EXTRACTION AND ANTIOXIDANT PROPERTIES OF PROPOLIS FROM CHINA, KOREA AND NORTHERN THAILAND

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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เชน ลิฮง:การสกัดและสมบัติการต้านออกซิเดชันของพรอพอลิสจากประเทศจีน เกาหลี และ จากภาคเหนือของประเทศไทย) .EXTRACTION AND ANTIOXIDANT PROPERTIES OF PROPOLIS FROM CHINA, KOREA AND NORTHERN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. รมณี สงวนดีกุล. อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ. ดร. สิริวัฒน์ วงษ์ศิริ, รศ. ดร. อุบลรัตน์ สิริภัทราวรรณ. 125 หน้า.

จุดมุ่งหมายหลักของการวิจัยในการศึกษาพรอพอลิสในครั้งนี้ คือการวิเคราะห์หาฟลาโวนอยด์ซึ่งเป็นสารประกอบหลักในการออกฤทธิ์ทางชีวภาพ โดยการใช้วิธีสเปกโตรโฟโตเมตรี และ ไฮเพอร์ฟอแมนซ์ - ลิควิดโครมาโตกราฟฟี (HPLC) เพื่อศึกษาสมบัติการต้านออกซิเดชัน และการต้านการเจริญของจุลินทรีย์ของพรอพอลิสที่มาจากแหล่งเลี้ยงผึ้งจากภาคเหนือของประเทศไทย พบฟลาโวนอยด์ทั้งหมดใน 17 ตัวอย่าง พรอพอลิสจากแหล่งต่างๆของประเทศไทยนั้น มีปริมาณน้อยกว่าพรอพอลิสจากสหราชอาณาจักร ประเทศเกาหลี ประเทศบราซิล และประเทศจีน อย่างไรก็ตาม มีเพียง 1 ตัวอย่างจากเชียงใหม่ที่มีค่าฟลาโวนอยด์ทั้งหมดสูงใกล้เคียงกับพรอพอลิสที่มาจากประเทศอื่น เมื่อวิเคราะห์ปริมาณของ ฟลาโวนอยด์หลัก 8 ชนิด ได้ผลดังนี้ รูติน 0.01-0.28 เปอร์เซ็นต์ ไมริซิติน 0.00-0.08 เปอร์เซ็นต์, เคอร์ซิติน 0.00-0.92 เปอร์เซ็นต์, เคมเฟอรอล 0.00-0.36 เปอร์เซ็นต์, อะพิจินิน 0.00-0.25 เปอร์เซ็นต์, พิโนเซมบริน 0.00-0.09 เปอร์เซ็นต์, ไครซิน 0.00-1.81 เปอร์เซ็นต์ และ กาเลนจิน 0.00-1.81 เปอร์เซ็นต์ ตัวอย่างจากเชียงใหม่ (CM 1) ตัวอย่างเดียวที่พบฟลาโวนอยด์ทั้ง 8 ชนิด ดังนี้ รูติน (0.06 เปอร์เซ็นต์) ไมริซิติน (0.08 เปอร์เซ็นต์) เคอร์ซิติน (0.92 เปอร์เซ็นต์) เคมเฟอรอล (0.36 เปอร์เซ็นต์) อะพิจินิน (0.25 เปอร์เซ็นต์) พิโนเซมบริน (0.01 เปอร์เซ็นต์)ไครซิน (1.81 เปอร์เซ็นต์) และ กาเลนจิน (1.81 เปอร์เซ็นต์) และพีคอื่นๆอีกหลายพีคในโครมาโตแกรมจาก HPLC ผลการทดสอบฤทธิ์การต้านออกซิเดชัน พบว่าพรอพอลิสจากประเทศไทยมีรีดิวซ์ซิ่งพาวเวอร์และกิจกรรม DPPH scavenging สูง แสดงว่ากิจกรรมการต้านอนุมูลอิสระของพรอพอลิสจากเชียงใหม่ (CM) เชียงราย (CR) ลำพูน (LP) และน่าน (N) อยู่ในระดับสูง ผลการศึกษา MIC_{50}ของสารสกัดพรอพอลิสต่อ *Staphylococcus aureus* เรียงจากน้อยไปมากดังนี้ บราซิล < N4 < N2 < CR1 < N1 < CR-MFU < CR7 < CR3 < CR4 < CM3 < LP สำหรับกิจกรรมการต้านการเจริญของจุลินทรีย์ต่อ *Candida albicans* พบว่า จาก 11 ตัวอย่างที่ทดสอบ มีเพียง 3 ตัวอย่าง คือ จากประเทศบราซิล เชียงราย (CR1) และน่าน (N4) ที่มีฤทธิ์ต้านจุลินทรีย์อย่างอ่อน

ภาควิชา.....-..... ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีการเกษตร..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา .....2554..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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LIHONG CHEN: EXTRACTION AND ANTIOXIDANT PROPERTIES OF PROPOLIS FROM CHINA, KOREA AND NORTHERN THAILAND. THESIS ADVISOR: ASST. PROF. DR. ROMANEE SANGUANDEEKUL.CO-ADVISOR: PROF. DR. SIRIWAT WONGSIRI, ASSOC. PROF. DR. UBONRAT SIRIPATRAWAN.

The main goal of this investigation is to determine the main flavonoid which is the main activity of Thai propolis by using spectrophotometric method and high-performance liquid chromatography (HPLC). Additionally, the antioxidant and antimicrobial activity of the propolis from different location in China, Korea and Northern Thailand are also determinated. The result shows that the total flavonoids in all 17 propolis from the different geographic locationin Thailand are lower than 2% and lower than the propolis samples from UK, Korea, Brazil and China. However, one sample (CM1) from Chiang Mai province of Thailand is higher (13.34%) and closer to the other countries. The contents of 8 major flavonoids in Thailand propolis are as follows:rutin ranges (w/w) 0.01–0. 28%, myricetin 0.00–0.08%, quercetin 0.00–0.92%, kaempferol 0.00–0.36% , apigenin 0.00–0.25%, pinocembrine 0.00–0.09%, chrysin 0.00–1.81% and galangin 0.00–1.81%. Only one propolis sample (CM1) from Chiang Maiidentified all 8 main flavonoids, rutin(0.06%), myricetin (0.08%), quercetin (0.92%), kaempferol (0.36%), apigenin (0.25%), pinocembrine (0.01%), chrysin (1.81%) and galangin (1.81%), and had many peaks in the HPLC figure.The antioxidant activity test showed that propolis had a strong reducing power and strong DPPH radical scavenging activity, indicating that the antioxidant activity of the propolis samples from Chiang Mai (CM), Chinag Rai (CR), Lamphun (LP) and Nan (N) of Thailand were all good. The result of study on MIC50 from various propolis extracts against *Staphylococcus aureus.* demonstrates that all the propolis extracts show the antimicrobial effect to *Staphylococcus aureus,* the sequence from low to high of MIC50 against S.aureus is Brazil<N4<N2<CR1<N1<CR-MFU<CR7<CR3<CR4<CM3<LP. For the antimicrobial activity against *Candida albicans,*among the 11 kinds of propolis samples, only three kinds of propolis, Brazil, Chiang Rai (CR4), and Nan4 (N4), show a weak antimicrobial activity against *Candida albicans.*

Department :…….. Student's Signature……
Field of Study :…….. Advisor's Signature……...
Acdemic Year :…….. Co-Advisor's Signature…….
Co-Advisor's Signature…….

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CHAPTER I.

INTRODUCTION

The term of Propolis derived from two Greek words “Pro” meaning “before” and “polis” meaning “city”----“before the city” or “defender of the city” (Steiner ,1977). The term “propolis” is often attributed to the famous Greek Philosopher Aristotle (384 - 322 B.C.). Propolis was used to constrict the entrance or opening of bees' cities - a measure undertaken by the insects to keep the hives safe from unwanted intruders (Crane, 1990).

Propolis is a sticky resinous substance collected by honeybees (*Apis mellifera*) from tree buds, tree bark, tree gum, or shrubs or other botanical sources. Honeybees carry it back to their hive and mix it with wax that secreted from their special glands. Honeybees use propolis as a building material to seal up any cracks or gaps where microorganisms could flourish, narrow the bee hive entry, and as a thin layer to varnish inside brood cells before the queen lay eggs into them; presumably this provides a strength, waterproof and hygienic unit for the developing larvae. Sometimes, bees use the propolis to wrap the bodies of intruding insects and other enemies in order to avoid rotting body. The volatile oils in propolis appear to serve as a kind of antiseptic air-freshener, keeping the hive bees clean homes and dry, ensuring a hygienic environment for the rearing of brood (Crane, 1990; Chen et al.2009, Banskota et al., 2001).

The main plant sources of propolis are poplar, betula, pines, cypress, willow and sumacs. There are also some minor sources distributed all over the country for bees to collect propolis, such as peach, plum, almond, eucalyptus, rubber plant, and helianthus etc .(Chen, 1993).

Natural (raw) propolis is a very complex mixture. It varies according to its resource (including botanical source, beeswax, geographic locations, season and climate, and so on). Some ingredients come from the honeybees themselves. Despite of that the different and intricate

composition of natural propolis from different countries and different regions, most of the propolis shares similar overall chemical compounds. Normally, natural propolis composition is approximately made of 50-55% resinous compounds, 30% beeswax, 10% essential oils, 5% bee pollen, and 5% others and impurities (Crane, 1990 and Fang, 1998). The main chemical component of propolis is flavonoid and phenol (Chen et al., 2010 and Fang, 1998). Therefore, analysis of the component of propolis is great importance in different location or different source of plants.

In recent years, propolis is extensively used in functional food, health care products and medicine to improve health and prevent diseases for instance inflammation, diabetes, heart disease, and cancer. Propolis is also used in animal husbandry, plant protection, food processing and cosmetics.

More over, there has been growing interest in functional foods. Functional food can be defined as food that produces a beneficial effect in one or more physiological functions, increases well-being and decreases the risk of suffering from a particular medical condition. The functionality of this food is usually related to some of the ingredients that it contains and at present consumers prefers these ingredients to come from a natural rather than synthetic origin. Thus, they are commonly extracted from plants, food by-products and other natural sources (Herrero et al., 2005). Among the functional ingredients, the group that is most widely studied is the family of antioxidants.

Interest in antioxidant compounds has increased nowadays in the light of recent evidence regarding the important role of antioxidants in human health. In fact, several preventative effects against different diseases such as cancer, coronary diseases, inflammatory disorders, neurological degeneration, aging, have been related to the consumption of antioxidants (Wollgast, 2002; Madhavi, 1996)

Flavonoid, phenolic compounds or polyphenols, are one of the most important groups of

compounds occurring in plants (Bravo, 1998). These compounds are reported to exhibit anti-carcinogenic, anti-inflammatory, anti-atherogenic, antithrombotic, immune modulating and analgesic activities, among others and exert these functions as antioxidants (Catapano, 1997 and Vinson et al., 1998).

Propolis is rich in phenolic compounds or polyphenols and flavonoids. In the past decade, there was a tremendous boom in the used of propolis products as a function health care products and folk medicine for treating diabetes, cardiovascular diseases, neurodegenerative diseases, and some cancers in China. It has been used in folk medicine from ancient times in many countries and has been extensively studied in European countries (Bankova, et al., 2000; Castaldo and Capasso, 2002, Chen et al.2009). Nowadays, the functional food has become a major topics of public interest and the propolis products also have become a popular products in Europe, Brazil, Argentina, Japan, China and more other countries. However, the native raw propolis has still not been researched and developed in Thailand.

The market for raw material and secondary products containing propolis has been increasing and continues to grow as they find more acceptances in medicine and as more cosmetic manufacturers have realized their benefits and marketing value in the world. There are several kinds of propolis products influx into marketsall over the world such as ointments, oil and nasal sprays, suntan lotions, syrups or honeys, tablets, capsule, and some cosmetics e.g. propolis shampoo, soap, creams, lotion, and toothpaste (Chen and Zhang 2002).

According to the above statment, it is extremely significant to analyze and determine the composition and the antioxidant activity and develop research on propolis in Thailand. It will contribute a great deal of benefits not only to apiculture but also to human health of Thai people.

This researchfocuseson extraction, antioxidant and antimicrobial properties of propolis from China, Korea, and northern Thailand.

CHAPTER II

Literature Review

The application of propolis on traditional medical have thousands year history. As early as 3000 years ago, the Egyptians used the propolis to cover the dead body as a mummy and proteced from decay (Wallis, 1901). In the Greek History of Alexander the great, the author cited that Aristotle who was his teacher, Alexander would like to use and no doubt about honey and propolis have the preserving properties (Ransome, 1986). It was also not until Greek times that the first specific reference was found to propolis, in Aristotle's pioneering natural scientific study of the honeybee (Wongsiri ,1985 and Wongsiri et al.1995). By around 400 BC, during the time of Pericles, 20,000 beehives were kept in Attica where became famous for its history on its honeybee, believed to possess medicinal properties on honey and propolis. Hippocrates (460-377 BC), considered the father of medicine, recognized the medicinal properties of propolis and used propolis to help heal sores and ulcers.The Greeks used propolis as the principal ingredient of a famous exquisite perfume named "polyanthus" mixed propolis with styrax, benzoin ad aromatic herbs (Murat, 1982). The Romans, Pliny in his 35-Volume Natural History illustrated a considerable knowledge of the bee world including a detailed knowledge and the medicinal properties of propolis, Ransome cited in 1986. The Arab famous doctor Abu Ali idn Sina (Avicenna) in his famous book The Canon of Medicine described the characteristics and applications of propolis (Makashvii,1978). In Europe, even though there are very few direct references to propolis are found in early British herbal literature, a great deal of to the anti-inflammatory properties of tree and plant resins.The basic raw maerials harvested by the bee to produce prpolis. Simultaneity, in southern and central Russia propolis was a familiar natural remedy. In Georgia, with the influence of Arab, Sul-han-Saba (1658-1725) the complier of a Georgian encyclopedic dictionary defined propolis as "a substance similar to wax from the

bottom of the hive" (Makashvii, 1978). A Georgian book of medicine published in the thirteenth century, called Carabadini, suggests a treatment for dental decay using propolis (Makashvii, 1978).

For thousands of year traditional natural medicine----herbs and plants to treat disease was the only medicine available to humans and had the Footprint of Propolis. These had made a great impact on modern time. Just as in EU, in the general and Russia, beekeeping has been an important agricultural activity and propolis a valued by-product of beekeeping and honey production. while the medicinal values of pollen have been discovered and used relatively recently by medical practitioners across the globe, it has been interesting to note that propolis or the bee glue was officially prescribed by pharmacopeias as well as physicians as early as in the 17th century. In particular, Europen continued to use propolis and other natural medicines long after they had been discarded in the west. The wild bees Caucasus produce large quantities of propolis not onl to help them survive the harsh continental winters but also to help generations local people to use propolis to treat a varies diseases such as coughs, colds, burns, wounds, chest and dental problem. In south afraica, propolis was used extensively to tread wounds and applied in many cases following surgery, and in particular following amputations during the Boer War. As same as in Russia, the Nepoleon's army doctors instead dressed the stump with the honey and propolis to assiste rapid and complete healing for amputation. During the Second World War, especially Russia doctors successfully experimented with the use of propolis ointment in treating wounds in order that propolis became known as Russian penicillin and so on many cases in the Europe especially in Russia, Poland , Romania, Bulgaria, Yugoslavia etc (Mansfield, 1994; James,2001).

Propolis has been officially used to treat several ailments since the 17th century, its usage became popular in the late 20th century both scientists as well as the laymen are now showing a renewed interest in the use of propolis. Literature on Europe, Japan, Korea, Brazil and China have

reported that propolis has various biological activities, such as antimicrobial, anti-pathogenic microbial activity, antioxidant activity, antiviral, anti–inflammatory, anticancer, antifungal and antitumor properties (Kujumgiev, 1999; Moreno et al.,2000; Joaquim, 2006).

2.1 Physical character of propolis

Propolis is a stick resinous substance collected by honeybees (*Apis mellifera*) from tree buds and gummy exudation from the cracks in the tree bark or other botanical sources, mainly from the poplar (Populus) , willow, betula,cypress, sumacs, and some from beech, horse chestnut, pines, conifer trees, and less from other plant such as peach, plum, almond, eucalyptus, rubber plant and helianthus etc. and mixed with beeswax and other secretion of bees (Chen et al.2009).

Raw propolis is very complex. It varies according to its source (including plants, hives, district, seasons, and so on). The natural propolis is an opaque solid with a rough or smooth surface. The color of propolis can vary enormously from different regions and climates. Normally the colour varies from golden brown, grayish brown, grayish green and dark green with luster, depending on its botanical source and region.Generally, natural propolis smells fragrant, with a milky fragrance when being burned. In temperate climates it ranges from a light yellow or brown to a dark brown colour, or with a reddish hue. In tropical climates it can range from the light brown-green to black and dark red. It also varies from the different trees and plants harvested as well as the types of the bees. Propolis collected by stingless bees tends to brown reddish and collected by black bees tends to be darker in color.

Raw propolis is sticky and pliant to the touch, with a slightly bitter and pungent taste. It will become hard, crisp and brittle at cold or frozen and turn soft and malleable while handled at moderate temperatures. Heated to 36°C, it becomes soft, pliable, plastic and sticky. When the

temperature rises to 60-70°C, it is melted into sticky liquid and bee's wax is extracted from it and turn to liquid at temperatures between 70-100°C (Crane 1990; Chen 1993).

Propolis is difficult to dissolvein water, slightly dissolved in turpentine, and easily dissolved in ether, chloroform and ethanol. When it dissolves in 95% alcohol it turns transparent and maroon in colour with sediment of granules.

2.2 Chemical composition of propolis

Propolis is a resinous substance collected by honeybees from buds and cracks in the bark of different plants (mainly from poplar, beech, horsechestnut, birch, conifer, Cypress， willow and sumach trees etc) and its possess majority of phenolic compounds. The plant origin of propolis determines its chemical diversity. Propolis chemical composition depends on the species of local flora present at the site of collection and its geographic and climatic characteristics (Bankova, 2005).

Natural propolis has an intricate composition, which is closely related to the types of plants from which it is collected. Some ingredients come from the honeybees themselves such as bees wax etc..In despite of that the different and intricate composition of natural (raw) propolis from different countries and different regions, most of the propolis share similar overall chemical compounds with the pleasant fragrance. Argentina, Australia, Brazil, China, Korea, Japan, Europe and other more countries have many researches show that most of the propolis share similar overall chemical compounds. Normally, nature (raw) propolis composition is approximatelyas follows (Chen1993; Crane, 1990; Fang, 1998; Hu, 2005):

Resinous compounds and balsam (aromatic oils)	55%
Beeswax	30%
Bee Pollen	10%
Others and Impurities	5%

2.3 The main composition of propolis

The main chemical components of propolis are flavonoid, phenolic, or polyphenol fraction in despite of the different and intricate composition of natural (raw) propolis from different countries and different regions (Li et al., 2007; Havsteen, 2002). And this fraction is considered to contribute more to the therapeutic effects than the other components of propolis (Nagy, 1996; Woisky, 1998; Castaldo,2002). This also becomes focus of propolis in international propolis research.

Flavonoids and phenolic acid esters, especially caffeic acid and ferulic acid, are known for their antimicrobial, antiviral and antioxidant activity (Pietta, 2000). Flavonoids are also reported to be the most abundant and most effective antioxidant in propolis. Flavonoids may reduce free radical formation and consequently might have a protective effect on serum lipids against oxidation (Moreno et al., 2000). European scientist Bonvehí and Coll (2000) analyzed the composition, bacteriostatic and ROO-scavenging potential activity of the propolis from China and Uruguay.

The main chemical component found in propolis extracts is as follows (Cao W., 2007; Li 2007; Yu, 2007).

- Flavonoids are the best important components of propolis, and 36 kinds of flavonoids have been identified in propolis, including: rutin myricetin, quercetin, apigenin, galangin, kaempferol, pinocembrin, pinostrobin, pinobanksin, chrysin, luteolin etc.
- Terpenes are another component of propolis extract, with 17 varieties identified in China (Li et al., 2007).
- Amino Acids: 18 kinds of amino acid, such as arginine etc.
- Minerals: 32 kinds of mineral have been identified.
- Other components: polyphenol and polysaccharide, alcoholic aldehyde, other

organic acids (including cinnamic acid, vanillin, caffeic acid, ferulic acid etc.), enzymes, vitamins etc (Zhang, 2007).

Therefore, the main composition, flavonoids will be the key study for the Thai propolis.Propolis is rich in flavonoids and phenolic compounds. In the past decade, there was a tremendous boom in the use of propolis products as a function health care products and folk medicine for treating diabetes, cardiovascular diseases, neurodegenerative diseases, and some cancers in China. It has been used in folk medicine from ancient times in many countries and has been extensively studied in European, south American and other countries of Asia etc. (Bankova, Castro and Marcucci, 2000; Castaldo and Capasso, 2002) .

Nowadays, the functional food has become a major topics of public interest and the propolis products also have become a popular products in Europe, Brazil, Argentina, Japan, China and more other countries. However, the Thailand propolis has still not been researched and reported. The researchers of Thai propolis are still blank in Thailand or in the world.

In view of above statement, it is of extremely significant to analyze and determine the composition and the biological activity and develop research on propolis of Thailand. It will contribute a huge amount of benefits not only to apiculture but also to human health of the Thai people.

2.4 Medical effects of propolis

In recent years, the growing interest in the health functions of food has led to increased research on the functions and efficacy of propolis. Research show that propolis possesses extensive pharmacological properties and functions in anti-bacterium, anti-inflammatory, anti-oxidative, anti-tumor and anti-cell toxin, prevented arteries hardening, improving blood microcirculation, enhancing immunity,and promoting tissue regeneration, etc. (Moreno et

al.,2000). Chinese Pharmacopoeia (2005) now includes propolis for medical applications.

2.4.1 Antioxidant effects

Recently, the marketing and consumption of antioxidant products have increased in light of evidence regarding the important role of antioxidants in human health (Wollgast, 2002; Madhavi, 1996). Flavonoids, the main component of propolis, are powerful antioxidants. Coffee acid, caffeic acid, and ester of propolis are also key anti-oxidants. The other components of propolis such as xanthine, and quercetinic acid have certain anti-oxidative properties. Another four furfuran lignans were isolated from Chinese propolis by column chromatography. They were identified by spectroscopic methods as sesamin, yangambin, (+)-pinoresinol and (+)-syringaresinol. Among them, (+)-pinoresinol and (+)-syringaresinol were isolated for the first time from propolis. The antioxidant activity was evaluated by measuring the inhibition of lipid peroxidation in rat liver microsomes (Cuiet al., 2002).

Numerous experiments have studied the anti-oxidative functions of propolis in aged mice. The results show that propolis indeed possesses good anti-oxidative properties that play a protective role for human health. Furthermore, these studies also demonstrated that the EEP extraction method demonstrates higher anti-oxidation (Wanget al., 2004; Huet al., 2005; Lang et al., 2006; Penget al 2005). Therefore, the previous studies suggest that propolis might be a kind of high quality food ingredient that could produce antioxidative functional food (Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002).).

Indeed, EEP was studied with lard oil, rapeseed oil, peanut oil, soybean oil and colza oil to determine their peroxide value (POV value) .The results showed that EEP not only could inhibit oil oxidation, but also the antioxidation effect of 0.5% EEP was 2~3 times to 0.5% VE

(Vitamin E), and better than butylated hydroxyanisole (BHA). It is also reported that propolis was a natural and safe antioxidant for food (Xuet al., 2007; Guoet al., 2007;Caoet al 2002).

2.4.2 Antimicrobial and anti-pathogenic microbial activity

Many of the tests suggest that propolis has widespread antimicrobial and anti-pathogenic microbial activity when used in various extracts and concentrations (Lan et al., 2006; Yang et al., 1999; Zhang et al, 1998;Xuan 2005; Hu et al., 1998)

Ethanol extract, for example, has an inhibitory effect on different food pathogenic bacteria (Zhang, 1998). Ethanol extract also has a positive inhibitory action on tooth decay pathogenesis (Wang, 1996).

For its anti-pathogenic microbial activity, propolis is used to treat clavus, tympanitis, oral erosions and ulcers. Dermatological direct external application of ethanol extracts or concentrated ointments (with up to 33% propolis) has given good results in veterinary use for healing wounds and sores. Plastic surgery employs extracts for improving wound healing and reducing scar tissue development. It is also effective in treating burn wounds.

2.4.3 Anti-inflammatory activity

Studies of propolis in mice have demonstrated anti-inflammatory and anti-pathogenic microbial activity. The studies show that propolis plays a positive role in assisting the treatment of arthritis, pleurisy, pneumonia and bronchitis (Li et al., 2002, Huet al.,2003). It is also appears to be very effective in treating gastric and/or duodenal ulcers.

2.4.4 Anti-tumor and anti-radiation activity

Since 2000, there has been increasing research on the inhibitive properties of flavonoids, caffeic acid, and terpenes of propolis on cancer. The studies found that in certain cases propolis possesses an inhibitory effect on cancer cell growth (Wu,2002) and was also found to actually kill human hepatocellular carcinoma cells (Zhen,2002). The propolis also has anti-mutational and anti-radiational effect, inhibiting and preventing production of carcinogens and potential carcinogens. (Hang, 2001). In addition, the anti-tumor mechanism of propolis was analyzed in China. It increasingly appears that there is a bright and positive future for the use of propolis in functional foods (Long et al., 2000; Chen et al., 2003; Liet al., 2006).

2.4.5 Antilipidic and hypoglycemic effect

The water extract (WEP) and ethanol extract (EEP) of propolis were also studied for the inhibitory effects on activities of α-glucosidase, which is responsible for the breakdown digestion of carbohydrate to monosaccharides in the process of intestinal absorption. When comparing WEP and EEP, it was found that both of them acted as inhibitors of α-glucosidase. Scientists observed the effect of propolis on blood glucose on mice. Studies suggest that propolis has both hypoglycemic effect and blood lipid regulatory effect (Wuet et al., 1998).

The function of propolis extract (EEP) on reducing blood lipids effect was also observed. SD rats with high levels of blood lipids were divided into 4 groups and orally administered propolis in different concentrations for 4 weeks. The blood lipids levels were then measured again and the results show that propolis has a regulating effect on blood lipid (Qian Ronghua 2003). Studies on the effects of WEP and EEP on blood lipids show that propolis extracted by either method had inhibitory effects on the level of triglyceride (TG), total cholesterol (TC), low-

density lip cholesterol (LDL-C) in serum, and TC and TG in liver. EEP also can regulate lipid metabolism to reduce TC, TG， LDL-C (Hu,et al 2003; Zheng et al., 2004; Hu et al., 2004; Zhang et al., 2005, and Zhao et al., 2005Zeng et al., 2006;).

2.4.6 Immunization activity

Researchers found that propolis has strong immunity adjustment functions. It can increase the lymphocyte proliferation in mice by 3.7 to 6 times (Tang et al., 2006; Shen et al., 1989), restrain red blood cell dissolution (Zhouet al., 2003), enhance macrophage vigor and strengthen phagocytosis (Zhaoet al., 2005; Zhanget al., 2005). Besides that, propolis was also found to enhance the killing action ofNatural killer (NK)cells .

2.4.7 Other effects

Propolis is also widely used in animal husbandry, plant protection, food processing, and cosmetics. Because of positive studies on its effects on tissue regeneration and renovation, the most popular use of propolis has been in cosmetic applications. Together with its bactericidal and fungicidal characteristics it provides many benefits in various applications in cosmetics(Chen, 1993).

Therefore, propolis is extensively used in functional food, health care products and medicine to improve health and prevent diseases for instance inflammation, diabetes, heart disease, and cancer. Propolis is used as well as in animal husbandry, plant protection, food processing and cosmetics (Banskota, 2001; Chen, 2009).

2.5 Methods of propolis compounds determination

2.5.1 Method to prepare propolis extract

Raw propolis cannot be used directly and must be purified after harvesting because it contains some useless impurities.

2.5.1.1 Ethanol extracted propolis (EEP)

EEP is the most common method for extracting propolis now especially in China of Asia. Extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components (Pietta, 2000). Before 2000, ethanol concentration of 70% was most commonly used to extract propolis (Bankova et al., 2000; Popova et al., 2004; Popova et al., 2005; Chen Weixian, 2006). Also some researchers used the 80% ethanol (Moreno et al., 2000). Subsequent research showed that 95% concentration was best. Some researches used various concentrations of ethanol as solvent and measured the absorption spectra of the different extracts. The 95% ethanol extraction yielded the highest concentration of flavonoids and the lowest amount of beeswax (Bosioet al., 2000; Wanget al., 2004; Sawaya, 2004; Ahn, 2004).

Preparation of raw propolis begins by freezing propolis in order to make a fine powder. In the next procedure, a weighed raw propolis powder is dissolved in ethanol (the most frequently used proportion is 1:10, w/v), left for 24h at room temperature. After filtering, this procedure is repeated several times. Alternatively, the raw propolis powder is dissolved by shaking at 70 C for 30min (National Standard of Propolis, 2007).

2.5.1.2 Soxhlet extraction method (SE)

Soxhlet Extraction Method also is used to extract raw propolis by some researchers. Raw propolis powder is dissolved in the Soxhlet extractor with 100ml ether. Heating and distillation turn it transparent(Wanget et al., 2007).

2.5.1.3 Ultrasonic wave extraction (UE)

For UE method, the raw propolis powder is dissolved in 50ml ether with ultrasonic for 10min and filtered; the residues are then put through the same procedure two more times. The UE method could extract flavonoids at a higher rate than the SE method (Guizhou Univ.). UE was shown to be the most efficient method based on yield and selectivity requires shorter time(Li et al., 2007).

2.5.1.4 Aqueous (water) extracted propolis (AEP) or steam-distilled extraction

The AEP method actually utilizes pure water or a lower concentration of ethanol such as 40% ethanol, at 80 C for 10min, which is then mixed into hot water. Some scientists use water and a small quantity of natural surfactant (Bankova et al., 2000), Although not all ingredients are water soluble, aqueous extracts have been shown to exhibit bactericidal and fungicidal effects.

2.5.1.5 Supercritical extracted propolis (SEP)

Supercritical Extraction is a newest method currently used in China of Asia. SEP was developed for the fractionation of propolis tincture to obtain flavonoids, terpenes and essential oil fractions. It removes high molecular mass components. Flavonoids are practically

insoluble in pureCO^2, but sufficiently soluble in CO^2ethanol to enable their separation from high molecular mass. Jiangsu University developed the method using of CO^2 or dimethylmethane as a solvent to extract propolis, also using microwave to assist in extracting the rest of the propolis material (Gao et al., 2000).

2.5.1.6 Others

Other extracted methods such as methanol, hexane and acetone and chloroform have also been used (Pietta et al., 2000; Gil et al., 1995).

2.5.2 Methods for analysis of the compounds of propolis

2.5.2.1 Spectrophotometric methods

The techniques reviewed will be based on spectrophotometer as well as analytical separation techniques such as gas chromatography, high-pressure liquid chromatography and capillary electrophoresis.

Rapid spectrophotometric methods are assumed to be especially useful for the routine control of propolis. These methods are aimed at the determination of total flavonoids and phenolics or total flavanones / dihydroflavonols and total flavones / flavonols (Moreno et al., 2000). It will be also used in this research.

2.5.2.2 HPLC methods

The complete characterization of propolis activity involves both qualitative and quantitative chemical analysis. Chromatographic techniques such as gas chromatography (GC) (Abd, Hady and Hegazi, 2002; Bankova, Castro and Marcucci 2000; Popova et al., 2005; Fontana et al., 2000), in particular, high-performance liquid chromatograph (HPLC) currently represents the most popular and reliable analytical technique to provide the profile and identification of the individual phenolic compounds of propolis (Marcucci et al.,2000; Pereira et al., 2000; Bruschi, Franco and Gremiao, 2003). Coupled mass spectroscopy (MS), are being used increasingly for routine work (Li et al., 2007). HPLC or HPLC-MS will be chosen as the main method of our research. The thin-layer chromatography as well as was used to separate apolar flavonoids (Jasprica et al., 2004; Medicet al., 2004).

HPLC method will be chosen for this research.

2.5.3 Methods of antioxidant capacity determination

Japanese scientists (Ahn et al., 2005) examined the antioxidant activity of propolis from various areas of China. They evaluated for the propolis antioxidant activities by using β-carotene bleaching, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging, and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assays. Their results showed that China propolis had relatively strong antioxidant activity accompanied by high total polyphenol contents.

Flavonoids and phenolic acid esters, especially caffeic acid and ferulic acid, are known for their antimicrobial, antiviral and antioxidant activity (Pietta, 2000). Flavonoids are also

reported to be the most abundant and most effective antioxidant in propolis. Flavonoids may reduce free radical formation and consequently might have a protective effect on serum lipids against oxidation (Moreno et al., 2000). European scientist Bonvehí and Coll (2000) analyzed the composition, bacteriostatic and ROO-scavenging potential activity of the propolis from China and Uruguay. They suggested that the quercetin, kaempferol, chrysin, caffeic acid in propolis had antioxidant activity.

In this study, the antioxidant activity of the Thai extracted propolis will be assessed through the scavenging effects on the free radical-scavenging activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and reducing power of iron (III)/ ferricyanide complex assays.

2.5.4 Methods of antimicrobial capacity determination

Bauer et al. some scientists used disc diffusion method to analysis the antimicrobial activity on different ethanol extracts of propolis (EEP) with different concentration such as 30%, 50%, 70% and 95%. They found that t each sample against seven Gram positive, four Gram negative bacteria and one fungus culture (Bauer et al., 1966; Murat, 2003; Popova, 2003).

Ozgur (2007) determined minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of EEP on the growth of test microorganisms by using agar dilution method. It was shown that propolis samples were more effective against Gram positive anaerobic bacteria than Gram negative ones, and the main compounds of EEPs were flavonoids such as pinobanksin, quercetin, naringenin, galangine, chrysin and aromatic acids such as cafeic acid. Because of increased antimicrobial resistance, propolis may be kept in mind in the treatment of oral cavity diseases.

Popova (2003) researched on Turkey propolis'antimicrobial activity with

Staphylococcus aureus, Escherichia coli.by Determination of the minimal inhibitory concentration (MIC). They found that *S. aureus* is susceptible to very low propolis concentrations. (Kujumgiev et al., 1999; Sforcin et al., 2000; Drago et al., 2000) also showed an efficient propolis antimicrobial action on *S.aureus.* The results confirm that the importance of propolis antimicrobial activity, which provides the hive with the best defense against microorganisms.

Popova (2003) also found that the typical poplar propolis samples displayed very similar phenolic and flavonoid content, and some other propolis sample were characterized by low phenolic and very low flavonoid concentrations. Qualitative analysis by GC-MS revealed that sample contained diterpenic acids, high percent of cinnamyl cinnamate, significant amounts of hydroxy fatty acids and triterpenic alcohols, and phenolic glycerides. The results confirm the importance of phenolics for propolis antimicrobial activity which provides the hive with the best defense against microorganisms.

2.6 Propolis productsmarket

With the research and development of propolis products have made rapid development during the past 20 years (Table1 and.2). Propolis research has promoted commercial applications in food, health care and medicine. Propolis preparations have also seen rapid development, from propolis liquid and ointment in the 1980s, to propolis spray and capsules in the 1990s. After the year 2000, there was development of propolis soft capsule products, and cosmetic products have seen dramatic growth.

In the honeybee consumer market, demand for propolis continues to grow, as it reaches mass consumption and wider retail outlets. Currently, a wide variety of propolis products can be found in supermarkets, shopping malls, and specialty shops, including propolis capsule, propolis tablets, propolis liquid, propolis oral membrane, propolis honey, propolis tincture, propolis dental

liquid, propolis sprayer and ointment etc.

Table 2.1 Preparation of propolis products for medical products

Propolis Preparation	Application
Propolis Ointment	Dermatology, Surgery, Gynecology (e.g.: Uses to treat scalds proliferation scars Eczema, dermatitis, belt-shaped blister measles etc.)
Propolis Tincture	
Propolis Ulcer spirit	Stomatology (Oral cavity ulcer, mucous membrane floccosoids)
Propolis Ulcer membrane	
Propolis Sprayers	Stomatology, Otorhinolaryngology (rhinitis, pharyngitis, laryngitis, sinusitis)
Propolis Drops	

Table 2.2 Preparation of propolis health food products

Preparations	**Application**
Propolis Soft Capsule	➢ Reduce blood Lipid and blood Sugar ➢ Hypoglycemic, diabetes ➢ Other cardiac, cerebra and vascular disease ➢ Inhibition of cancer. ➢ Enhance immuno competence ➢ Protect from colds
Propolis tablet	
Propolis Extract Liquid	
Propolis Hard Capsule	

With continued research and development, the rage of propolis cosmetic products has increased: propolis skin cream, propolis jelling cream,propolis astringent, propolis facial cleanser,

propolis moisturizers, propolis body lotion, propolis hand wash,propolis body wash,propolisshampoo, etc., especially in China. The Chinese government has shown strong interest in the technological development of propolis. Over the past decade, the Ministry of Health of China approved 138 kinds of propolis food products and 5 medical products (MOH 2005; Fig1).

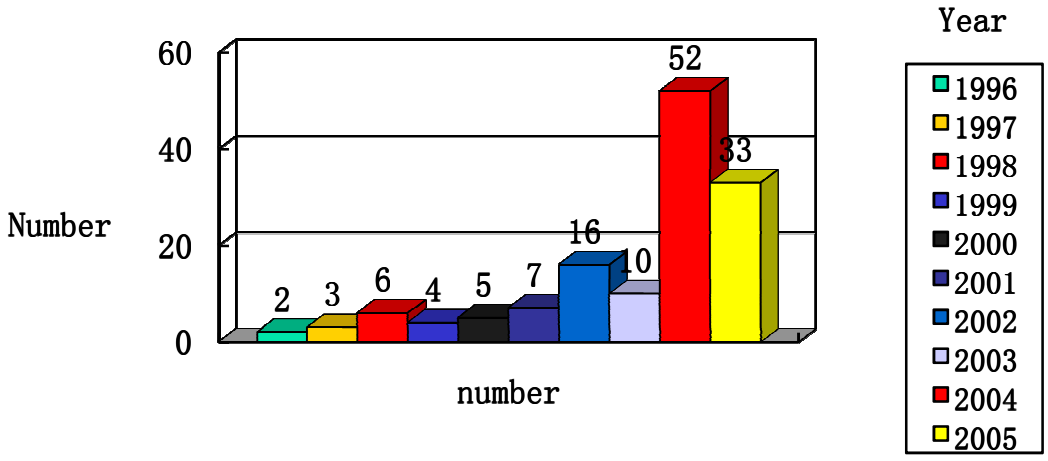


Fig 2.1 The number of propolis health care food and medicines approved by the China government(1996-2005)

Propolis products have also enjoyed steady growth, especially in 2004 and 2005 in China. These products take the following forms: 72% capsules, 17% liquid propolis preparations, 9% tablet, 1.5% ointment, and 0.5% membrane (Bulletin 1996-2005, Fig2). After 2000, the National Science Committee has elevated propolis research into the Key Science and Technology Attack Project of Ministry of Agriculture (MOA) of China. MOA also gives large amounts of funds to support propolis research and development.Propolis also gained entrance to the “Chinese Pharmacopoeia 2005”.

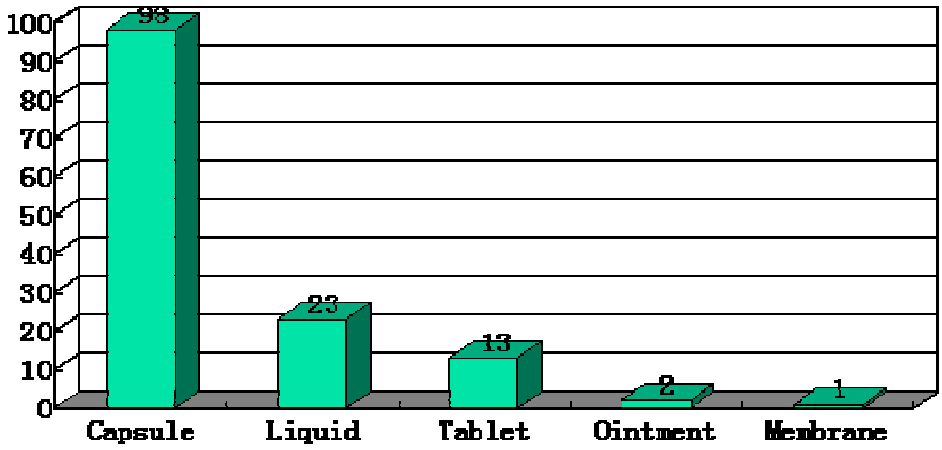


Fig2. 2 Forms of propolis products approved by Chinese government (1996-2005)

CHAPTER III

METHODOLOGY

3.1 **Materials**

3.1.1 Sample

All propolis samples in this research were collected from *Apis mellifera*.

3.1.1.1 Propolis samples from different locationin northern Thailand

The total 18 samples, 17 samplesfrom Thai propolis were collected from the top or entrance or between the flam of bee hive of different bee farms of thefour main beekeeping provinces of Thailand. Three samples were collected from3 different bee farm of different location of Chiang Mai;one was collectd from a company (Bee Products Health Co.) of Chiang Mai. Six samples were collected from 6 different bee farm of different location of Chiang Rai. Four sampleswere collected from 4 different bee farm of different location of Nan by Prof. Siriwat Wongsiri and two samples were collected from 2 different bee farm of different location of Lamphun. The collected time of all samples of Thailand was during August 2009 to March 2010. The detail was showed in Table 3.1,Fig. 3.1 and Fig.3.2. The other oneSample is from UK collected by Prof. Siriwat Wongsiri. All samples were kept in the freezer under -18°C until analysis.

Fig 3.1.Nature (raw) propolis samples from different location of Thailand

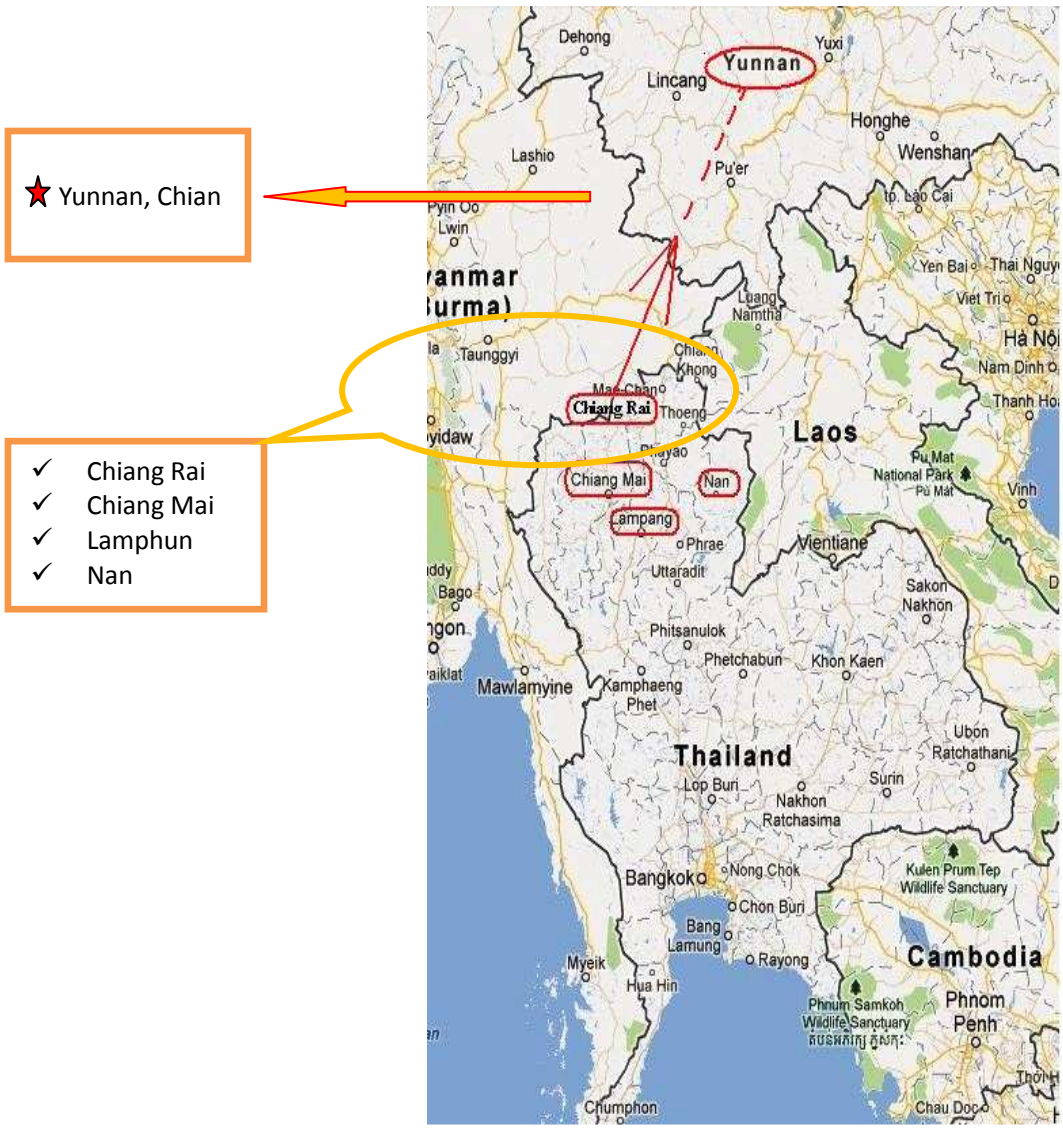


Fig 3.2. Different location of the propolis sample collected in Thailand

Table3.1 Propolis samples from the different location of Thailand

	Sample code	Collected area	Collecte time
1	LP1	Lamphun(Banpahaew)	9 Aug. 2009
2	LP2	Lamphun(Paheao)	9 Aug. 2009
3	CM4-Pay2	Chiang Mai(Pa Pao)	9 Aug. 2009
4	CM2	Chiang Mai(Mae Wang)	9 Aug. 2009
5	CM3	Chiang Mai(Radin BeeFarm)	9 Aug. 2009
6	CM1	Chiang Mai (Health Co.)	19Aug.2008
7	CR-Mfu	Chiang Rai(MFU)	11Aug.2009
8	Nan1	Nan	March,2010
9	Nan2	Nan	March,2010
10	Nan3	Nan	March,2010
11	Nan4	Nan	March,2010
12	CR1	Chiang Rai (Maesuai)	9 Aug. 2009
13	CR2	Chiang Rai(SipangDang)	9 Aug. 2009
14	CR3	Chiang Rai(Pangha)	9 Aug. 2009
15	CR4	Chiang Rai(Ban San)	9 Aug. 2009
16	CR6	Chiang Rai(MaeSai)	9 Aug. 2009
17	CR7	Chiang Rai(HK Bee Farm, Chiang Sean)	9 Aug. 2009
18	UK	England	Nov.2009

3.1.1.2 Propolis samples from different location of Korea

Eleven propolis sampleswere collected by Professor Woo, the vice-presidentof Asia Apicultural Association, from different bee farms of different location of Korea. They are from GokSung, BoSung, JangHeung, Kang Wen, Wen Do, Jung Eub, YiChun,ChungJu, ChungJu, JeJu, Jun Buk (Talbe 3.2; Fig 3.3). All samples were kept in the freezer under -18°C until analysis.

Table3.2 Propolis samples from the different location of Korea (2009)

Sample	Sample code	Collected location
1	GS	GokSung
2	BS	BoSung
3	JH	JangHeung
4	KW	KangWen
5	WD	Wen Do
6	JE	JungEub
7	YC	YiChun
8	CJ1	ChungJu
9	CJ2	ChungJu
10	JJ	JeJu
11	JB	Jun Bu

Fig 3.3. Nature (raw) propolis sample from different location of Korea (2009)

3.1.1.3 Propolis samples from different location of Brazil

There are 4 samples collected from different bee farms of Brazil by the group of Apicultural Science Association of China while they visited Brazil after the Apimondia Congress in Argentina on September 2011.

Fig 3.4. Powder of the nature (raw) propolis sample of Brazil (2011)

3.1.1.4 Propolis samples from different location of China

The 68 of propolis samples were collected from top or entrance or fram or propolis collector of bee hive from different location of China, such as Anhui, Beijing, Hebei, Henan, Guizho, Heilongjiang, Hubei, Jilin, Jiangsu, Liaoning, Ningxia, Qinghai, Shandong, Shanxi, Shanghai, Sichuan and Xinjiang, 20 provinces (Fig 3.6) during March 2008 to March 2010 (Table 3.3). All samples were kept in the freezer under -18°C until analysis.

Fig 3.5. Different location of the propolis samplescollected in China

Table3.3 : Propolis sample from different location of China

	Sample code	Collected location	Collected time
1	AH1	Anhui I	Mar.2008
2	AH2	Anhui II	Mar.2008

3	AH3	Anhui III	Mar.2008
4	AH4	Anhui IV	Mar.2008
5	AH5	Anhui V	Mar.2008
6	AH6	Anhui VI	Mar.2008
7	BJ1	Beijing	May 2008
8	BJ2	Beijing	May 2008
9	HB1	Hebei I	May 2010
10	HB2	Hebei II	May 2010
11	HB3	Hebei III	May 2010
12	HB4	Hebei IV	May 2010
13	HB5	Hebei V	May 2010
14	HB6	Hebei VI	May 2010
15	GZ	Guizhou I	Mar. 2009
16	HN1	Henan I	June 2010
17	HN2	Henan II	June 2010
18	HN3	Henan III	June 2010
19	HN4	Henan IV	June 2010
20	HN5	Henan V	June 2010
21	HN6	Henan VI	June 2010
22	HLJ1	Heilongjiang I	July 2010
23	HLJ2	Heilongjiang II	July 2010
24	HLJ3	Heilongjiang III	July 2010
25	HLJ4	Heilongjiang IV	July 2010

26	HLJ5	Heilongjiang V	July 2010
27	HLJ6	Heilongjiang VI	July 2010
28	HB1	Hubei I	Mar. 2010
29	HB2	Hubei I	Mar. 2010
30	HB3	Hubei II	Mar. 2010
31	HB4	Hubei II	Mar. 2010
32	JL1	Jilin I	June. 2009
33	JL2	Jilin I	June. 2009
34	JL3	Jilin II	June. 2009
35	JL4	Jilin III	June. 2009
36	JL5	Jilin III	June. 2009
37	JS1	Jiangsu I	Mar. 2010
38	JS2	Jiangsu II	Mar. 2010
39	JS3	Jiangsu III	Mar. 2010
40	LN1	Liaoning I	Nov.2009
41	LN2	Liaoning I	Nov.2009
42	LN3	Liaoning II	Nov.2009
43	LN4	Liaoning III	Nov.2009
44	LN5	Liaoning III	Nov.2009
45	LN6	Liaoning IV	Nov.2009
46	NX	Ningxia	Nov.2009
47	QH	Qinghai	Aug.2010
48	SD1	Shandong I	May 2010

49	SD2	Shandong I	May 2010
50	SD3	Shandong II	May 2010
51	SD4	Shandong III	May 2010
52	SD5	Shandong IV	May 2010
53	SD6	Shandong V	May 2010
54	SX1	Shanxi I	April 2010
55	SX2	Shanxi II	April 2010
56	SX3	Shanxi III	April 2010
57	SH	Shanghai I	Mar. 2010
58	SC1	Sichuan I	Aug.2010
59	SC2	Sichuan I	Aug.2010
60	SC3	Sichuan II	Aug.2010
61	XJ1	Xinjiang I	Sep.2010
62	XJ2	Xinjiang II	Sep.2010
63	XJ3	Xinjiang II	Sep.2010
63	YN1	Yunnan	Aug.2010
64	YN2	Yunnan	Aug.2010
65	YN3	Yunnan	Aug.2010
66	ZJ1	Zhejiang I	Mar.2010
67	ZJ2	Zhejiang I	Mar.2010
68	ZJ3	Zhejiang II	Mar.2010

Fig 3.6 Nature (raw) propolis sample from different botanic source and location of China

3.1.2 Chemical Reagents

1) Rutin standard: 50μg/mL was made by weighing 5.0mg Rutin (purity≥99%), dissolved in methanol to 100ml.

2) Standards sample of flavonoids: Rutin, myricetin, quercetin,kaempferol, apigenin, pinocembrine, chrysin and galangin (purity≥99%) were purchased from Sigma (St. Louis, MO, USA).

3) Phosphoric acid, guaranteed reagent grade, was from Beijing chemical reagent company, Beijing, China.

4) Ethanol, 95%, analytical reagent grade, was purchased from Beijing chemical reagent company, Beijing China.

5) Methanol, analytical reagent (≥95%) HPLC grade reagents, was from DIMA (DIMA Technology Inc., Richmond, USA).

6) Polyamide powder (100-200 mesh) was purchased from Beijing chemical reagent company, Beijing China.

7) Deionized water was purchased from Beijing chemical reagent company, Beijing China.

8) DPPH was purchased from Sigma (U.S.A).

9) Fluconazole was purchased from Guang Dong Yuelong Pharmaceutical Co., Ltd of China.

10) Penicillin potassium Tablets was purchased from Hu Nan Dinuo Pharmaceutical Co., Ltd of China.

11) Potassium ferricyanide, analytical reagent, was purchased from Beijing chemical reagent company, Beijing China.

12) Chloroacetic acid, analytical reagent, was purchased from Beijing chemical reagent company, Beijing China.

13) Ferric chloride, analytical reagent, was purchased from Beijing chemical reagent company, Beijing China.

14) Disodium hydrogenphosphate was purchased from Beijing chemical reagent company, Beijing China.

15) polyamide powder (100-200 mesh)

16) Rutin standard: 50μg/ml was made by weighing 5.0mg Rutin (purity≥99%), dissolved in methanol to 100ml.

17) Sodium hydroxide solution (1 mol/l)

18) Sodium nitrite: analytical reagent

19) 5% Sodium nitrite solution was made by weighing 5g sodium nitrite, dissolved in pure water to 100ml.

20) Aluminum nitrate: analytical reagent

21) 10% aluminum nitrate solution was made by weighing 10g aluminum nitrate, dissolved in pure water to 100ml.

3.1.3 Apparatus

1) HPLC, high performance liquid chromatography system withUVD170U UV-VIS Detector and PDA-100 detector was from Shimadzu of Japan.

2) Column: 350mm (length), 15mm (interior diameter) with stopcock, sand core, and round-bottom flask.

3) UV-VIS-2550 Spectrophotometer (Shimadzu, Japan).

4) KQ-500E Ultrasonic cleaning (China)

5) BS124S Analytical balance (China)

6) HH-S thermostat water bath (China)

7) RE-52c Rotary evaporator (China)

8) SPX-250B Biochemical incubator (China)

9) SPX-250B Biochemical incubator (China)

10) MJ-180B Mold incubator (China)

11) AUW220D electronic analytical balance (Shimadzu, Japan)

12) Ultrasonic bath (SY, Shanghai, China)

3.2 Method

3.2.1 Optimization of extraction procedure

In order to obtain optimal extraction efficiency, extraction solvents and extraction time were investigated. Various solvents including, ethanol, ethyl acetate, butyl alcohol, ether, acetone and water were tested for the extraction of raw propolis sample. Ethanol was particularly suitable to obtain dewaxed propolis extracts (Pietta, Gardana et al. and Pietta, 2002). Aqueous ethanol was the preferred choice of extraction solvent as a variety of compounds with different polarity. In this study, 95% aqueous ethanol (v/v) was chosen as the extraction solvent not only because the raw propolis could be efficiently dissolved but also the bee wax could be remarkably separated from the raw propolis solutions. The ultrasound,reflux, and Soxhlet extraction were compared in parallel experiments using 95% ethanol as the solvent. The ultrasound-assisted extraction method showed the greatest extraction ability.

This study select the ethanol with ultrasound-assisted as the extraction method.

3.2.2 Preparation of samples

3.2.2.1 Preparation of the samples for Spectrophotometer determination

The crude propolis samples were powdered by food processer and sieved through 40meshes.Powderedsample5.0 g was extracted by 200 ml ethanol with ultrasound extraction (Power: 100 W, Frequency: 40 kHz) for 25 min, the extraction process was repeated 3 times and all extracts obtained were combined in the volumetric flask and then filtered through a filter paper. This ethanol extraction of propolis (EEP) was kept at room temperature forSpectrophotometer determination.

Fig 3.7. Powder of the Nature (raw) propolis samples

3.2.2.2 Preparation of the samples for HPLC determination

Five grams of powder propolis was extracted by 200 ml ethanolusing ultrasoundassisted extraction(Power: 100 W, Frequency: 40 kHz)for40min.This extraction process was repeated and extracts obtained were combined in the flask. The extracts were then filtered through a filter paper to remove macro and micro-molecular components such as minerals and bees-wax and transferred into a 250 ml pear-shape flask. Then, filtrates were evaporated to near dryness with a rotary evaporator below 35–40 °C. Residue (refined propolis) was freeze-dried and the dry powder (0.1 g) was extracted by ultrasonic technique with 20 min by adding 50 mL methanol into pear-shape flask and filtered through a 0.45 μm filter for HPLC–UV analysis.

This method is according to the national standards of China for determination of the 8 main flavonoids in propolis (AQSIQ, 2003).

3.2.3 Preparation of standard solutions

Standard solutions were prepared within the range 0.1–0.8 mg/mL by dissolving eight flavonoids, rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine,chrysin and galangin, in methanol. The concentration of mixed standard solution was selected according to the level of the flavonoids expected in the propolis samples. Working mixed standard solutions were made daily by gradual dilution with methanol to the required concentration, which was based on the sensitivity of detection and the linearity range of the study. All of the standard solutions were stored at -18 °C in darkness and could be used for two months

3.2.4 Spectrophotometer determination of total flavonoids

One millilitre of supernatant was pipette into glass evaporating dish, 5ml ethanol and 1g polyamide powder were added and mixed up well, volatilized the ethanol in 60 °C water bath, then transferred into closed column. Benzene solution of 20 ml was used to wash the glass evaporating dish three times, and then transferred into column too. After 15 min, the stopcock was opened and benzene solution was eliminated and the stopcock was closed. Methanol of 20 ml was used to wash the glass evaporating dish three times, and transferred into column. After 15min, the flavone was eluted into 25ml volumetric flask, methanol was added to 25ml and shook well for determination by spectrophotometric method at 360 nm. All sampleswere analysed in triplicate replications.

This research regards rutin as standard andused standard curve method to quantitatethe content of total flavonoidsin Thai propolis EEP.This method is according to the

national standards of the propolis in China (Standardization Administration of PRC, 2009).

3.2.5 Calculation of total flavonoids

The total flavonoids were calculate asc follows:

$$X = \frac{A \times V_2 \times 100}{V_1 \times M \times 1000}$$

X—total content of flavonoid in the propolis of Thailand, mg/100g

A—content of flavonoid in the solution calculated by standard curve (μg)

M—samples weight (g)

V_1—volume of supernatant extraction (EEP, ml)

V_2—total volume of samples, constant volume (ml)

Two significant figures were reserved in the result. This method is according to the national standards of the propolis in China (Standardization Administration of PRC, 2009).

3.2.6 HPLC chromatographic conditions

The high performance liquid chromatography system consisting of a P680 quaternary pump, UVD170U UV–VIS Detector and PDA-100 detector, ASI-100 automated sample injector and thermo stated column compartment was used for quantitative analysis. The separation column used was a Mightysil RP-18 3 um, 150 x 4.6 mm. The mobile phase was methanol + water (65 + 35), phosphoric acid (PH=3) and a flow rate was 0.7 ml/min. The detection wavelength was set at 270 nm. Injection volume of solution was 10uL.

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks. Different mobile phase compositions were optimized: formic

acid, acetic acid and phosphoric acid were added to the aqueous phase of mobile phase to enhance the resolution, restrain the ionization of flavonoids and eliminate the peak tailing of target compounds. As a result, mobile phases containing phosphoric acid were selected. Mobile phase with methanol has a satisfactory resolution and stable baseline. To acquire better selectivity and higher efficiency, eluent pH over the range of 2.0–5.0 was tested and different concentrations of phosphoric acid in the aqueous phase were also investigated. In the end, the mobile phase consisting of methanol and 0.4% phosphoric acid (pH 3.0) were chosen for the determination of eight flavonoids in propolis.

On the ultraviolet spectra with chromatograms of HPLC–UV of eight flavonoids in propolis and reference standards, maximum absorbance values around 280 nm and 350 nm were observed. More detectable peaks could be obtained and the baseline was well improved around 270 nm at which the better characterization of flavonoids can be attributed. Hence, characteristic chromatographic patterns were obtained by using 270 nm as the detection

All propolis extracts were analyzed under the above HPLC conditions. Each sample was analyzed in triplicate to determine the mean contents of eight major flavonoidswavelength.

3.2.7 Method validation

The injection precision was determined by replicating injection of the same sample solutionfor five times in a day. The sample stability test precision was determined with one sample during five days. The repeatability was assessed by analyzing five independently

prepared samples of propolis samples. During this period, the solution was stored at room temperature.

3.2.8 Antioxidant activity of Thai propolis

3.2.8.1 Determination of the reducing power(Klompong, *et al*. 2007)

The EEP (0.4, 0.8, 1.2, 1.6 and 2.0 ml) at a concentration of 300 μ g/mL were separately added to each tube, and added phosphate buffer solutions(0.2 mol/l, pH 6.6) to 2.5 ml total volume. Then, 5ml of 1% potassium ferricyanide solutionwas added. Then reacting in 50°Cconstant temperature water bath for 20 min, mixed with 5 mL 10% trichloroacetic acid. The mixtures should be shaken up and standing for 5min. The supernatant 2.5ml mixed well with 2.5 ml diluted water and 0.5ml 1% ferric chloride FeCl3. Absorbance at 700nm was measured using the mixture without EEP as a blank and with ascorbic acid instead of EEP as positive control. The determination was analyzed in triplicate.

3.2.8.2 Determination of DPPH free radical scavenging

The DPPH free radical method is based on the determination of the concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at steady state in ethanol solution, after adding the mixture of antioxidants. DPPH absorbs at 517 nm, and as its concentration is reduced by the existence of an antioxidant, the absorption gradually disappears with time (MĂRGHITAS L, 2009). A UV-VIS-2550 Spectrophotometer (Shimadzu) was used and the quantity of the mixture of antioxidants needed to reduce by 50 % the initial DPPH concentration was evaluated. This characteristic parameter is called inhibitory concentration (IC50) or oxidation index. The lower

the IC50, higher is the antioxidant activity of the examined compound. The DPPH radical-scavenging activity in terms of percentage was calculated according to the following equation. DPPH scavenging activity (%) = {1- (Abs517 sample / Abs517 DPPH solution)} × 100 %The EEP samples were mixed with absolute ethanol and 0.2 mmol/L DPPH. The determination was analyzed in triplicate (MĂRGHITAS L, 2009).

3.2.9 Antimicrobial activity of Thai propolis

3.2.9.1 Determination of the inhibition zone –the filter paper method

3.2.9.1.1 Preparation of the microbial suspension

3.2.9.1.1.1 Preparation of *Staphylococcus aureus* suspension

One or two loops of *Staphylococcus aureus* was picked from agar and put into broth and incubate at 37°C

3.2.9.1.1.2 Preparation of *Candida albicans* suspension

The suspension of *Candida albicans* was prepared in Sabouraud dextrose broth and incubate at 28°C for 48h.Then the concentration of the cell suspension was adjusted with sterile saline to $1 \times 10^7 \sim 1 \times 10^8$ cfu / ml.

3.2.9.1.2 Preparation of the bacterial agar plate

3.2.9.1.2.1 Preparation of the *Staphylococcus aureus* agar plate

The agar was sterilized and cooled to about 50°C and pour

into sterile petri dishes to be solidified. One ml of*Staphylococcus aureus* bacterial suspension was added on agar plate and spreaded evenly. Same thing was done for *Candida albicans* on Sabouraud dextrose agar plate.

3.2.10.1.2.2 Preparation of the aseptic filter paper

Antimicrobial susceptibility disk with diameter 6mm was made from filter paper and autoclaved at 120 °C for 20 min.

3.2.9.1.3 Preparation of the sample solution

The EEP sample was evaporated to remove the ethanol in rotary evaporator. The solutions to the concentration 0.25 mg/mL， 0.5 mg/mL， 1.0 mg/mL， 2.0 mg/mL， 4.0 mg/mL and 8.0 mg/mL were prepared with Dimethyl sulfoxide (Aseptic, every container sterilized in advance).

3.2.9.1.4 Determination of the inhibition zone

Firstly, the agar plate was divided into six districts, each district was marked by adding the sample name and concentration. Secondly, sterile filter paper pieces were placed to the middle of each district on top of plate (Aseptic operation). Thirdly, 10μl of various concentrations of sample solutionwas pipetted on to the sterile filter paper. Then plate was inculated at 37°C for 30h for *Staphylococcus aureus*and at 28°Cfor 48 h for *Candida albicans*. The experiment was done in duplicate.

At the same time, positive control and negative control were done too. Penicillin potassium salt was used as the positive control against the *Staphylococcus*

aureus bacteriostasis and fluconazole was used positive control against for*Candida albicans.*

3.2.9.2 Determination of MIC_{50}

Sample solution (100 μl)was pipetted to the plate which coated evenly with the cultures and prepared according to 3.2.10.1.2.1 andincubated at 37 ℃for 30 h. Viable colony were counted. The inhibition rate of each sample solution with different concentration against *Staphylococcus aureus* and the *Candida albicans* were calculated. Repeat two times. The inhibition rate was calculated as follows:

$$X_1 = \frac{A-B}{A} \times 100\%$$

X_1 is the inhibition rate with percentage. A means colony count of the negative control sample (dimethyl sulfoxide). B means colony count of the sample solution.

Inhibitory concentration with 50% inhibition was the half of the minimum inhibitory concentration (MIC_{50}).An exploratory data analysis was made tothe most appropriate statistical investigation.Using SPSS software10.0. Thelevel of significance for statistical tests was$p<0.05$.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Analysis of Total Flavonoid of Propolis Extraction

4.1.1 Analysis of total flavonoids of Thai propolis extraction

Recently propolis has attracted much attention as a useful substance applied in medicine, health care and cosmetics due to its antimicrobial and antioxidant activities (Burdock, 1998).Among constituents with biological activity, flavonoids contribute more than others to the observed effect of propolis.The flavonoids are most commonly known for their antioxidant activity and a nature antioxidant (Marcucci, 1995; Burdock, 1998). Flavonoids and phenolic acid esters are known for their antimicrobial, antiviral and antioxidant activity (Pietta, 2000). Flavonoids may reduce free radical formation and consequently might have a protective effect on serum lipids against oxidation (Moreno et al., 2000).Flavonoids are also reported to be the most abundant and most effective antioxidant in propolis(Kumazawa et al., 2004; Dobrowolski et al., 1991; Kujumgiev et al., 1999; Castaldo and Capasso, 2002; Russo et al., 2004).European scientists Bonvehí and Coll (2000) analyzed the composition, bacteriostatic and free radical-scavenging potential activity of the propolis from China and Uruguay. That is why it is the main goal to determine the flavonoids in thisresearch.

The result of the total flavonoids of 18 propolis samples from the main beekeeping provinces of the northern of Thailand was shown in Table 4.1. To view the results of the of 17 Thai propolis samples, the total flavonoids components of Thai propolis samples are 0.12 to 13.34%: 7 samples from Chiang Rai 0.12-0.53%, 4 samples from Nan 0.24-0.34%, 4 from Chiang Mai 0.23-13.4%, and 2 from Lamphun 0.25-0.61%. Only one sample from Chiang Mai has rwlatively high content of total flavonoid 13.4%.It is similar to those from UK, Korea and

China.Most of the Thai samples, 16 Thai propolis samplesrange 0.12% to 0.61%which are very low(<1%) when compared with propolis from China, Brazil, Japan, Argentina, Turkey, UK, Italy and other Europen countries(Abd et al., 2002; AQSIQ, 2003; ASAC, 2004; Bankova et al., 2000; Bonvehí et al., 2000; Bonvehí et al., 2000; Claudio et al., 2007; Nicola et al., 2005; Alencar et al., 2007; Havsteen 2002; Jasprica et al., 2004; Chen et al., 2000; Cao 2007; Chen 2010).As we know that the flavonoids of Brazilian propolis is relatively low but it is also higher than Thai. It is probably due to the tropical climate, temperature, botanic resource, or some other reason.

In this study,one sample from UK had a high content of the total flavonoids(14.35%). It is similar to the China and Korea.

However, there is no report on Thai propolis sample before. Therefore, there is no experimental data published to compare with this result. The present work should be repeated and compare with other Asian country.

Table4.1Total flavonoid of Thai propolisextraction (g/100g)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample	LP1	LP2	CM4(Pay2)	CM2	CM3	**CM1**	CR-Mfu	Nan1	Nan2
Flavonoid(%)	0.61	0.25	0.23	0.32	0.38	**13.34**	0.12	0.24	0.31
	10	11	12	13	14	**15**	16	**17**	18
Sample	Nan3	Nan4	CR1	CR2	CR3	CR4	CR6	CR7	**UK**
Flavonoid(%)	0.34	0.28	0.19	0.14	0.2	0.14	0.53	0.25	**14.35**

4.1.2 Comparison of the total flavonoids of Thai propolis with Chinese propolis

The result of this study is shown in Table4.2 that the total flavonoids components of propolis sample from China ranged from 11.29% (Hebei) to 31.66% (Sichuan). The propolis samples from Anhui province range 10.02% - 30.69%, Beijing 17.09% - 17.61%, Hebei 11.29% - 25.04% , Guizhou 14.41%, Henan 26.65% - 13.8%, Heilongjiang 11.25 - 29.21%, Hubei 16.97%-29.24%, Ningxia 22.22%, Qinghai 12.02%, Shandong 19.25% - 28.64%, Shanxi 17.44% -19.93%, Shanghai 17.46%, Sichuan 22.8% - 31.66%, Xinjiang 18.37% -20.83%, Yunnan 13.06% - 16.245, Zhejiang 17.97% -24.09%. All the total flavonoids of the samples from China, even for Yunan province nearest to Thailand, are higher than Thai propolis which are ranged from 0.1% - 0.53 % (<1%), except one sample CM1 from Chiang Mai which have 13.01% (>11%) . Due to the fact that Yunan is the nearest to Thailand, we collect the propolis sample from Yunnan bee farms in order to determinate the flavonoids and compared with Thai propolis. We hope its result will be similar to Thai. But certify to the fact, it is also different from Thai propolis. According to the scientist Gardana et al. (2007) suggestion, all of the Chinese propolis samples including Yunnan's propolis sample are all good (>11%) and the one sample, CM1 from Chiang Maiof Thailand, is good too. But the other 16 Thai propolis samples are not good at flavonoids with unknown reason which might be because of the difference in the botanical resources and climate (Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002;Zhao et al. , 2005.; Li et al. 2007; Wang X.P. and Lin L. 2007).

Table4.2Comparison of the total flavonoids of Thai propolis with Chinese propolis

	Sample code	Collected area	Collected time	Flavonoids(%)
1	AH1	Anhui I	2010.3	21.60
2	AH2	Anhui II	2010.3	22.50
3	AH3	Anhui III	2010.3	30.69
4	AH4	Anhui IV	2010.3	23.15
5	AH5	Anhui V	2010.3	12.02
6	AH6	Anhui VI	2010.3	20.60
7	BJ1	BeijingI	2010.5	17.09
8	BJ2	BeijingII	2010.5	17.61
9	HB1	Hebei I	2010.5	25.04
10	HB2	Hebei II	2010.5	18.02
11	HB3	Hebei III	2010.5	17.92
12	HB4	Hebei IV	2010.5	11.29
13	HB5	Hebei V	2010.5	19.99
14	HB6	Hebei VI	2010.5	23.21
15	GZ	Guizhou I	2009.3	14.41
16	HN1	Henan I	2010.6	26.65
17	HN2	Henan II	2010.6	22.32
18	HN3	Henan III	2010.6	19.39
19	HN4	Henan IV	2010.6	23.99
20	HN5	Henan V	2010.6	21.11
21	HN6	Henan VI	2010.6	13.8

22	HLJ1	Heilongjiang I	2010.7	19.22
23	HLJ2	Heilongjiang II	2010.7	15.44
24	HLJ3	Heilongjiang III	2010.7	11.25
25	HLJ4	Heilongjiang IV	2010.7	16.23
26	HLJ5	Heilongjiang V	2010.7	13.23
27	HLJ6	Heilongjiang VI	2010.7	29.21
28	HB1	Hubei I	2010.3	16.97
29	HB2	Hubei I	2010.3	22.2
30	HB3	Hubei II	2010.3	23.91
31	HB4	Hubei II	2010.3	29.24
32	JL1	Jilin I	2009.6	24.35
33	JL2	Jilin I	2009.6	19.64
34	JL3	Jilin II	2009.6	19.23
35	JL4	Jilin III	2009.6	17.79
36	JL5	Jilin III	2009.6	30.33
37	JS1	Jiangsu I	2010.3	22.59
38	JS2	Jiangsu II	2010.3	30.95
39	JS3	Jiangsu III	2010.3	23.98
40	LN1	Liaoning I	2009.11	21.24
41	LN2	Liaoning I	2009.11	22.07
42	LN3	Liaoning II	2009.11	23.01
43	LN4	Liaoning III	2009.11	24.5
44	LN5	Liaoning III	2009.11	22.86

45	LN6	Liaoning IV	2009.11	22.84
46	NX	Ningxia	2009.11	22.22
47	QH	Qinghai	2010.8	12.02
48	SD1	Shandong I	2010.5	21.93
49	SD2	Shandong I	2010.5	22.12
50	SD3	Shandong II	2010.5	23.25
51	SD4	Shandong III	2010.5	28.64
52	SD5	Shandong IV	2010.5	22.87
53	SD6	Shandong V	2010.5	19.25
54	SX1	Shanxi I	2010.4	18.67
55	SX2	Shanxi II	2010.4	19.93
56	SX3	Shanxi III	2010.4	17.44
57	SH	Shanghai I	2010.3	17.46
58	SC1	Sichuan I	2010.8	22.49
59	SC2	Sichuan I	2010.8	22.8
60	SC3	Sichuan II	2010.8	31.66
61	XJ1	Xinjiang I	Sep.2010	18.37
62	XJ2	Xinjiang II	Sep.2010	19.47
63	XJ3	Xinjiang II	Sep.2010	20.83
63	YN1	Yunnan	2010.8	13.06
64	YN2	Yunnan	2010.8	15.69
65	YN3	Yunnan	2010.8	16.24
66	ZJ1	Zhejiang I	2010.3	19.95

67	ZJ2	Zhejiang I	2010.3	24.09
68	ZJ3	Zhejiang II	2010.3	17.97
69	LP1	Lamphun(Banpahaew)	9 Aug. 2009	0.53
70	LP2	Lamphun(Paheao)	9 Aug. 2009	0.23
71	CM4(Pay2)	Chiang Mai (Pa Pao)	9 Aug. 2009	0.21
72	CM2	Chiang Mai (Mae Wang)	9 Aug. 2009	0.29
73	CM3	Chiang Mai (Radin BeeFarm)	9 Aug. 2009	0.35
74	CM1	Chiang Mai (Health Co.)	19Aug.2008	13.01
75	CR-Mfu	Chiang Rai(MFU)	11Aug.2009	0.14
76	Nan1	Nan	March,2010	0.24
77	Nan2	Nan	March,2010	0.3
78	Nan3	Nan	March,2010	0.31
79	Nan4	Nan	March,2010	0.31
80	CR1	Chiang Rai (Masuai)	9 Aug. 2009	0.21
81	CR2	Chiang Rai(SipangDang)	9 Aug. 2009	0.12
82	CR3	Chiang Rai(Pangha)	9 Aug. 2009	0.19
83	CR4	Chiang Rai(Ban San)	9 Aug. 2009	0.13
84	CR6	Chiang Rai(MaeSai)	9 Aug. 2009	0.49
85	CR7	Chiang Rai(HK Bee Farm)	9 Aug. 2009	0.26

The results also show that the total flavonoids are different from with different provinces, or different location of the same province, or different botanic resource.

In total 85 propolis samples, the 68 propolis samples from China all have a good quality and most of these samples from China have best quality on total flavonoids according to the evaluation of the Italian scientist Gardana et al. (2007). For the Thai propolis samples, only one sample CM1 from Chiang Mai has a good quality on total flavonoids (13.01%). The others 16 propolis samples from Thailand are lower on total flavonoids. This might be due to the tropical climate, temperature, botanic resource, or some other reasons (Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002).

Although this thesis is the first time to analyse Thai propolis, this comparing test maybe further indicate that the data of total flavonoids of Thai propolis are low, as there is no experimental data published to compare with this result. In order to confirm the data the analysis will be repeated and compared with other Asian country again. For the further research, the comparison will be made with the propolis from Brazil as every one knows in the propolis industry that the Brazilian propolis also has a lower flavonoid than China, Korea, UK and the other countries but also has a good quality for human health (Marcucci1995; Marcucci2000; Bankovaet al., 2000; Bonvehet al., 2000; Laura et al., 2006; Estheret al., 2008; Yoshimiet al., 2007; Claudioet al., 2007; Alencaret al., 2007; Chen 2010) .

4.1.3 Analysis of total Flavonoids of the Korea Propolis

The contents of total flavonoids in 11 propolis samples of Korea from different geographic location are range from 1.46 to 25.14 (Table 4.3). The result shows that most of Korean propolis, 9 samples, possess abundant flavonoids(14.21-25.14%).According to the evaluation suggestion of

Italianscientist Gardana et al. (2007), the 9 Korean propolis samples have a good quality on total flavonoids (>11%). It is higher than Thai too. However, the result also shows that the total flavonoids of the other two samples are lower likely Thai propolis. Maybe it is due to the different botanic resource, location and climate. The result is as similar as the Korean and other countries' propolis which of previously published studies(Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002).

Table 4.3 Total flavonoids content (g/100 g) in Korean propolisextracts

Sample	Sample code	Weight (mg)	Absorbant (360nm)	Total Flavonoid(%)
1	GS	82.7	0.291	15.11
2	BS	86	0.344	17.14
3	JH	91.5	0.303	14.21
4	KW	50.5	0.319	25.14
5	WD	101.3	0.038	1.75
6	JE	92.5	0.343	15.89
7	YC	94.5	0.313	14.21
8	CJ1	81.1	0.403	21.26
9	CJ2	87.5	0.443	21.64
10	JJ	95.2	0.029	1.46
11	JB	94.7	0.333	15.07

4.1.4 Comparison of the total flavonoids of Thai propolis with Brazilian propolis

The result of total flavonoids Thai propolis (10 samples) compared withBrazilian propolis(4 samples) is showed as Table4.4. The result shows that the total flavonoids of Brazilian propolisare range from 5.79% to 8.22%. With the evaluation suggestedby Gardana et al. (2007), the total flavonoids of Brazilian propolish is low (<11%), but it wastillhigher than Thai. Unfortunately, the result further indicate that the total flavonoids in Thaipropolis also too low(<1%).

Table 4.4 Total flavonoid of Thai propolisand Brazilian propolis (g/100g)(method 2)

	Sample	Collected area	Collecte time	Flavonoids (%)
1	CM3(P1)	Chiang Mai (Health Co.)	19Aug.2008	0.27
2	CM4(P2)	Chiang Mei(Mae Wang)	9 Aug. 2009	0.17
3	CR1(P4)	Chiang Mei(Radin BeeFarm)	9 Aug. 2009	0.23
4	CR7(P5)	Chiang Rai(HK Bee Farm)	9 Aug. 2009	0.39
5	CR3(P6)	Chiang Rai (Masuai)	9 Aug. 2009	0.35
6	LP2(P7)	Lamphun(Banpahaew)	9 Aug. 2009	0.34
7	CR-Mfu	Chiang Rai(MFU)	11Aug.2009	0.26
8	Nan1	Nan	March,2010	0.32
9	Nan2	Nan	March,2010	0.36
10	Nan4	Nan	March,2010	0.25
11	B1	Brazil	Nov.2010	5.79
12	B2	Brazil	Nov.2010	7.84
13	B3	Brazil	Nov.2010	8.22
14	B4	Brazil	Nov.2010	7.73

4.1.5 Comparison of the total flavonoids of Thai propolis with Brazilian propolis by different method

Due to the result of the total flavonoid of the Thai propolisshowed lower. We continued to analysis it in different method, the Al (III) method. It is also used to determine the flavone content in general. It is based on a neutral or slightly alkaline or sodium nitrate solution condition, flavonoids react with aluminum salt to form stable chelate complex. The complex became orange-red color with sodium hydroxide solution and has maximal absorption peak at the wavelength of 530nm as similar as rutin. Rutin was used as control.

The result of the total flavonoids of the Thai propolis(10 samples) and Brazilian propolis (4 samples) analyzed by this method were showed as Table 4.5. Unfortunately, the result also shows lower of the total identified flavonoids (<1%) of the Thai propolis sample. But total flavonoids of Brazilian propolis were higher. Comparing last result, we found that the total flavonoid in Thai propolis wasstill low.

Table 4.5 Total flavonoids of Thai propolisand Brazilian propolis(g/100g) (method 2)

	1	**2**	**3**	**4**	**5**	**6**	**7**
Sample	CM3 (P1)	CM4 (P2)	CR1 (P4)	CR7 (P5)	CR3 (P6)	LP2 (P7)	CR-Mfu
Flavonoid(%)	0.55	1.45	0.81	1.21	0.54	1.25	0.46
	10	**11**	**12**	**13**	**14**	**15**	**16**
Sample	**N1**	**N2**	**N4**	**B1**	**B2**	**B3**	**B4**
Flavonoid(%)	0.70	0.47	0.40	13.66	15.65	15.08	14.90

Comparing with the Korea, China, UK and Brazil, the total flavonoids of the propolis samples from the 3 countries are good and higher then Thai propolis. Chinese is the best, Thai is the lowest. It is varies from different plants, different location and different climate, especially the tropical climate. It is possible that there will be some phenolic compounds in Thai propolis.

4.2 Determination of the flavonoids by HPLC-UV

4.2.1 Determination of the 8 mainflavonoids in Korean Propolis

All 11propolis extracts were analyzed under the above HPLC conditions. Each sample was analyzed in triplicate to determine the mean contents of eight major flavonoids in Korean propolis samples. The Korean propolisare characterized by the presence of the most abundant of the content of 6 major flavonoids were rutin (0.00–0.97%), quercetin (0.00–1.41%), kaempferol (0.00–2.25%), pinocembrine(2.78–6.33%), chrysin (0.03–1.78%) and galangin (0.02– 1.85%) respectively. The myricetin and apigenin were not detected in the all Korea sample. Only one sample of the Korean propoliscontains rutin. The significant variability might be caused by different and mixed botanical origin in various samples of the same province. The results of HPLC analysis were showed (Fig4.3-4.14).

The results also showed that the eight flavonoids in Korean propolis samples were difference from different origin of Korea. The variability might be caused by different and mixed botanical origin in various samples of the different location.

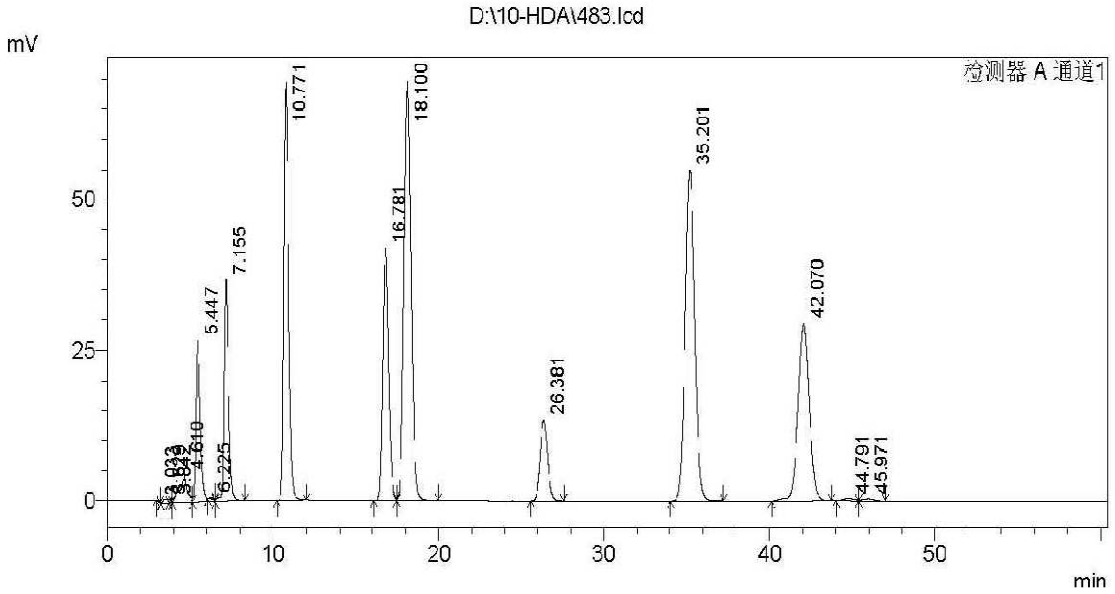


Fig 4.1 HPLC chromatogram of 8 standard flavonoids

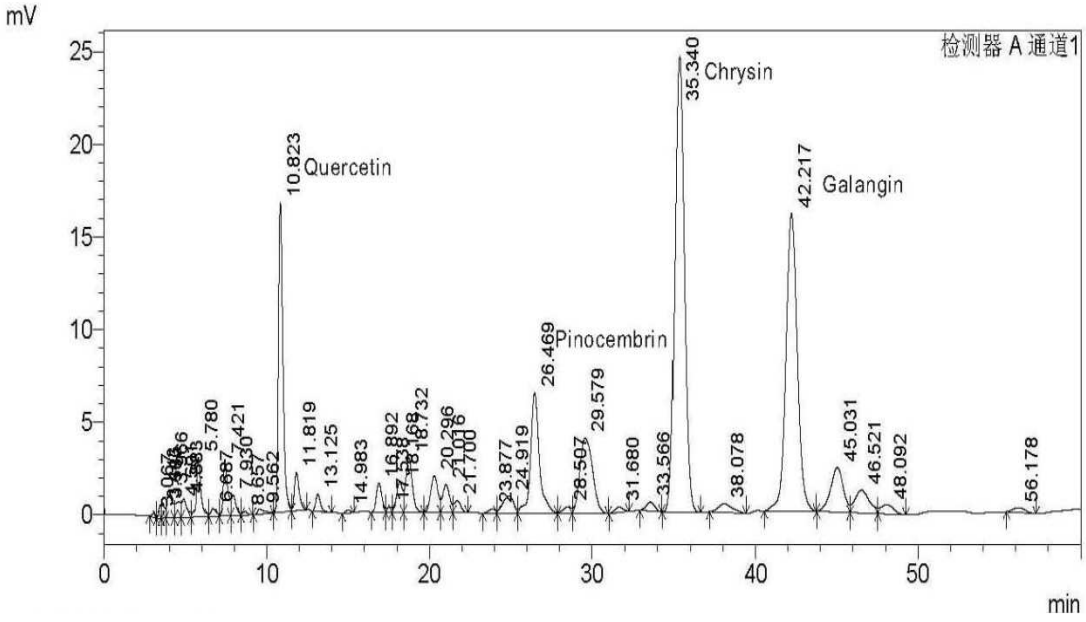


Fig4.2 HPLC chromatogram of Korean propolis sample 1

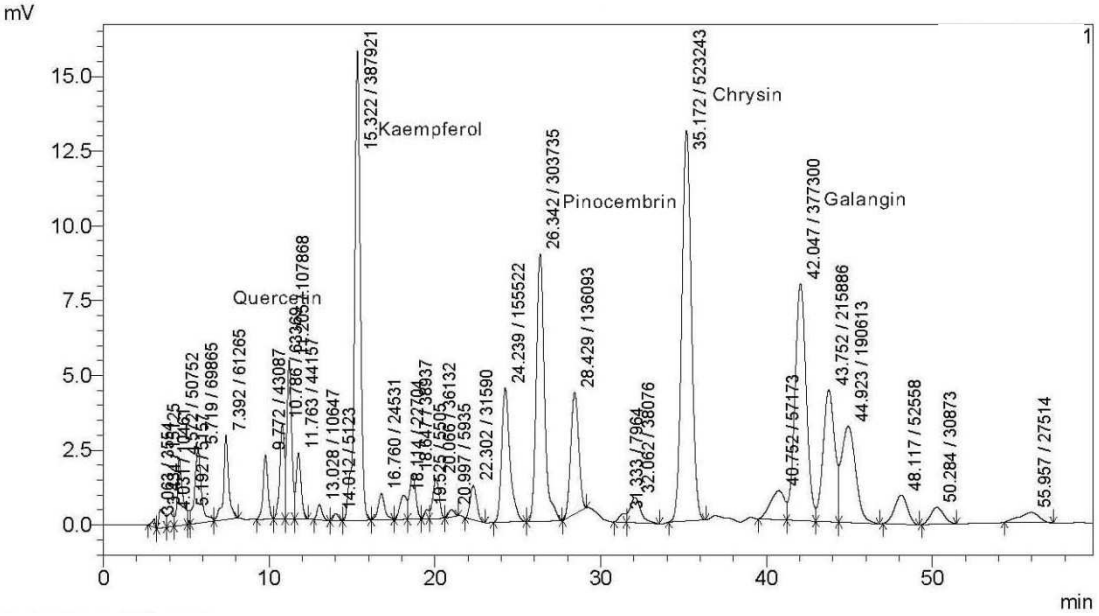


Fig4.3 HPLC chromatogram of Korean propolis sample 2

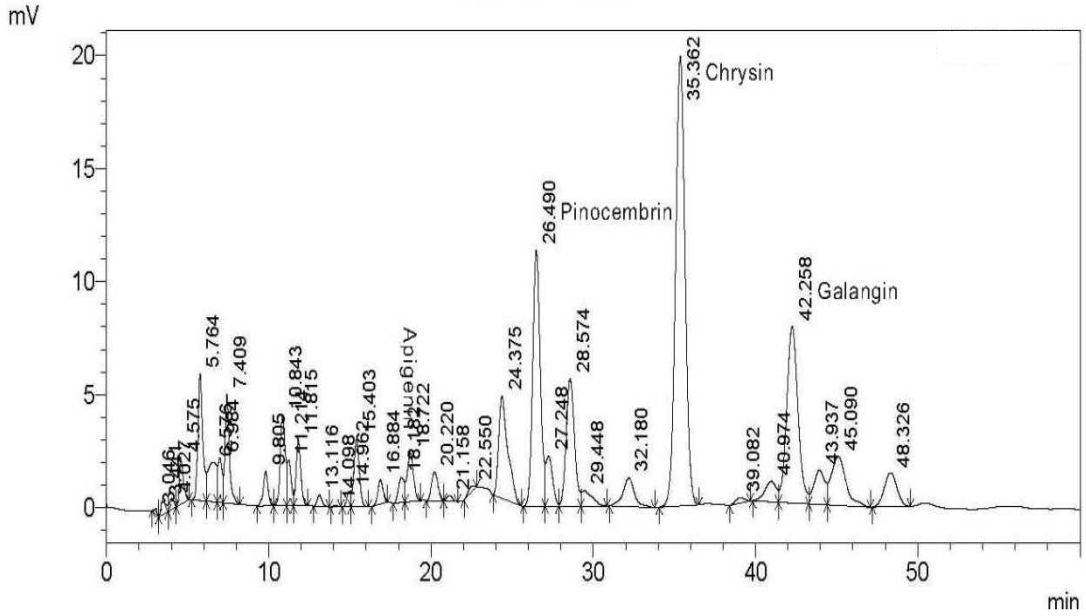


Fig4.4 HPLC chromatogram of Korean propolis sample 3

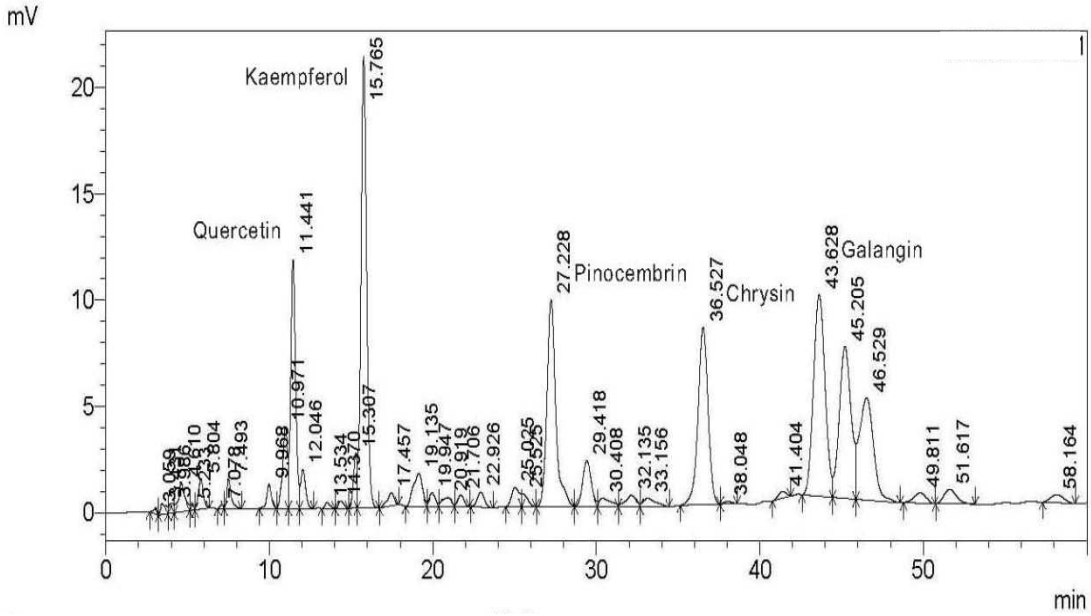


Fig4.5 HPLC chromatogram of Korean propolis sample 4

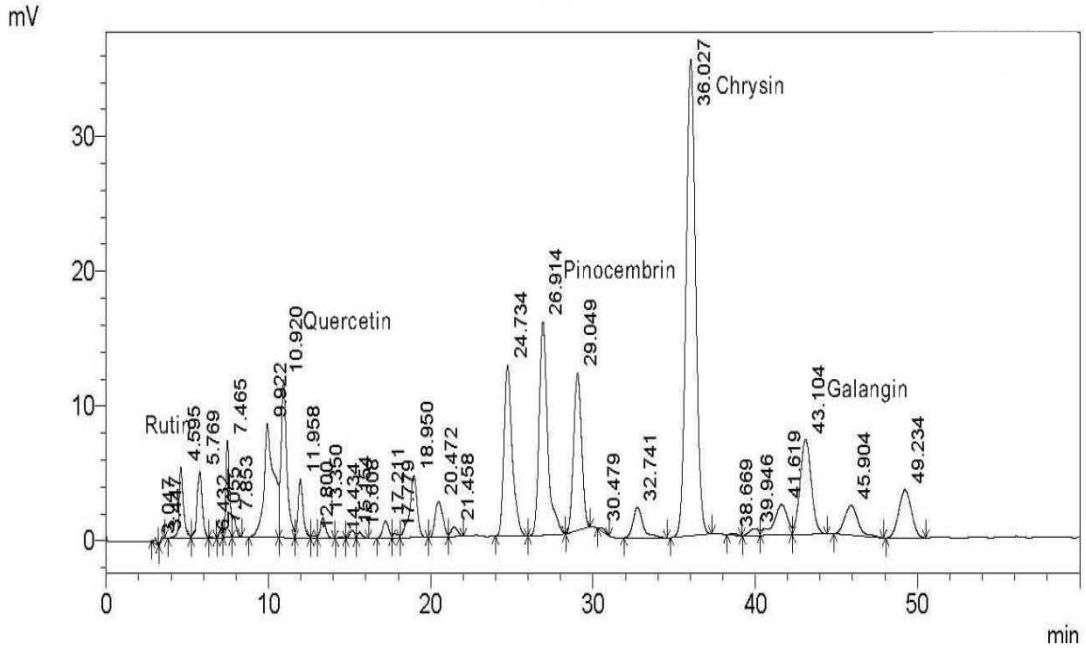


Fig4.6 HPLC chromatogram of Korean propolis sample 5

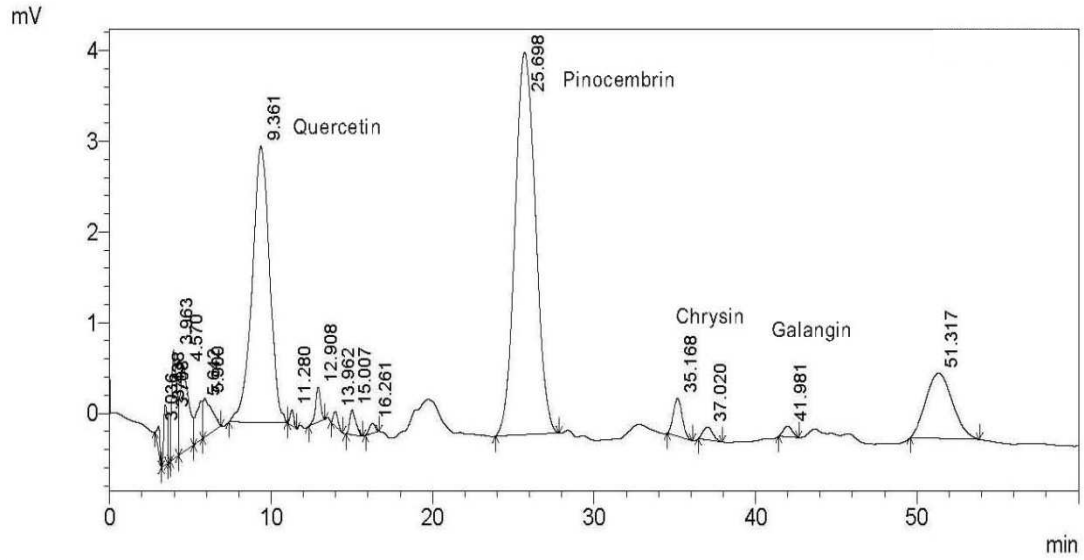


Fig4.7 HPLC chromatogram of Korean propolis sample 6

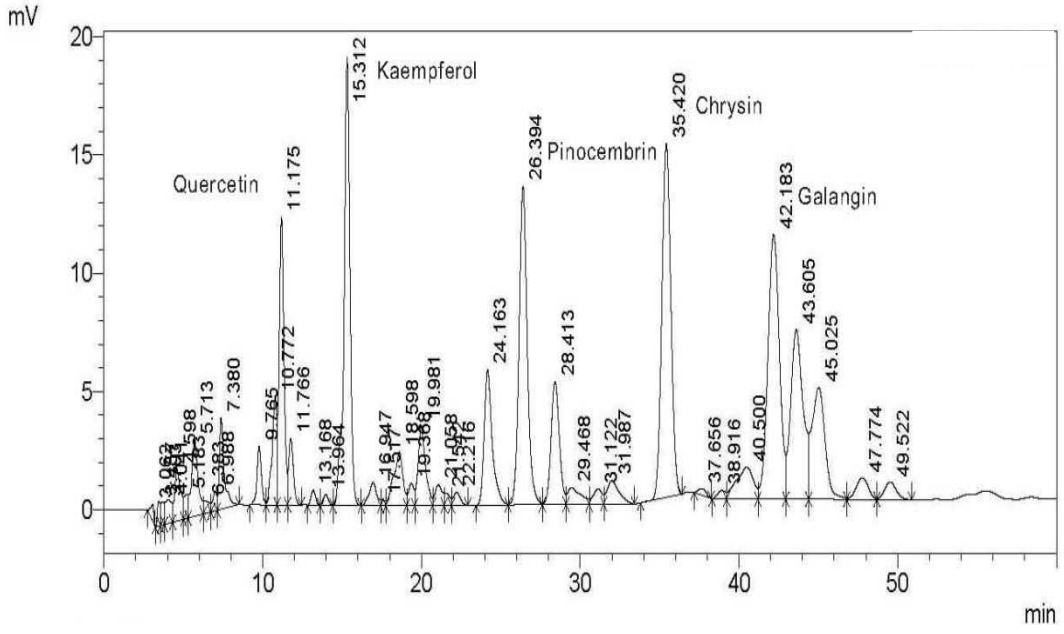


Fig4.8 HPLC chromatogram of Korean propolis sample 7

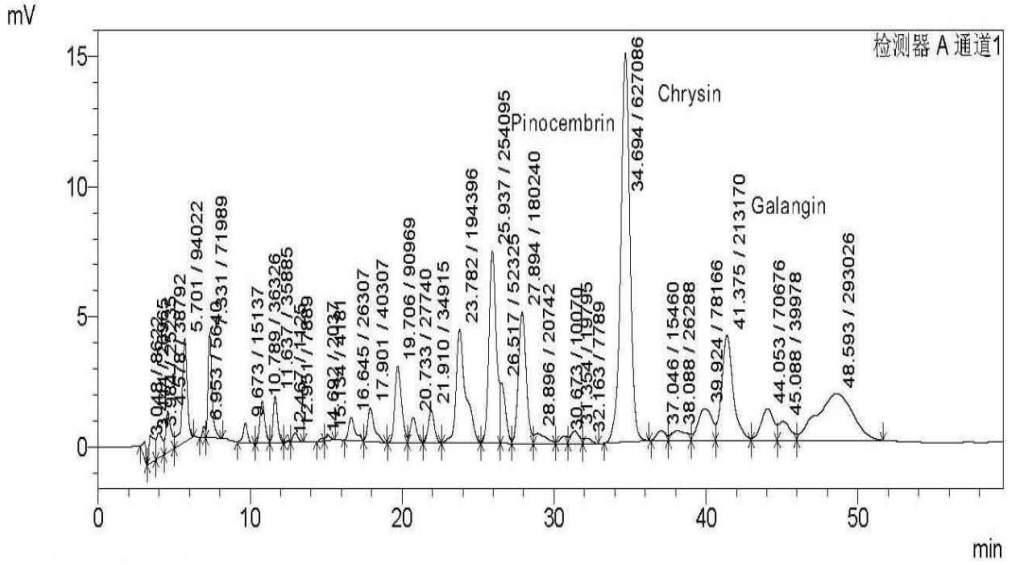


Fig4.9 HPLC chromatogram of Korean propolis sample 8

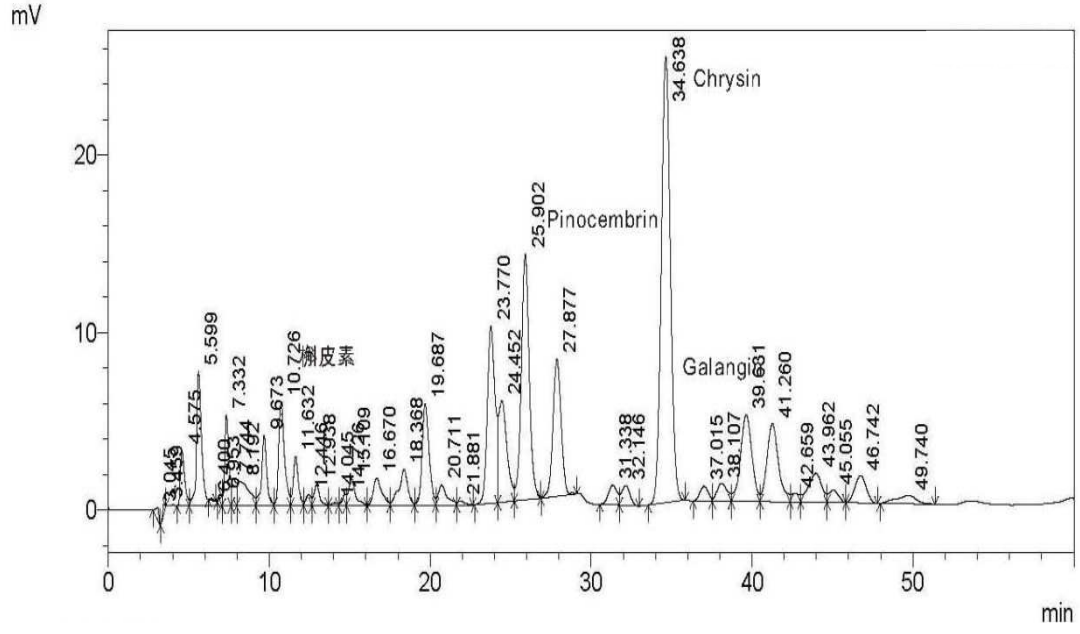


Fig4.10 HPLC chromatogram of Korean propolis sample 9

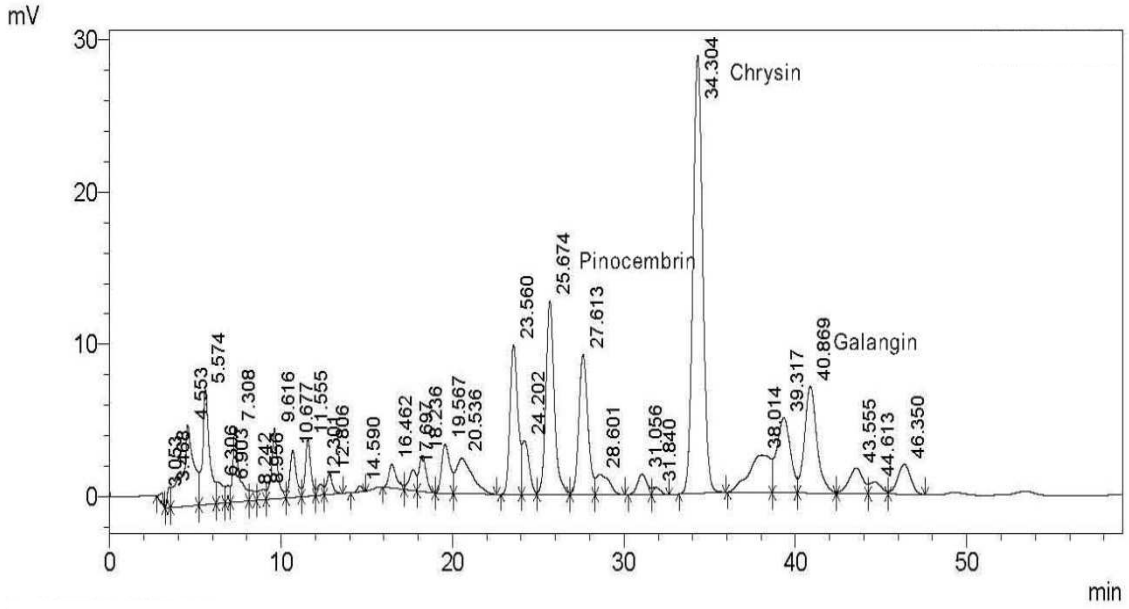


Fig4.11 HPLC chromatogram of Korean propolis sample 10

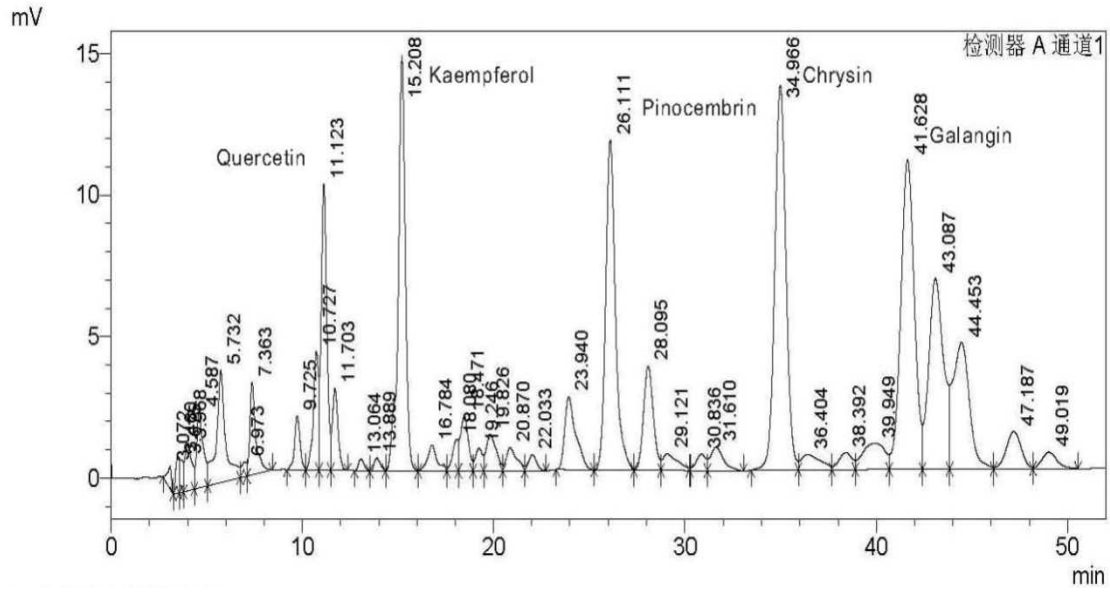


Fig4.12 HPLC chromatogram of Korean propolis sample 11

4.2.2 Determinattionof the 8 main flavonoids inThai propolis

In the HPLC chromatogram, peak areas of 8 flavonoids standards were presented with the relatively retention time (Fig4.13), retention time of each flavonoid is as follows, rutin 4.536 min, myricetin 5.673 min, quercetin 7.860 min, kaempferol 11.374 min, apigenin 12.297 min, pinocembrin 18.090 min, chrysin 23.527 min and galangin 26.618 min.

The 18 Thai propolis samples were also analyzed using the established extraction method under the above HPLC conditions. Each sample was analyzed in triplicate to determine the mean contents of eight major flavonoids in Thai propolis samples. The peaks in HPLC chromatograms were identified based on retention time and UV adsorption spectra comparison. The HPLC chromatogram of the Thai propolis, sample 1 to 17 and sample 18 from UK are shown as follows (Fig4.14 –Fig31).

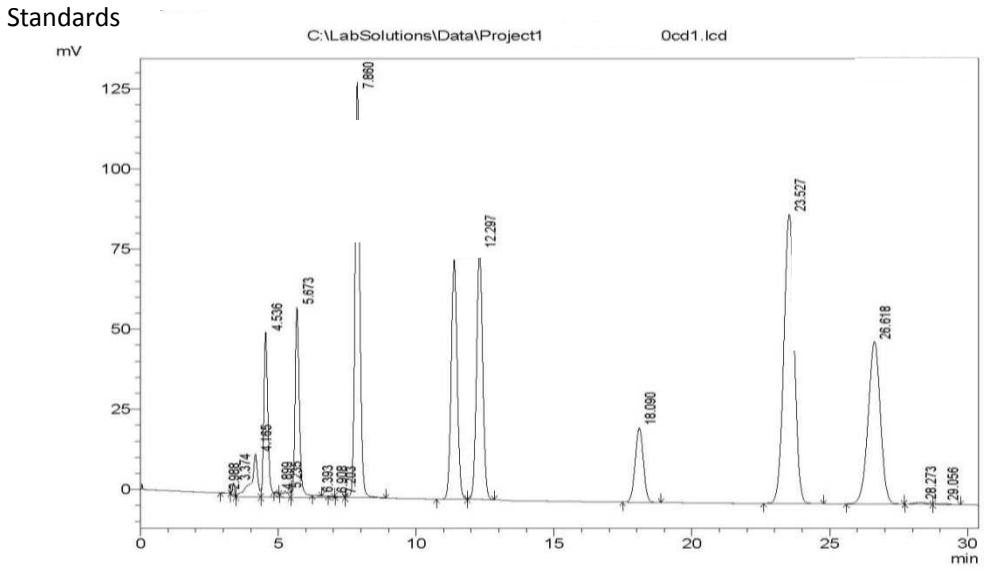


Fig.4.13 HPLC chromatogram of the 8 main flavonoids standards

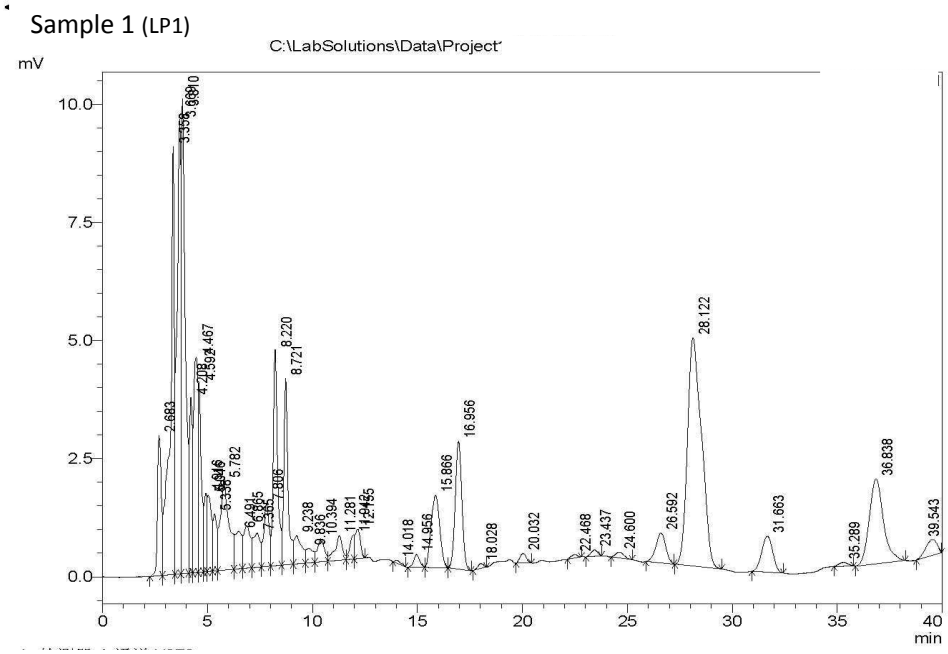


Fig.4.14 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 1

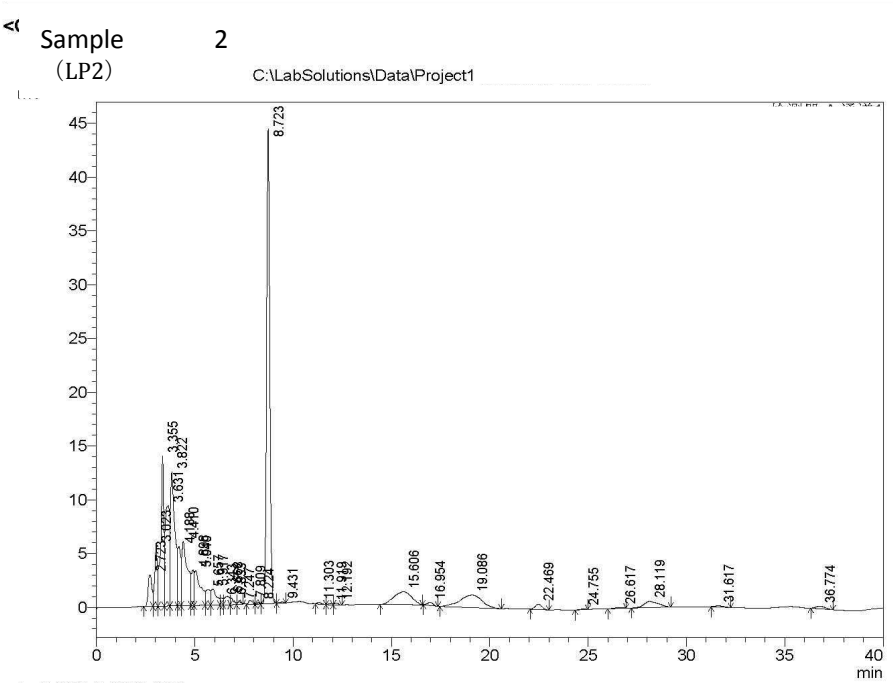


Fig.4.15 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 2

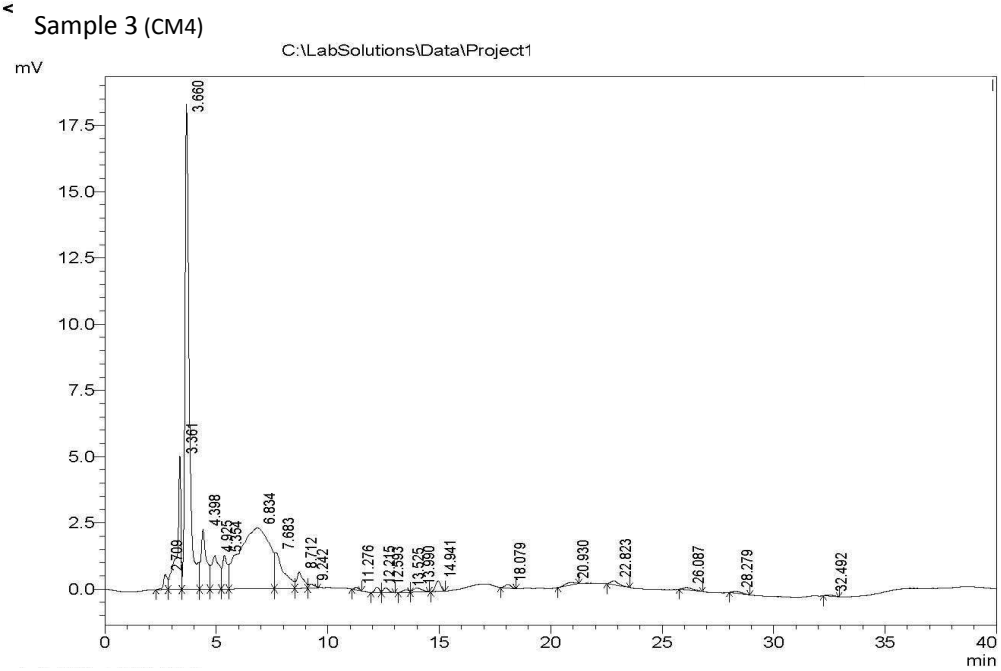


Fig.4.16 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 3

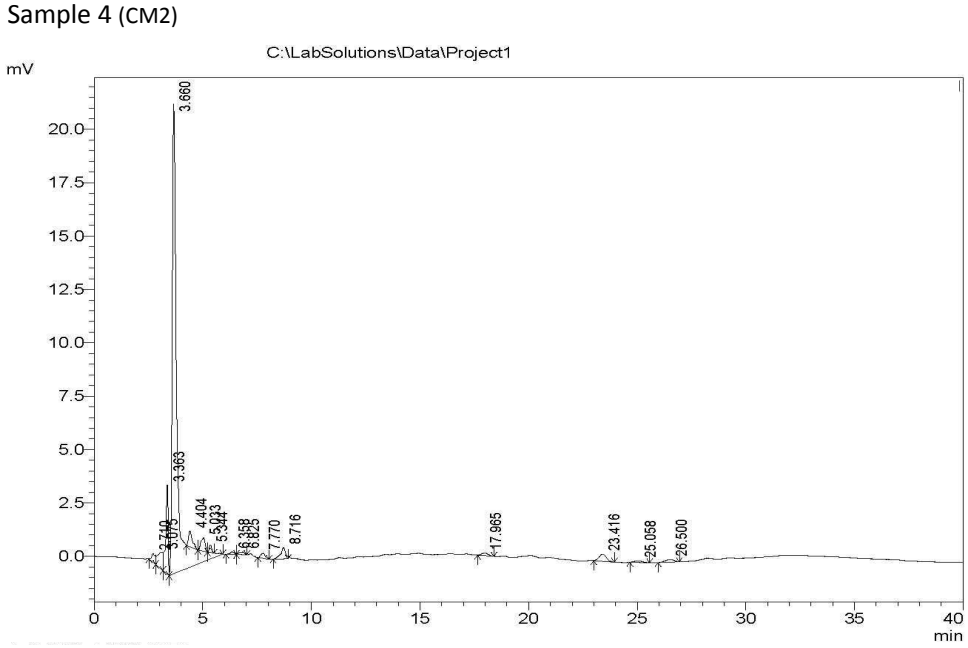


Fig.4.17 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 4

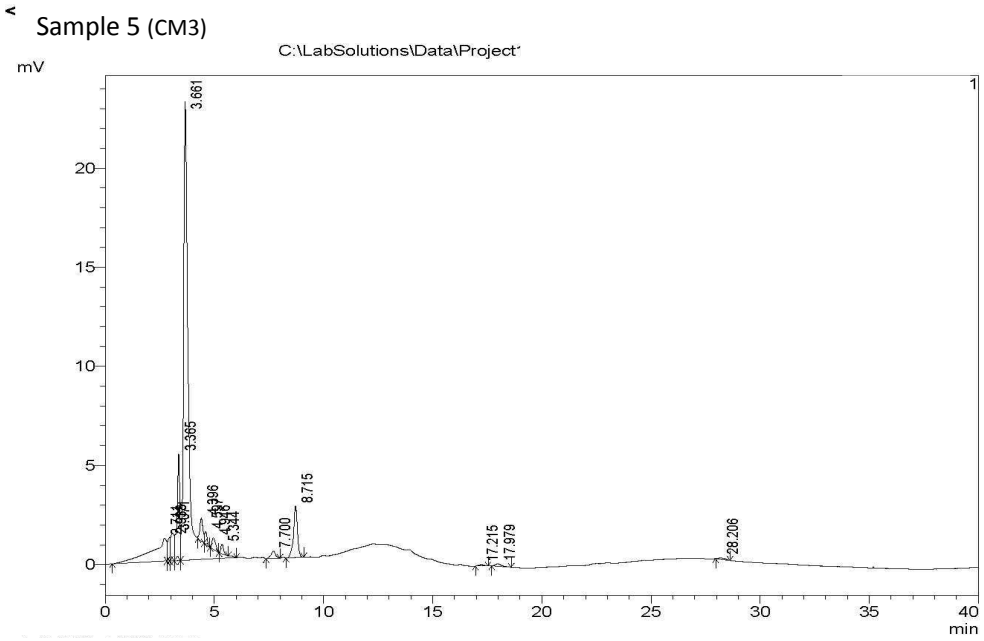


Fig.4.18 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 5

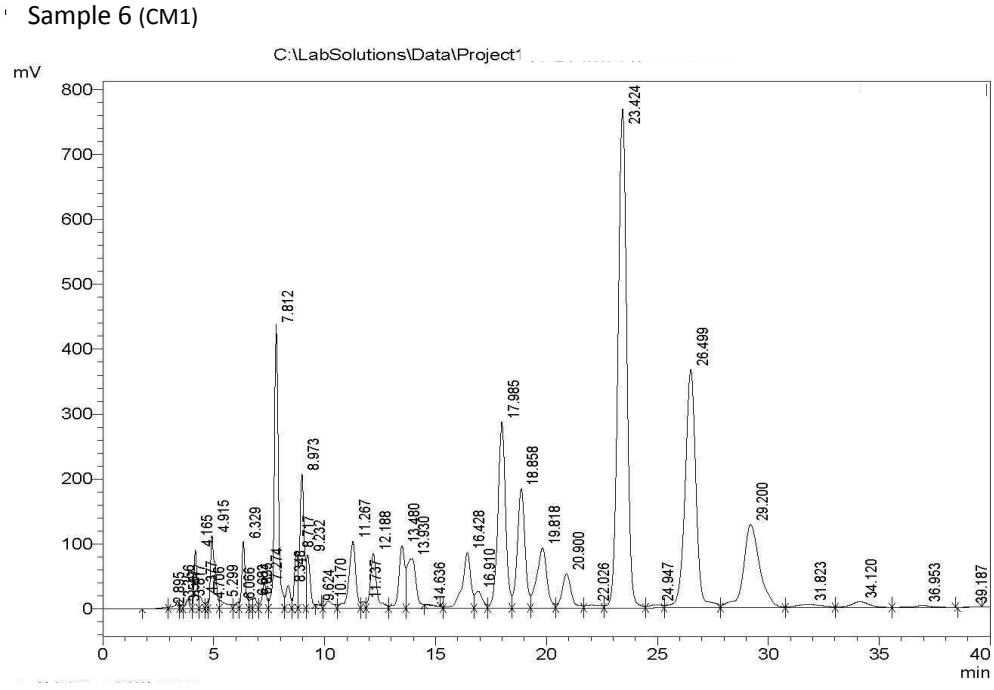


Fig.4.19HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 6

Sample7 （CR-MFU）

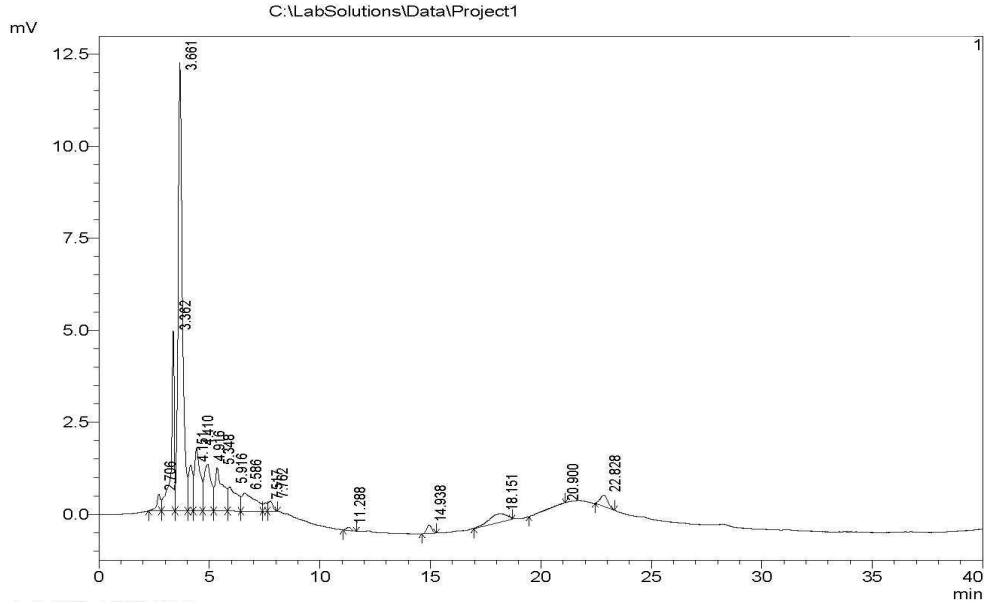


Fig.4.20 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 7

Sample 8 (Nan 1)

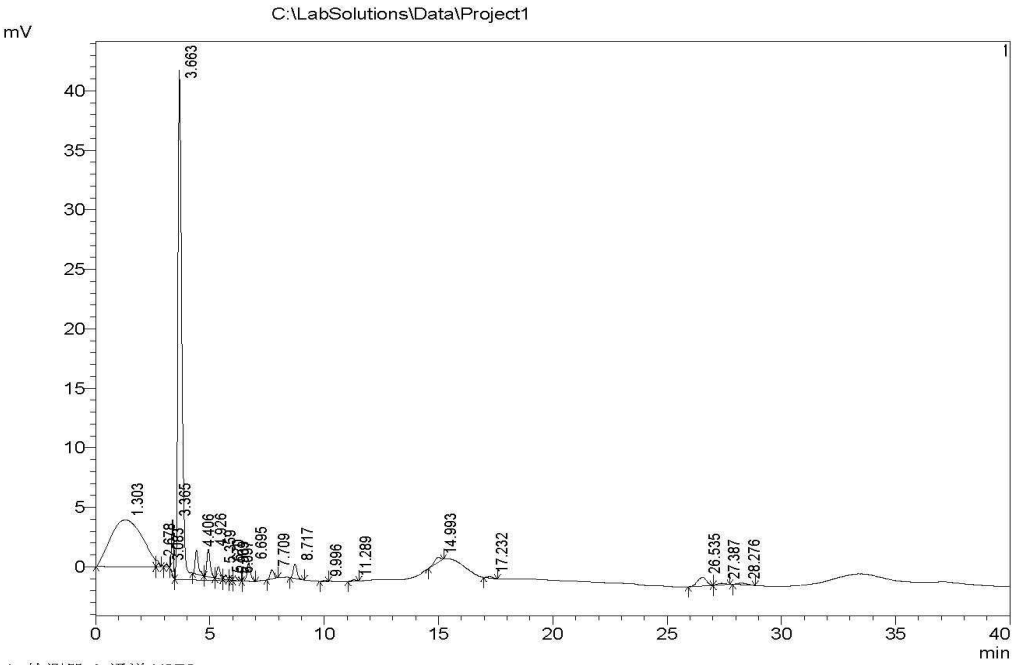


Fig.4.21 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 8

Sample 9 (Nan2)

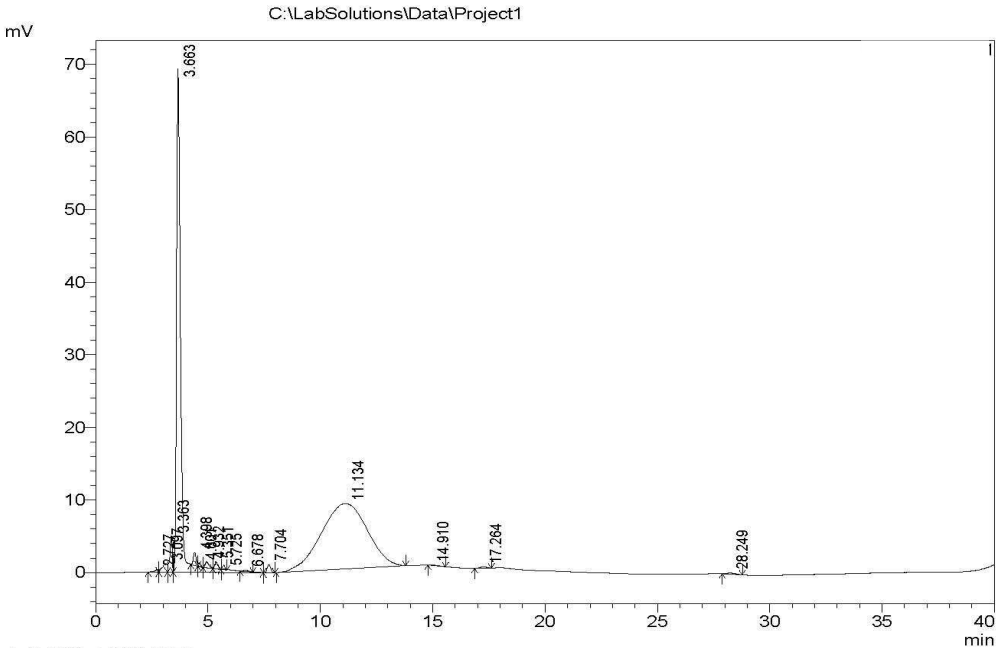


Fig.4.22 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 9

Sample 10 (Nan 3)

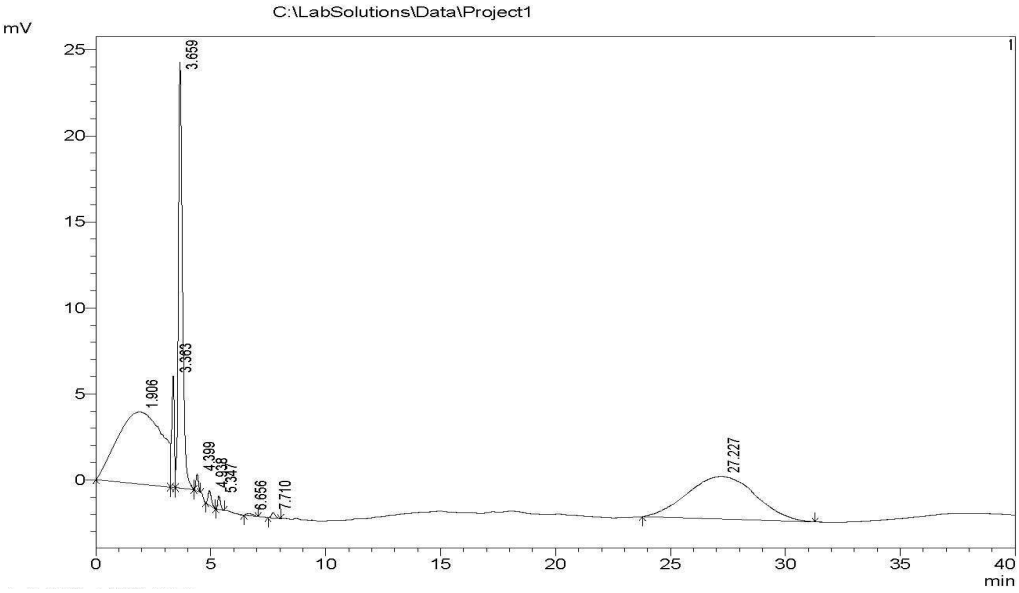


Fig.4.23 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 10

Sample 11 (Nan4)

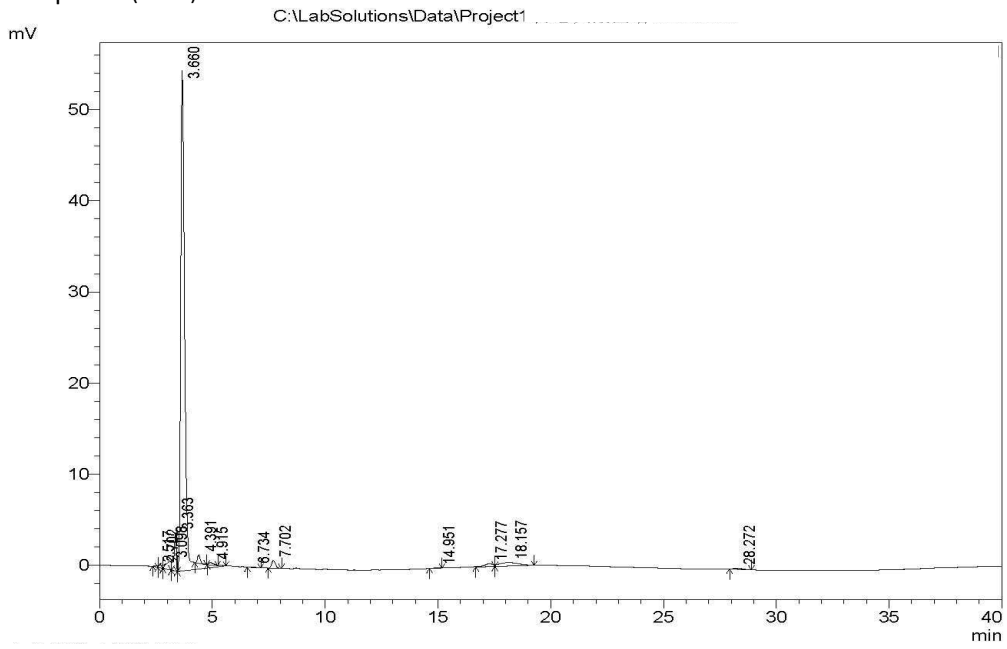


Fig.4.24 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 11

Sample 12 (CR1)

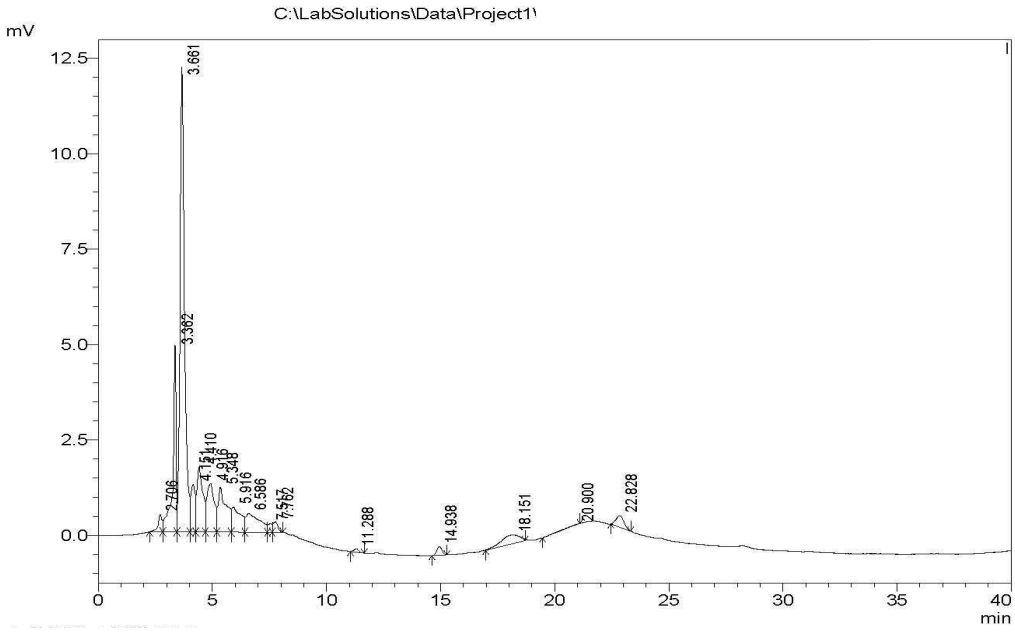


Fig.4.25 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 12

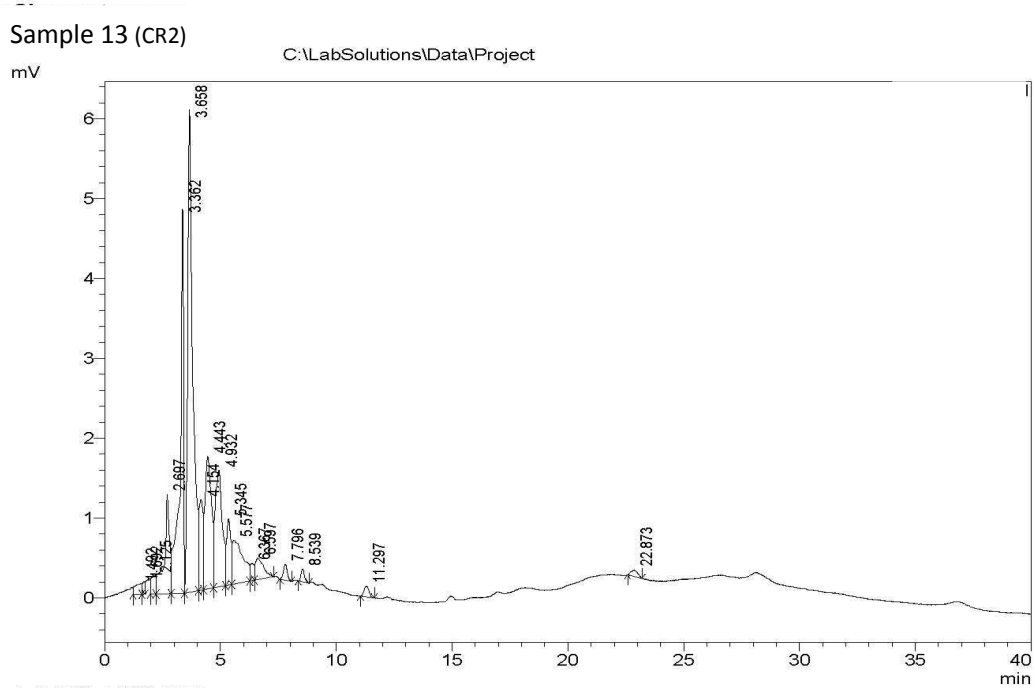


Fig.4.26 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 13

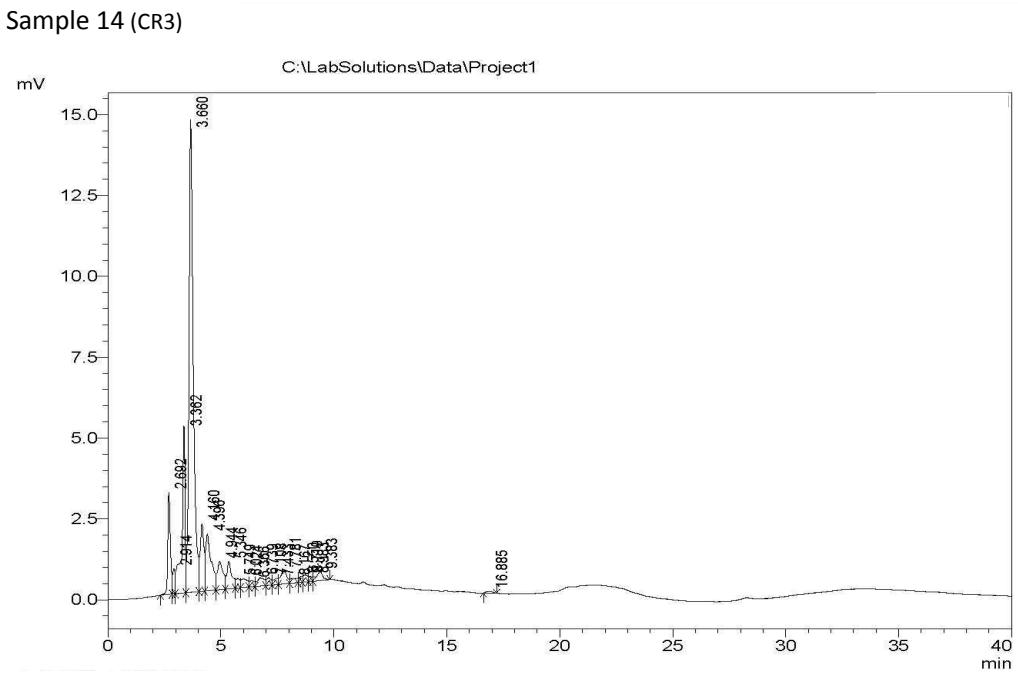


Fig.4.27HPLC chromatogram of 8 main flavonoids in Thain propolis sample 14

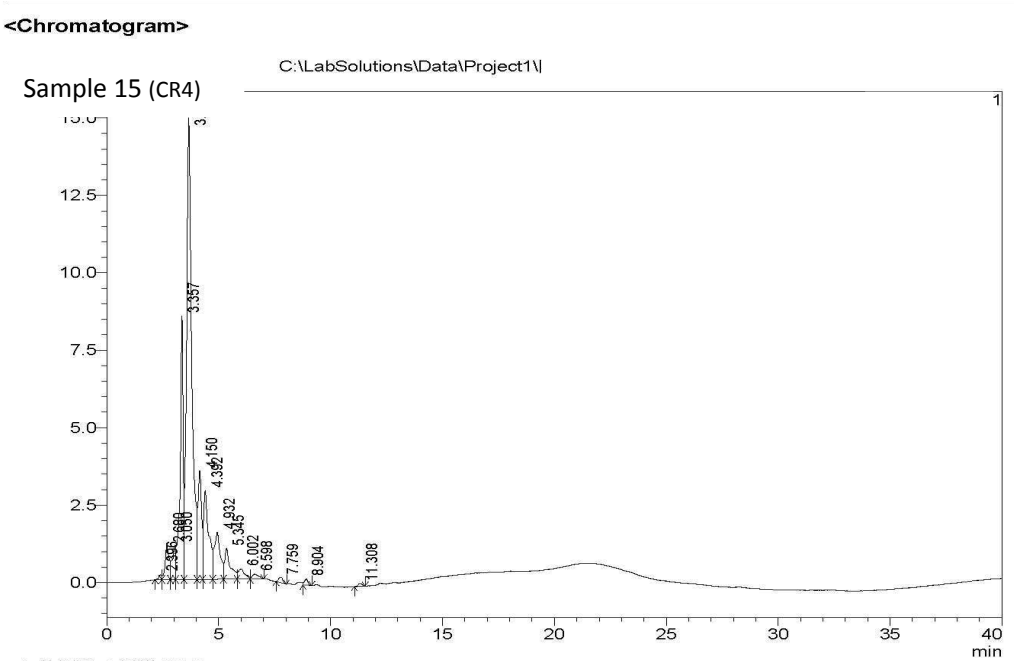


Fig.4.28 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 15

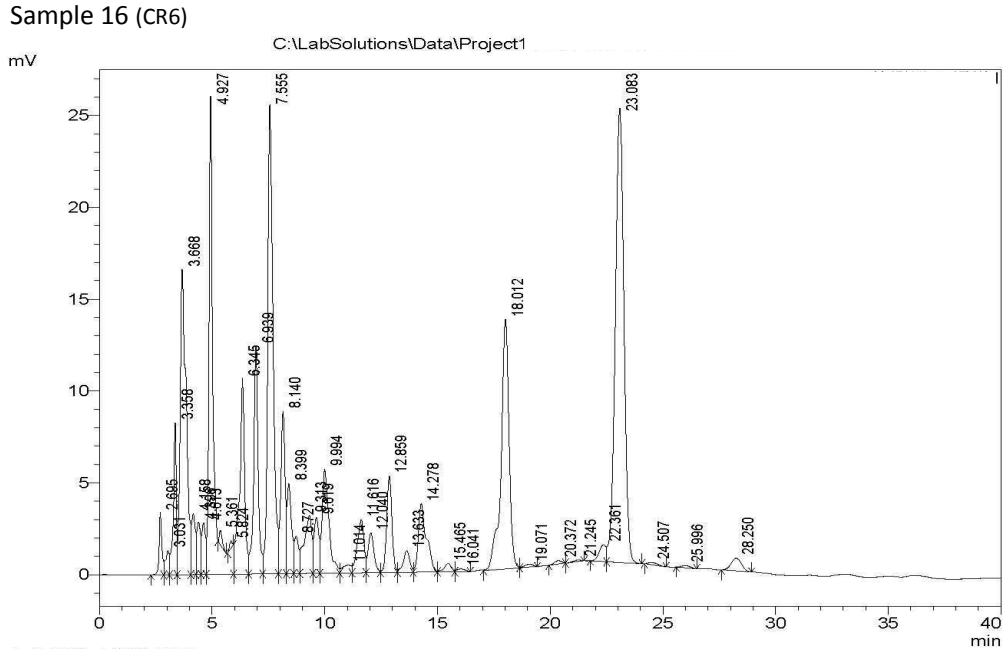


Fig.4.29 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 16

Sample 17 (CR7)

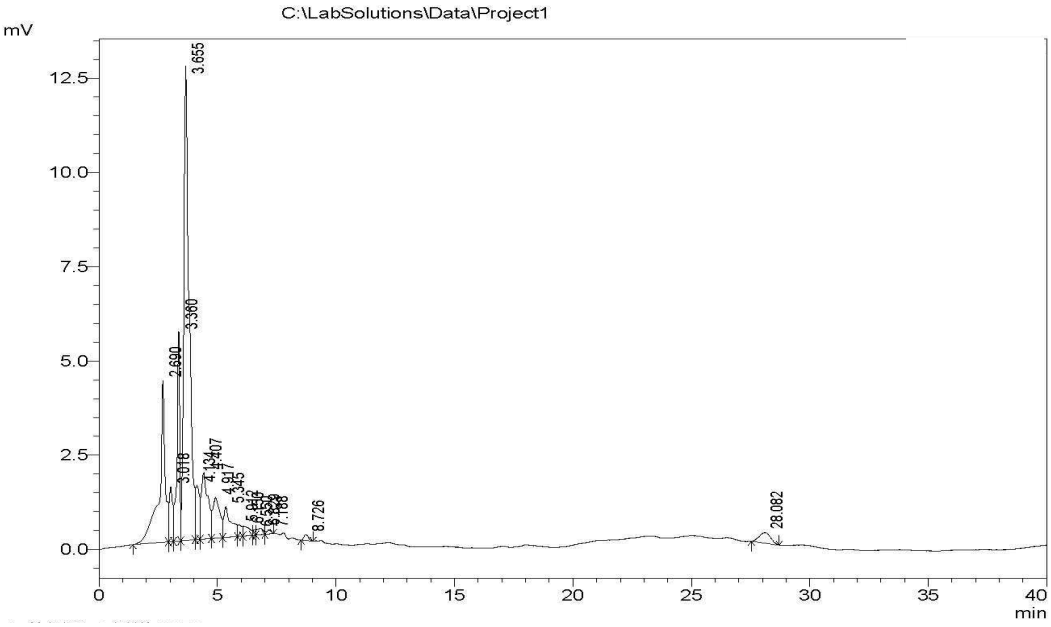


Fig.4.30 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 17

Sample 18 (UK)

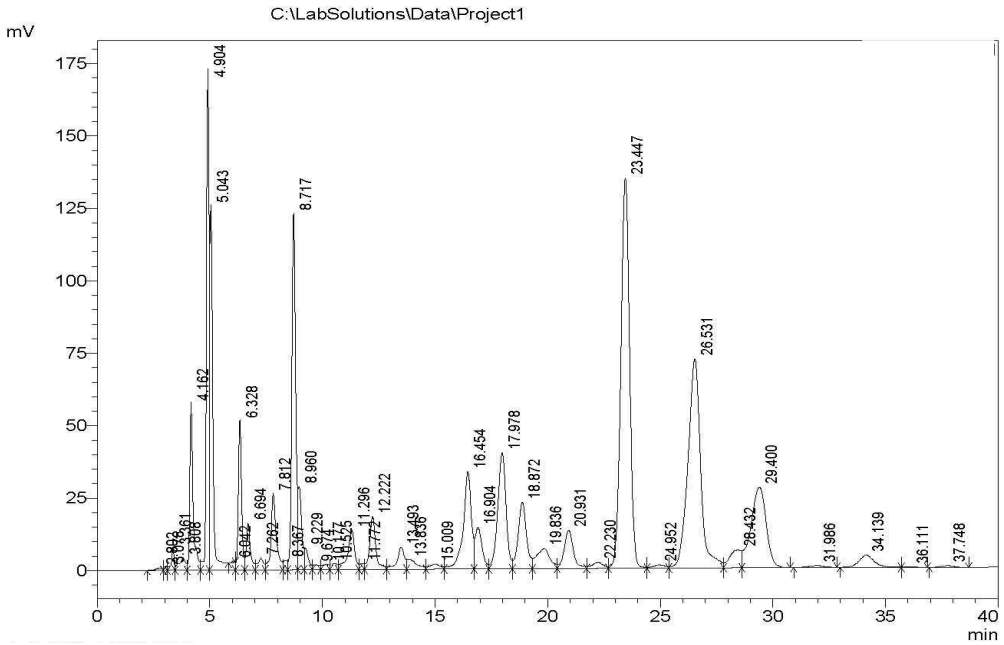


Fig.4.31 HPLC chromatogram of the 8 main flavonoids in Thai propolis sample 18

Table4.6. The8 main flavonoids content identified by HPLC-UV in propolis samples of Thailand

	Sample	Figure	Weight (mg)	Rutin	Myricetin	Quercitin	Kaempferol	Apigenin	Pinocembrin	Chrysin	Galangin
1	**LP1** 伟泰	ocd 5	1058.9	0.01	0.01	-	-	-	-	-	-
2	**LP2** 伟泰	ocd 6	1033.2	0.04	-	-	-	-	-	-	-
3	**Pay2**	ocd 7	1005.8	0.01	-	-	-	-	-	-	-
4	**CM2**	ocd 8	1008.3	-	-	-	-	-	-	-	-
5	**CM3**	ocd 9	1007.5	-	-	-	-	-	-	-	-
6	**CM1**	**ocd 10**	**1022.1**	**0.06**	**0.08**	**0.92**	**0.36**	**0.25**	**0.01**	**1.81**	**1.81**
7	**CR-Mfu**	ocd 11	1063.4	0.28	-	0.36	0.03	-	-	-	0.05
8	**Nan1**	ocd 12	1055	-	-	-	-	-	-	-	-
9	**Nan2**	ocd 13	847.8	-	-	-	0.26	-	-	-	-
10	**Nan3**	ocd 14	750.6	-	-	-	-	-	-	-	0.1
11	**Nan4**	ocd 15	627.3	-	-	-	-	-	-	-	-
12	**CR1**	ocd 16	681.6	0.01	0.01	-	-	-	-	-	-
13	**CR2**	ocd 17	537.3	0.02	-	-	-	-	-	-	-
14	**CR3**	ocd 18	782.2	0.01	-	-	-	-	-	-	-
15	**CR4**	ocd 19	508.5	0.03	-	-	-	-	-	-	-
16	**CR6**	ocd 20	664.7	0.02	-	0.11	-	-	0.09	-	-
17	**CR7**	ocd 21	713.8	0.02	-	-	-	-	-	-	-
18	**UK**	**ocd 22**	**229**	**0.79**	**1.4**	**0.31**	**0.22**	**0.24**	**0.05**	**1.39**	**1.83**

The results showed that the mean contents of eight major flavonoids of the studied samples from different location of Thailand are lower than UK and Korea. The content of 8 major flavonoids in Thai propolisis as follows: rutin ranges0.01–0.28%, myricetin 0.00–0.08% , quercetin 0.00–0.92%, kaempferol 0.00–0.36% , apigenin 0.00–0.25% , pinocembrine 0.00–0.09%, chrysin 0.00–1.81% and galangin 0.00–1.81%, respectively (table 4.1). only one propolis sample (cm1) from the healthy product co. ltd. of chiang mai of thailand identified all 8 main flavonoid, rutin(0.06), myricetin (0.08), quercetin (0.92), kaempferol (0.36), apigenin (0.25), pinocembrine (0.01), chrysin (1.81), galangin (1.81), and have many peaks in the HPLC chromatogram. This result is as similar as the result of UK and the Korea. That is also similar to the previously published studies mentioned before (Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002; Zhao et al. , 2005.; Li et al. 2007; Wang X.P. and Lin L. 2007). The total flavonoid of CM1 is also higher, 13.34%, closed to UK and Korea. Rutin was identified in 8 samples from Chiang Rai province of Thailand, CR-MFU, CR1 to CR7 in the range of 0.01 - 0.28%. Myricetin was identified in only CR at 0.01%, and quercetin in CR6and CR-MFU at 0.11% and 0.36 respectively. Kaempferol,pinocembrine,galangin were identified in only CR-MFU,CR6 ,CR-MFU at 0.035%, 0.09%, 0.05%, respectively.Apigenin and chrysin not detected. In two samples from Lamphun province of Thailand rutin were identified at 0.01-0.04% and myricetin in LP at 0.01 % and the HPLC chromatogram of LP1 have some unkown peaks. For examples from Nan province, the 8 flavonoids studied could not be identified in these samples but only kaempferol identified in Nan 2at 0.26%. There are some unkown peaks in the HPLC chromatogram of LP1 and CR propolis, sample1, 2 and 16. Most HPLC chromatogram of Thai

samples showed a common characteristic of the peak at the retention time3.655min, but no standard to identify the peak.

Among the experiment of the 8 main flavonoids, rutin was more likely to be detected in the all propolis samples of Thailand. The propolis sample from MFU of Chiang Rai has a good content of rutin 0.28%. The propolis samples from Chiang Rai, Lamphun, and Chiang Mai (CM1) have a less rutin (0.01-0.04%). In this case, the repeat and comparison with China testing will be carried out.

4.2.3 Determination of the 8 main flavonoids of Chinese propolis

The results were showed in Table 4.7. and the Appendix B. the contents of eight major flavonoids of the studied samples from different location of China are all good.There are abundant and higher amount of the 8 main flavonoids in Chinese propolis than in Thai.The results also indicate that the 8 flavonoids varies from different location, different botanic resource. The testing result has a good consistance with the previous published studies (Abd et al., 2002; Hegazi et al., 2002;Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002).

According to the results of 4.2.1, 4.2.2 and 4.2.3, testing results indicate that the 8 flavonoids contents in Korea, China and UK are higher than that in Thailand. And the Chinese is the good and highest, than UK and Korea, the last one is Thai. However, there is also an abundant of the 8 flavonoids rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin in the only one sample form Chiang Mai company of Thailand. It is as similar as Korea,

UK and China and the other countries in previous published researches (Abd et al., 2002; Hegazi et et al., 2002; Bankova et al., 2000; Popova et al., 2005; Bankova et al., 1995; Salomão et al., 2004; Park et al., 1998; Park et al., 2002;Zhao et al. , 2005.; Li et al. 2007; Wang and Lin. 2007).All Thai samples were detected rutin (0.01–0.28%), even if it is lower than Korea, UK and China as a whole, some sample is higher than the sample of China and Korea (4.2.1; 4.2.3). And in a whole, the other 7 flavonoids myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin contents are lower in the 16 samples except the one from Chiang Mai company. Maybe it is due to the tropical climate, different botanic, location and some other reasons.May be there will be some other kinds of flavonoids As you know, flavonoids are a large family of polyphenolic compounds synthesized by plants. Flavones are divided into four groups such as flavone (Luteolin, Apigenin, Tangeritin, et al.), flavonol (Quercetin, Kaempferol, Myricetin, Fisetin Rhamnazin, Isorhamnetin, Pachypodol, et al.,), flavanone(Eriodictyol, Homoeriodicty Hesperetin, Naringenin, et al.) and flavanonol (Taxifolin, et al.). Terpenes are also another component of propolis extract, with 17 varieties identified in China (Li et al., 2007). Maybe there will be some other phonolic compounds, phenols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones et al. For example Pinostrobin chalcone, hexamethoxy, pinostrobin, pinobanksin 3-acetate, naringenin, dihydrocinnamic acid, cinnamic acid, p-coumaric acid, isoferulic acid, ferulic acid, caffeic acid in Egyptian propolis (Abd et al., 2002; Hegazi et et al., 2002;); pentenyl caffeates, benzyl caffeates, phenethyl caffeate acid in European propolis (Bankova et al., 2002); Cinnamic acid, benzyl cinnamate, cinnamyl cinnamate, pinobanksin, pinobanksin 3-acetate, phenylethyl caffeate, cinnamyl caffeate, vanillin, p-coumaric acid, ferulic acid, caffeic acid, dehydroabietic acid in Turkish propolis (Popova et al., 2005); Cinnamic acid, vanillin, ethyl cinnamate, vanillic acid, p-coumaric acid, ferulic acid, ethyl ferulate, 3-methylbut-2-enyl ferulate, 3-methylbut-3-enyl ferulate in New Zealand propolis (Bankova et al., 1995); Ethyl hydrocinnamate, hydrocinnamic

acid, inositol, cinnamic acid, ferulic acid, caffeic acid, pinostrobin, Coumaric acid, ferulic acid, pinobanksin, isosakuranetin, dimethylallyl caffeic acid, pinobanksin 3-acetate, kaempferide, pinobanksin 3-acetate, rhamnetin, sakuranetin, kaempferide, tectochrysin, acacetin and tectochrysin in Brazilian and Bulgarian propolis (Salomão et al., 2004; Park et al., 1998; Park et al., 2002) .

The further determination should be testing the antioxidant and antimicrobial activity of Thai propolis. If the antioxidant and antimicrobial activity is good, it maybe worth to carry on research of the other individual flavonoids and phnolic compositioncontinually Due to the time and the finance supports, we are very sorry that the further analysis on should be researched continually in the future.

Table 4.7: The 8 Main Flavonoids Content in propolis samples of China by HPLC-UV

	Sample code	Collected area	Rutin	Myricetin	Quercetin	Kaempferol	apigenin	pinocembrine	chrysin	galangin	Total
1	AH 1	Anhui I	1.65	0.58	0.43	0.33	0.25	3.87	4.57	3.72	15.4
2	AH 2	Anhui II	0.77	0.92	0.62	0.28	0.36	4.47	4.54	4.39	16.35
3	AH 3	Anhui III	0.64	0.33	1.47	0.83	0.47	5.54	3.54	5.78	18.6
4	AH 4	Anhui IV	1.38	0.83	0.64	0.37	0.38	2.77	3.7	2.37	12.44
5	AH 5	Anhui V	0.46	0.17	0.1	0.57	0.02	1.92	0.45	2.28	5.97
6	AH 7	Anhui VII	0.63	0.08	0.59	0.11	0.2	2.72	0.27	0.97	5.57
7	AH 10	Anhui X	0.79	0.51	0.7	0.65	0.48	5	4.11	4.56	16.8
8	AH 10	Anhui X	0.58	0.56	0.76	0.39	0.43	4.2	4.01	4.21	15.14
9	AH 10	Anhui X	1.02	0.69	0.86	0.43	0.43	3.91	0.89	4.08	12.31
10	AH 11	Anhui XI	1.88	0.12	0.58	0.23	0.14	3.19	0.45	1.81	8.4
11	HB 1	Hebei I	0.83	0.55	0.9	0.31	0.25	8.59	3.42	4.48	19.33
12	HB 4	Hebei IV	0.18	0.0042	0.72	0.0017	0.07	2.59	0.64	4.68	8.89

13	HB 5	Hebei V	1.27	0.69	0.3	0.39	0.21	4.96	2.68	2.68	13.18
14	HB 6	Hebei VI	1.15	0.71	0.34	0.24	0.24	1.69	2.56	2.34	9.27
15	HLJ 1	Heilongjiang I	1.58	0.28	0.51	0.23	0.2	4	0.43	5.06	12.29
16	HLJ 2	Heilongjiang II	2.27	1.03	0.09	0.09	0.11	2.36	2.45	2.15	10.55
17	HLJ 5	Heilongjiang V	0.61	0.13	0.16	0.069	0.0017	1.18	0.3	5.21	7.66
18	HLJ 6	Heilongjiang VI	0.75	0.06	0.1	0.48	0.2	4.55	3	5.17	14.31
19	HN 3	Henan III	1.18	0.71	1.02	0.46	0.41	3.69	3.72	4.19	15.38
20	JS 2	Jiangsu II			0.62	0.69	0.37	5.08	3.58	5.65	15.99
21	JL 1	Jilin I	0.16	0.98	1.42	0.43	0.45	5.51	3.95	4.58	17.48
22	JL 2	Jilin I	0.78	0.11	0.57	0.36	0.25	6.56	2.33	2.11	13.07
23	JL 3	Jilin II	0.8	0.68	0.06	0.25	0.18	3.28	0.37	5.03	11.07
24	JL 4	Jilin III	0.66	0.31	0.83	0.4	0.28	7.67	2.7	3.32	16.17
25	LN 3	Liaoning III	1.24	0.76	0.24	0.38	0.25	1.27	2.49	3.76	10.39
26	LN 3	Liaoning III	0.78	0.45	0.28	0.11	0.41	1.63	2.41	2.68	8.75
27	LN 4	Liaoning IV	1.78	0.98	1.12	0.53	0.42	9.96	4.11	4.6	23.5
28	SX 3	Shaanxi III	1.46	1.13	0.96	0.27	1.32	2.93	3.04	3.3	14.41

29	SD 1	Shandong I	0.63	0.3	0.13	0.27	0.13	3.43	1.68	1.99	8.56
30	SD 2	Shandong II	0.84	0.3	0.61	0.28	0.32	2.49	4.13	3.19	12.16
31	SD 4	Shandong IV	0.69	0.47	0.63	0.39	0.53	4.12	4.21	4.22	15.26
32	SD 6	Shandong V	2.33	1.09	1.09	0.4	0.42	3.67	3.88	3.05	15.93
33	SD 7	Shandong VI	1.39	0.6	0.64	0.23	0.24	2.79	2.96	2.76	11.61
34	SD 7	Shandong VI	1.61	0.73	0.14	0.35	0.37	3.55	3.45	2.3	12.5
35	SD 7	Shandong VII	0.56	0.37	1.13	0.22	0.83	2.41	3.16	2.39	11.07
36	SC 1	Sichuang I	0.96	0.43	0.15	0.35	0.11	1.5	1.6	5.64	10.74
37	SC 2	Sichuang I	0.74	0.37	0.03	0.41	0.17	1.67	0.31	5.38	9.08
38	SC 3	Sichuang II	0.71	1.02	1.27	0.5	0.36	3.76	2.65	5.28	15.55
39	ZJ 1`	Zhejiang I	0.29	0.36	0.51	0.47	0.25	3.46	2.02	3.48	10.84

4.3 Antioxdant activity of Thai propolis

4.3.1 Determination of reducing power

There is an obvious relativity between the reducing power/capacity and antioxidant activity. The reducing power/capacity can indirectly reflect the antioxidant activiity. Figure 4.35 to Figure 4.38 and Table 4.6 show that the entire EEP sample of propolis has a strong reducing power, and has dose-effect relationship. Reducing power of the EEP sample of propolis in Brazil and P1(CM3), followed by reducing power of the P2(CM4), P18(CM-MFU) and the weakest N1 propolis, P5(CR7), P7(LP2), and P4(CR1) propolis.This result also inticated that different types and location of propolis are different on reducing power activity.

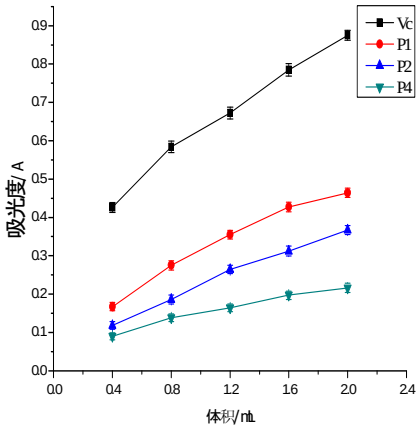


Fig.4.32 Reducing power of P1,P2 and P4

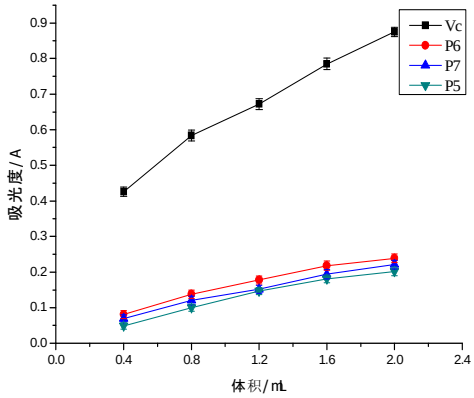


Fig.4.33 Reducing power of P5,P6 and P7

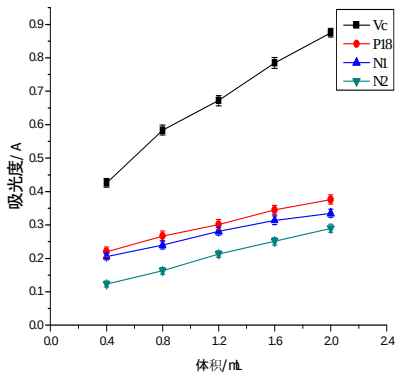


Fig.4.34 Reducing power of P18,N1 and N2

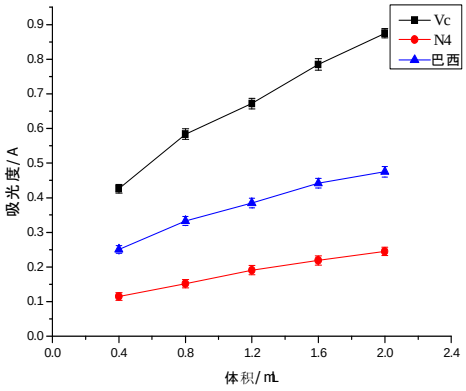


Fig.4.38 Reducing power of Brazil and N4

Table4.8 The reducing power/capacity of the antioxidant activity of Thai propolis

Propoli extract	Concentration (μg/mL)	Antioxidant effect		Propolis extract	Concentration (μg/mL)	Antioxidant effect	
		Reducing power	DPPH Scavenging rate (%)			Reducing power	DPPH Scavenging rate (%)
P1	50	0.142±0.010	37.90±0.8	P18	50	0.201±0.014	20.90±0.9
	100	0.257±0.012	50.93±0.9		100	0.264±0.015	36.30±0.8
	200	0.361±0.012	73.20±1.0		200	0.307±0.015	56.22±0.9
	300	0.446±0.012	83.90±0.7		300	0.376±0.014	68.56±1.0
	400	--	85.32±0.9		400	--	73.01±0.9
	500	--	86.11±0.8		500	--	74.42±1.0
P2	50	0.109±0.010	24.32±0.8	Brazil	50	0.249±0.011	35.90±0.8
	100	0.245±0.012	34.76±0.9		100	0.330±0.013	52.98±0.9
	200	0.313±0.011	56.72±0.8		200	0.391±0.014	76.17±1.0
	300	0.367±0.010	72.73±0.8		300	0.475±0.015	85.29±0.9
	400	--	77.38±1.0		400	--	86.99±0.9
	500	--	81.74±0.9		500	--	87.89±1.0
P4	50	0.087±0.009	20.20±0.9	N1	50	0.203±0.010	19.32±0.8
	100	0.133±0.010	30.20±0.9		100	0.238±0.012	31.76±1.0
	200	0.175±0.010	51.51±1.0		200	0.287±0.011	54.72±0.9
	300	0.216±0.012	66.95±0.8		300	0.335±0.012	67.51±0.9
	400	--	74.37±1.0		400	--	71.19±1.0
	500	--	78.21±0.9		500	--	73.92±1.0

P5	50	0.047±0.009	17.12±0.8	N2	50	0.121±0.009	17.22±0.8
	100	0.095±0.010	31.25±0.9		100	0.159±0.010	29.25±0.9
	200	0.155±0.010	47.51±1.0		200	0.218±0.010	47.51±0.9
	300	0.202±0.011	57.95±0.8		300	0.290±0.011	65.95±1.0
	400	--	60.43±1.0		400	--	70.23±0.9
	500	--	63.75±0.9		500	--	73.35±1.0
P6	50	0.080±0.011	25.90±0.8	N4	50	0.113±0.011	17.41±0.8
	100	0.136±0.012	34.30±0.9		100	0.148±0.012	29.76±0.9
	200	0.183±0.011	51.22±1.0		200	0.199±0.013	52.72±0.9
	300	0.239±0.013	62.16±1.0		300	0.245±0.012	65.51±0.9
	400	--	67.02±0.9		400	--	68.87±1.0
	500	--	71.11±1.1		500	--	71.29±1.0
P7	50	0.068±0.010	21.32±0.8	Positive control Vc	50	0.423±0.013	41.20±1.0
	100	0.120±0.012	30.76±0.9		100	0.581±0.015	60.00±1.2
	200	0.165±0.011	46.72±0.8		200	0.675±0.015	80.00±1.2
	300	0.221±0.010	57.73±0.9		300	0.875±0.013	93.00±1.2
	400	--	63.19±1.0		400	--	96.30±1.2
	500	--	65.74±1.0		500	--	96.40±1.2

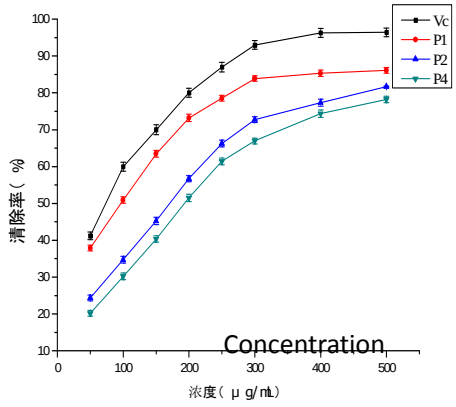
Note: The sample P1=CM1,P2=CM4, P4=CR1, P5=CR7, P6=CR3, P7=LP2, P18 is P8 =CR-MFU

4.3.2 Determination of DPPH free radical scavenging

DPPH is stable free radicals in organic solvents and has strong absorption at about 517 nm (dark purple). When a free radical scavenger exists, the DPPH lone pair electrons are

paired, and the absorption at 517 nm will be reduction or disappearance. The free radical scavenger activity can be evaluated by measuring the extent of absorption weakened. Therefore DPPH method is extensively used for the study of free radical scavenger activity.

As can be seen from Figure 4.36-4.39 all of the propolis sampleshave a strong activity of DPPH radical scavenging. Different types and location of propolis are different on DPPH clearance rate. The results show that the antioxidant activity of Brazil and CR3 (P1) propolis are the best, closer Vc. The clearance rate of DPPH of CR7(P5) and LP (P7) lead lowest.



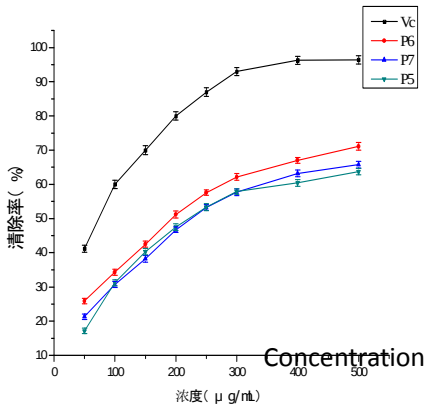
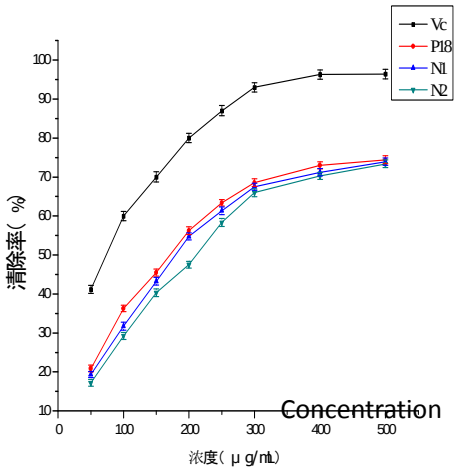


Fig.4.36 DPPH Scavenging Rate of P1,P2 and P4 Fig.4.437 DPPH Scavenging Rate of P5,P6 and P7



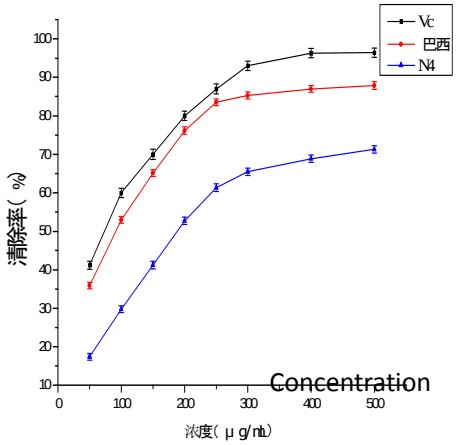


Fig.4.38 DPPH Scavenging Rate of P18,N1,N2Fig.4.39 DPPH Scavenging of Brazil and N4

Note: The sample P1=CM1,P2=CM4, P4=CR1, P5=CR7, P6=CR3, P7=LP2, P18 is P8 =CR-MFU

Table4.9: IC50 values of propolis extracts on DPPH radical scavenging rate

Propolis extracts	IC_{50} （μg/mL）	Propolis extracts	IC_{50} （μg/mL）
P1	89.335	P18	163.321
P2	160.794	Brazil	82. 074
P4	187.563	N1	180.645
P5	232.952	N2	201.794
P6	192.499	N4	193. 374
P7	231.892	Vc	70.636

The experimental results of the antioxidant areas similar as previously studies on the propolis antioxidant activity of Brazilian propolis and other contries'(Strehl, 1994; Abd, 2002; Ahn,2004; Ahn,2005; Vinson, 1998; Cao Wei, Wei, 2002; Cui, 2002; Dillon, 2006; Guo And Li2007; Hu Chunsheng; Hu, 2005; Herrero, 2005; Moreno, 2000; Peng and Zhang. 2005; Chen et al., 2009).

In a word, there are strong antioxidant activity in all of the Thai propolis. It is worth to developusing on human health, medical and comestics field. The functional food, health care and conestics of Thai propolis will be researched and developed in the future.

4.4 Antimicrobial activity of propolis from the main beekeeping locations of Thailand

The experimental results of the antimicrobial activity experiment of different varieties of propolis extract can be check in Table 4.10. It can be seen from Table 4.10 that dimethyl sulfoxidehad no inhibitory effect on both *Staphylococcus aureus* and *Candida albicans.* And

positive control fluconazole and penicillin potassium salt had a strong antimicrobial effect. It means that this test method is reasonable and valid.

The results showed that all propolis sample, 1 ~ 8 mg / mL concentration, had the good ability to inhibit *Staphylococcus aureus.* In contrast, the Brazilian propolis exhibits the strongest antimicrobial activity against *Staphylococcus aureus,* whereas Thai propolis P7 is the weakest. This testing result was consistent with that of previously published studies and reported in the literature that propolis has antimicrobial activity on Gram-positive bacteria.

However, the 11 samples of propolis extract had no inhibition effect on *Candida albicans,* but the propolis sample from Brazil and Chiang Rai and Nan of Thailand propolis sample at 1 ~ 8 mg / mL concentration had a weak inhibitory effect.

The result of inhibition zone test showed that the propolis extracts had significant inhibitory activity against *Staphylococcus aureus* (Table4.11), in order to further determine the inhibitory concentration, the minimum inhibitory concentration (MIC) were tested. The results showed that there was no bacterial growth of the propolis extract at the concentration of 8 mg / mL. According to this, half of the minimum inhibitory concentration (MIC50) was tested by culture plate dilution method

Table 4.11 showd the MIC50 of various propolis extracts against *Staphylococcus aureus*. The result demonstrates that all the propolis extracts show antibacterial effect against *S. aureus,* the sequence from low to high of MIC50 against *S.aureus* is as follows: Brazil<N4<N2<P4<N1<P18<P5<P6<P2<P1<P7. The MIC50 of Brazilian propolis is the lowest (0.093mg/mL) among all these, followed by N4, N2 and P4, they are 0.15mg/mL, 0.70mg/mL, 0.77mg/mL respectively. The MIC50 of P7 is the highest (7.69mg/mL). The lower the MIC50 the better the antimicrobial effect, so the antibecterial activity of Brazil sample is the best, and then

followed the Thai propolis from Nun and Chiang Rai province of Thailand, the sample from Lamphun is the last one. In a word, all propolis has the antibacterial activity against Gram positive *Staphylococcus aureus*. This result is consistent with preiously published studies(Ghisalberti, 1979; Mochida et al., 1985; Zhang et al, 1998; Hu etal, 1998; Yang et al., 1999;Velikova et al., 2000; Pepeljnjak et al., 1985; Lan et al., 2006). That means it is worth to develop Thai propolis application in medical and health care area, as well as cosmetics field.

Among the 11 propolis samples, eight propolis extracts did not exhibit antibacterial activity against *Candida albicans* in the 0.25 to 8 mg / ml concentration range, only Brazil, sample P4 from Chiang Rai, and sample N4 form Nun, three kinds of propolis on Candida albicans show a weak antibacterial activity. Maybe it is due to the difference in botanic resource and location. It validates the theoretical of previous research of Brazil and the other countries on the propolis is complex, it varies from the difference plants, regions and climates(Bosio, 1994; Bauer, 1966; Schneidewind et al., 1979; Ochida, 1985; Hu 1990;Marcucci, M.C. 1995; Keskin, 1998; Park, 1998;Zhang And Chui, 1998;Kujumgiev,1999; Bosio,2000; Bonvehíand Coll, 2000; Sforcin,2000; Velikova, 2000; Keskin, 2001;Popova,2005; Park1998; Marcucci,2001;Bonvehíand Coll, 2001; Abd, 2002; Chen et al, 2009).

Table 4.10 The antimicrobial activity of Thai propolisextract

sample	Concentr-ation (mg/ml)	Microbial strain		sample	Concentr-ation (mg/ml)	microbial strain	
		Candida albicans(mm)	*S. aureus*(mm)			*candida albicans*(mm)	*S. aureus*(mm)
P1	0.25	-	-	P18	0.25	-	8.9±0.3
	0.5	-	-		0.5	-	9.1±0.5
	1.0	-	7.1±0.3		1.0	-	10.0±0.3
	2.0	-	9.2±0.4		2.0	-	10.2±0.5
	4.0	-	10.5±0.4		4.0	-	11.4±0.3
	8.0	-	11.8±0.5		8.0	-	12.2±0.2
P2	0.25	-	7.8±0.4	Brazil	0.25	-	8.0±0.3
	0.5	-	8.2±0.5		0.5	-	8.2±0.4
	1.0	-	10.3±0.1		1.0	7.8±0.2	10.6±0.5
	2.0	-	10.8±0.4		2.0	8.0±0.1	12.1±0.2
	4.0	-	11.3±0.8		4.0	8.0±0.3	12.4±0.3
	8.0	-	12.0±0.5		8.0	8.2±0.5	12.5±0.2
P4	0.25	-	9.8±0.1	N1	0.25	-	7.9±0.2
	0.5	-	12.0±0.3		0.5	-	8.2±0.3
	1.0	-	12.5±0.2		1.0	-	9.7±0.4
	2.0	6.7±0.1	12.2±0.1		2.0	-	10.2±0.7
	4.0	7.0±0.3	12.6±0.6		4.0	-	10.5±0.5
	8.0	7.4±0.4	12.4±0.1		8.0	-	11.6±0.8
P5	0.25	-	-	N2	0.25	-	8.2±0.5

	0.5	-	-		0.5	-	8.4±0.3
	1.0	-	10.0±0.3		1.0	-	8.9±0.4
	2.0	-	13.6±0.2		2.0	-	9.7±0.1
	4.0	-	14.0±0.4		4.0	-	10.0±0.7
	8.0	-	14.2±0.5		8.0	-	10.5±0.2
	0.25	-	-		0.25	-	7.8±0.1
	0.5	-	-		0.5	-	8.5±0.4
	1.0	-	-		1.0	-	9.8±0.2
P6				N4			
	2.0	-	9.0±0.5		2.0	6.7±0.4	10.3±0.4
	4.0	-	9.8±0.4		4.0	7.8±0.2	12.0±0.7
	8.0	-	12.2±0.6		8.0	8.1±0.1	12.7±0.3
	0.25	-	7.6±0.2	dmso			
					10µl	-	-
	0.5	-	8.2±0.2	control			
	1.0	-	8.5±0.4	Fluconazole	400		
						22.5±2.1	---
P7	2.0	-	8.2±0.5	control	µg/ml		
	4.0	-	8.4±0.2	penicillin			
					20		
	8.0	-	8.4±0.1	potassium		---	25.8±2.8
					mg/ml		
				salt			

Note: The sample code is as follows: P1=CM3, P2=CM4, P4=CR1, P5=CR7, P6=CR3, P7=LP, P18 is P8=CR (MFU).

Talbe4.11 Inhibitory effect on *Staphylococcus aureus*

Propolis Sample	Concen-tration (mg/ml)	Colony (cfu)	Inhibition rate （%）	Propolis sample	Concen-tration (mg/ml)	Colony (cfu)	Inhibition rate （%）
P1	0.25	20000±700	3.85±3.37	P18	0.25	18600±500	10.58±1.44
	0.5	19800±300	4.81±1.44		0.5	14200±300	31.73±2.40
	1.0	18400±200	11.54±0.96		1.0	9600±620	53.85±2.98
	2.0	15600±400	25.00±1.92		2.0	7200±480	65.38±2.31
	4.0	12400±600	40.38±2.88		4.0	5040±190	75.77±0.91
	8.0	6240±240	69.23±1.15		8.0	3520±200	83.08±0.96
P2	0.25	19200±400	7.69±1.92	Brazil	0.0078	19500±500	6.25±1.44
	0.5	18800±300	9.62±1.44		0.0156	18900±800	9.13±3.84
	1.0	17600±500	15.38±2.40		0.0313	15800±400	24.04±1.92
	2.0	16000±400	23.07±1.92		0.0625	12700±700	38.94±3.37
	4.0	8640±120	58.46±0.58		0.125	8040±470	61.35±2.26
	8.0	6240±360	70.1500±1.73		0.25	1600±60	92.31±0.28
P4	0.25	18100±700	12.98±3.37	N1	0.25	13400±1000	35.58±4.81
	0.5	11800±800	43.27±3.84		0.5	12800±900	38.46±4.33
	1.0	9300±130	55.29±0.64		1.0	9200±840	55.77±4.04
	2.0	6400±120	69.23±0.58		2.0	7000±540	66.35±2.60
	4.0	4300±80	79.33±0.38		4.0	6700±500	67.79±2.40
	8.0	2560±90	87.69±0.43		8.0	5120±250	75.38±1.20
P5	0.25	19800±400	4.81±1.92	N2	0.25	15600±700	25.00±3.37

	0.5	13600±900	34.62±4.32		0.5	12000±600	42.31±2.88
	1.0	11200±300	46.15±1.44		1.0	8000±540	61.54±2.60
	2.0	8000±420	61.54±2.01		2.0	6200±410	70.19±1.97
	4.0	5760±460	72.31±2.21		4.0	4800±530	76.92±2.55
	8.0	4800±430	76.92±2.07		8.0	3200±180	84.62±0.87
P6	0.25	17600±600	15.38±2.88	N4	0.03125	19600±760	5.77±3.65
	0.5	15200±300	26.92±1.44		0.0625	14800±880	28.85±4.23
	1.0	14400±800	30.77±3.84		0.125	11600±580	44.23±2.79
	2.0	13400±700	35.58±3.37		0.25	6400±120	74.19±0.58
	4.0	8640±350	58.46±1.68		0.5	5500±230	77.82±1.11
	8.0	4000±410	80.77±1.97		1.0	4800±400	80.65±1.92
P7	0.25	20600±200	0.96±0.96	DMSO control	100μL	20800±900	/
	0.5	18400±600	11.54±2.88				
	1.0	15200±200	26.92±0.96				
	2.0	13200±400	36.54±1.92	Penicillin potassium salt	20	0	100
	4.0	12800±500	38.46±2.04				
	8.0	10200±800	50.96±3.84				

Note: The sample code is as follows: P1=CM3, P2=CM4, P4=CR1, P5=CR7, P6=CR3, P7=LP and P18 is P8=CR(MFU)

Table4.12The MIC50 of various propolis extracts against S. aureus.

Propolis extract	MIC_{50} （mg/mL）	Propolis extract	MIC_{50} （mg/mL）
P1	5.33	P18	0.91
P2	3.52	Brazil	0.093
P4	0.77	N1	0.83
P5	1.25	N2	0.70
P6	3.00	N4	0.15
P7	7.69		

Note: The sample code is as follows: P1=CM3, P2=CM4, P4=CR1, P5=CR7, P6=CR3, P7=LP and P18 is P8=CR (MFU)

CHAPTER V

CONCLUSIONS

In this dissertation, we mainly focus on the research of the main components, total flavonoids and main flavonoids such as rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin, including the activity of the antioxidant and antimicrobials of Thai propolis, at the same time, comparing with China, Korea,UK and Brazil.

Firstly, the EEP with the ultrasonic UE is the best method to extract the propolis than that by EEP or UE alon and AEP. And using the 95% ethanol to extract the propolis sample is better than that other consentration such as 60%, 70%, 75%, 80%, 90%.

Secondly, the total flavonoids components of propolis samples from the main beekeeping provinces of the northern of Thailand are very low contents. The 16 samples are < 1%. Only one sample from Chiang Mai has a good content of total flavonoid, 13.4%. We are open to doubt that maybe the sample contaminated due to import from China, because the Healthy Product Company is the Thai- Taiwan company. Comparing with the China, Korea , Brazil and UK, they are all higher than Thailand propolis, China propolis samples are range from 12.02% to 30.09%; Korea's are range from 14.21% to 25.14%, only two sample is 1.46% and 1.75%; Brazil's are between 13.66-15.65%; one UK sample is 14.35%.

The third, from the HPLC experiment for the 8 main flavonoids , the 8 flavonoids standards were presented with the relatively retention time, retention time of rutin is 4.536min, myricetin is5.673min, quercetin 7.860, kaempferol11.374， apigenin12.297， pinocembrin 18.090， chrysin 23.527 and galangin 26.618. The content of 8 major flavonoids in Thailand propolis are : rutin ranges (w/w) 0.01–0. 28%, myricetin 0.00–0.08% , quercetin 0.00–0.92%,

kaempferol 0.00–0.36% , apigenin 0.00–0.25% , pinocembrine 0.00–0.09%, chrysin 0.00–1.81% and galangin 0.00–1.81%, respectively (Table 2). Only one propolis sample (CM1) from the Healthy Product Co. Ltd. Of Chiang Mai of Thailand identified the all 8 main flavonoid, rutin(0.06), myricetin (0.08), quercetin (0.92), kaempferol (0.36), apigenin (0.25), pinocembrine (0.01), chrysin (1.81), galangin (1.81), and have many peaks in the HPLC figure. The total flavonoids of CM1 is also high,13.34%, closed to UK[Detected and reported in this report II,2010] . The other 3 sample of CM almost were not identified any one of the 8 flavonoid, but the rutin 0.01 from CM4. The eight samples from Chiang Rai province of Thailand, CR-MFU,CR1 to CR7, were identified rutin (0.01 - 0.28); myricetin 0.01 (only CR1), quercetin 0.11 (CR6) and 0.36 (CR-MFU)； kaempferol 0.03(only CR-MFU); pinocembrine 0.09 (only CR6); galangin 0.05(only CR-MFU); apigenin and chrysin not detected. Two samples from Lumphun province of Thailand were identified rutin 0.01-0.04 and myricetin 0.01 (LP1), and the HPLC figure of LP1 have some peaks. Fore samples from Nan province were not identified the 8 flavonoids but the kaempferol 0.26(only Nan 2). There are some peaks in the HPLC Figure of LP1 and CR propolis, sample1, 2 and 16, but unknowns the name. Most HPLC figure of the Thai samples show a common characteristic of the peak at the retention time3.655min， but unknown the name. The results showed that the mean contents of eight major flavonoids of the studied samples among different region of Thailand are lower than UK , Korea and China (the data see chapter).Maybe they will be rich on other phenolic constituents. That should be recomented to continue to study in the future.

And then, the antioxidant activity test showed that propolis has a strong reducing power and strong DPPH radical scavenging activity, indicating that the antioxidant activity of the propolis samples is near to the international standard. During the 11 kinds of propolis samples for

determination to the antioxidant activity, Brazilian propolis is the best, followed by the propolis samples from Chiang Mai and MFU of Chinag Rai, then Nan, HK Bee Farm, Chiang Sean of Chiang Rai, and Lumphun antioxidant activity has weakest result. The results showed that the everage of the content of flavonoids in Thai propolis are lower than the international standard,but still have some antioxidant properties. The antioxidant activity is different with different origin and different kind of plants. Probably Thai propolis belongs to new tropical propolis zone that is different from the other countries; imply the composition outside of the flavonoid at antioxidant effects. This result will need to be further more study and research in the future.

Finally, the result also demonstrates that all the propolis extracts shows anti-bacteria effect to*Staphyllococcusaureus*, the MIC50 of Brazil Propolis is the lowest (0.093mg/ml) , followed by the propolis from Nan and Chiang Mei, Chiang Rai and Lumpoon of Thailand . They are 0.15mg/ml, 0.70mg/ml, 0.77mg/ml respectively. However, the MIC50 of Lumpoonis the highest (7.69mg/mL).

And during the 11 kinds of propolis samples, there are eight kinds of propolis extract was not found in the 0.25 to 8 mg / ml concentration range of its antibacterial activity against *Candida albicans*, only Brazil,Chiang Rai, and Nan, three kinds of propolis on *Candida albicans* show a weak antibacterial activity.

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APPENDIX

APPENDIX A

METHODS

A.1 Standard curve of rutinfor determination at 500nm

Rutin control solution 0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml was piped into 25ml volumetric flask respectively, 6ml de-ionized water and 1ml 5% Sodium nitrite solutionwere added and shook up. After 6min standing, 1ml 10% aluminum nitrate solutionwasadded and shook well too. 6min later, added 10ml 1 mol/L sodium hydroxide solution and de-ionized water to 25ml, shook up well and left for 15min for determination of the absorption by spectrophotometric method at 500 nm. Standard curve was drawn and regression equation was calculated.

A.2 Calibration curves

This research linearity was determined by analysis of six different concentrations of the rutin standard solutions. The standard curves were obtained by absorbancy (y) vs. different concentration (x) that was fitted to the linear regression $y = ax + b$. Concentrations of the flavonoid in samples were calculated from this regression analysis.

A.3 Standard curve of rutin for total flavonoid analysis

The standard curve of rutin was prepared as shown in 3.2.2. The standards curves of rutin were showed in Fig A.1 and Fig A.2.

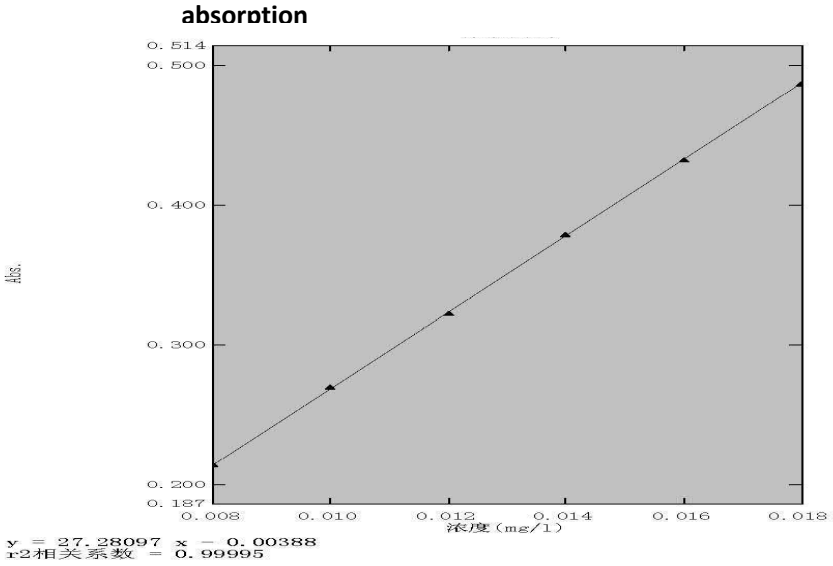

Fig A.1

Standard curve of rutin (360nm)

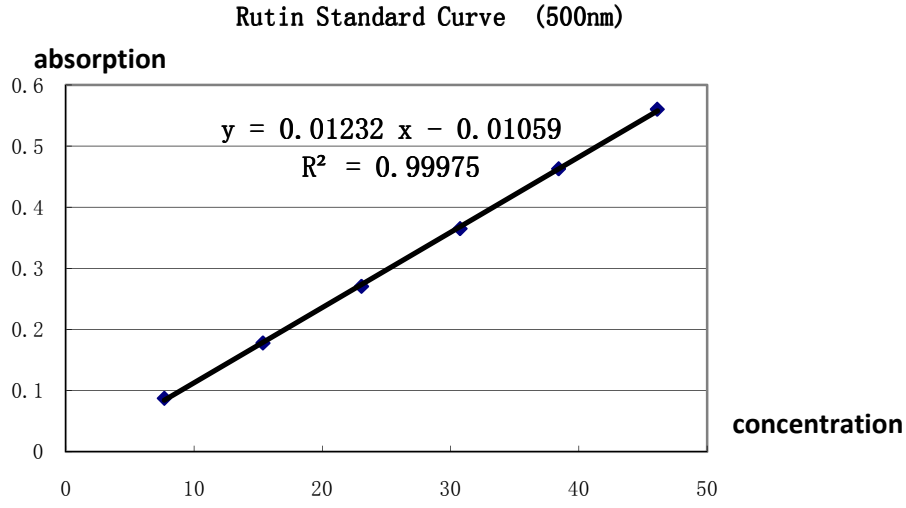

Fig A.2 Standard curve of rutin (500nm)

APPENDIX B

SeriesHPLC Figure of the flavonoids in 39 Chinese propolis

Fig B.1HPLC chromatogram of 8 main flavonoids Standard

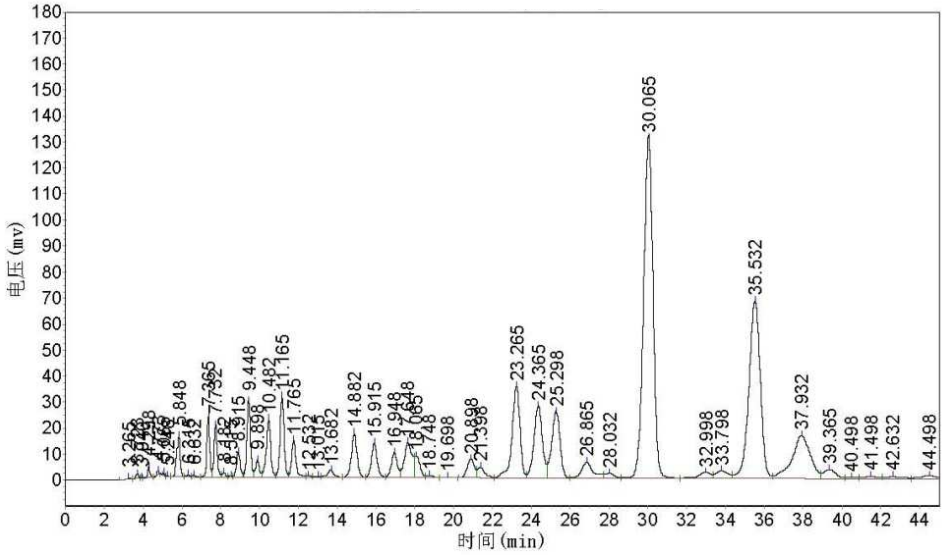

Fig B.2HPLC chromatogram of 8 flavonoids in Chinese sample (AH4)

Fig B.3HPLC chromatogram of 8 flavonoids in Chinese sample (AH5)

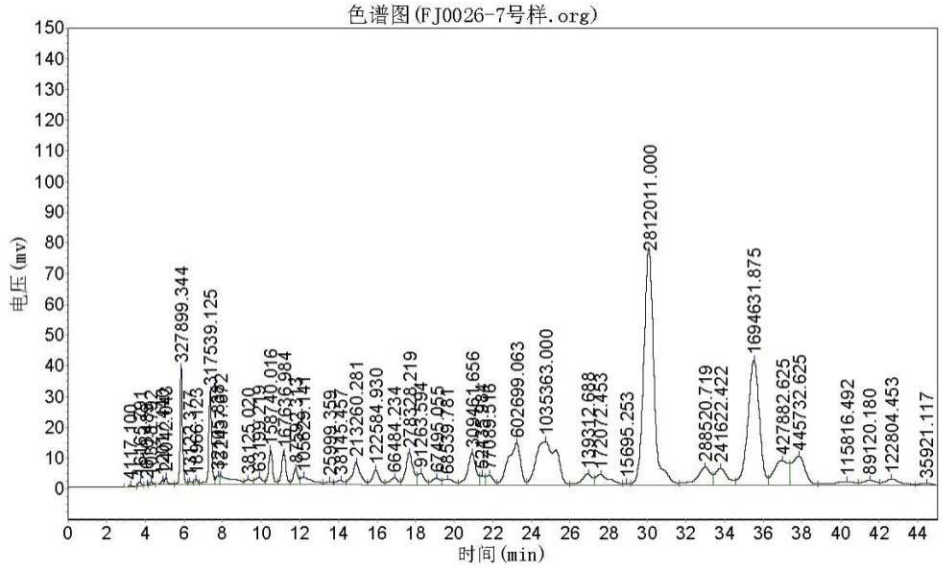

Fig B.4 HPLC chromatogram of 8 flavonoids in Chinese sample(HB5)

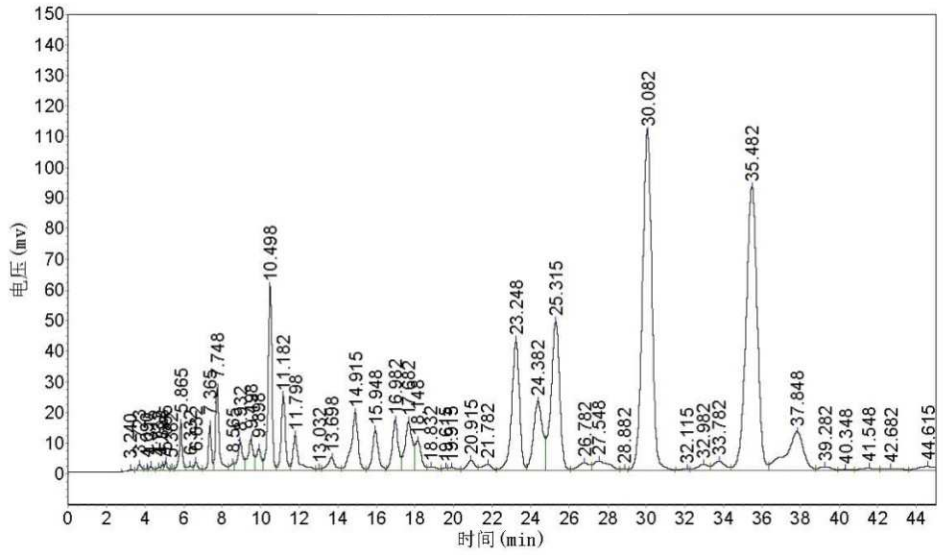

Fig B.5 HPLC chromatogram of 8 flavonoids in Chinese sample (AH3)

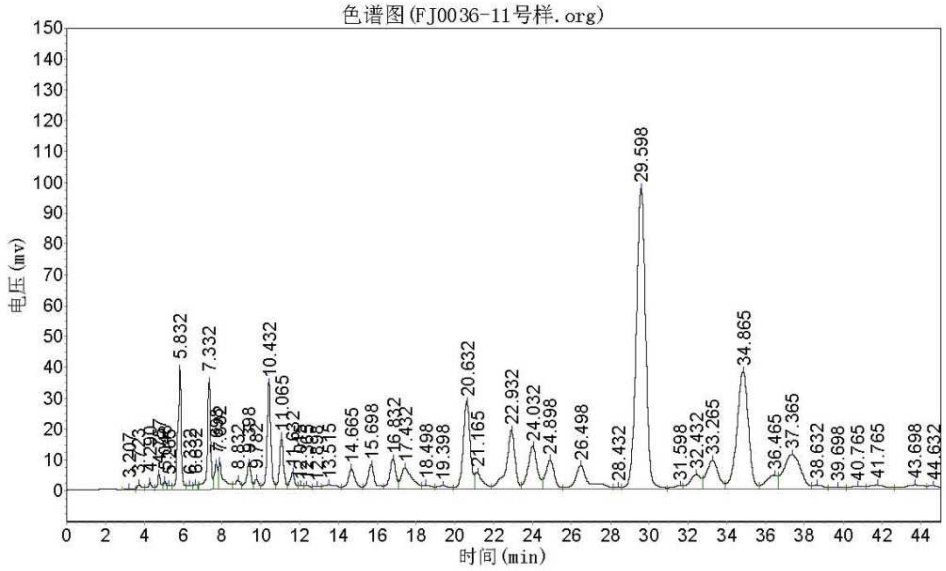

Fig B.6HPLC chromatogram of 8 flavonoids in Chinese sample(SD6)

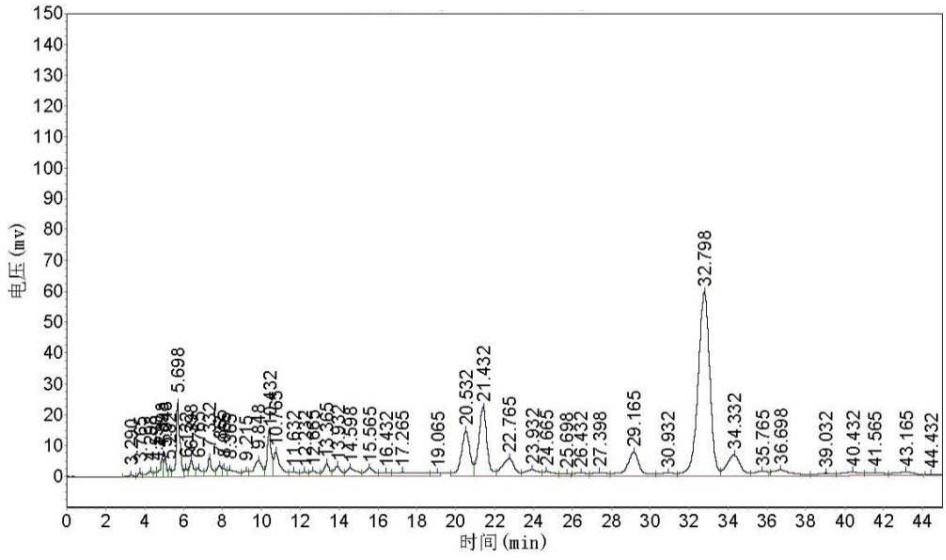

Fig B.7 HPLC chromatogram of 8 flavonoids in Chinese sample (HLJ1)

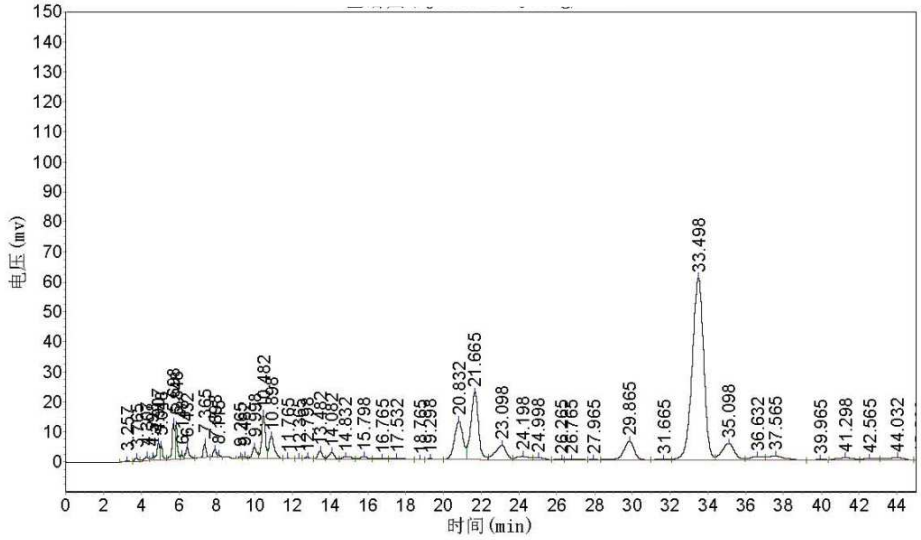

Fig B.8 HPLC chromatogram of 8 flavonoids in Chinese sample(HLJ5)

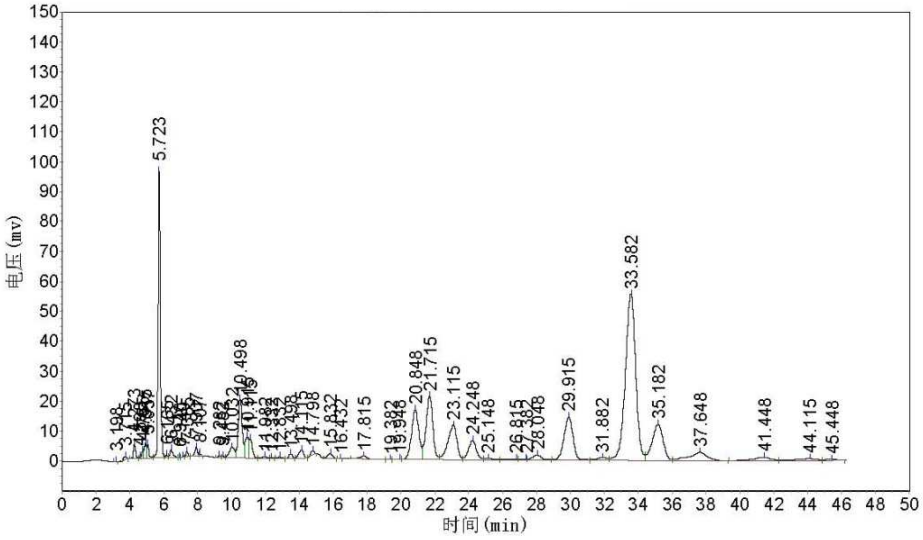

Fig B.9 HPLC chromatogram of 8 flavonoids in Chinese sample(HB4)

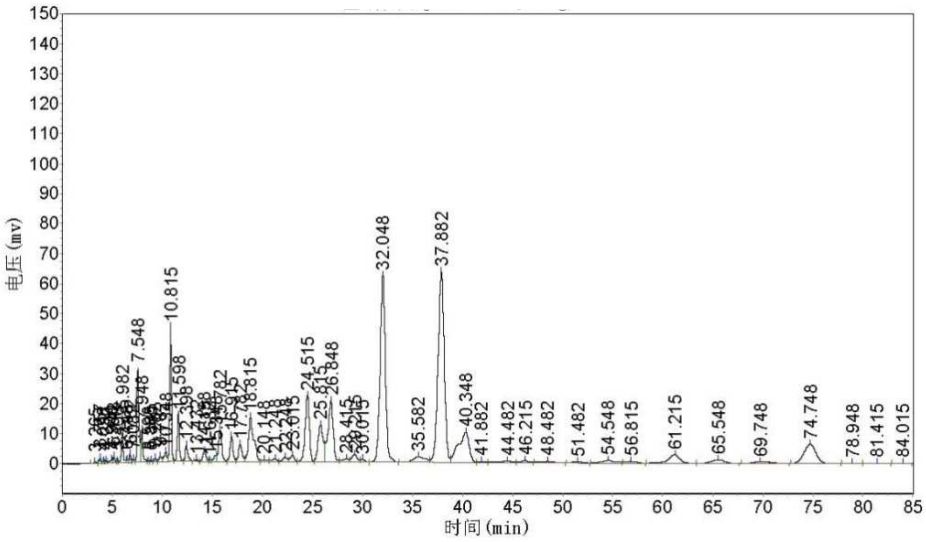

Fig B.10 HPLC chromatogram of 8 flavonoids in Chinese sample(SC3)

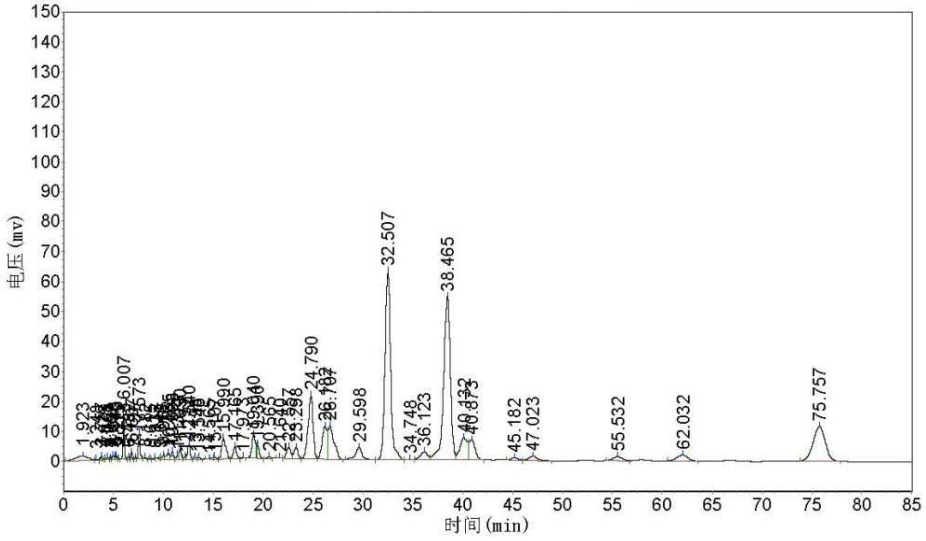

Fig B.11 HPLC chromatogram of 8 flavonoids in Chinese sample(HLJ6)

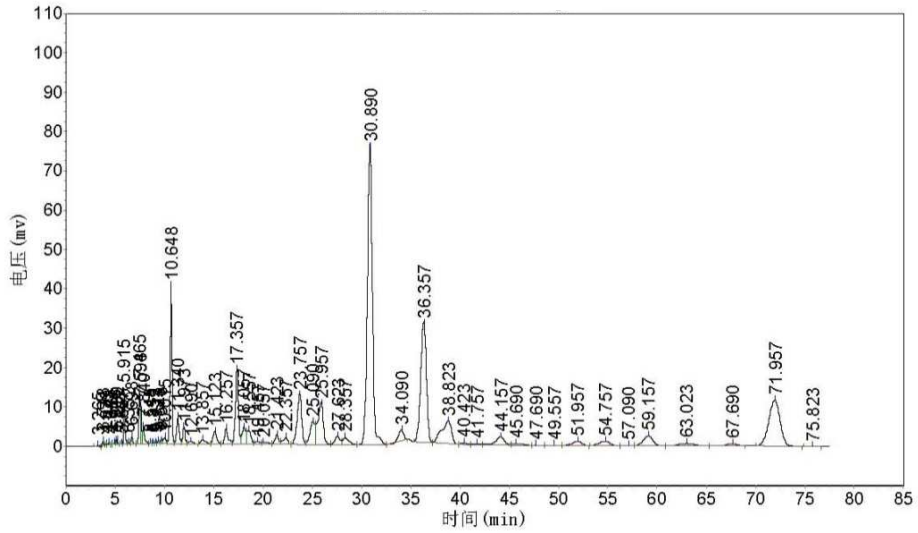

Fig B.12 HPLC chromatogram of 8 flavonoids in Chinese sample(SD7)

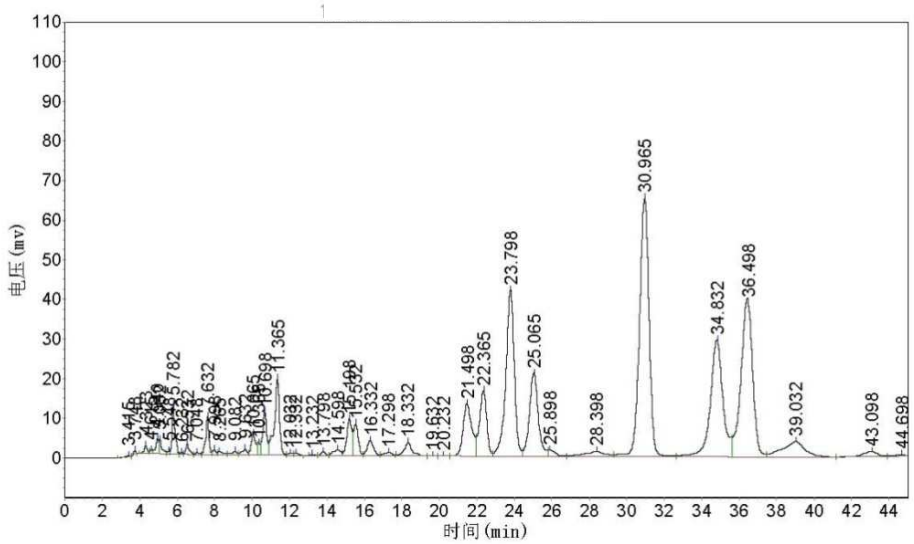

Fig B.13 HPLC chromatogram of 8 flavonoids in Chinese sample(JL4)

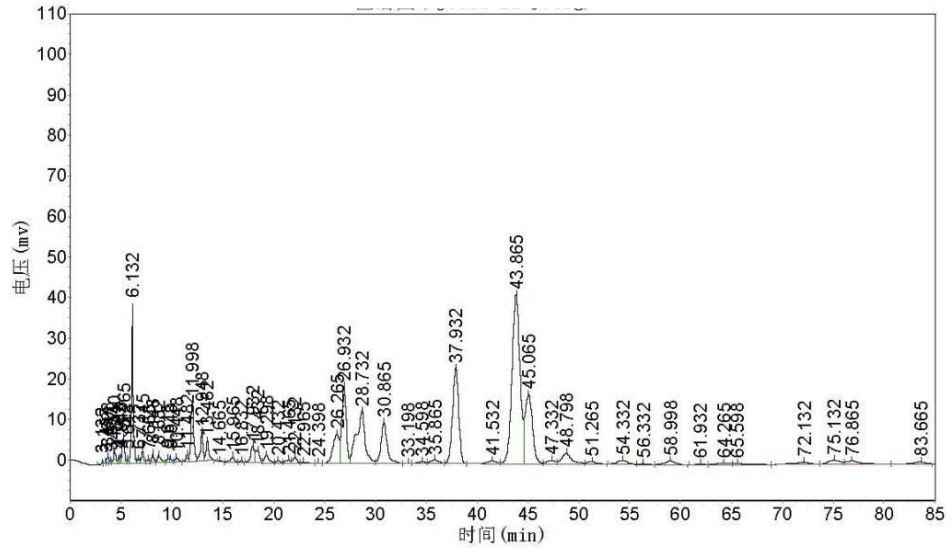

Fig B.14 HPLC chromatogram of 8 flavonoids in Chinese sample(AH11)

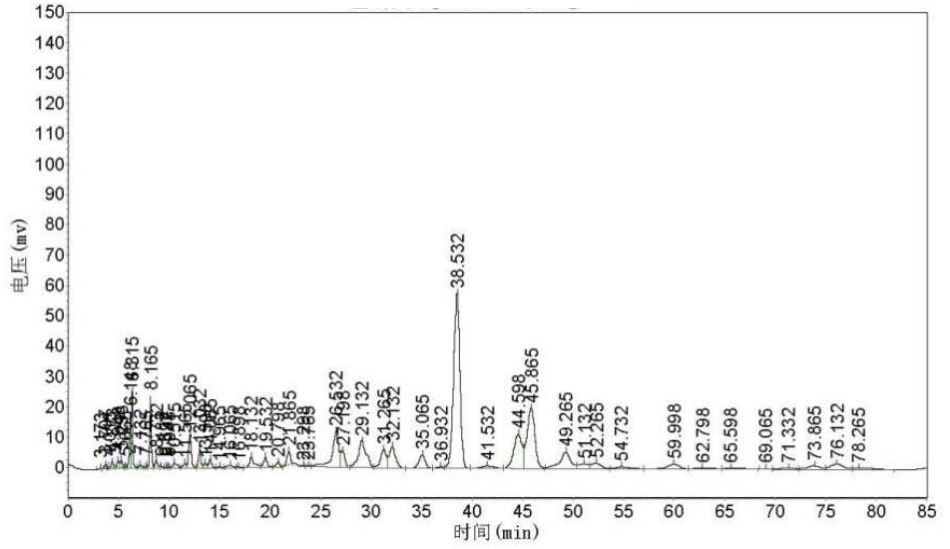

Fig B.15 HPLC chromatogram of 8 flavonoids in Chinese sample(JL3)

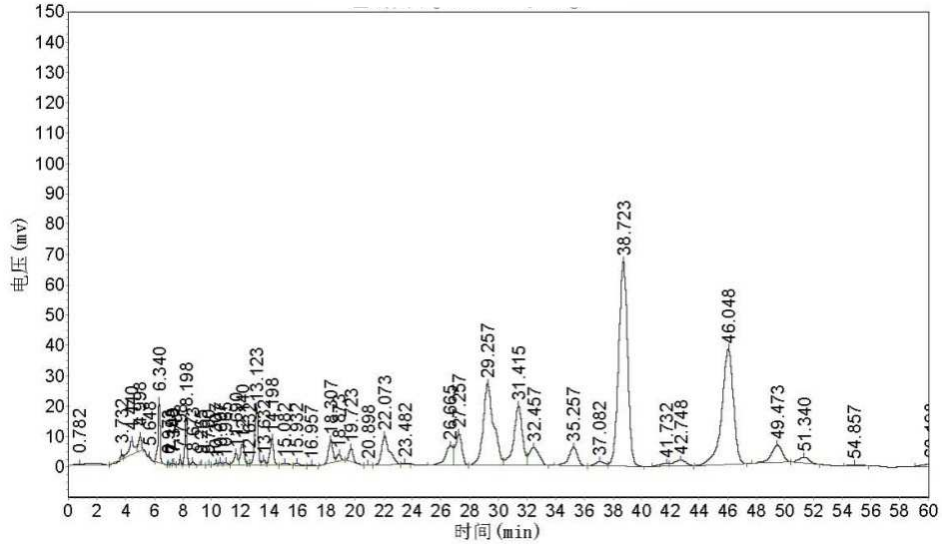

Fig B.16 HPLC chromatogram of 8 flavonoids in Chinese sample(SC1)

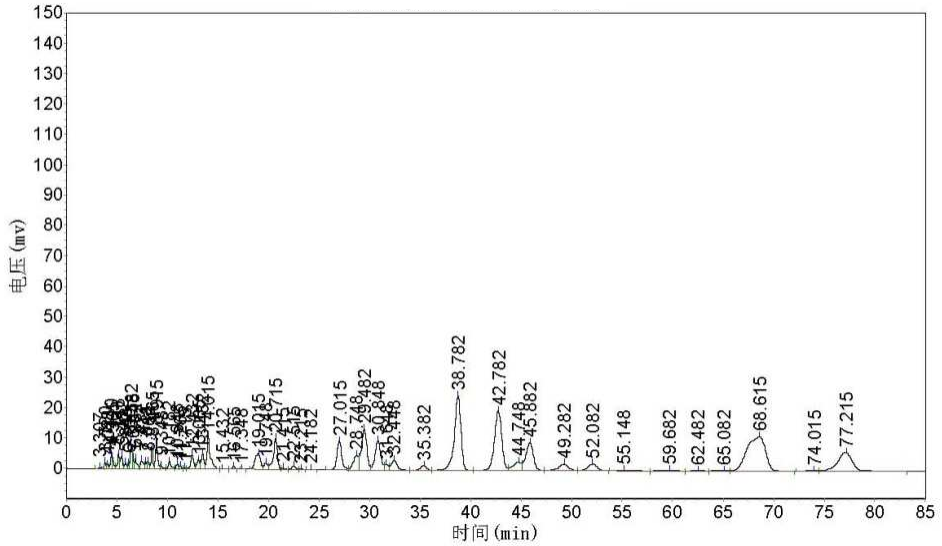

Fig B.17 HPLC chromatogram of 8 flavonoids in Chinese sample(AH5)

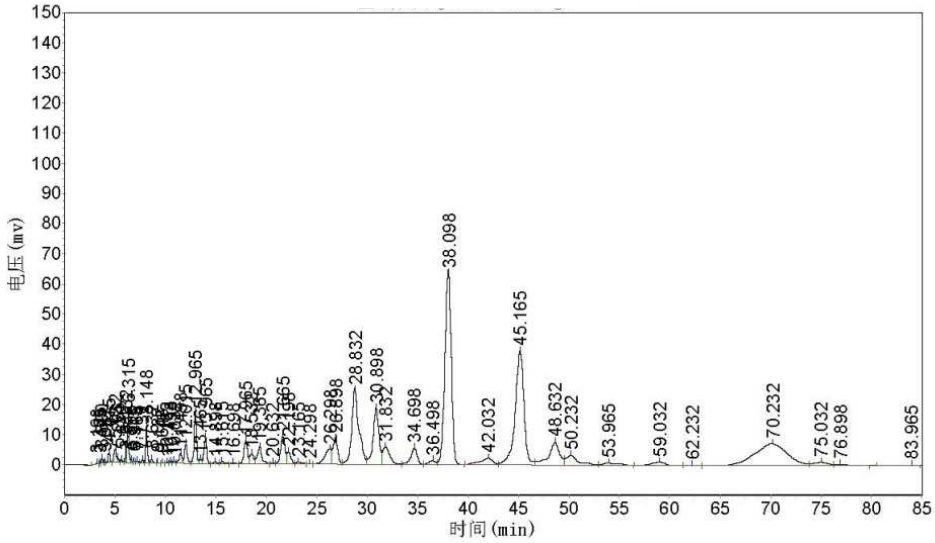

Fig B.18 HPLC chromatogram of 8 flavonoids in Chinese sample(SC2)

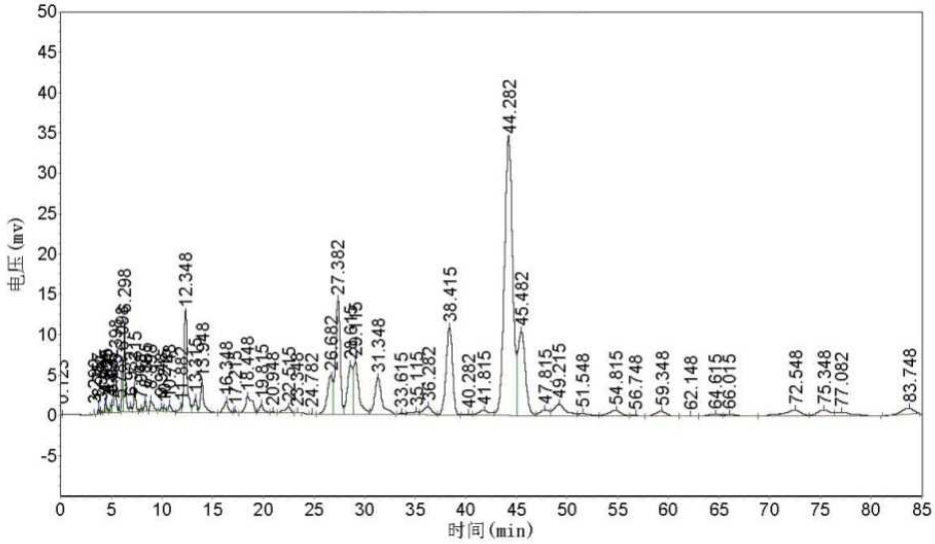

Fig B.19 HPLC chromatogram of 8 flavonoids in Chinese sample(AH7)

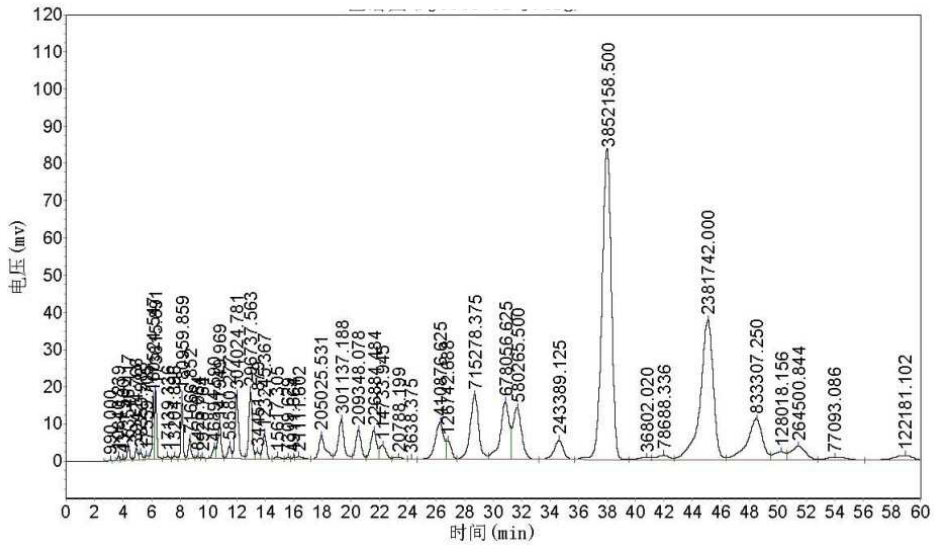

Fig B.20 HPLC chromatogram of 8 flavonoids in Chinese sample(SD4)

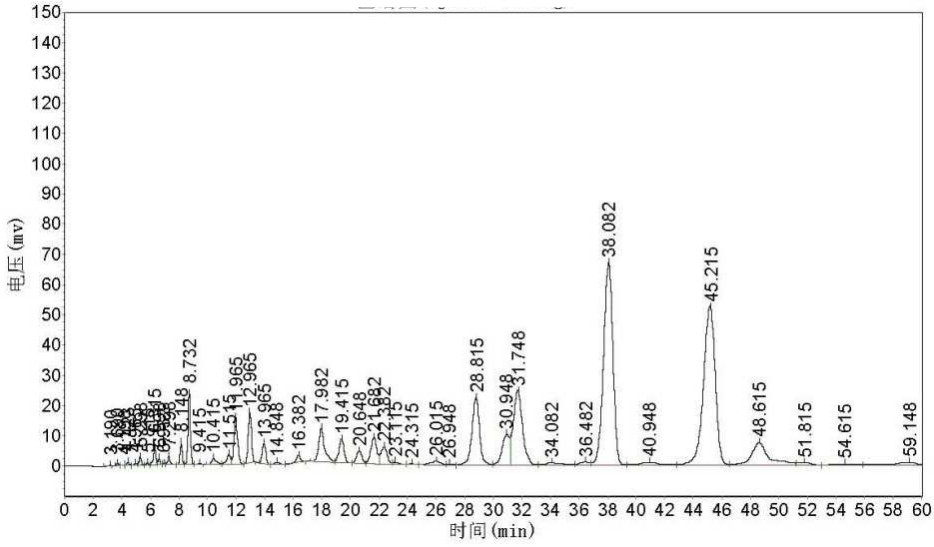

Fig B.21 HPLC chromatogram of 8 flavonoids in Chinese sample(JS2)

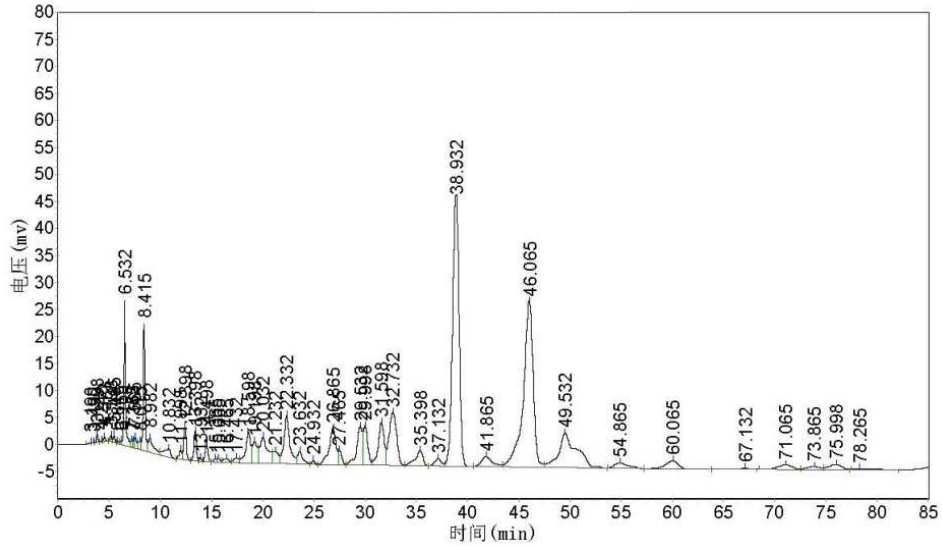

Fig B.22 HPLC chromatogram of 8 flavonoids in Chinese sample(LN3)

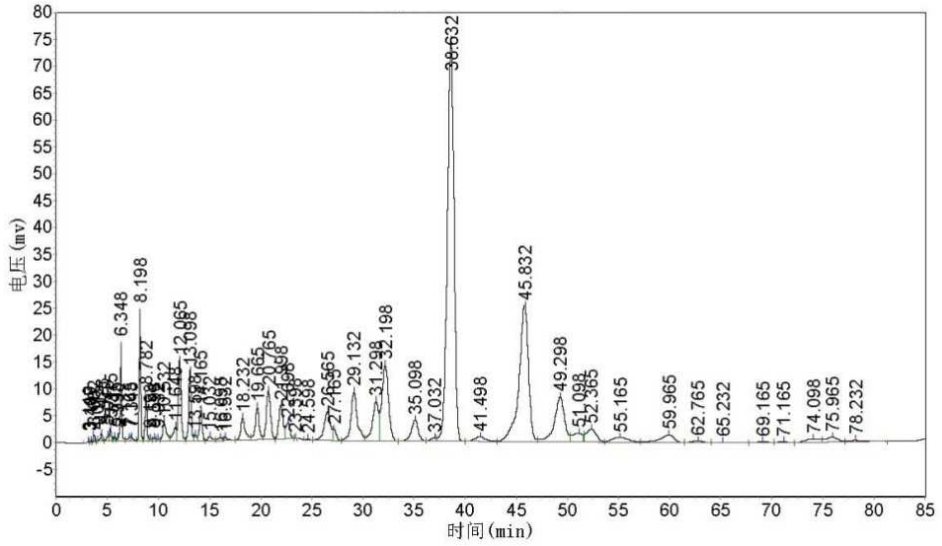

Fig B.23 HPLC chromatogram of 8 flavonoids in Chinese sample(SD2)

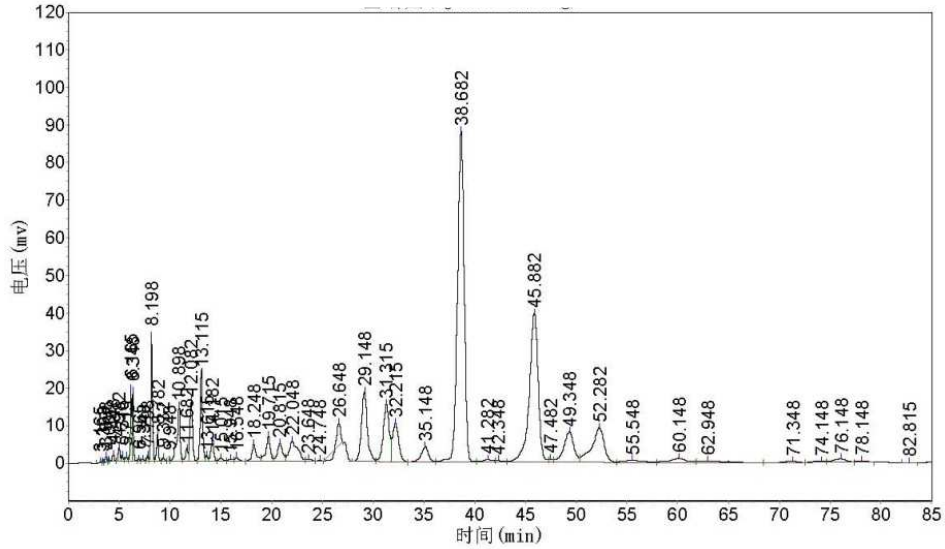

Fig B.24 HPLC chromatogram of 8 flavonoids in Chinese sample(AH2)

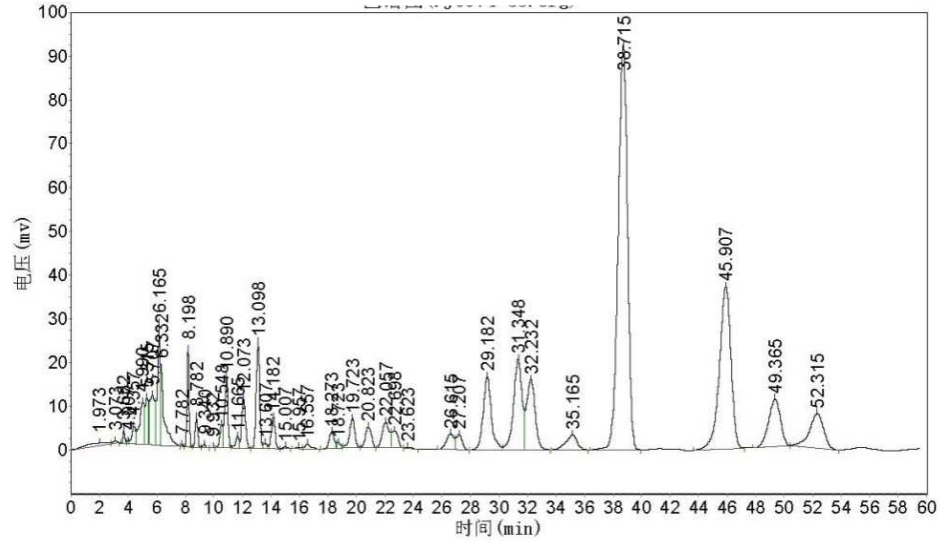

Fig B.25 HPLC chromatogram of 8 flavonoids in Chinese sample(AH1)

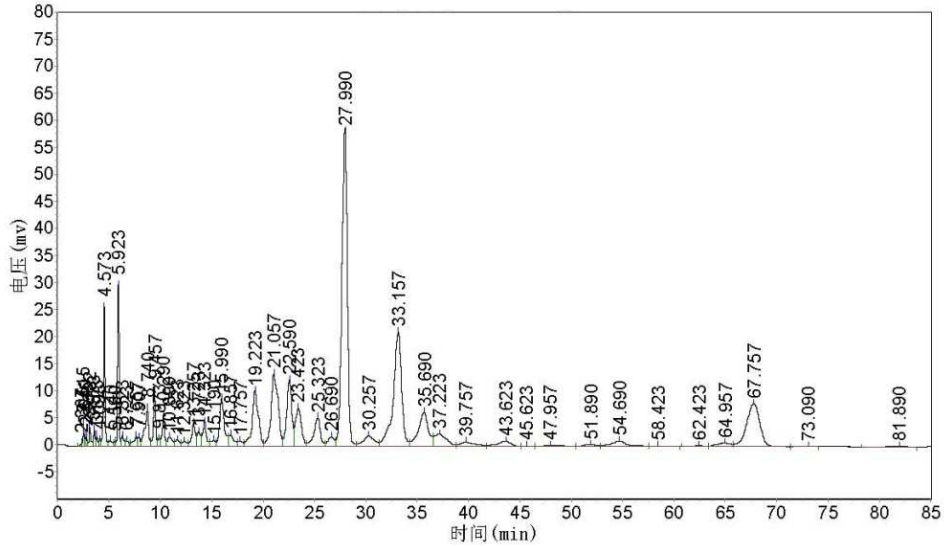

Fig B.26 HPLC chromatogram of 8 flavonoids in Chinese sample(HB6)

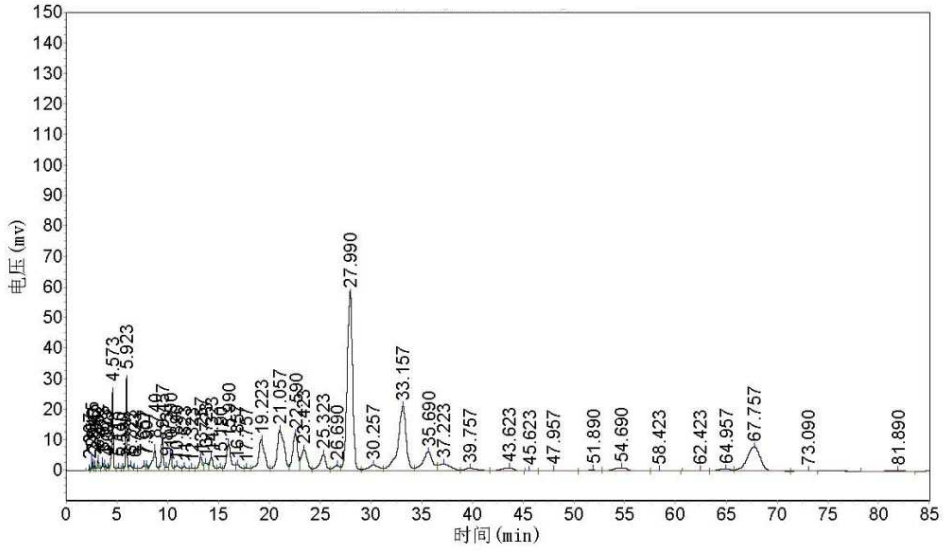

Fig B.27 HPLC chromatogram of 8 flavonoids in Chinese sample(HB6)

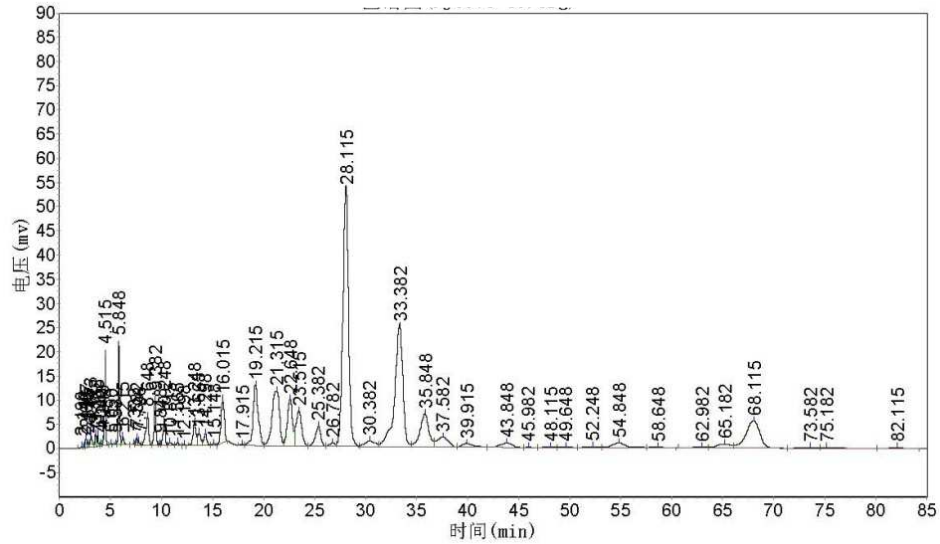

Fig B.28 HPLC chromatogram of 8 flavonoids in Chinese sample(LN3)

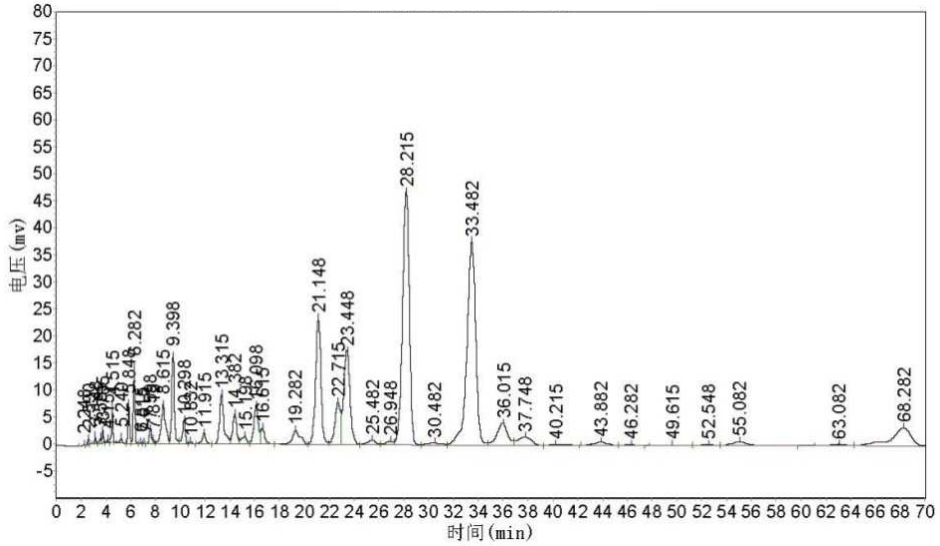

Fig B.29 HPLC chromatogram of 8 flavonoids in Chinese sample(ZJ1)

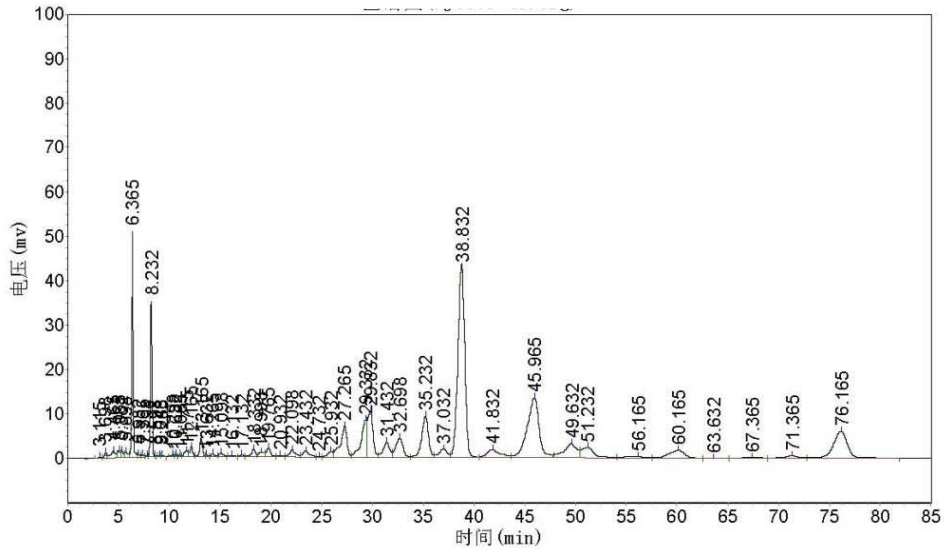

Fig B.30 HPLC chromatogram of 8 flavonoids in Chinese sample(HLJ2)

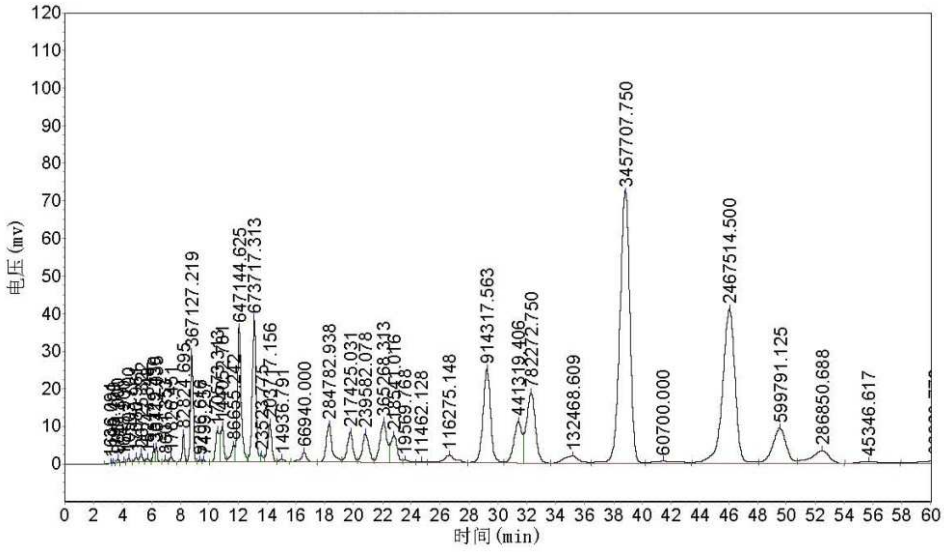

Fig B.31 HPLC chromatogram of 8 flavonoids in Chinese sample(JL1)

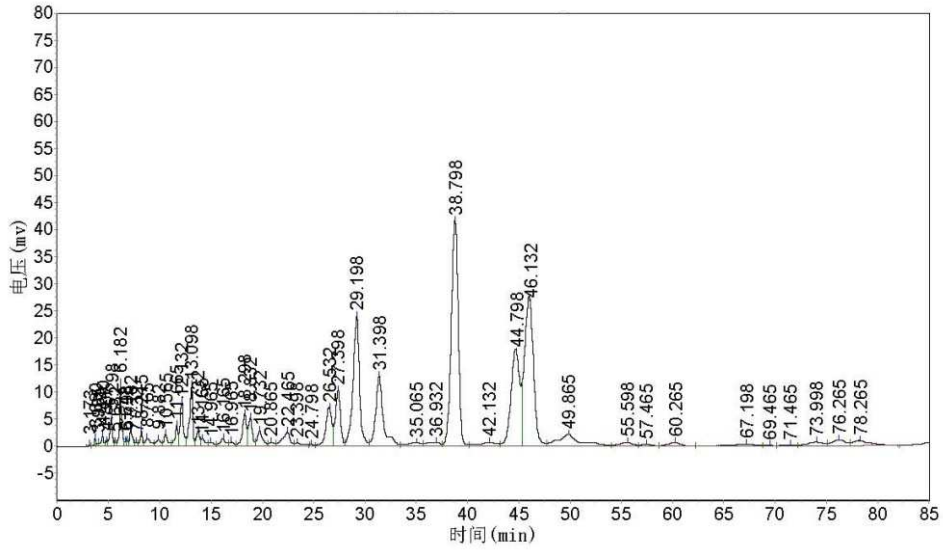

Fig B.32 HPLC chromatogram of 8 flavonoids in Chinese sample(JL2)

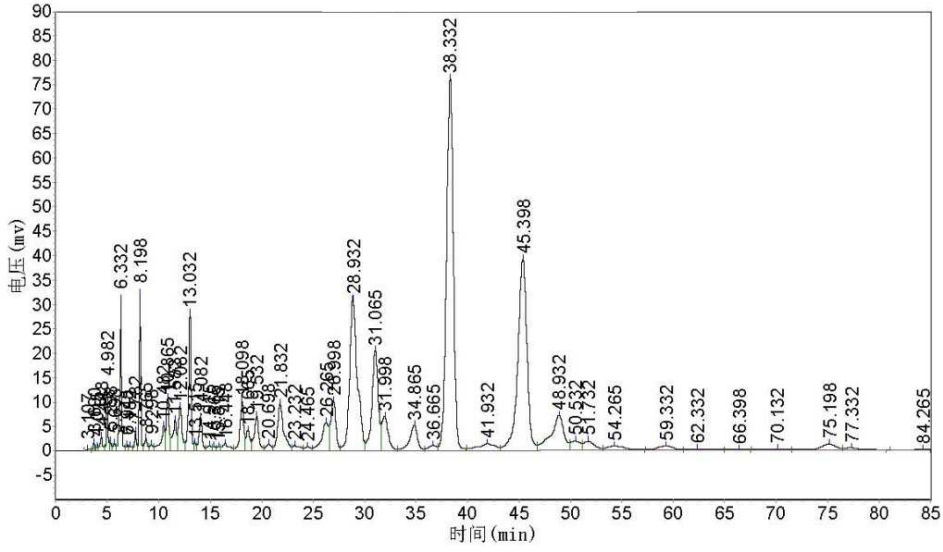

Fig B.33 HPLC chromatogram of 8 flavonoids in Chinese sample(LN4)

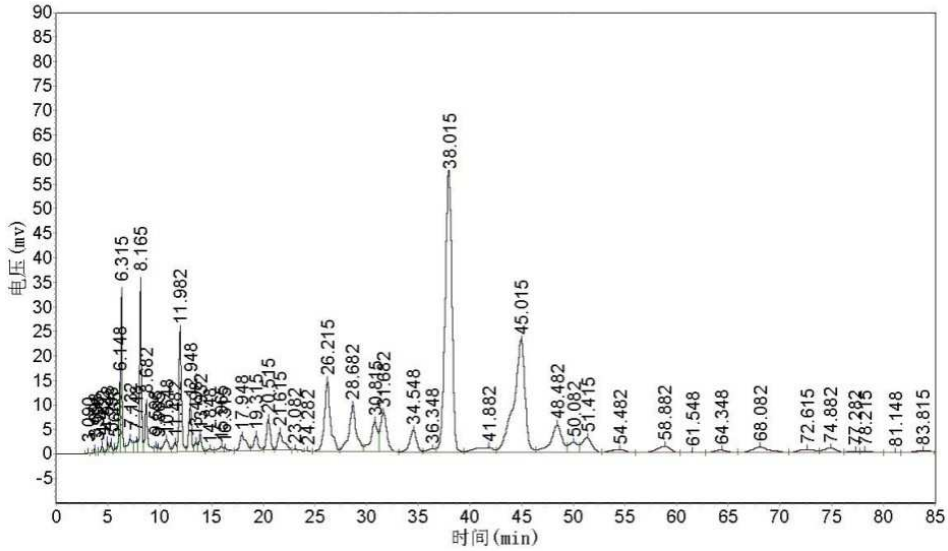

Fig B.34 HPLC chromatogram of 8 flavonoids in Chinese sample(SX3)

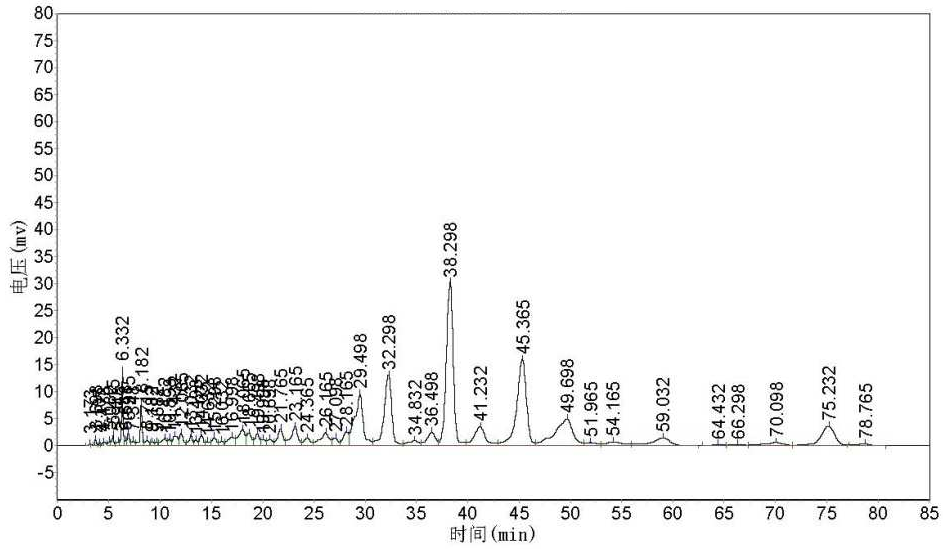

Fig B.35 HPLC chromatogram of 8 flavonoids in Chinese sample(SD1)

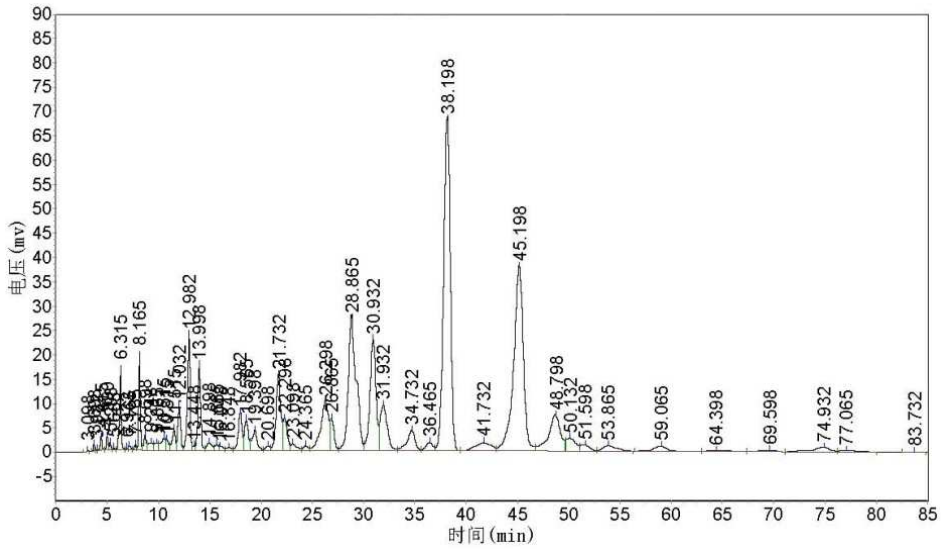

Fig B.36 HPLC chromatogram of 8 flavonoids in Chinese sample(HB1)

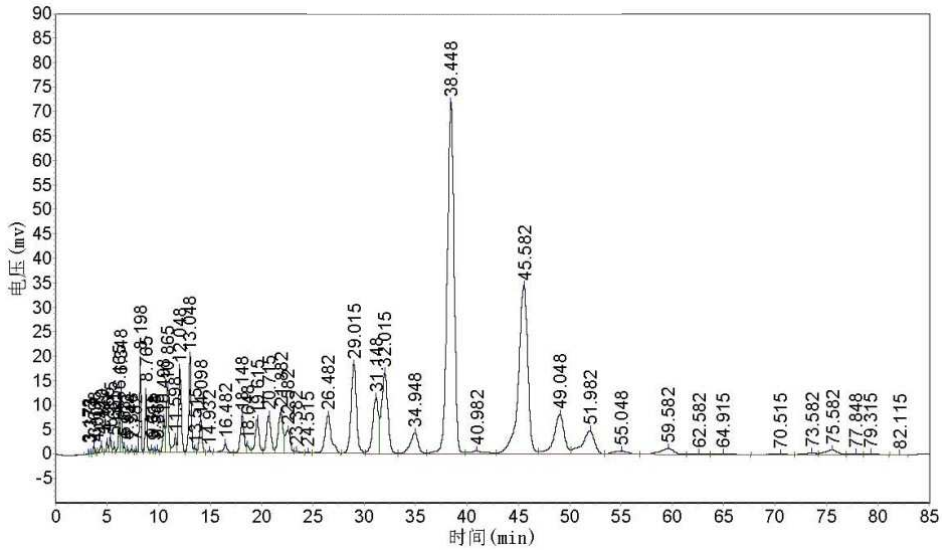

Fig B.37 HPLC chromatogram of 8 flavonoids in Chinese sample(AH10)

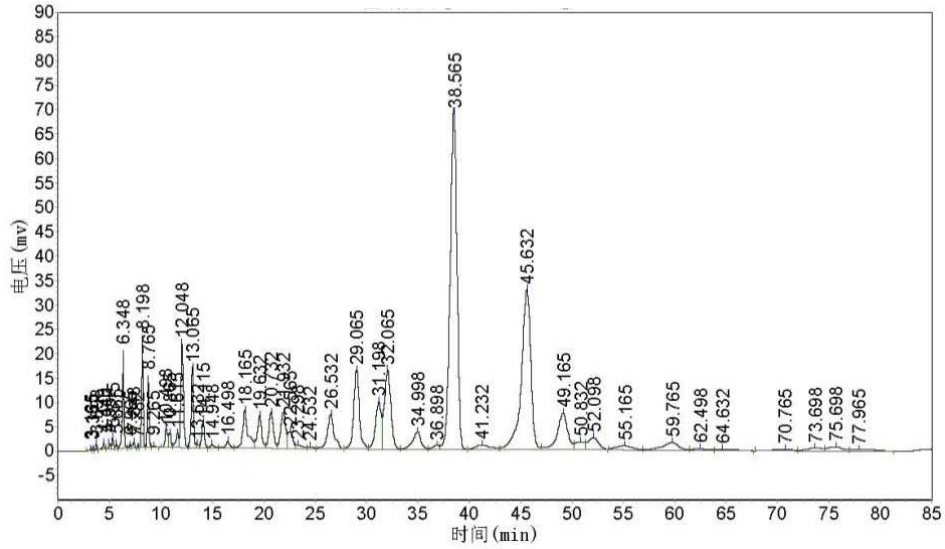

Fig B.38 HPLC chromatogram of 8 flavonoids in Chinese sample(AH10)

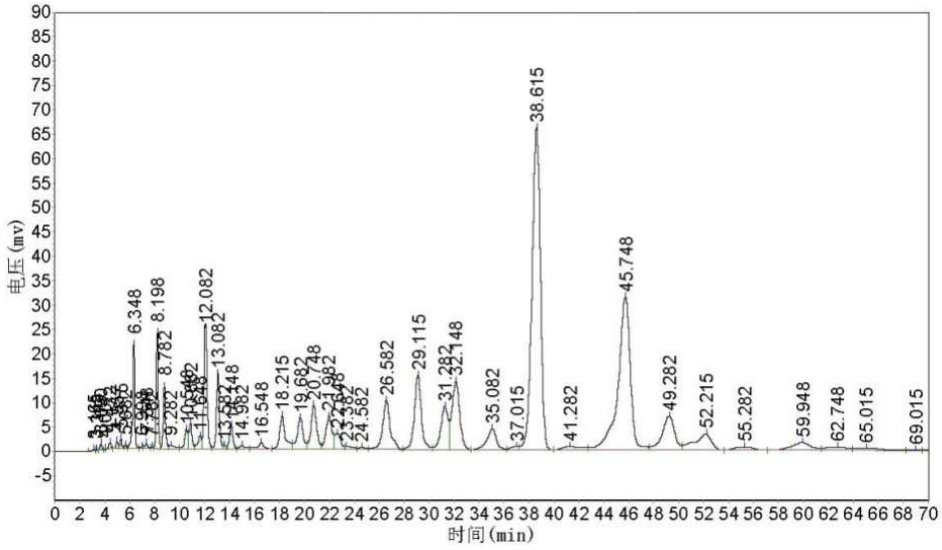

Fig B.39 HPLC chromatogram of 8 flavonoids in Chinese sample(AH10)

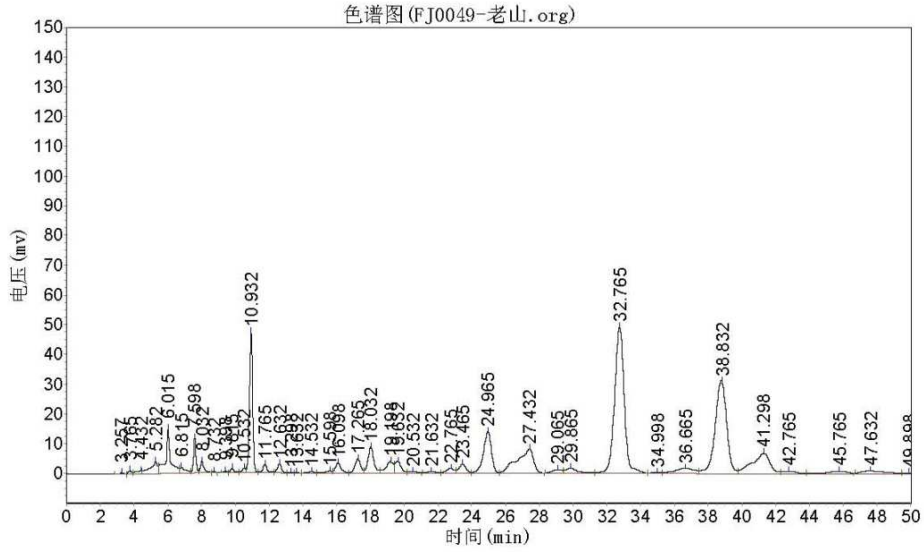

Fig B.40 HPLC chromatogram of 8 flavonoids in Chinese sample(HB4)

VITAE

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