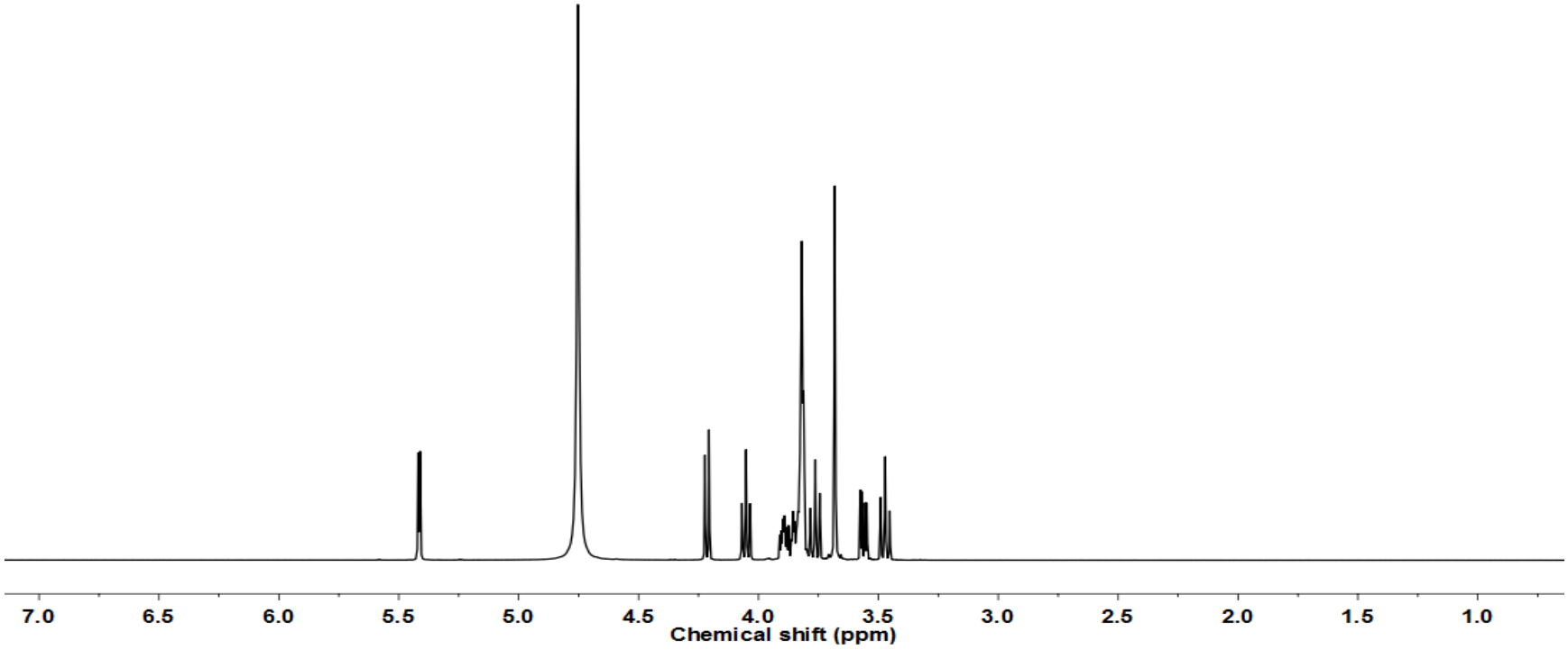


IR spectrum of neoandrographolide (KBr)

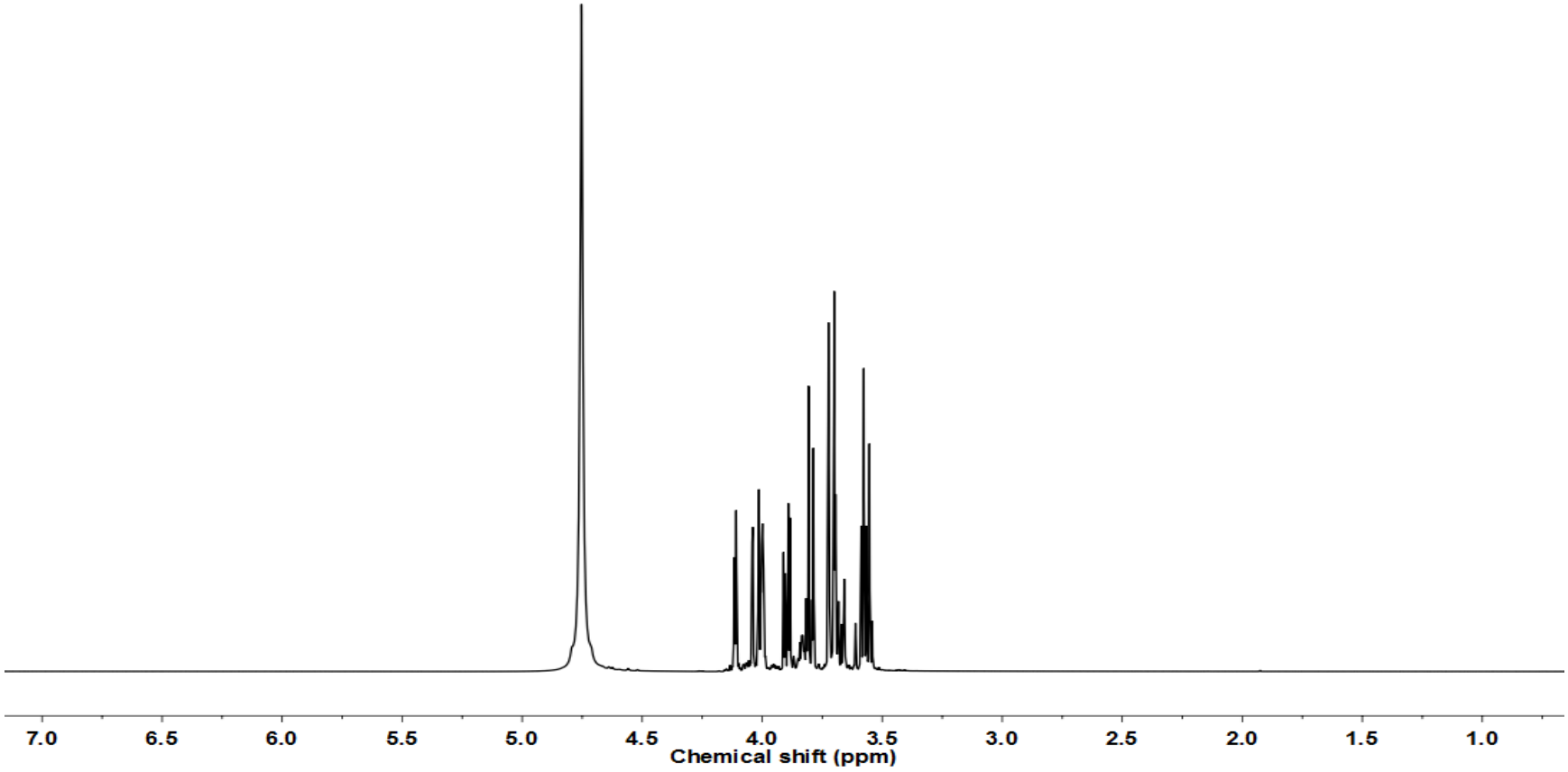
APPENDIX B



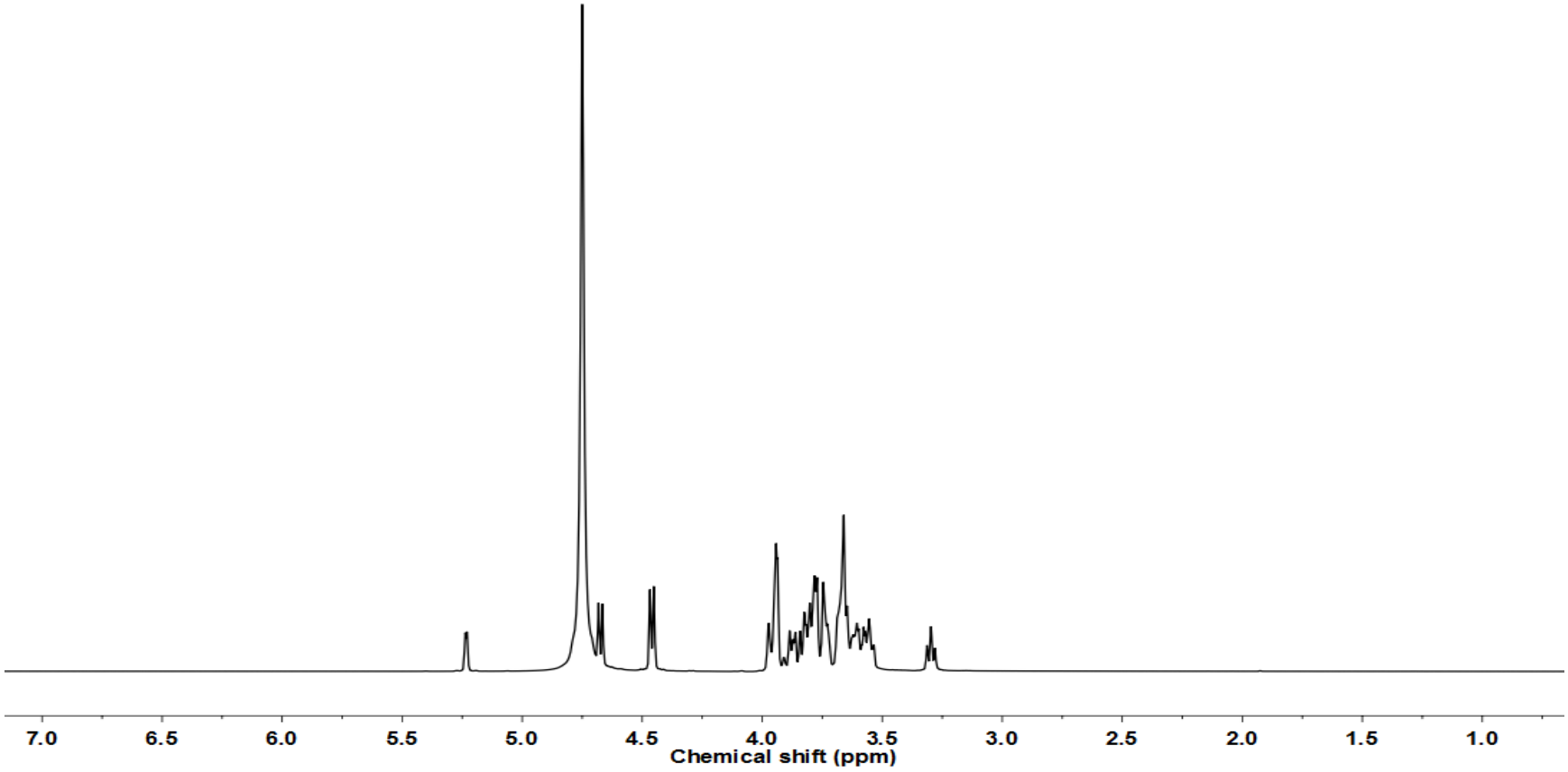
^{1}H NMR spectrum of glucose (500 MHz, D_2O)



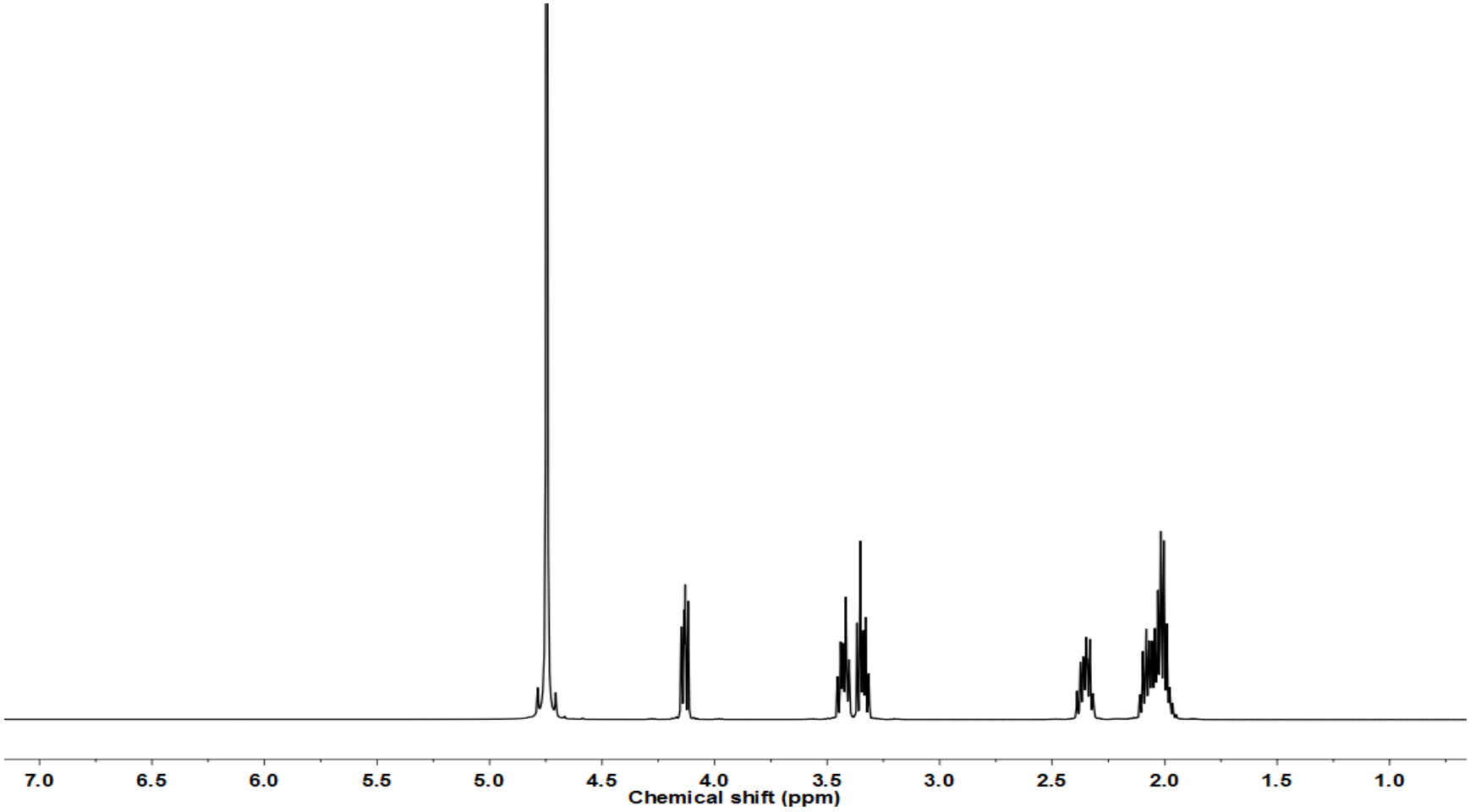
^{1}H NMR spectrum of sucrose (500 MHz, D_2O)



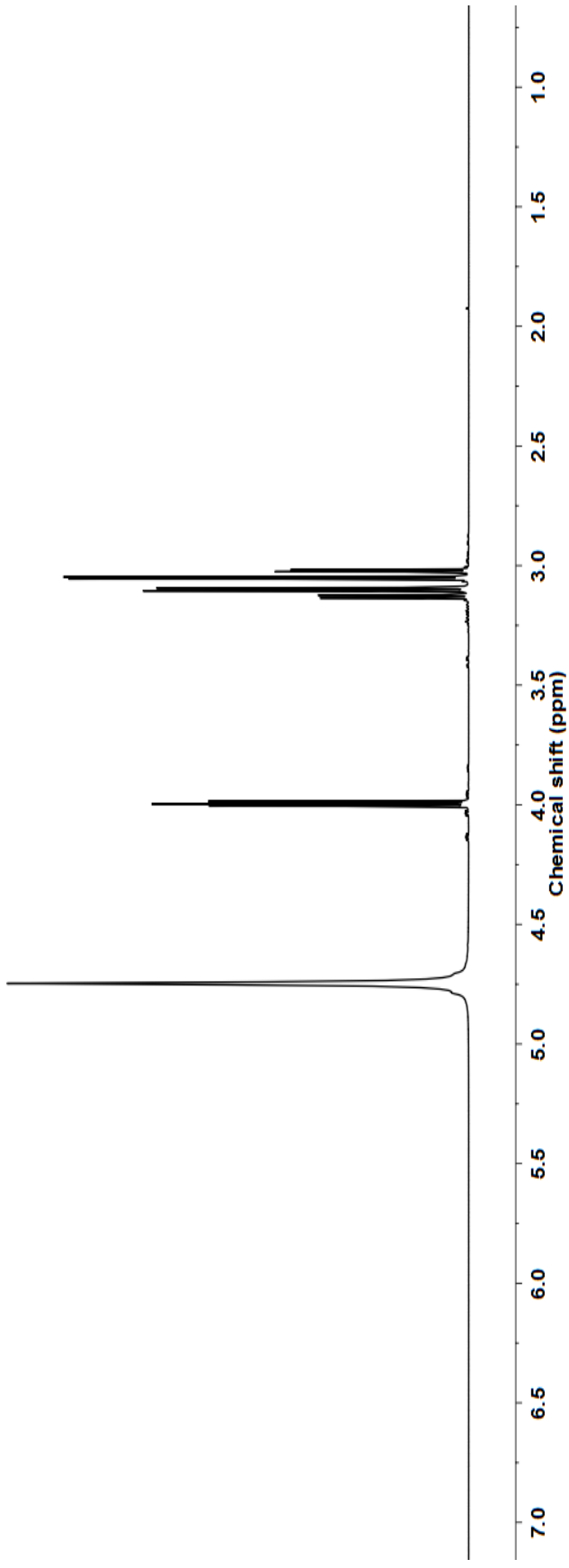
^{1}H NMR spectrum of fructose (500 MHz, D_2O)



^{1}H NMR spectrum of lactose (500 MHz, D_2O)



^{1}H NMR spectrum of proline (500 MHz, D_2O)



^{1}H NMR spectrum of cysteine (500 MHz, D_2O)

VITAE

Name Mr. Abdulaziz Wadeng

Student ID 5510720025

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Chemistry)	Prince of Songkla University	2013

Scholarship Awards during Enrolment

The Discipline of Excellence in Pharmacy, phase 2, Faculty of Pharmaceutical sciences, and the Graduate School Research Support Funding for Thesis.

List of Publication and Proceeding

Wadeng A, Plubrukarn A. Chemical profile of Andrographis herb from different geographical sources. The 2nd international conference on herbal and traditional medicine. 2017, Asia hotel, Bangkok, Thailand.