การวิเคราะห์หาชนิดและปริมาณสารสำคัญที่มีฤทธิ์แก้ไขภาวะบกพร่องของการเรียนรู้และความจำในสารสกัดจากเมล็ดลำไย

นางสาว ชุติพันธ์ หนูสุก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ACTIVE PRINCIPLES HAVING POSITIVE EFFECT ON MEMORY DEFICIT IN LONGAN SEED EXTRACT

Miss Chutipan Nusuk

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutical Chemistry
Department of Food and Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
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Thesis Title	QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ACTIVE PRINCIPLES HAVING POSITIVE EFFECT ON MEMORY DEFICIT IN LONGAN SEED EXTRACT
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Field of Study	Pharmaceutical Chemistry
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ชุติพันธ์ หนูสุก: การวิเคราะห์หาชนิดและปริมาณสารสำคัญที่มีฤทธิ์แก้ไขภาวะบกพร่องของการเรียนรู้และ ความจำในสารสกัดจากเมล็ดลำไย (QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ACTIVE PRINCIPLES HAVING POSITIVE EFFECT ON MEMORY DEFICIT IN LONGAN SEED EXTRACT) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ภก.ดร. ชำนาญ ภัตรพานิช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ภก.ดร. บดินทร์ ติวสุวรรณ, 141หน้า.

ลำไยเป็นสมุนไพรที่มีการศึกษาพบว่ามีฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำ ในงานวิจัยนี้พบว่าสิ่งสกัดเมล็ดลำไยในชั้นเอทิลอะซิเทต มีฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำที่ถูกเหนี่ยวนำให้เกิดในหนูเมาส์ ด้วยวิธีอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้าง และเมื่อนำสิ่งสกัดเมล็ดลำไยในชั้นเอทิลอะซิเทต มาแยกสารบริสุทธิ์ด้วยวิธีคอลัมน์โครมาโตกราฟีและพิสูจน์เอกลักษณ์ของสารที่ได้ทางกายภาพและวิธีสเปกโตรสโกปีพบว่าแยกสารได้4ชนิดคือ เคอร์ซิทิน, กาลิค แอซิด, โพรพิว กาเลต และ รูทีน เมื่อนำสารทั้ง4ชนิดไปทดสอบฤทธิ์แก้ไขภาวะบกพร่องทางการเรียนรู้และความจำ พบว่ารูทีนเป็นสารที่มีฤทธิ์ดีที่สุดตัวหนึ่ง ในการศึกษานี้ได้เสนอใช้รูทีนเป็นสารบ่งชี้ (chemical marker) ในสารสกัดมาตรฐาน (standardized herbal extract) และได้พัฒนาวิธีการเตรียมสารสกัดมาตรฐาน ใช้วิธีสกัดโดยอาศัยความเป็นกรดและเบสของสารที่มีความแตกต่างกัน (acid-base extraction) เพื่อกำจัดสารที่เสียง่ายออกจากสารสกัด ทำให้ได้สารสกัดมาตรฐานของเมล็ดลำไยที่คงตัว เพื่อนำไปใช้ในการศึกษาทางเคมีและพรีคลินิกต่อไป

ในการศึกษาครั้งนี้ได้พัฒนาวิธีวิเคราะห์เพื่อวิเคราะห์รูทีนในเมล็ดลำไยโดยใช้วิธี ไฮเปอฟอแมนลิควิด โครมาโตกราฟี ใช้คอลัมน์ รีเวอร์สเฟสเป็นวัฏภาคนิ่ง ใช้ระบบไอโซเครติกเป็นวัฏภาคเคลื่อนที่ และใช้สารเฮสเปอริดินเป็นสารมาตรฐานอินเทอร์นอล วิธีวิเคราะห์ดังกล่าวได้ผ่านการตรวจสอบความใช้ได้ตามข้อกำหนดไอซีเอช ซึ่งพบว่าความเป็นเส้นตรงมีค่าสัมประสิทธ์สหสัมพันธ์เท่ากับ0.9940 ช่วงความเป็นเส้นตรงในช่วง0.31 ถึง1.54ไมโครกรัมต่อมิลลิลิตร ร้อยละของการกลับคืนอยู่ในช่วง 99.30-101.98 และร้อยละของความเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 2 ลิมิตการตรวจวัดและลิมิตการวิเคราะห์เชิงปริมาณ มีค่าเท่ากับ0.13และ 0.38 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ

ในการศึกษายังได้พัฒนาวิธีทินแลโครมาโทกราฟี เพื่อวิเคราะห์รูทีนในเมล็ดลำไย โดยใช้แผ่นซิลิกาเจลเป็นวัฏภาคคงที่ และ บิวทานอลต่อเกลเซียลอะซิติกแอซิด (9:3) เป็นวัฏภาคเคลื่อนที่ โดยมีสารเฮสเปอริดิน เป็นสารมาตรฐานอินเทอร์นอล การตรวจวัดจุดของสารโดยฉีดพ่นด้วยสารละลายแอนโทรนในเอทานอล วิธีวิเคราะห์ดังกล่าวได้ผ่านการตรวจสอบความใช้ได้ตามข้อกำหนดของไอซีเอช เป็นเส้นตรงมีค่าสัมประสิทธ์สหสัมพันธ์เท่ากับ0.9950 ช่วงความเป็นเส้นตรงในช่วง 0.82 ถึง2.83 มิลลิกรัมต่อมิลลิลิตรซึ่งพบว่าปริมาณร้อยละของการกลับคืนอยู่ในช่วง 98.41-100.46 และ ร้อยละของความเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 2 ลิมิตการตรวจวัดและลิมิตการวิเคราะห์เชิงปริมาณ มีค่าเท่ากับ 0.21 และ 0.62 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ จากการศึกษาพบว่าวิธีวิเคราะห์ทั้ง2วิธีดังกล่าวมีความเหมาะสมและได้ผ่านการตรวจสอบความใช้ได้ของวิธีตามข้อกำหนดไอซีเอช

ภาควิชา อาหารและเภสัชเคมี ลายมือชื่อนิสิต……..

สาขาวิชา เภสัชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……..

507 65600 33: MAJOR PHARMACEUTICAL CHEMISTRY
KEYWORDS: DIMOCARPUS LONGAN/ ISOLATION/ /LONGAN SEED/ RUTIN/HPLC /TLC DENSITROMETRIC

CHUTIPAN NUSUK: QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ACTIVE PRINCIPLES HAVING POSITIVE EFFECT ON MEMORY DEFICIT IN LONGAN SEED EXTRACT.
URBAN. ADVISOR: ASST. PROF. CHAMNAN PATARAPANICH, Ph.D.,
CO-ADVISOR: BODIN TUESUWAN, Ph.D., 141 pp.

Several studies of chemicals in longan (*Dimocarpus longan* Lour.) seed have been suggested to have positive effect on deficit in learning and memory. In this study, the extraction methods from longan seeds found that ethyl acetate extracts showed the positive effect on learning and memory impairment after bilateral carotid arteries (2-VO) occlusion in mice. Using the fractionation of the ethyl acetate part by conventional chromatographic technique together with the screening activities, we obtained four major compounds; quercetin, gallic acid, propyl gallate and rutin. Rutin showed to be a one of the most active principle compounds at dose 100mg/kg and used to be a chemical marker for the standardized herbal extract. All the compounds were identified by physical and spectroscopic properties. Furthermore, we prepared relatively stable standardized herbal extract from longan seeds by removing the problematic phenolic portion.

High performance liquid chromatography technique (HPLC technique) for determination of rutin in longan seed extract was developed and validated. This method was validated according to ICH guideline. The rutin in longan seed extract was determined using reverse phase column as stationary phase, an isocratic as mobile phase and hesperidin as internal standard. The method shows linearity range of 0.31-1.54µg/ml of rutin (r^2 = 0.9940).The percentage recovery (%R) was in the range of 99.30-101.98 and the relative standard determination (%RSD) was not more than 2. The limit of detection and limit of quantification of rutin were 0.13 and 0.38 µg/ml, respectively.

A thin-layer chromatographic (TLC) method was developed to determine rutin by using silica gel plate GF_{254} as stationary phase and butanol: glacial acetic acid (9:3) as developing solvent, hesperidin as internal standard. The detection of the TLC spot was developed by spraying with anthrone in ethanol. This method was validated according to ICH guideline. The method shows linearity range of 0.82-2.83mg/ml of rutin (r^2= 0.9950) .The percentage recovery was in the range of and percentage of 98.41-100.46 .RSD was not more than 2. The limit of detection and limit of quantification of rutin were 0.21 and 0.62mg/ml, respectively.Proper validation of two analytical methods and according to ICH guidelines.

Department: Food and Pharmaceutical Chemistry Student's Signature:……

Field of Study: Pharmaceutical chemistry Advisor's Signature:……...

Academic Year: 2010 Co-advisor's Signature:……

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AR	Analytical reagent
^{o}C	Degree Celsius
cm	Centrimeter
g	Gram
IR	Infrared
l	Liter
mg	Milligram
ml	Milliliter
mm	Millimeter
min	Minute
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
nm	Nanometer
No.	Number
Std	Standard
SD	Standard deviation
sec	second
%R	Percent recovery

rpm	sound per minute
TLC	Thin Layer Chromatography
UV	Ultraviolet
µg	Microgram
µl	Microliter
HPLC	High performance liquid chromatography

CHAPTER I

INTRODUCTION

Alzheimer's disease (AD) is a degenerative disease of the brain. The disease is the most prevalent from of dementia, and the progress of AD is slowly, inexorably and no recovery. The disease attacks nerve cell in all parts of the brain, thereby impairing a person's abilities to govern emotions, recognize errors, coordinate movement and remember at the last and affected person loses all memory and mental functioning. AD is incurable and leads to death within and arrange of 8 years after diagnosis **[1-3]**. The disease current indicated show that twenty-nine million people live with AD worldwide, a figure expected rise exponentially over the coming disease. It has been recently estimated that the worldwide costs for dementia care are $315.4 billion annually **[3-5]**. Certain major pathological changes have been identified such as (1) brain atrophy (2) neuronal chemical change (cholinergic deficiency, serotonergic deficiency, increased neurotoxic glutamate, increase monoamine oxidase type B) (3) microscopic changes (neurofibrillary tangles, neutritic plaques and synaptic loss) (4) biochemical changes (free radicals, inflammation and estrogen deficit)**[5-6]**.

Two types of drugs are currently approved by the U.S. food and drug administration (FDA) to treat cognitive symptoms of AD. The first type, cholinesterase inhibitors, are designed to prevent the breakdown of acetylcholine, a chemical messenger important for memory and learning. Three cholinesterase

inhibitors are commonly used to treat mild to moderate AD (1) donepezil (approved in 1996) (2) rivastigmine (approved in 2000) (3) galantamine (approved in 2001). The second type of drug works by regulating the activity of glutamate, a different messenger chemical involved in information processing. Mementine (approved in 2003) is the only currently available drug in this class and approved for treatment of moderate to severe AD **[4-6]**.

There are a variety of natural AD treatments that are proven to be effective in slowing the progression of the disease. While there is currently no cure of AD but can offer similar results, without the risk of side effects. Several herbs have a history of traditional use in enhancing cognitive performance. For example, Withania (*Withania somnifera* L.) root, Horse balm, Rosemary, Dandelion, Fava Beans, Fenugreek, Sage, Ginkgo, Brazil Nut, Stinging Nettle, Willow as well as Gotu Kola are all helpful and commonly used in the treatment of AD as they stimulate acetylcholine production in the brain **[7]**. Some herbs can be used daily as a dietary supplement. This practice is encouraged **[7-9]**.

Dimocarpus longan Lour.(Sapindaceae), commonly known as longan or Lamyai (Thailand) is natively grown in tropical countries such as Indonesia, China, Vietnam and Thailand. This plant continues to be used as crude medicinal herb within the framework of folk medicine as an effective remedy **[10-11]**. The dried pulp of longan has been used traditionally to treat symptoms such as deficiency of spleen and heart, palpitations. Its fruit extract is used to treat insomnia and forgetfulness in traditional Chinese medicine **[12-17]**. In addition, the flesh of fruit is administered as a stomachic, febrifuge, vermifuge, and regarded as an antidote for poison **[10-13].** The

substances found in longan were polyphenolic compounds such as tannin (corilagin, gallic acid and ellagic acid as the major component) **[12,18-19]**, flavone derivative (such as quercetin and kaempferol) **[19]**, anthocyanidins **[20]** and cathechin **[20]**. In addition, volatile compounds (such as cis-β-ocimen and trans-β-ocimene) **[21]** and fatty acid (dihydrosterculic acid) **[22]** have been found also.

In 2007, Nattanan Losuwannarak , *et al* reported the effect of ethanolic extracts of longan seed on scopolamine induced learning and memory deficit in mice with reference to saline-treated group, scopolamine-treated mice demonstrated impairment of learning and memory in morris water maze test and reduction of latency and an increased numbers of error in step-down test. Performances of mice receiving longan seed extract at the dose of 300 mg/kg/day weight once daily were not different from those of the scopolamine treated group. However, oral administrations of longan seed extract at the dose of 1000 mg/kg/day significantly reduced the latency to find the platform in morris water maze test but showed no improvement on the positive avoidance task. Additionally, injection of scopolamine depressed locomotor activity which were unaffected by the administration of longan seed extract **[23]**. This extract is matrix of natural compounds including tannin, glycoside and some other compounds.

These studies indicate that longan seed extract may be a new source of anti-amnesic. However, there is limited information the pharmacologically chemical marker compound and the processes employed to prepare the standardized herbal extracts. The objectives of the present study were to develop standardized extracts to identifying and quantifying the chemical marker compound from longan seed. To

isolate and identified the chemical marker, conventional column chromatography were used to fractionate the matrix base on the polarity basis, each fractions collected were tested for activity. The active fraction was then subjected to further purification to obtain pure substance which was then confirmed by the activities testing.

To quantify the principle active compound, an appropriate analytical method which can simultaneously, detect those analyze must be developed. A simple, rapid and accurate thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) method were developed for the quantitative and qualitative the active compound in longan seed. For this HPLC analysis based on comparison between the retention time and peak areas of the samples with authentic standard which can be measured, either manually or electronically. The analysis by TLC-densitometry due to lack of chromatographic functional of in the bioactive analyze structure, an anthrone spray reagent was used to develop for colorimetric analysis by densitometry. HPLC and TLC-densitometry were developed and validated according to ICH guideline. Finally the developed method will be applied to analyze the interesting compounds in plant samples.

CHAPTER II

LITERATURE REVIEW

Dimocarpus longan Lour.(Sapindaceae), the synonyms *Euphoria longan* Steud is known as longan, lungan, dragon eye (English), longán, longana (Spanish), leng-keng (Indonesia, Malaysia), lamyai pa (Thailand), nhan (Vietnam), etc. It is a cultivated plant in the family Sapindaceae that Native to Southeast Asia. Cultivated throughout the tropics and subtropics, but primarily in Thailand, China, Taiwan, Vietnam, Australia and in the USA, Florida and Hawaii **[10-11]**.

2.1. Botanical description

The longan tree is large, erect, to 30 or 40 ft (9-12 m) in height and to 45 ft (14 m) in width, with rough-barked trunk to 2 1/2 ft (76.2 cm) thick and long, spreading, slightly drooping, heavily foliaged branches. The evergreen, alternate, paripinnate leaves have 4 to 10 opposite leaflets, elliptic, ovate-oblong or lanceolate, blunt-tipped; 4 to 8 in (10-20 cm) long and 1 3/8 to 2 in (3.5-5 cm) wide; leathery, wavy, glossy-green on the upper surface, minutely hairy and grayish-green beneath. New growth is wine-colored and showy. The pale-yellow, 5- to 6-petalled, hairy-stalked flowers, larger than those of the lychee, are borne in upright terminal panicles, male and female mingled. The fruits, in drooping clusters, are globose, 1/2 to 1 in (1.25-2.5 cm) in diameter, with thin, brittle, yellow-brown to light reddish-brown rind, more or less rough (pebbled), the protuberances much less prominent than those of the lychee. The flesh (aril) is mucilaginous, whitish, translucent, somewhat musky,

sweet, but not as sweet as that of the lychee and with less "bouquet". The seed is round, jet-black, shining, with a circular white spot at the base, giving it the aspect of an eye **[10-11]**.

Figure 2.1 Picture of *Dimocarpus longan* Lour.

2.2. Cultivars and Varieties

Although there are numerous longan cultivars in Southeast Asia, the cultivar diversity is lower in other regions. The following cultivars are the most commonly cultivated in tropics and subtropics countries.

2.2.1 Biew Kiew: From Thailand, The fruit is large, of good quality, and with a small seed. requires cool winters to flower well

2.2.2 Diamond River (Phetsakon): A tropical longan from Thailand which has more reliable production in tropical areas. The fruit is of medium size, good quality and early.

2.2.3 Kohala: Originally from Hawaii, the most important cultivar in Florida, but has erratic production in tropical regions. The fruit is early, large, of good quality, and with a small seed.

2.2.4 Sri Chompoo: From Thailand, with more consistent fruiting in Hawaii than ‘Kohala’, but still requires cool winters to flower well. Large,good quality fruit.

Other cultivars include ‘Egami’, ‘E Wai’, ‘Edo’, ‘Edau’ and ‘Tiger Eye’. *Dimocarpus longan* var. *malesianus*, the mata kuching, is a subspecies of longan that is better adapted to tropical conditions **[11]**.

2.3. Chemical constituents

2.3.1 Polyphenolic compounds

2.3.1.1 Tannins

Polyphenolic compounds were extracted with 70% methanol or hot water from peel, pulp and seed of longan fruit and the major components were identified as gallic acid, corilagin, and ellagic acid **[12, 18-19, 26]**.

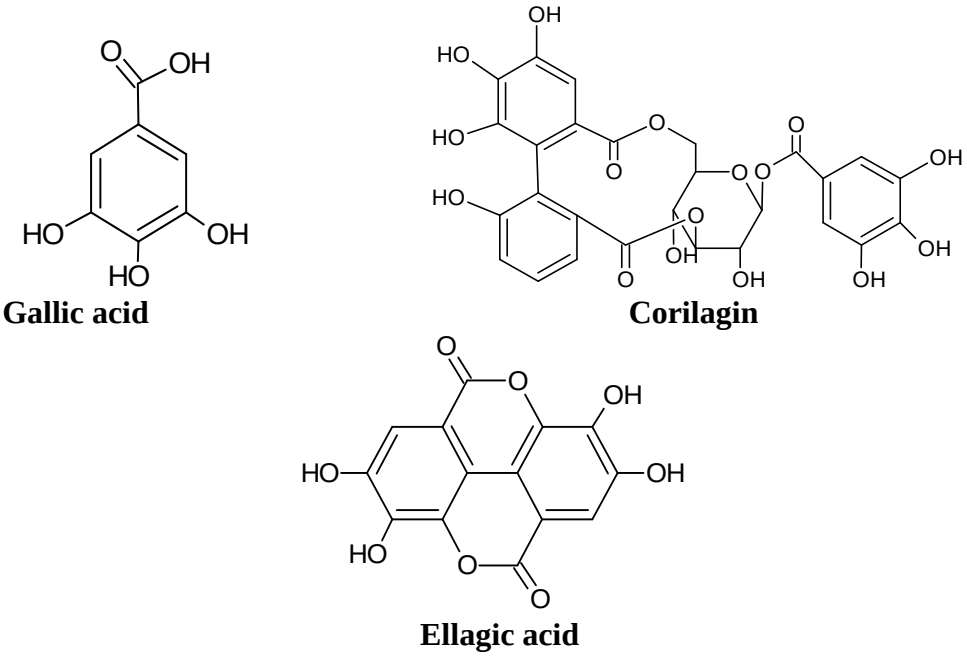
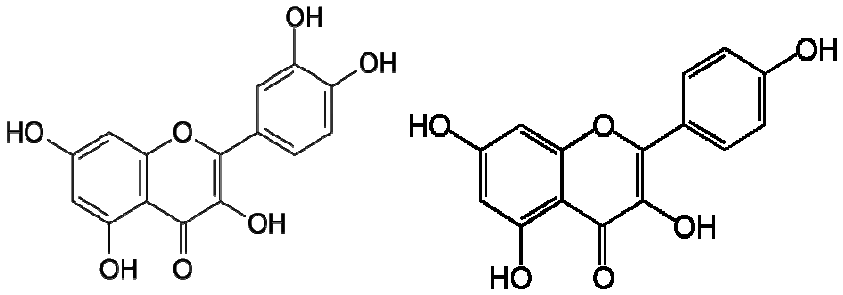


Figure 2.2 The structure of major polyphenolic compounds from *Dimocarpus longan* Lour. . Lour.

2.3.1.2 Flavonoid derivertive

2.3.1.2.1. Flavone derivatives

Acid hydrolyzed extracts of longan peel showed that the majority of the flavones were glycosides of quercetin and kaempferol **[19]**.



Quercetin Kaempferol

Figure 2.3 The structure of kaempferol and quercetin from *Dimocarpus longan* Lour.

2.3.1.2.2 Anthocyanidins

Proanthocyanidin has been found in dried longan flower extracts **[20]**.

2.3.1.2.3 Catechins

(-)-Epicatechin has been found in dried longan flower extracts **[20].**

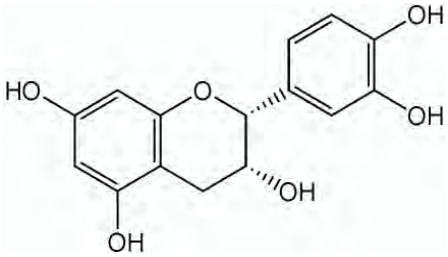


Figure 2.4 The structure of (–)-epicatechin from *Dimocarpus longan* Lour.

2.3.2 Volatile compounds

Major volatile compounds detected in fresh longan were cis-β-ocimene, trans-β-ocimene **[21]**.

2.3.3 Fatty acid

Major fatty acid compound detected in seed of *Dimocarpus longan* was dihydrosterculic acid **[22]**.

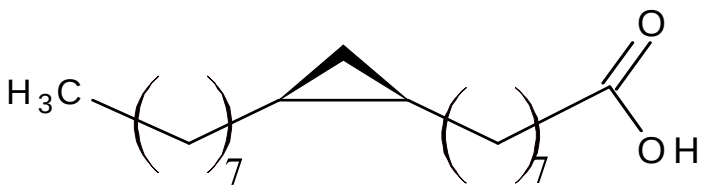


Figure 2.5 The structure of dihydrosterculic acid from longan seed.

2.4 Medicinal uses: uses described in folk medicine, not supported by clinical data or experimental.

The flesh of the fruit is administered as a stomachic, febrifuge and vermifuge **[10-17]**, and is regarded as an antidote for poison **[10].** A decoction of the dried flesh is taken as a tonic and treatment for insomnia and neurasthenic neurosis **[10-13].** In both North and South Vietnam, the "eye" of the longan seed is pressed against a snakebite (maybe it will absorb the venom) **[10-17].**

Leaves, seed and flowers are sold in Chinese herb markets but are not a part of ancient traditional medicine. The seeds are administered to counteract heavy sweating and the pulverized kernel, which contains saponin, tannin and fat **[10]**.

2.5 Experiment and clinical pharmacology

2.5.1 Effect on memory deficit

Oral administrations of longan seed extract in scopolamine-treated mice at the dose of 1000 mg/kg/day significantly reduced the latency to find the platform in morris water maze test but showed no improvement on positive avoidance task. Additionally, injection of scopolamine depressed locomotor activity which were unaffected by administration of longan seed extract **[23]**.

Additionally, Park, *et al* reported the step-through latency in the aqueous extract of longan seed was significantly increased compared with that in the vehicle-treated control ($p<0.05$) in the passive avoidance task. Immunohistochemical studies revealed that the number of cells was significantly increased in the hippocampal

dentate gyrus and CA1 regions (involved in learning and memory) after longan seed treatment for 14 days, and also enhanced immature neuronal survival **[24].**

2.5.2 Effect on cardiovascular system

The results of longan extract on heart indicated that longan pulp and seed extracts did not affect the heart rate. However, longan pulp and seed extracts significantly inhibited the effect of norepinephrine induced force of contraction in isolated descending aorta. This may be due to the active compounds such as corilagin that can act as vasodilatation agent. Previous studies reported that corilagin found in longan seed has been shown to lower blood pressure of spontaneously hypertensive rats (SHR) by blocking noradrenaline release and (or) by direct vasorelaxation **[12]**.

2.5.3 Effect on antityrosinase activities

Both fresh and dried longan seed clearly showed tyrosinase inhibitory activity in a concentration-dependent manner. The IC_{50} values for fresh and dried seed extracts were 2.9 and 3.2 mg/ml, respectively. However, the inhibitory activity of these extracts was weaker than a reference inhibitor, kojic acid (IC_{50}= 8.9µg/ml) **[18]**.

2.5.4 Effect on antimalarial activities

Both dichloromethane and water fractions of longan seed extract inhibited *P. falciparum in vitro* with IC_{50}34.06 and 36.57µg/ml, respectively. The active compound responsible for this antimalarial activity may be ellagic acid present at high levels in these two longan fractions **[12]**.

2.5.5 Effect on free radical scavenging

In 2007,Nuchanart Rangkadilok , *et al* present the studies examined the free radical scavenging activity of longan seed extract by using three different assay methods. In the scavenging activity of DPPH and superoxide radicals, longan seed extract was found to be as effective as Japanese green tea extract while dried longan pulp and mulberry green tea extracts showed the least scavenging activities. The result of ORAC assay, both fresh and dried longan seed also had higher activity than dried pulp and whole fruit. However, the results demonstrate that three polyphenolics may not be the major contributors of the high antioxidant activity of longan water extracts but this high activity may be due to other phenolic/flavonoid glycosides and ellagitannins present in longan fruit **[18, 26]**.

2.5.6 Effect on anti-glycated activity

Ultrasonic wave was used to extract the polysaccharides of longan fruit pericarp (PLFP) .The anti-glycated activity of PLFP was evaluated (Glycation has been confirmed to have a significant role in diabetic complications and normal aging). This study show the optimal ultrasonic conditions for the highest anti-glycated activity of PLFP were 276 W, 24 min and 69°C. The predicted value of the anti-glycated activity was 60.4% **[25].**

2.6 Overview of Alzheimer's disease

Neurodegenerative disorders are diseases that result in the deterioration of nervous system cells in the brain and spinal cord. They can be divided into two subclasses based on whether the loss of function is associated with movement (motor function) or memory (cognitive function).These disorders include, but are not limited to, Alzheimer's, Parkinson's, Huntington's and Lou Gehrig's diseases. As of 2008, the most common neurodegenerative disorder was Alzheimer's, which affects a total of 5.2 million Americans of which 5 million are over the age of 65. Not only is there a significant number of individuals who suffer from this disease, but a great deal of money is required to provide long term medical care. It was estimated in 2010 that $160 billion was paid by Medicare and $24 billion by state and federal Medicaid for the medical expenses of beneficiaries that suffer from AD. Although these numbers are already significant, they have the ability to increase dramatically over the next few years. The average age for the onset of AD is 65. As the size of the population over the age of 65 increases so will the occurrence of this disease. For these reasons and more importantly, for the emotional welfare of those afflicted and their families, the development of pharmacological treatments that will potentially delay the onset and progression of this disease needs to be pursued. Currently, there is no cure for Alzheimer's, but medicines that treat the symptoms associated with this disease are available **[27-31]**.

AD is characterized by the presence of protein deposits called amyloid plaques and tangle deposits of the tau protein, which are referred to as neurofibrillary tangles. These deposits in the brain cause the interruption in neuronal communication

and function that result in cognitive dysfunction. Also characteristic of AD is the reduction of acetylcholine levels in the brain, which may be linked to the death of cholinergic neurons that are responsible for producing acetylcholine.There are three different hypotheses used to describe the pathophysiology of AD. They are the amyloid, tau and cholinergic hypotheses. The various hypotheses vary on the basis of what is the primary initiating factor in the development of AD. The amyloid hypothesis suggests that the aggregation of Aβ42 to form plaques triggers the formation of neurofibrilliary tangles, cell death and reduction in acetylcholine levels, while the tau hypothesis proposes that the hyperphosphorylation of tau initiates the disease progression.The cholinergic hypothesis assumes that AD is merely the result of the deficiency in the production of the vital neurotransmitter acetylcholine. Regardless of how the sequence of events take place that leads to AD, the formation of extracellular β-amyloid plaques, neurofibrillary tangles and reduction in acetylcholine levels are factors in the disease that can be targeted for drug development **[27]**.

The amyloid plaque consists of a β-amyloid protein (Aβ) core surrounded by dystrophic neurites. Aβ42, the toxic species of Aβ that is composed of 42 amino acid residues, is the product of the proteolytic cleavage of β-amyloid precursor protein (βAPP). The sequential cleavage of βAPP is performed by β- and γ- secretase, where β-secretase is responsible for the initiation of the Aβ42 formation process **(**Figure2.6).

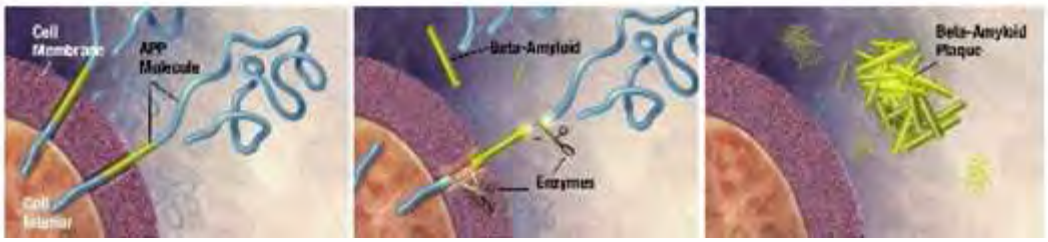


Figure2.6 The proteolytic cleavage of βAPP by β-and γ-secretase to yield Aβ42 which accumulates to form the β-amyloid plaque that it proposed to be the inhibitor in AD.

Numerous drugs have been developed for the inhibition of APP cleavage and the reduction in Aβ-aggregation to form β-amyloid plaques. Several are currently undergoing clinical trials. These drugs consist of anti-Aβ aggregation, anti-Aβ antibodies, and selective β-and γ-secretase inhibitors. Alzhemed TM (Tramiprosate) by Neurochem is an anti-Aβ aggregation treatment, currently in phase III of clinical trials, which is responsible for hindering the conversion of soluble Aβ to the insoluble fibrous plaque. The function of the anti-Aβ antibodies is to reduce the amount of β-amyloid plaques present in the brain. Drugs of this class in late stage clinical testing include Bapineuzumab from Wyeth/Elan and LY-2062430 by Eli Lilly **[27-30].**

Tramiprosate is a glycosaminoglycan (GAG) mimetic drug that interacts with the Aβ peptide to inhibit the transition from random structures to organized β-sheets. It was determined that at least four proteoglycans (PG), heparan sulfate, keratan sulfate, dermatan sulfate and chondriton sulfate, which are characterized by there GAG chains, are associated with both amyloid plaques and neurofibrillary tangles. Once bound to a given protein, PGs and GAGs allow noncovalent interactions with other PGs, proteins and GAGs, thereby improving the strength and stability of the fibrous structure formed. This is the same interaction that occurs between the GAG

and Aβ-peptide. Once the Aβ-peptide develops into a fibril its solubility decreases, which leads to the accumulation of the β-amyloid plaques. The GAGs are shown to bind to the 13-16 region (HHQK) of Aβ-peptide. A drug that is capable of binding to this same region would be capable of hindering the process by which soluble Aβ is converted to the insoluble form. This is the manner in which Tramiprosate functions. The functional groups that are present in Tramiprosate, which are similar to those found in GAGs associated with amyloid plaques and neurofibrillary tangles in AD, enable it to bind to this same region of the Aβ-peptide. This leads to a competitive interaction between GAGs and Tramiprosate for the binding region. Once bound to this region, Tramiprosate hinders the conversion of Aβ to the β-sheet conformation, due to a decrease in noncovalent interactions, and formation of the fibrous plaque. This allows the Aβ-peptide to remain in a soluble state. The Aβ-Tramiprosate complex can then be transported to the kidney or liver to be eliminated. This course of action will hopefully reduce the formation and accumulation of additional β-amyloid plaques. The structure and synthetic routes of preparation of Tramiprosate are given below in Figure2.7 **[27]**.

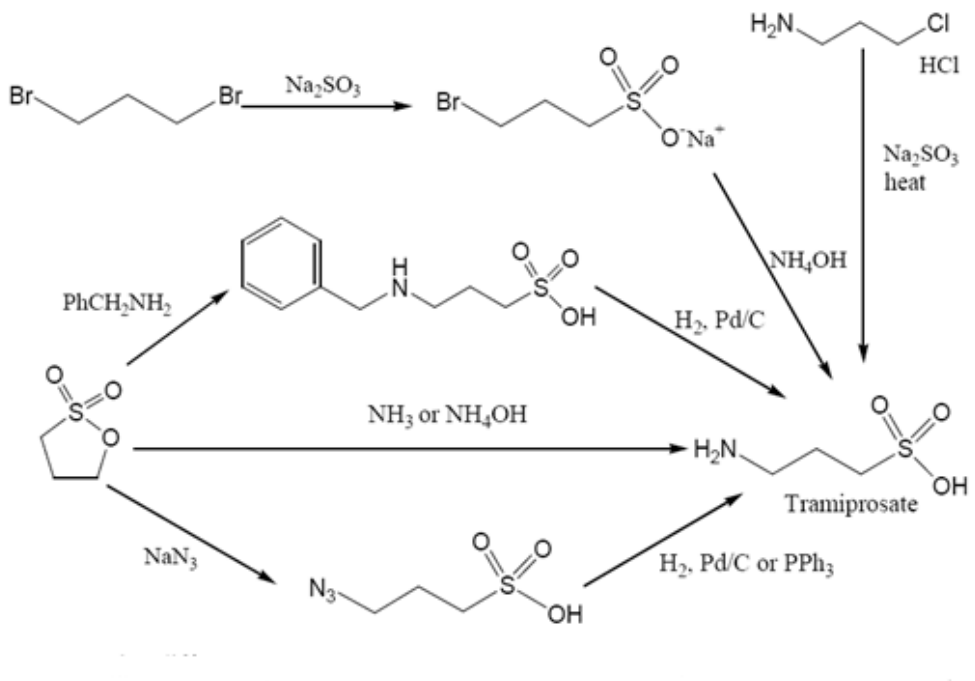


Figure 2.7 Five different synthesis routes for the preparation of Tramiprosate (Alzhemed ^{TM})

Another approach to reducing the production of Aβ peptide is to use β- or γ-secretase inhibitors. β-secretase (BACE1) is an aspartic-acid protease, which is responsible for the initial extracellular cleavage of APP to generate the Aβ40 and Aβ42 peptide. Transmembrane cleavage to complete the generation of the Aβ peptides is performed by γ-secretase. By inhibiting the β- or γ- secretase enzyme, proteolysis of βAPP will be hindered. A major drawback from this approach is that all the other functions of β- and γ-secretase will be disrupted. β-secretase is also responsible for the formation of myelin sheaths in peripheral nerve cells, while γ-secretase is critical in the processing of the Notch protein. Currently, Eli Lilly has a γ-secretase inhibitor LY-450139 that is in the late stages of clinical testing for AD **[27]**.

The availability of three-dimensional structural information for β-secretase and information regarding how inhibitors coordinate with its active binding site has allowed for the development of new potent inhibitors. The X-ray crystal structure shown in Figure 2.8 is a representation of the catalytic unit of BACE1 complexed with the BACE1 inhibitor OM-99-1, which is a peptidomimetic. The design of this class of inhibitors is based on the substrate that BACE1 is known to process, APP, and therefore the bonding interaction are similar to those of the BACE1 and protein substrate complex. Other classes of BACE1 inhibitors are 2-amino-3,4-dihydroquinazolines and substituted aminopyridines. These nonpeptidomimetic inhibitors are designed based on the 3D pharmacophore map of BACE1. The interactions between inhibitor and enzyme include both hydrophobic and hydrogen bonding interactions. Therapies associated with β-secretase inhibition are currently being pursued by GlaxoSmithKline **[27].**

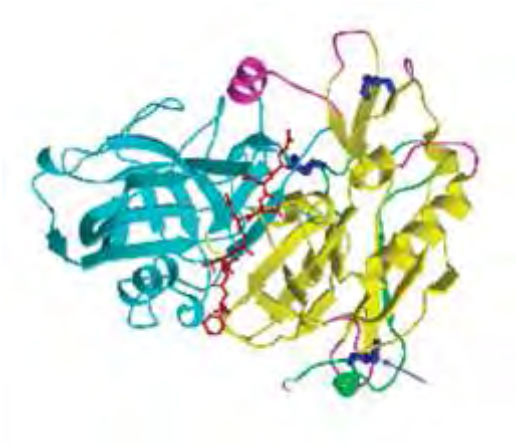


Figure2.8 X-ray crystal structure of BACE1 complexed with inhibitor OM-99-1(red). Arrows indicate the β-strands, while ribbons represent the α-helices. The blue molecules designated by the arrow are disulfide bridges.

The formation of intracellular neurofibrillary tangles is another causative agent in the development of AD. Once the tangles are formed, the neuron loses all its remaining cytoskeleton which results in cell death. After cell death, the mass that remains is termed an extracellular, or “ghost”, neurofibrillary tangle. These neurofibrillary tangles result from the hyperphosphorylation of the tau protein, which is the protein responsible for the stabilization of microtubules. Once the tau protein is phosphorylated, its binding affinity for microtubules is reduced and hyperphosphorylation leads to the formation of paired helical filaments, which aggregate to form the tangles. Cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3-β (GSK3β) are putatively responsible for the phosphorylation of protein tau.Brain permeable drugs that can selectively inhibit these enzymes by binding to their ATP pocket would potentially reduce the likelihood of tau phosphorylation and, in turn, the formation of neurofibrillary tangles. Pfizer has two

compounds, CP-668863 and CP-681301 that are currently being assessed as CDK5 inhibitors **[30].**

Having knowledge of the GAG binding pocket of the Aβ-peptide, the active pocket of β-secretase and the enzymes responsible for the hyperphosphorylation of tau provides a variety of drug targets that will allow for the development of numerous drugs that have great potential in fighting AD. However, until the research is completed so that the drugs can be prescribed to Alzheimer's patients, their value cannot be fully appreciated **[28**].

The drugs which are currently available to Alzheimer's patients only provide palliative care. They are based on the cholinergic hypothesis and treat the symptoms associated with cognitive dysfunction.These drugs include Donepezil (Aricept), Galantamine (Razadyne formerly Reminyl), Rivastigmine (Exelon), Tacrine (Cognex) and Memantine (Namenda) (Figure 2.9).The first four are acetylcholinesterase inhibitors and they treat symptoms related to memory, thinking, language, judgment and other thought processes. While Memantine is a NMDA receptor antagonist that hinders apoptosis triggered by a high influx of metal ions into the cell **[27-28]**.

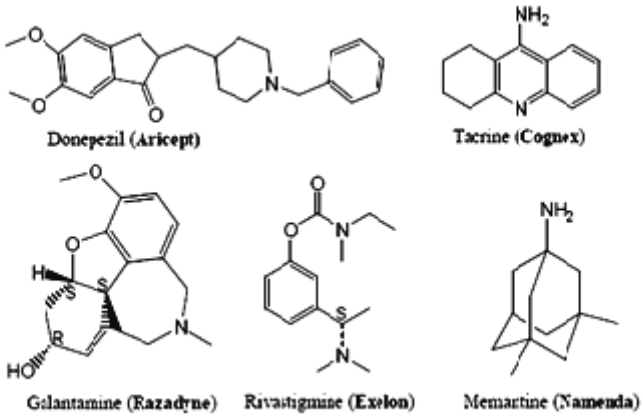


Figure2.9 FDA approved treatments for Alzheimer's disease

Acetylcholine (Ach) is synthesized from choline and acetyl coenzyme A in the nerve ending of the presynaptic neuron, a reaction catalyzed by the enzyme acetyltransferase. It is then incorporated into membrane bound vesicles and transported to the cell membrane, where it is released into the synaptic gap upon arrival of a nerve signal and opening of the calcium ion pump. Upon crossing the synaptic gap, Ach binds with the cholinergic receptor, resulting in the stimulation of a second nerve cell. Acetylcholine is released from the receptor and moves to acetylcholinesterase, which is located in proximity to the receptor in the postsynaptic nerve cell. Acetylcholine undergoes hydrolysis once it is bound in the active site of acetylcholinesterase. Binding to the active site involves both ionic bonding between the quaternary nitrogen of acetylcholine with the aspartate residue and hydrogen bonding between the ester region of acetylcholine and a tyrosine residue (Figure 2.10). The hydrolysis mechanism involves the aspartate, histidine (acid-base catalyst) and serine (the nucleophile) residues in the active site. The function of the inhibitor is

to hinder the binding and hydrolysis of acetylcholine, thereby allowing it to remain available for the reactivation of the cholinergic receptors **[27-28]**

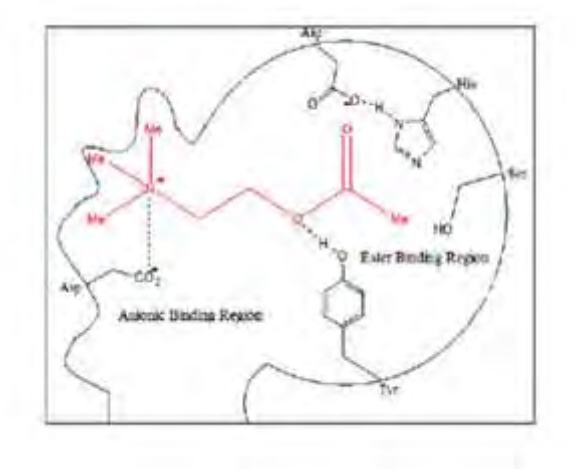


Figure2.10 Binding interactions between acetylcholine and the active site of acetylcholinesterase.

The acetylcholinesterase inhibitors hinder the hydrolysis of acetylcholine by occupying the active site of the enzyme for an extended period of time. Rivastigmine (Exelon), a physostigmine analogue, undergoes hydrolysis in the same manner as acetylcholine once in the active site. However, generation of a stable carbamoyl intermediate prolongs hydrolysis and release of serine, therefore rendering the active site temporarily unavailable for the hydrolysis of acetylcholine (Figu Rivastigmine shows high selectivity for the brain and has a short half life, reducing the toxic risk associated with accumulation or drug-drug interactions **[27].**

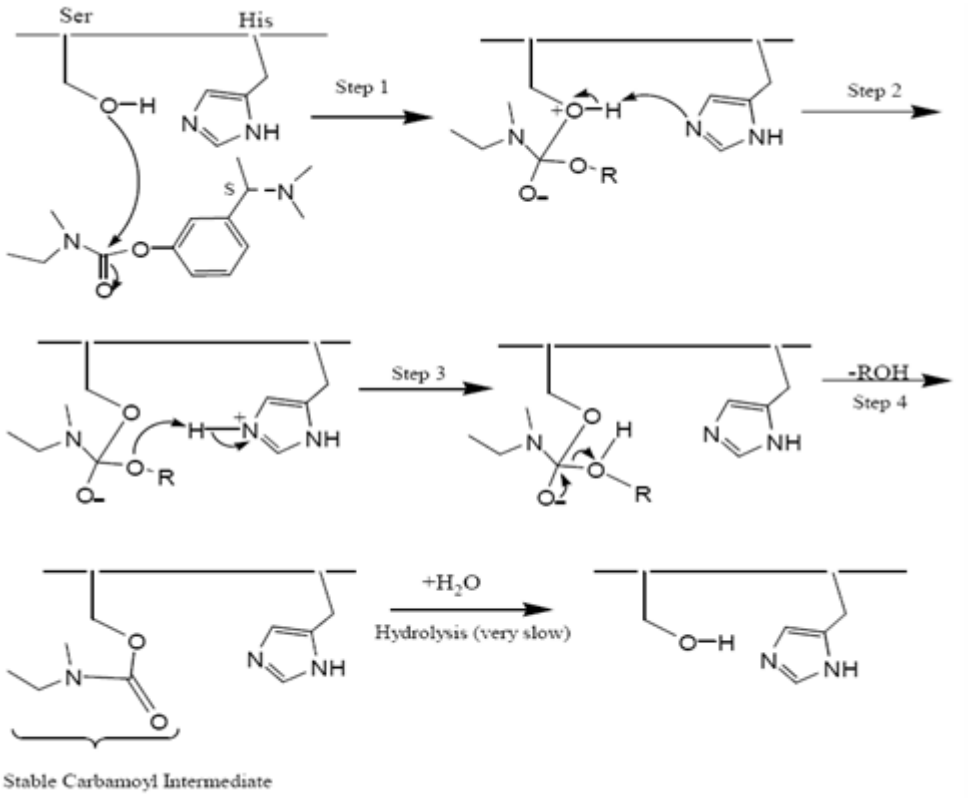


Figure2.11 Mechanism of AchE inhibition by rivastigmine

Memantine is an uncompetitive glutamatergic *N*-methyl-D-aspartate (NMDA) receptor antagonist. The NMDA receptor is an ionotropic receptor, which allows for the transfer of electrical signals between neurons in the brain and spinal column. The NMDA receptor is activated (opened) once glycine and the neurotransmitter glutamate bind to the receptor. This allows the influx of Na+ and Ca2+ ions into the cell. Memantine inhibits this influx of ions by blocking the ion channel (Figure 2.12). It is important to control the concentration of Ca2+, because it activates numerous enzymes within the cell that can cause cell damage and death. This process is called neuronal excitotoxicity. Because memantine is an uncompetitive inhibitor, it can be removed from the channel to allow normal function to resume. Controlling the ion

concentration within the cell is necessary in AD, because it will reduce the number of neurons that are lost and damaged by a process independent of the formation of β-amyloid plaques and neurofibrillary tangles. The more neurons that are available, the more efficient neurotransmission will be, thereby potentially resulting in improved cognitive function. Figure 2.12. Schematic showing the action of memantine in the ion channel pore **[27-28]**.

Figure2.12 Schematic showing the action of memantine in ion channel pore.

Although the drugs that are currently available to treat Alzheimer's disease reduce the cognitive dysfunction associated with AD, it is not known how long these drugs are capable of providing relief from the symptoms associated with the disease. For a chronic neurodegenerative disorder like AD, it is desirable to develop treatments that can halt or significantly impede the progression of the disease. This would alleviate some of the socioeconomic burden associated with providing long term care for Alzheimer's patients. It is less expensive to provide drug treatments alone rather than in concert with 24 hr supervision and care. Of course, a key

consideration is how the side effects associated with these treatments will affect the quality of life of the Alzheimer's patient **[27-28]**.

Oxidative stress hypothesis in Alzheimer's disease

The major hurdle in understanding AD is a lack of knowledge about the etiology and pathogenesis of selective neuron death. In recent years, considerable data have indicating that the brain in AD is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death in this disorder. The direct evidence supporting increased oxidative stress in AD is: (1) increased brain Fe^{2+} and Cu^{2+}in AD, capable of stimulating free radical generation; (2) increased lipid peroxidation and decreased polyunsaturated fatty acids in the AD brain, and increased 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid; (3) increased protein and DNA oxidation in the AD brain; (4) diminished energy metabolism and decreased cytochrome c oxidase in the brain in AD; (5) advanced glycation end products (AGE), malondialdehyde, carbonyls, peroxynitrite, heme oxygenase-1 and SOD-1 in neurofibrillary tangles and AGE, heme oxygenase-1, SOD-1 in senile plaques; and (6) studies showing that amyloid beta peptide is capable of generating free radicals. Supporting indirect evidence comes from a variety of in vitro studies showing that free radicals are capable of mediating neuron degeneration and death. Overall, these studies indicate that free radicals are possibly involved in the pathogenesis of neuron death in AD. Because tissue injury itself can induce reactive oxygen species (ROS) generation, it is not known whether this is a primary or secondary event. Even if free radical generation is secondary to other initiating causes, they are deleterious and part of a cascade of events that can lead to neuron

death, suggesting that therapeutic efforts aimed at removal of ROS or prevention of their formation may be beneficial in AD **[27-32]**.

2.7 Overview of Cerebral ischemia model

2.7.1 Cerebral ischemia

Cerebral ischemia resulted from a transient or permanent reduction in cerebral blood flow that is restricted to major brain artery. That reduction in flow is, in most cases, caused by the occlusion of cerebral artery either by an embolus or by local thrombosis. The major pathologic mechanisms of this cascade include excitotoxicity, peri-infarct depolarization, inflammation and programmed cell death **[33]**.

Brain tissue has a relatively high consumption of oxygen and glucose, almost exclusively used in an oxidative phosphorylation for energy production. Impairment of blood flow restricts the delivery of substrates, oxygen and glucose, and impairs the energy required to maintain ionic gradients. With energy depletion, membrane potential is lost and depolarization of neuron and glia occur. Consequently, somatodendritic as well as presynaptic voltage-dependent Ca^{2+} channels become activated and excitatory amino acids are released into the extracellular space **[34]**. At the same time, the energy-dependent processes, such as presynaptic reuptake of excitatory amino acids, are impeded, which further increases the accumulation of glutamate in the extracellular space. Activation of nmda receptors and metabotropic glutamate receptors, via phospholipase C and IP_3 signaling, contribute to Ca^{2+} overload. As a result of glutamate-mediated over activation, Na^+ and Cl^- enter the neuron via channels for monovalent ions such as AMPA. Water follow passively, as

the influx of Na^+ and Cl^- is much larger than the efflux of K^+. the ensuring edema can affect the perfusion of regions surrounding the core of the perfusion deficit negatively, and also have remote effects that are produced via increased intracranial pressure. Brian edema, which gives rise to earliest markers for the ensuing pathophysiology, studied with MRI and CT sacn, is one of major determinants of whether the patient survives beyond the first few hours after stroke **[35]**.

An increase in the universal second messenger, Ca^+, is thought to initiate a series of cytoplasmic and nuclear events that impact the development of tissue damage profoundly, such as activation of proteolytic enzymes that degrade cytoskeletal proteins, extracellular matrix proteins. Activation of phospholipase A_2 and cytooxygenase generates free radical species that overwhelm endogenous scavenging mechanisms, producing lipid peroxidation and membrane damage **[36]**. The important role of oxygen free-radicals in cell damage associated with stroke is underscored by the fact that even delayed treatment with free radical scavengers can be effective in experimental focal cerebral ischemia. Oxygen free radicals also serve as important signaling molecules that trigger inflammation and apoptosis. Nitric oxide (NO) synthesized by the Ca^+-dependent enzyme, neuronal nitric oxide synthase (NOS) reacts with a superoxide anion to from the highly reactive oxygen species, peroxynitrite, which promotes tissue damage **[37]**.

In transient cerebral ischemia, when blood flow is restored (reperfusion), oxygen can enhance the biochemical reactions that generate free radicals in the cytosolic compartments or subcellular organelles and mitochondria. Moreover, during reperfusion, the endogenous antioxidative defense is likely to be perturbed as a result

of overproduction of oxygen radicals **[38]**. Another component that contributes to cell damage is inflammation. In inflammatory phase, endothelial adhesion receptors are upregulated and white cells adhere to the wall of blood vessels, invade the parenchyma and release cytotoxic cytokines, such as tumor necrosis factor, interleukin(IL) 1, and IL6 **[39]**.

2.7.2 Cerebral ischemia model

The goal of cerebral ischemia model is to reduce oxygen and glucose supply to brain tissue. This process produces brain injury via variety of cellular and molecular mechanisms that impair the energy required to maintain ionic gradients. The mechanisms involve a complex series of pathophysiolgy events that are dependent on the severity, duration, and location of ischemia within the brain. Cerebral ischemia experimental models are characterized as global, focal, and multifocal ischemia. Global ischemia occurs when cerebral blood flow (CBF) is reduced throughout most or of brain, whereas focal ischemia is represented by a reduction in blood flow to a very distinct, specific brain region. In multifocal ischemia, there are is patchy pattern of reduced CBF **[40]**.

Global cerebral ischemia in rodent is an established model in experimental research on cerebral ischemia which is characterized morphologically by a selective neuronal damage in the hippocampus, striatum and cortex. At present, there are three experimental models which are commonly used:(1) the four-vessel ocllusion(4VO) where the vertebral arteries are permanently occluded and the carotid arteries are clamped transiently for 10-30 min, (2) the two vessel occlusion plus hypotension (2VO+hypo) which is produced by transient occlusion of both common carotid

arteries and withdrawal of blood, (3) In gerbils a transient occlusion of common carotid arteries (2VO) because these animals display an incomplete circle of Willis **[41]**.

The two-vessel (2VO) model was initially used to characterize cerebral energy state following incomplete ischemia. In this model, bilateral common carotid artery occlusion is coupled with systemic hypotension to produce a reversible ischemia. Furthermore, in this model, both of the ischemia and reperfusion which has ischemic/reperfusion injury to neurons are almost immediate. Moreover, following reperfusion hypoperfusion has been demonstrated. With this model, injury occurs within selectively vulnerable areas such as the CA1 of hippocampus, caudoputamen, and neocortex **[40]**.

2.7.3 The Morris Water Maze (MWM)

The Morris water maze, a commonly used test of spatial memory, was devised by Richard Morris about 20 years ago. It developed to test abilities to learn, remember and go to the place in space defined only by its position relative to cues **[42]**. This spatial version of the task is dependent on the hippocampus; it hippocampal biochemical mechanisms have been partly identified. It must be borne in mind that even in the spatial version of this task there is a procedural learning embedded. The learning of where to escape to is declarative information that hinges on or is superposed upon the swimming-to-escape knowledge **[43]**.In this test, animal is placed in pool filled with water. Submerged just below the surface in one location is a small platform that allows animal to escape. Typically, an animal learns to escape from the water by locating the hidden platform with the help of visual cues around the

pool. The relative simplicity of the MWM task is undoubtedly one of the reasons for its continuing success. The MWM task has often been used in the validation of rodent models. Though its much application, MWM testing gained a position at the very core of contemporary neuroscience research **[44]**.

2.7.4 Passive avoidance

One of the most common animal tests in memory research is the inhibition to imitate activities or learn habits. This task relies heavily on the dorsal hippocampus, where it uses a sequence of molecular events very remindful of those of long-term potentiation; but it also depends on the entorhinal and parietal cortex and is intensely modulated by the basolateral nucleus of the amygdale, in all of which it uses other sequences of molecular events **[43].** The term passive avoidance is usually employed to describe experiments in which animal learns to avoid a noxious event by suppressing a particular behavior **[45]**. Step-down test is one of the most frequently used tests for passive avoidance. The test is usually based on electric current as source of punishment. In many tests, the floor of the apparatus is made up by a grid that can be electrified. After habituation to this apparets the animal were placed into the platform for an acquisition trial and a foot shock is delivered as soon as the animal enters the grid, and 24 hr later the retention trial were performed. The latency to refrain from performing the punished act expresses the ability to avoid **[41].**

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1 Dichloromethane (Lab scan)

3.1.2 Ethyl acetate (Lab scan)

3.1.3 Glacial acetic acid (Scharlau)

3.1.4 Hexane (E. Merck)

3.1.5 Butanol(Lab scan)

3.1.6 Methanol, AR grade (Lab scan)

3.1.7 Sodium Hydroxide (E. Merck)

3.1.8 Sulfuric acid concentrate (Lab scan)

3.1.9 Hydrochloric acid (Lab scan)

3.1.10 Sodium bicarbonate (Lab scan)

3.1.11 Filter paper, Number1 (Whatman)

3.1.12 Silica gel 60 (number 7734), particle size 0.063-0.200 nm (70-230 mash ASTM)

3.1.13 Sephadex

3.1.14 KBr (Merk)

3.1.15 DMSO (Merk)

3.1.16 TLC plate silica gel 60 F254 (E. Merck)

3.2 Instruments

3.2.1 Hot air oven (OMRON)

3.2.2 Rotary evaporator (BUCHI)

3.2.3 Ultraviolet (UV) absorption spectra (Milton Roy Spectronic 3000 array)

3.2.4 Infrared (IR) Absorption Spectra (Shimatsu IR 440 infrared Spectrometer)

3.2.5 Melting point (Buchi glasscapillary apparatus)

3.2.6 High- performance liquid chromatography (Shimadzu)

3.2.7 Densitometer (Shimadzu:CS9301PC)

3.3 Plant materials

Longan seed (cultivars Edor) were freshly harvested from the garden, Chiangmai province, Thailand in June, 2008 .The seed was washed and dried in the air at room temperature for one week and then ground it in to powder.

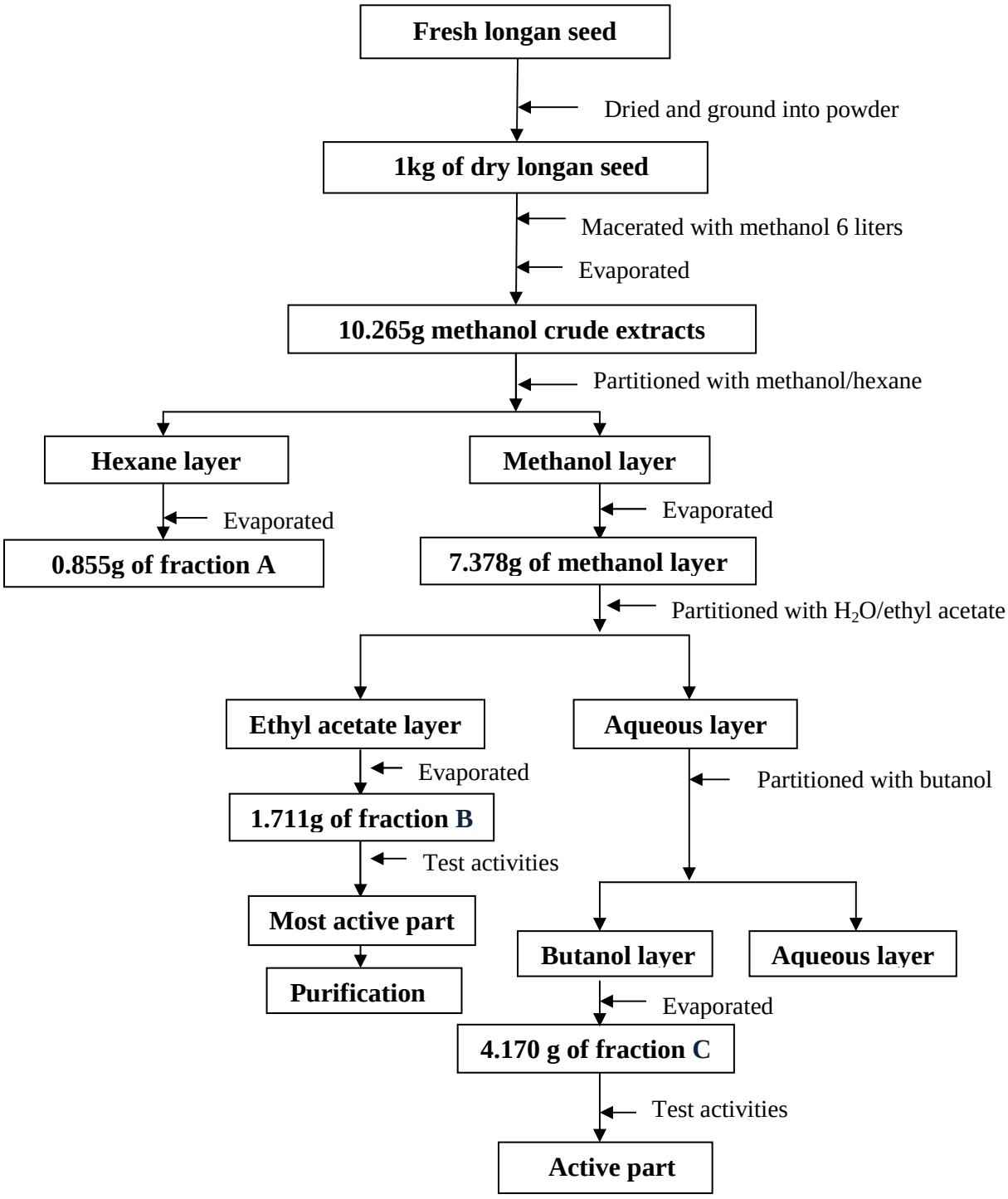
3.4 Extraction, isolation and determination of the positive effect on learning and memory impairment from longan seed.

3.4.1 Extraction and Separation

Diagram of extraction and isolation is shown in scheme 3.1. The whole dried ground seed of longan (1 kg) was macerated four times in 6 liters with methanol, each time for three days. The collected filtrate was concentrated under reduced pressure at temperature not over 60° C, giving the 10.265g yellow -brown precipitate residue.

The marc was dissolved with methanol and repeatedly partitioned with hexane for three times. The collected hexane and methanol were concentrated under reduced pressure at temperature not over 60° C, giving the 0.855g yellow oil residue of hexane fraction (fraction A) and 7.378g yellow-brown precipitate of methanol fractions. The methanol residue was dissolved with distilled water and repeatedly partitioned with ethyl-acetate for three times. The collected ethyl-acetate was concentrated under reduced pressure at temperature not over 60° C, giving the 1.711g yellow- orange precipitate residue (fraction B). The collected distilled water residue was partitioned with butanol for three times. The butanol extract was concentrated and evaporated under reduced pressure at temperature not over 60° C, thus giving the 4.170g brown precipitate residue (fraction C). Each fraction (hexane, ethyl-acetate

and butanol) was tested for the effect on learning and memory impairment after bilateral carotid arteries occlusion (2-VO) in mice. The ethyl-acetate fraction is the most active principle (at dose 200mg/kg B.W.).



Scheme 3.1 Extraction scheme for the positive effect on learning and memory impairment after bilateral carotid arteries occlusion from longan seed.

3.4.2 Isolation of chemical compound

Fraction B (0.701g), the most active part was separated on sephadex column chromatography. The column was eluted with methanol and 50ml of fraction was collected. Methanol was used for eluting column until the eluate was clear. Each fraction was examined by TLC.

The fraction, giving the same chromatographic pattern, were combined and concentrated to give a portion as shown in table 3.1.

Table 3.1 The combined portions from fraction B

Fractions	Combined portions	Weight (g)
1-15	A-1	0.075
16-28	A-2	0.143
29-48	A-3	0.345
49-55	A-4	0.075
Methanol eluted	A-0	0.031

Fraction 1-15 (A-1) 0.075g, the yellow-orange precipitate .It was separated on column chromatography (Silica gel). The column was eluted with the mixture of hexane and ethyl acetate by increasing polarity and 10 ml of fraction was collected. The eluent were used as table 3.2.

Table 3.2 The eluent were used from fraction A-1

Eluting solvent	Volume (ml)	Fractions
Hexane	100	1-10
Hexane-Ethyl acetate (9:1)	200	11-31
Hexane-Ethyl acetate (7:3)	200	32-52
Hexane-Ethyl acetate (4:6)	200	53-73
Ethyl acetate	300	74-103

Methanol was used for eluting column until the eluate was clear. Each fraction was examined by TLC chromatography .The fraction, giving the same chromatographic pattern were combined.

Table 3.3 The combined portion from fraction A-1

Fractions	Combined portions	Weight (g)
1-50	A-1.1	0.015
51-69	A-1.2	0.012
70-92	A-1.3	0.031
93-methanol	A-1.4	0.007

Fraction 70-92 (A-1.3) 0.031g, the white-yellow crystal was precipitated. It was separated on fresh column chromatography (silica gel). The column was eluted with the mixture of dichloromethane and methanol by increasing polarity and 5 ml of fraction was collected. The eluent were used as table 3.4.

Table 3.4 The eluent were used from fraction A-1.3

Eluting solvent	Volume (ml)	Fraction
Dichloromethane	50	1-10
Dichloromethane-Methanol (9:1)	100	11-31
Dichloromethane-Methanol (7:3)	100	32-52
Dichloromethane-Methanol (4:6)	100	53-73

Methanol was used for eluting column until the eluate was clear. Each fraction was examined by TLC chromatography.

From table 3.4 the combined portions 45-54 (A-1.3.3), the yellow crystal which was identified as quercetin. The combination of gave 11.0 mg.

From table 3.1 0.143g of fraction A-2,the yellow-white precipitate. It was separated on column chromatography. The column was eluted with the mixture of hexane and ethyl acetate by increasing polarity and 5 ml of fraction was collected. The eluent were used as table 3.5.

Table 3.5 The eluent were used from fraction A-2

Eluting solvent	Volume (ml)	Fraction
Hexane	50	1-10
Hexane-Ethyl acetate(9:1)	100	11-30
Hexane-Ethyl acetate(7:1)	100	31-50
Hexane-Ethyl acetate(5:1)	100	51-70
Hexane-Ethyl acetate(3:1)	100	71-90
Hexane-Ethyl acetate(1:1)	100	91-110
Hexane-Ethyl acetate(1:3)	100	111-130
Hexane-Ethyl acetate(1:5)	100	131-150
Hexane-Ethyl acetate(1:7)	100	151-170
Ethyl acetate	200	171-200

Methanol was used for eluting column until the eluate was clear.

From table 3.5 the combined fraction 158-182 (A-2.2), the white crystal which was identified as gallic acid. The combination of A-2.2 gave 63.0mg.

From table 3.5 the combined fraction 139-145(A-2.1), the white-yellow crystal was precipitated which combination of A-2.1 gave 25 mg. It was separated on fresh column chromatography. The eluant was mixture of dichloromethane: methanol (4:1) and 5ml of fraction was collected. Each fraction was determined by TLC. The fraction which showed the same pattern of chromatogram were combined, concentrated and assigned as shown in table 3.6.

Table 3.6 The combined portions from fraction A-2.1.

Fractions	Portions	Weight(g)
1-7	A-2.1.1	0.007
8-17	A-2.1.2	0.005

Fraction 1-7 (A-2.1.1) was identified to propyl gallate. The combination of A-2.1.1 gave 7.0mg.

From table 3.1 fraction 29-48(A-3) was performed for future isolation by sephadex column chromatography. The eluant was mixture of dichloromethane: methanol (1:1) and fractions based on the color band (approximate 25 ml) were collected. Each fraction was determined by TLC. The fraction which showed the same pattern of chromatogram were combined, concentrated and assigned as shown in table 3.7.

Table 3.7 The combined portions from fraction A-3.

Fractions	Portions	Weight (g)
1-10	A-3.1	0.101
11-16	A-3.2	0.042
17-25	A-3.3	0.115

Fraction 11-16 (A-3.2), the yellow-green crystal was precipitated. It was separated by fresh column chromatography.

The eluant was mixture of dichloromethane: methanol: water (4:1:0.1) was collected. Each fraction determined by TLC, combined, concentrated and assigned as shown in table 3.8.

Table 3.8 The combined portions from fraction A-3.2.

Fractions	Portions	Weight (g)
1-6	A-3.2.1	0.011
7-16	A-3.2.2	0.009
17-22	A-3.3.3	0.007

Fraction 7-16 (A-3.2.2), the yellow-green crystal was identified as rutin. The combination of A-3.2.2 gave 9.0mg.

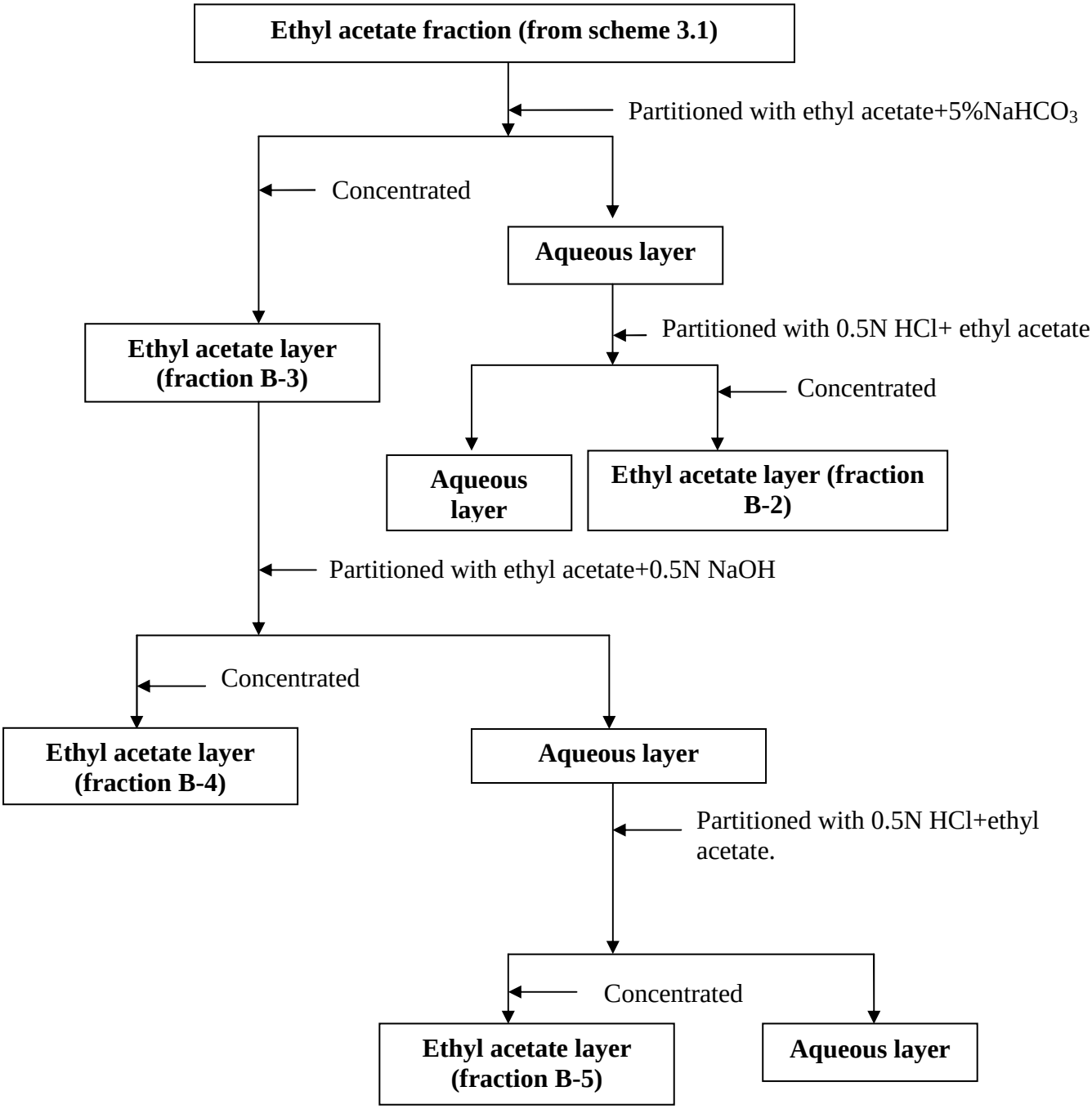
Each pure compound was test for the positive effect on memory impairment and neuronal cell death after bilateral carotid arteries occlusion. The chemical structure of the pure compound was characterized by spectrophotometric method (IR and ^{1}H and ^{13}C NMR).

The result shown rutin is the active principle (at dose 100mg/kg B.W.) and will be used as chemical marker for both qualitative and quantitative assessment.

Scheme 3.2 is the extraction method for developed standardized herbal extract, rutin as a chemical marker compounds. Acid-base extraction is a useful method to separate components in this study and to remove unwanted compounds (gallic acid and propyl gallate) from a extraction, the chemical marker were separated and was the standardized herbal extract.

The ethyl acetate fraction (scheme 3.1) was dissolved in ethyl acetate and then partitioned with 5% sodium bicarbonate for three times. The ethyl acetate layer was concentrated and evaporated under reduced pressure at temperature not over 60°C, thus giving the yellow-brown precipitate (fraction B-3). The fraction B-3 was dissolved in ethyl acetate and partitioned with 0.5 N sodium hydroxide for three times, giving as yellow precipitate (fraction B-4). The aqueous extract was partitioned with 0.5N hydrochloric acid and partitioned with ethyl acetate for three times. The collected was concentrated and evaporated under reduced pressure at temperature not over 60°C, giving the yellow-brown precipitate (fraction B-5).

Another fraction, the aqueous layer was partitioned with 0.5N hydrochloric acid and then partitioned with ethyl acetate three times. The ethyl acetate layer was concentrated and evaporated under reduced pressure at temperature not over 60°C, giving the yellow precipitate (fraction B-2). The active principle compound (rutin) was obtained in fraction B-4, as a standardized herbal extracts for identifying and quantifying the chemical marker compounds.



Scheme 3.2 Preparation of standardized herbal extract

The weight of each ethyl acetate part (B-2, B-4 and B-5) from obtained shown in table 3.9

Table 3.9 The weight of each ethyl acetate part extracts from Scheme 3.2

Ethyl acetate part	Weight	Mean ±SD
Ethyl acetate part from scheme 3.1	20.2019,19.7731, 20.1011	20.0253±0.1294
B-2	6.0111,5.6118, 5.9211	5.8480±0.2094
B-3	12.5640,12.9293,11.9981	12.4971±0.4692
B-4	2 .7051, 2.8592, 2.7878	2 .7840±0.0445
B-5	8.3590, 8.1701, 8.1996	8.2429±0.1016

3.4.3 Learning and memory impairment after bilateral common carotid arteries occlusion (2-VO) in mice test.

The effects of longan seed extracts and isolated compounds on learning and memory impairment after bilateral common carotid arteries occlusion (2-VO) in mice test was carried out at Department of Physiology, Faculty of Pharmaceutical sciences, Chulalongkorn University.

Mice were subjected to cerebral ischemia induced by 2-VO plus hypotension **[56]** and were divided into three groups as followed (n=6-10 per group)

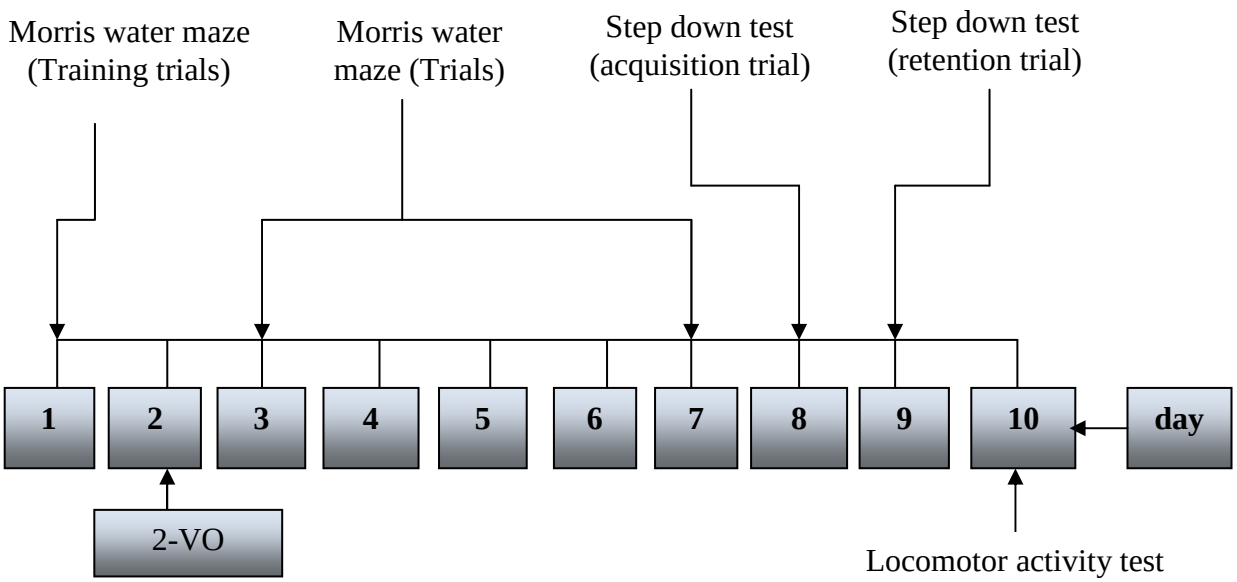
(1) Sham operated group administered with 0.5% CMC.

(2) 2-VO group administered with 0.5% CMC.

(3) 2-VO group administered with longan seed extracts and isolated compounds.

Longan seed extracts (from scheme 3.1) and isolated compounds (quercetin, gallic acid, propyl gallate and rutin) were suspended in vehicle (0.5% w/v Carboxy methyl cellulose (CMC)) and given orally to mice once daily. The control animals were orally administered with vehicle.

To study the effects of longan seed extracts and isolated compounds on 2-VO mice **[56]** received tested substance by orally for 8 consecutive days. Three behavioral tested, Morris water maze (MWM), Step-down test and test for spontaneous locomotor activity were performed **[56].** MWM was tested for 5 consecutive days. The step-down test was performed 6 days after 2-VO. Spontaneous locomotor activity test 8 days after 2-VO as shown in scheme 3.3.



Scheme3.3 Diagram of experimental schedule.

3.4.4 Chromatographic and spectroscopic studies.

3. 4.4.1 Thin layer chromatographic method (TLC method).

Each extract and isolated compounds were dissolved in methanol. The solution was applied on a silica gel plate, which was then developed in suitable mobile phase, after that, the R_f value of each compounds were detected with UV at 365 nm, anthrone and ferric chloride spray reagents.

Spray reagent

2) Ferric chloride spray reagent: A 1 mg of ferric chloride was dissolved in methanol and diluted to 100 ml.
3) Anthrone spray reagent: 100mg of anthrone was dissolved in concentrate 5ml sulfuric acid and diluted with ethanol to 25 ml.

TLC condition

Stationary phase: silica gel plate GF_{254} 10 x 10 cm

Developing solvent (Mobile phase):

System 1) Butanol : Acetic acid (9:3)

System 2) Chloroform : Methanol : Acetic acid (30 : 15 :5)

System 3) Methanol: Ethyl acetate: acetic acid (1:6:3)

Detector: 1) UV at 365 nm.

2) Ferric chloride spray reagent

3) Anthrone spray reagent (Heated at 120 ^{o}C for 20 minutes before detecting the compounds.)

3.4.4.2 Infrared Spectroscopic method (IR).

IR test was carried out at Scientific and Technological Research Equipment Centre Foundation (STREC), Chulalongkorn University.

Approximately 2 or 3 mg of each isolated compounds was mixed and grounded with about 15 mg. of previously dried potassium bromide (KBr). The solid mixture was compressed to thin film KBr disc and scanned with IR spectrophotometer.

3.4.4.3 Nuclear-Magnetic Resonance (^{1}H and ^{13}C-NMR).

NMR test was carried out at Scientific and Technological Research Equipment Centre Foundation (STREC), Chulalongkorn University.

Each isolated compounds was weighed about 2-4 mg. and dissolved with deuterated DMSO in a NMR tube. The mixture was measured in the NMR spectrospin.

3.5 Determination of percentage purity of rutin

3.5.1 Preparation of reference standard solution

The rutin reference standard was obtained from the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The standard purified by crystallization with methanol-water before using.

Accurately weighed about 3.0mg of reference standard and dissolved in methanol. The mixture was adjusted volume to 100.0 ml to obtain the 3µg/ml of standard solution.

3.5.2 Preparation of sample solution

Accurately weighed about 250 mg of fraction B-4 (from scheme3.2) and dissolved in methanol. The mixture was adjusted volume to 100.0 ml

3.5.3 Analysis of standard and sample solutions

The solution from 3.5.1 and 3.5.2 were injected onto a HPLC system, the retention time of working standard substances was compared to their reference standards and the purity of working standard was determined and calculated from the peak area as chromatographic purity. The HPLC system used was the same as in 3.6.1.

3.6 Development of High Performance Liquid Chromatography analytical method (HPLC analytical method) and Thin Layer Chromatography analytical method with densitometer (TLC analytical method-densitometer).

3.6.1 HPLC analytical method

3.6.1.1 HPLC system

3.6.1.1.1 Instrument

HPLC:	Shimadzu
Pump:	System module LC-10ADvp
Auto sample:	SIL-10Dvp
Degasser:	DGU-14A
Detector:	Diode-array detector SPL-10Avp
Controller:	System controller SCL-10Avp
Software:	Class VP

3.6.1.1.2 Chromatographic Conditions.

Column:	Alltech (Alltima C18, 4.6x150mm,5um)
Mobile phase:	0.5% Formic acid: Acetonitrile
Flow rate:	0.9 ml/min.
Mobile phase ration:	30:70 % v/v.
Detection:	Photodiode array at 365 nm.
Injection quantity:	20 µl.

3.6.1.2 Preparation of the standard solution.

3.6.1.2.1 Rutin standard stock solution

Accurately weighed 15.0 mg of rutin into a 50.0 ml volumetric flask. Add 25.0 ml of methanol and sonicated until the clear solution was obtained. Adjust to volume with methanol and mixed. Pipette 1.0 ml of stock solution into a 100.0 ml volumetric flask. Adjust to volume with methanol and mixed. The final concentration of the solution is 3.0 µg/ml.

3.6.1.2.2 Internal standard stock solution (IS)

Several criteria were purposed in finding the suitable internal standard as following; stable compound, no interaction with an analyze, detected by detector (photo diode array at 365 nm.), resolved from the other component in the matrix sample. The following compounds were studied in process of finding internal standard; hesperidin, quercetrin, diosmin. Only hesperidin gave optimum resolution in the HPLC analysis and served as internal standard.

A 20.0 mg hesperidin accurately weighed, into a 50.0 ml volumetric flask. Add 25.0 ml of methanol and sonicated until the clear solution was obtained. Adjust to volume with methanol and mixed. Pipette 1.0 ml. of stock solution into 50.0 ml. volumetric flask. Adjusted to volume with methanol and mixed. The final concentration of the solution is 8.0µg/ml.

3.6.1.2.3 Calibrated standard solution

The standard stock solution was quantitatively diluted with methanol to the concentration of 0.3, 0.6, 0.9, 1.2 and 1.5µg/ml and added internal standard solution 1.0 ml (concentration to 0.8µg/ml), adjusted volume to 10.0ml with methanol as shown in table 3.10.

Table 3.10 Concentration of calibrated standard solutions.

Items	Solutions				
	CS_1	CS_2	CS_3	CS_4	CS_5
Rutin standard stock solution (ml.)	1.0	2.0	3.0	4.0	5.0
Final volume (ml.)	10.0	10.0	10.0	10.0	10.0
Concentration of rutin working solution (µg/ml.)	0.3	0.6	0.9	1.2	1.5

3.6.1.3 Preparation of sample solutions

3.6.1.3.1 Sample stock solutions

Accurately weighed about 250 mg. of dried powder of B-4 (from sheme3.2) into a 50.0 ml.volumetric flask. Add 25.0 ml. of methanol and sonicated until the clear solution was obtained. Adjust to a 50.0 ml. volumetric flask with methanol to make up the sample stock solution.

3.6.1.3.2 Sample solutions

For each five sample solutions, sample solutions (E_1, E_2, E_3, E_4 and E_5) were prepared by pipetting 3.0 ml. of sample stock solution (3.5.1.3.1), then added 1.0, 2.0, 3.0, 4.0, 5.0 ml of rutin standard stock solutions and add stock internal standard solution 1.0 ml ,the final volume was 10.0 ml.

Another sample solution (without standard addition, E_0) was prepared by pipetting 1.0 ml. of stock sample solution and added 1.0 ml. of internal stock standard solution and adjusted volume to 10.0 ml. with methanol.

Table 3.11 Concentration of rutin spiked in sample solutions.

Items	Concentration of rutin spiked in sample solutions (µg/ml.)					
	E_0	E_1	E_2	E_3	E_4	E_5
rutin	0	0.3	0.6	0.9	1.2	1.5
IS	0.8	0.8	0.8	0.8	0.8	0.8

3.6.1.4 Validation of HPLC analytical method.

The developed HPLC analytical method was validated according to the International Conference on Harmonization guideline (ICH guideline) on following parameters; specificity, linearity, accuracy, precision, detection limit, quantitation limit.

3.6.1.4.1 Specificity

The specificity of the method was determined by injecting rutin standard solution and sample onto the chromatographic system previously described.

3.6.1.4.2 Linearity

Three replications of calibrated standard solutions (CS_1, CS_2, CS_3, CS_4 and CS_5) were applied to the HPLC analytical system (scheme 3.6). The standard calibration curve was plotted between peak area ratio (area of rutin/area of IS) versus the concentration of standard solution (mg/ml.). The equation of the standard calibration curve was determined with the coefficient of determination (r^2), and then $r^2 \geq 0.99$ was accepted.

3.6.1.4.3 Accuracy

Six replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied 20 µl to HPLC system and calculated the accuracy by using below equation.

The accuracy was determined in term of percent recovery (%R).

%R = (Analytical found/Actual added) x 100

96-104 %R was accepted.

3.6.1.4.4 Precision

Six replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied 20 µl. to HPLC analytical system in the same days. The percent recoveries of each of sample solution were calculated as repeatability precision (or intra-day precision) by using the equation below.

Three replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied 20 µl. to HPLC system for three days. The percent recoveries of each of sample solution in three days were calculated as intermediate precision (or inter-day precision) by using below equation.

The precision was determined in term of percent of coefficient of variation (%CV) or relative standard deviation (%RSD).

$$\%RSD = [SD / \bar{X}] \times 100$$

Where

SD = standard deviation of percent recovery

$\bar{X}$ = mean of percent recovery

%RSD≤2% was accepted

3.6.1.4.5 Detection limit (DL) .

Detection limit (DL) is generally determined by the analysis of samples with known concentrations of analyze and by establishing the minimum level at which the analysis can be detected. The detection limit was expressed as

$$DL = \frac{3.3\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

3.6.1.4.6 Quantitation limit (QL)

Quantitation limit (QL) is generally determined by the analysis of samples with known concentrations of analyze and by establishing the minimum level at which the analysis. The quantitation limit was expressed as

$$QL = \frac{10\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

3.6.2 TLC -densitometer analytical method

3.6.2.1 TLC system

3.6.2.1.1 Densitometer parameter

Photo mode	Reflection
Scan mode	Linear
Set zero mode	At start
Beam size	0.4 x 0.5 mm.
Wavelength	365 nm.

3.6.2.1.2 Chromatographic condition

TLC plate : Silica plate, GF 254, 10 x 20 cm

Developing solvent : butanol-glacial acetic acid (9:3)

Detection : Spray with anthrone in ethanol, heat at 120 ^{o}C for 20 min and keep at room temperature for 10 min , Densitometer at wavelength 365 nm.

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3.6.2.2 Preparation of standard solution

3.6.2.2.1 Rutin standard stock solution

Accurately weighed 1000.0 mg of rutin and dissolved in methanol and adjusted the volume to 50.0 ml as a stock standard solution (20.0 mg/ml).

3.5.2.2.2 Internal standard stock solution (IS)

Accurately weighed 50 mg of hesperidin and dissolved in methanol and adjusted the volume to 25.0 ml. (2.0 mg/ml).

3.6.2.2.3 Standard solution

Six working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5 and WS_6) were prepared according to table 3.12 and adjusted volume to 10.0 ml with methanol.

Table 3.12 Preparation of working standard solutions for method validation.

Items	Solutions					
	WS_1	WS_2	WS_3	WS_4	WS_5	WS_6
Stock standard rutin solution (ml)	2.0	3.0	4.0	5.0	6.0	7.0
Final volume (ml)	10.0	10.0	10.0	10.0	10.0	10.0
Concentration of rutin working solution (mg/ml)	4.0	6.0	8.0	10.0	12.0	14.0

3.6.2.3 Preparation of sample solutions

3.6.2.3.1 Sample stock solutions

200 mg of fraction B dissolved in methanol and adjusted the volume to 100.0 ml as a sample stock solution.

3.6.2.3.2 Sample solutions

For each five sample solutions, sample solutions (SE_1, SE_2, SE_3, SE_4 and SE_5) were prepared by pipetting 6.0 ml of sample stock solution, then added 2.0 ml of each working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5) and add stock internal standard solution 2.0 ml, the final volume was 10.0 ml.

Another sample solution (without standard addition, SE_0) was prepared by pipetting 6.0 ml of stock sample solution and added 2.0 ml of internal stock standard solution and adjusted volume to 10.0 ml with methanol.

Table 3.13 Concentration of rutin spiked in sample solutions

Items	Concentration of rutin spiked in sample solutions (mg/ml)					
	SE_0	SE_1	SE_2	SE_3	SE_4	SE_5
rutin	0	0.8	1.2	1.6	2.0	2.4
IS	0.4	0.4	0.4	0.4	0.4	0.4

3.6.2.4 Validation of the TLC method

3.6.2.4.1 Specificity

In this TLC-densitometric analytical system, representative spots should be used to demonstrate specificity. Specificity was demonstrated by the hR_f of the components which elute closest to each other.

The standard and sample solutions were applied 2 µl to TLC-densitometric analytical system. The detecting spot of all analyzes in sample solution were compared with developing spot in standard solution.

The specificity was determined by anthrone spray reagent . No other spot from sample solution overlap with the analytical spot in this TLC condition.

3.6.2.4.2 Linearity

Three replications of calibrated standard solutions (CSs_1, CSs_2, CSs_3, CSs_4, CSs_5 and CSs_6) were applied to the TLC-densitometric analytical system. The calibrated standard solutions were prepared by pipetting 2.0 ml of each working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5 andWS_6) and added stock internal standard solution 2.0 ml, adjusted volume to 10.0 ml with methanol, as shown in table 3.14, and applied to TLC-densitometric analytical system. The standard calibration curve was plotted between peak area ratio (area of rutin/area of IS) versus the concentration of standard solution (mg/ml).

The equation of the standard calibration curve was determined with the coefficient of determination (r^2), and then $r^2 \geq 0.99$ was accepted.

Table 3.14 Concentration of calibrated standard solutions

Items	Concentration of calibrated standard solutions (mg/ml)					
	CSs_1	CSs_2	CSs_3	CSs_4	CSs_5	CSs_6
rutin	0.8	1.2	1.6	2.0	2.4	2.8
IS	0.4	0.4	0.4	0.4	0.4	0.4

3.6.2.4.3 Accuracy

Six replications of each sample solution (SE_0, SE_1, SE_2, SE_3, SE_4 and SE_5) were applied 2 µl to TLC-densitometric analytical system and calculated the accuracy by using below equation.

The accuracy was determined in term of percent recovery (%R)

%R= (Analytical found/Actual added) x 100

96-104%R was accepted

3.6.2.4.4 Precision

Six replications of each sample solution (SE_0, SE_1, SE_2, SE_3, SE_4 and SE_5) were applied 2 µl to TLC-densitometric analytical system in the same days. The percent recoveries of each of sample solution were calculated as repeatability precision (or intra-day precision) by using the equation below.

Three replications of each sample solution (SE_0, SE_1, SE_2, SE_3, SE_4 and SE_5) were applied 2 µl to TLC-densitometric system for three days. The percent recoveries of each of sample solution in three days were calculated as intermediate precision (or inter-day precision) by using below equation.

The precision was determined in term of percent of coefficient of variation (%CV) or relative standard deviation (%RSD).

$$\%RSD = [SD / \bar{X}] \times 100$$

Where

SD = standard deviation of percent recovery

$\bar{X}$ = mean of percent recovery

2%RSD ≤ was accepted

3.5.2.4.5 Detection limit (DL) .

Detection limit (DL) is generally determined by the analysis of samples with known concentrations of analyze and by establishing the minimum level at which the analysis can be detected. The detection limit was expressed as

$$DL = \frac{3.3\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

3.5.2.4.6 Quantitation limit (QL)

Quantitation limit (QL) is generally determined by the analysis of samples with known concentrations of analyze and by establishing the minimum level at which the analysis. The quantitation limit was expressed as

$$QL = \frac{10\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

3.7 Quantitative analysis of rutin in dried longan seed extracts.

A 5 g of dried ground longan seed (LG1-LG3) was macerated with methanol 70ml for three days. The extract was filtered, and finally applied to HPLC system (as in content 3.6.1). The contents of rutin were determined.

3.8 Quantitative analysis of rutin in standardized herbal extract from fraction B-4 (scheme 3.2)

250.0 mg of dried powder of B-4 (from sheme3.2) into a 50.0 ml volumetric flask and adjust to a 50.0ml with methanol, then pipetting 3.0 ml solution in to volume 10.0ml,final volume was 10.0ml. finally applied to HPLC system (as in content 3.6.1). The contents of rutin were determined.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extraction and Isolation and preparation the standardized extract.

A 1000 g of ground dried longan seed was extracted and isolated of the quercetin, gallic acid, propyl gallate and rutin as mentioned in scheme 3.1 and 3.3 to yield 11.0 mg. (0.0027% w/w), 63.0 mg. (0.0154 %w/w), 7.0 mg. (0.0017% w/w) and 9.0 mg. (0.0022% w/w),respectively.

In the extract for developed standardize herbal extract to generate more stable extract and easy to maintain the chemical consistency of herbal, the study removed unwanted compounds from herbal extract by acid-base extraction. The chemical marker (rutin) was obtained in fraction B-4, as a standardized herbal extracts for identifying and quantifying the chemical marker compounds.

4.2 Identification of the positive effect on learning and memory impairment after bilateral carotid arteries occlusion.

4.2.1 Morris water maze (MWM) test

24 hours after cerebral ischemia the MWM test was performed. Longan seed extract (ethyl acetate, butanol part from scheme 3.1 at 200 mg/kg. B.W.) and isolated compounds (gallic acid, quercetin, propyl gallate and rutin at 100 mg/kg. B.W.) were given orally to animal 30 minutes before testing. In the results, the administration of longan seed extract in ethyl acetate part and rutin significantly

improved learning and memory performance on day 5. On day 5 the escape latencies of ethyl acetate treated mice at 200 mg/kg. B.W. were 19.43±2.12 seconds, whereas it was 18.11±2.09 seconds in rutin (figure 4.10 And 4.11).

4.2.2 Passive avoidance in mice (Step down latency and step down error test)

Impairment of learning and memory in step down test was observed after cerebral ischemia. In longan seed extracts treated mice step down latency of ethyl acetate part was 156.00±0.09 seconds and butanol part was 104.33± 0.06 seconds. Number of errors average of ethyl acetate was 1.30 times and butanol was 2.51 times for the dose of 200 mg/kg B.W. (figure 4.12 and 4.14).

In isolated compounds treated group. Gallic acid, quercetin, propyl gallate and rutin treated mice step down latencies were 100.43±1.22, 58.98±1.79, 72.43±3.47 and 156.00±0.04 seconds, respectively. Number of error was 3.14, 3.17, 3.29 times and 1.38, respectively (figure 4.13 and 4.15).

As the figure4.12and4.13, no differences in either initial step down latency or number of error were noted among different treatment groups in acquisition test. However, on second day 2-VO mice significantly shortened the step down latency and increased number of error when compared other groups. The results showed rutin (100mg/kg B.W.) was the longest step down latency and less of error when compare others.

All results show rutin is the most positive effect on learning and memory impairment after bilateral carotid arteries occlusion in mice (2-VO mice) (100 mg/kg B.W), rutin was the chemical marker for qualitative and quantitative.

Additionally, The MWM of these isolated compounds (100mg/kg B.W.) increases with their in the order Rutin > Ethyl acetate > Gallic acid >Quercetin > Propyl gallate .

Step down latency increases with their in the order Rutin > Ethyl acetate >Gallic acid > Propyl gallate > Quercetin.

4.2.3 Effects of longan seed extracts and isolated compounds on spontaneous locomotor activity in mice.

Administration of ethyl acetate part (at dose 200mg/kg B.W.) and rutin (at dose 100mg/kg B.W.) had no effect on spontaneous locomotor in 2-VO mice.

The previous study correlated that rutin may potential prove to have a neuroprotective effect and revealed protective the memory impairment, such as ;

1) In 2009,Tomoko koda, *et al* they showed that rutin has a protective effect on trimethyltin (TMT)-induced memory dysfunction in rats. The results suggested that neuroprotective effect of rutin on TMT-induced spatial memory impairment could be attributable to inhibitory effect against microglial activation and its role in synapse formation via neurotropic factors in hippocampus **[46].**

2) Fengling Pu, *et al* investigated the effects of (-)-epigallocatechin gallate (EGCG), cathechin, rutin and quercetin on spatial memory impairment and neuronal death induced by repeated cerebral ischemia in rats. Both rutin and

quercetin (50mg/kg×2 times) improved spatial memory impairment in the 8-arm radial maze task and neuronal death in the hippocampal CA1 area. The study showed the effects of rutin on spatial memory impairment were slightly stronger than those of quercetin, but the neuroprotective ability of quercetin was slightly stronger than that of rutin **[47].**

3) Mer-Hsien Lee, *et al* reported four natural flavonoid, quercetrin, isoquercetin , rutin and quercetin that exhibited an inhibitory effect on monoamine oxidase type B (MAO-B) and IC_{50} values were 19.06, 11.64, 3.89 and 10.89µM, respectively. MAO-B activity is elevated in certain neurological diseases such as Alzheimer's, Parkinson's and Huntington's disease. The result showed that rutin (IC_{50}=3.89 µM) is the most active principle and may used for preventing some neurodegenerative disease in the future **[48].**

4) Joseph Torel, *et al* studied about autooxidation of linoleic acid and methyl linolenate is inhibited by flavonoids. They reported antioxidant efficiency of these flavonoids increases with their concentration and in the order fustin <catechin < quercetin <luteolin < kaempferol < morin and rutin. They found the antioxidant efficiency of rutin is more effective than quercetin and kaempferol **[49].**

5) In 1990, Chen Yoting, *et al* studied the superoxide anions scavenging activity and lipid peroxidation of 7 flavonoids ; quercetin, rutin, morin, acacetin, hispirudin, hisperidin and naringin. They reported superoxide anions scavenging activity of flavonoids followed the order rutin (IC_{50}56±3.5µM) >narigin> (IC_{50}192±6.7) quercetin (IC_{50}207±15.9) > morin (IC_{50}1400±152.6) and inhibition of lipid peroxidation activity followed the order: rutin (IC_{50}3.2±0.4µM) > morin (IC_{50}4.4±0.2µM) > quercetin (IC_{50}5.2±0.3µM) > acacetin (IC_{50}13.8±1.7µM) >

hispirutin (IC_{50}64±2.1µM) .The study found rutin was the strongest, and quercetin and naringin the second **[50].**

6) Concepción Sánchez-Moreno, *et al* studied about free radical scavenging capacity and inhibition of lipid peroxidation of wine, grape juices and related polyphenolic constituent. They found the inhibition of lipid peroxidation of standards followed the order: rutin = ferulic acid > tannic acid = gallic acid = resveratrol > BHA = quercetin > DL-α tocopherol > caffeic acid. The study exhibited rutin was the strongest antioxidant activity **[51]**.

4.3 Identification with chromatographic and spectrophotometric method.

The isolated quercetin, gallic acid, propyl gallate and rutin can be identified by both chromatographic techniques and the spectrophotometric technique.

4.3.1 Identification with thin layer chromatography method (TLC method).

Three chromatographic methods (TLC) were selected by comparing the R_f value.TLC method was performed to screen the isolated rutin by the system as mentioned in 3.4.4 The R_f values of extracted and isolated compounds (quercetin, gallic acid, propyl gallate and rutin) were showed as table 4.1, 4.20 and figure 4.9

Table 4.1 R_f values and characteristic of spots on TLC-plate of quercetin, gallic acid, propyl gallate and rutin.

System	Detector	R_f values and color of spots.			
		Quercetin	**Gallic acid**	**Propyl gallate**	**Rutin**
1 (butanol:acetic acid, 9:3)	UV_{365}	0.71 (yellow)	0.55 (yellow)	0.62 (yellow)	0.42 (yellow)
	Anthrone	0.71 (yellow)	-	-	0.42 (green)
	Ferric chloride	-	0.55 (green-black)	0.62 (green-black)	-
2 (chloroform:methanol:acetic acid,30:15:5)	UV_{365}	0.73 (yellow)	0.58 (yellow)	0.67 (yellow)	0.50 (yellow)
	Anthrone	0.73 (yellow)	-	-	0.50 (green)
	Ferric chlorid	-	0.58 (green-black)	0.67 (green-black)	-
3 (methanol:ethyl acetate:acetic acid,1:6:3)	UV_{365}	0.81 (yellow)	0.65 (yellow)	0.74 (yellow)	0.59 (yellow)
	Anthrone	0.81 (yellow)	-	-	0.59 (green)
	Ferric chloride	-	0.65 (green-black)	0.74 (green-black)	-

4.3.2 Structure evaluation with spectrophotometric method.

4.3.2.1) Compound A-1.3.3; Compound A-1.3.3 was yellow crystal that give positive result with UV 365 nm. Its melting point was 317-318ºC (melting point of quercetin is 316 ºC **[57]**). The IR spectrum of A-1.3.3 (Figure4.16) showed the pattern of the functional group as table 4.2.

Table 4.2 The comparison of IR spectra (KBr disc) of quercetin and unknown that isolate from longan seed.

Functional groups	Vibration	Wave number (cm^{-1})	
		Quercetin **[52]**	Unknown
-OH	OH-stretching	3419	3406.95
C=O	C=O-stretching	1666	1663.56
C=C	C=C- stretching	1516	1521.75

The ^{1}H NMR and ^{13}C NMR (Figure4.17,4.18and4.19) showed the assignment as table 4.3.

Table 4.3 The comparison of ^{1}H NMR (300 MHz,DMSO-*d6*) and ^{13}C NMR (125 MHz, DMSO- *d6*) data of **quercetin** and unknown that isolate from longan seed.

compounds	Quercetin (DMDO-*d6*) **[53]**			Unknown isolated from longan seed (DMSO-*d6*)		
Position	δ *C*(ppm)	δ *H*(ppm)	*J*(Hz)	δ *C*(ppm)	δ *H*(ppm)	*J*(Hz)
2	146.8			146.783		
3	135.7			135.713		
4	175.8	12.53(s,5-OH)		175.822		
5	160.7	6.22(d)		160.700	12.474(s,5-OH)	
6	98.1			98.165	6.17(d)	2.1
7	163.9	6.44(d)		163.9		
8	93.3		1.8	93.328	6.39(d)	1.9
9	156.1			156.117		
10	103.0			102.986		
1’	121.9	7.71(d)		121.931		
2’	115.0		2.0	115.040	7.66(d)	2.2
3’	145.0			145.039		
4’	147.7	6.92(d)		147.679		
5’	115.6	7.58(dd)	8.5	115.579	6.87 (d)	8.55
6’	119.9		8.5	119.956	7.52 (dd)	

All data supported that **A-1.3.3** was the quercetin (Figure 4.1).

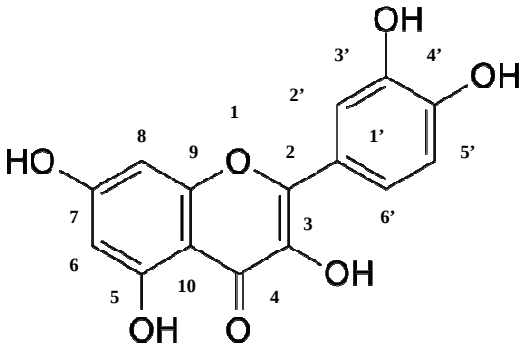


Figure 4.1 The structure of A-1.3.3 (Quercetin).

4.3.2.2) Compound A-2.2; Compound A-2.2 was white crystal that give positive result with ferric chloride spray reagent (green-black spot). Its melting point was 251-252ºC (melting point of gallic acid is 250 ºC **[58]**). The IR spectrum of A-2.2 (Figure4.20) showed the pattern of the functional group as table 4.4.

Table 4.4 The comparison of IR spectra (KBr disc) of gallic acid and unknown (A-2.2) that isolate from longan seed.

Functional groups	Vibration	Wave number (cm^{-1})	
		Gallic acid **[52]**	Unknown
-OH	OH-stretching	3367.74	3363.74
-CH	CH-stretching	3064	3064.53
C=O	C=O-stretching	1702	1702
C=C	C=C- stretching	1541	1540.88
C-O	C-O-bending	1246	1247.48

The ^{1}H NMR and ^{13}C NMR (Figure 4.21, 4.22 and 4.23) showed the assignment as table 4.5.

Table 4.5 The comparison of ^{1}H NMR (300 MHz,DMSO-*d6*) and ^{13}C NMR (125 MHz, DMSO- *d6*) data of **gallic acid** and unknown that isolate from longan seed.

compounds	**Gallic acid** (DMDO-*d6*) **[54]**			Unknown isolated from longan seed (DMSO-*d6*)		
Position	δ *C* (ppm)	δ *H* (ppm)	*J* (Hz)	δ *C* (ppm)	δ *H* (ppm)	*J*(Hz)
1	120.3			120.440		
2,6	108.5	6.90(s,2H)		108.724	6.904(s)	
3,5	145.2	9.21		145.145	9.165 (s)	
4	137.8	8.73		137.997	8.807 (br s)	
7	167.2			167.476	12.203 (br s)	

All data proposed that **A-2.2** was the gallic acid (Figure 4.2).

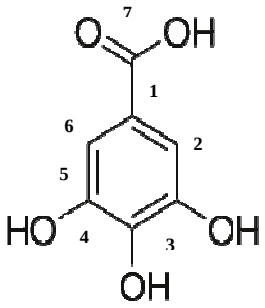


Figure 4.2 The structure of A-2.2 (Gallic acid).

4.3.2.3) Compound A-2.1.1; Compound A-2.1.1 was white crystal that give positive result with ferric chloride spray reagent (green-black spot). Its melting point was 146-149°C (Melting point of propyl gallte is 148-151°C **[59]**). The IR spectrum of A-2.1.1 (Figure4.24) showed the pattern of the functional group as table 4.6.

Table 4.6 The IR spectra (KBr disc) of unknown (A-2.1.1) that isolate from longan seed.

Functional groups	vibration	Wave number (nm^{-1})
-OH	OH-Stretching	3500-3000
C=O	C=O-Stretching	1700-1690
C=C	C=C-Stretching	1620-1600, 1550-1460
C-O	C-O-Stretching	1190-1330

The ^{1}H NMR and ^{13}C NMR (Figure 4.25, 4.26 and 4.27) showed the assignment as table 4.7.

Table 4.7 The spectra of ^{1}H NMR (300 MHz,DMSO-*d6*) and ^{13}C NMR (125 MHz, DMSO-*d6*) unknown (A-2.1.1) that isolate from longan.

compounds	Unknown isolated from longan seed (DMSO-*d6*)		
Position	δ *C*(ppm)	δ *H*(ppm)	*J*(Hz)
1	119.603		
2,6	108.490	6.944 (s)	
3,5	145.582	9.240 (s)	
4	138.373	8.902 (s)	
7	165.902		
8	65.462	4.101(triplet)	6.5
9	21.725	1.66(sextet)	6.9
10	1.409	0.9(triplet)	7.3

All data proposed that **A-2.1.1** was the propyl gallate (Figure 4.3).

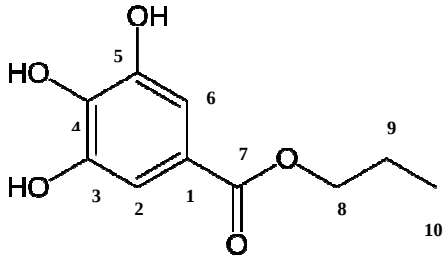


Figure4.3 The structure of A-2.1.1 (Propyl gallate).

4.3.2.4) Compound A-3.2.2; Compound A-3.2.2; Compound A-3.2.2 was yellow crystal that give positive result with anthrone spray reagent. Its melting point was 178-183ºC (melting point of rutin is 174-177 ºC **[60]**). The IR spectrum of A-3.2.2 (Figure4.28) showed the pattern of the functional group as table 4.8.

Table 4.8 The comparison of IR spectra (KBr disc) of **rutin** and unknown that isolate from longan seed.

Functional groups	Vibration	Wave number (cm^{-1})	
		Rutin **[52]**	Unknown
-OH	OH-stretching	3423	3426.92
-CH	CH-stretching	2938,2909	2924.40,2854.60
C=O	C=O-stretching	1656	1655.79
C=C	C=C-stretching	1505	1504.47

The ^{1}H NMR and ^{13}C NMR (Figure4.29,4.30,4.31and 4.32) showed the assignment as table 4.9.

Table 4.9 The comparison of ^{1}H NMR (300 MHz,DMSO- *d6*) and ^{13}C NMR (75.4 MHz, DMSO- *d6*) data of **rutin** and unknown that isolate from longan seed.

compounds	Rutin (DMSO-*d6*) **[55]**			Unknown isolated from longan seed (DMSO-*d6*)		
Position	δ *C*(ppm)	δ *H* (ppm)	*J*(Hz)	δ *C*(ppm)	δ *H*(ppm)	*J*(Hz)
Aglycone						
2	156.4			156.458		
3	133.3			133.302		
4	177.4			177.369		
5	161.2			161.223		
6	98.7	6.20 (d)	2.1	98.744	6.18	1.95
7	164.1			164.268		
8	93.6	6.39 (d)	2.1	93.637	6.37	2.10
9	156.6			156.610		
10	104.0			103.922		
1’	121.2			121.170		
2’	115.2	7.55 (m)		115.254	7.53	2.4
3’	144.7			144.79		
4’	148.4			148.472		
5’	116.3	6.85(d)	8.9	116.277	6.81	8.0
6’	121.6	7.55(m)		121.606	7.52	2.39
Glucose						
1’’	101.2	5.35(d)	7.3	101.218	5.30	7.511
2’’	74.0			74.090		
3’’	76.3			76.461		
4’’	70.6			70.577		
5’’	75.9			75.914		
6’’	67.0			67.009		
Rhamnose						
1’’’	100.7	4.40(d)	1.2	100.766	4.30	1.2
2’’’	70.4			70.387		
3’’’	70.3			70.014		
4’’’	71.8			71.862		
5’’’	68.2			68.262		
6’’’	17.7	1.00(d)	6.1	17.748	0.9(d)	6.0

All data proposed that **A-3.2.2** was the rutin (Figure 4.4).

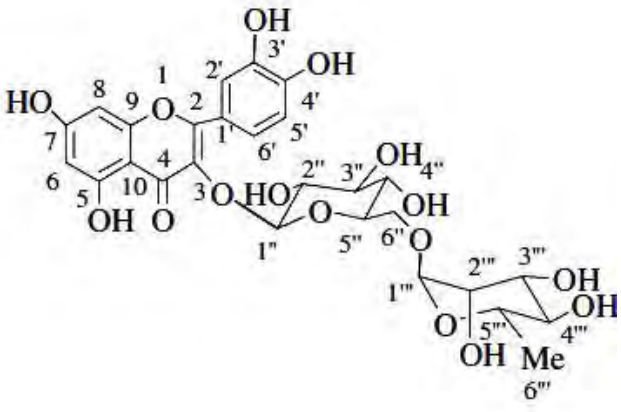


Figure 4.4 The structure of A-3.2.2 (Rutin).

4.4 Determination of percentage purity of rutin

Chromatographic purity of rutin was 77.9 when compared the area of substances to peak area of their standard and calculated by HPLC software.

4.5 Development of HPLC analytical methods

4.5.1 Development of HPLC method: The HPLC condition which give best chromatogram were

Column:	Alltech (Alltima C18, 4.6x150mm,5um)
Mobile phase:	0.5% Formic acid: Acetonitrile
Flow rate:	0.9 ml/min
Mobile phase ration:	30:70 % v/v
Detection :	Photodiode array at 365 nm
Injection quantity:	20 µl

4.5.2 Selection of internal standard

Of all compounds tested for the internal standard, only the hesperidin showed a chromatographic peak appearance in between the peak of rutin and the other peak without interference the other compound, as shown in figure 4.5 Therefore, hesperidin was chosen as internal standard**.**

4.6 Validation of HPLC analytical method.

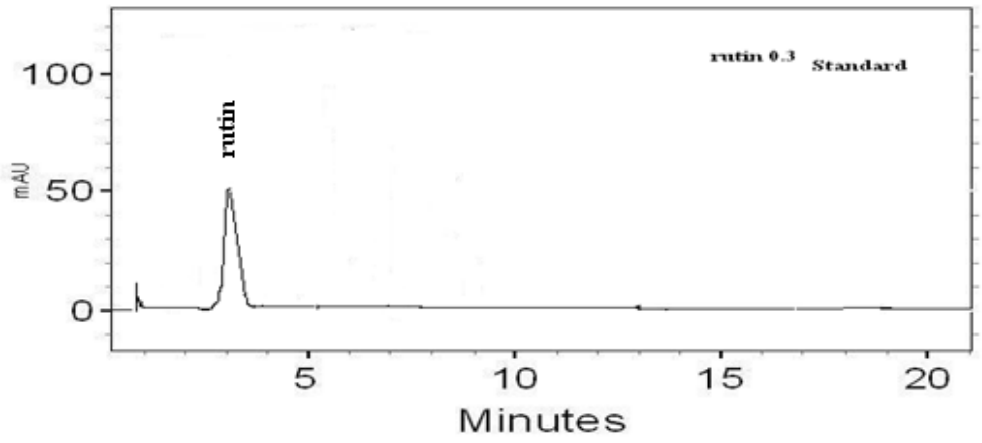
Specificity, linearity, accuracy, precision detection limit and quantification limit were considered in the method validation study.

4.6.1 Specificity

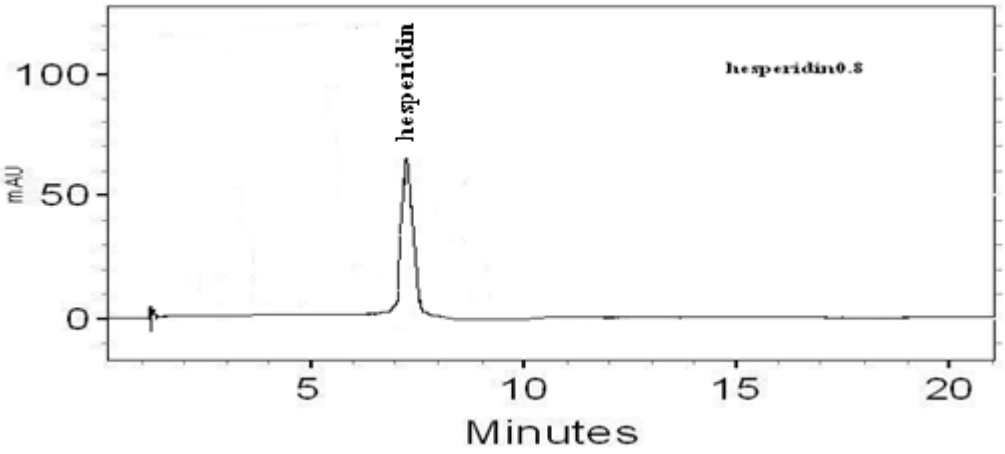
Specificity is the ability to assess unequivocally the analyze in the presence of components which may be expected to be present.

Figure4.5 represented the HPLC chromatogram from high performance chromatography (HPLC) instrument of each compound in standard solution and sample solution. Chromatogram of each compound in sample solution was identical to chromatogram in standard solutions. The chromatographic show retention time of rutin was at 2.97 min while that of hesperidin used as internal standard was at 7.09min.

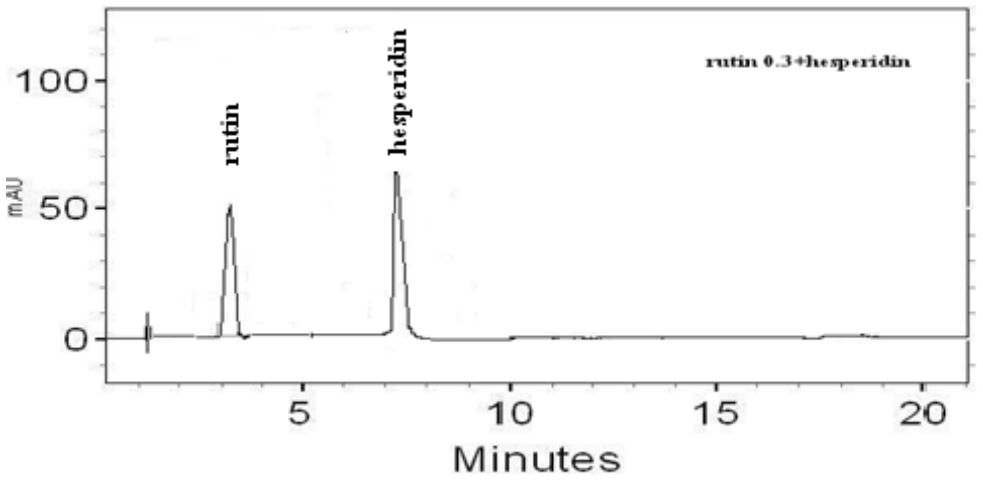
Figure 4.5 HPLC chromatogram of rutin standard **(a)**, IS (hesperidin) **(b),** rutin+IS (hesperidin) **(c),** longan seed extract **(d)** and longan seed extract+IS (hesperidin) **(e)** detected.



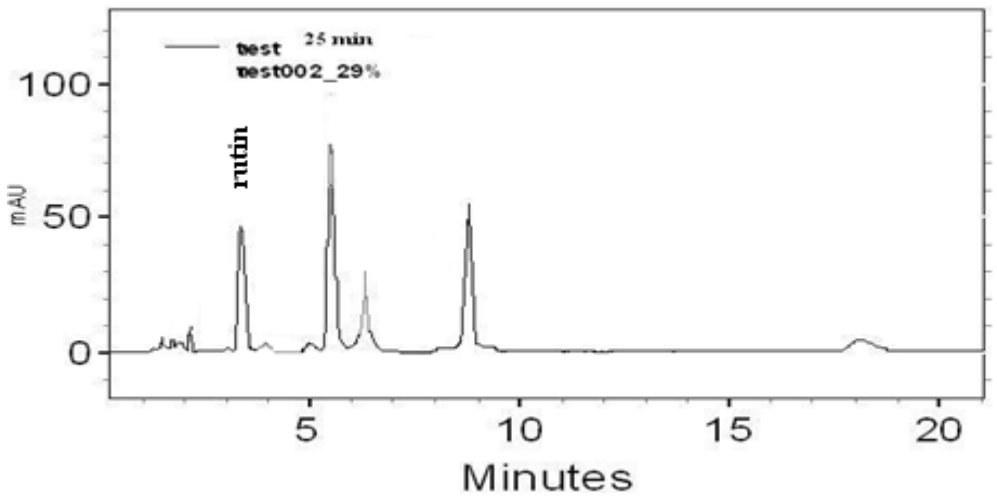
(a)


hesperidin0.8
hesperidin
mAU
100
50
0
5
10
15
20
Minutes

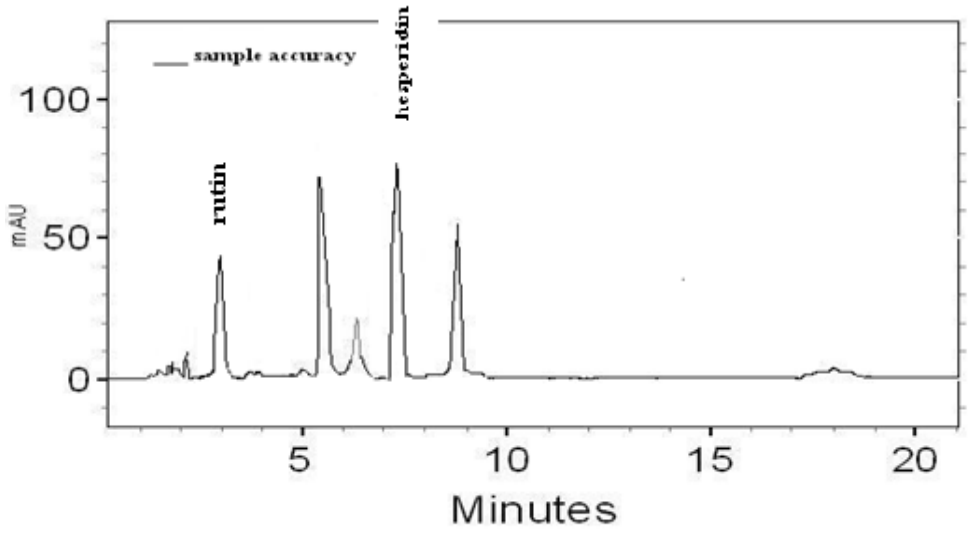
(b)


rutin 0.3+hesperidin
rutin
hesperidin
mAU
100
50
0
5
10
15
20
Minutes

(c)


test 25 min
test002_29%
rutin
mAU
100
50
0
5
10
15
20
Minutes

(d)



(e)

4.6.2 Linearity and range

The standard calibration curve of rutin standard were plot between peak area ratio (rutin/IS) and concentration of standard rutin solutions (Data of table 4.10). The equation coefficient of determination was y = 1.000x+0.095. The equation coefficient of determination (r^2) was 0.9940, as shown in figure 4.6. The concentration of standard calibration curve was in the range 0.31-1.54 µg/ml.

Concentration of rutin (µg/ml) ,CS1-CS5	Peak area of Rutin (n=3)	Peak area of internal std (n=3)	Peak area ratio of rutin and Is (PAR)	
0.3	1364665	3605972	0.3784	
	1363691	3606773	0.3781	0.3783
	1364077	3606092	0.3783	
0.6	2444778	3606799	0.6778	
	2455467	3606659	0.6808	0.6798
	2454999	3606771	0.6807	
0.9	3695300	3606999	1.0245	
	3694279	3606540	1.0243	1.0244
	3694521	3606601	1.0244	
1.2	4846599	3606701	1.3438	
	4849993	3606791	1.3447	1.3444
	4850019	360655	1.3448	
1.5	5964729	3606799	1.6537	
	5964599	3606777	1.6537	1.6537
	5963999	3606741	1.653	

Table 4.10 Data of linearity and range test of HPLC analytical method of rutin.

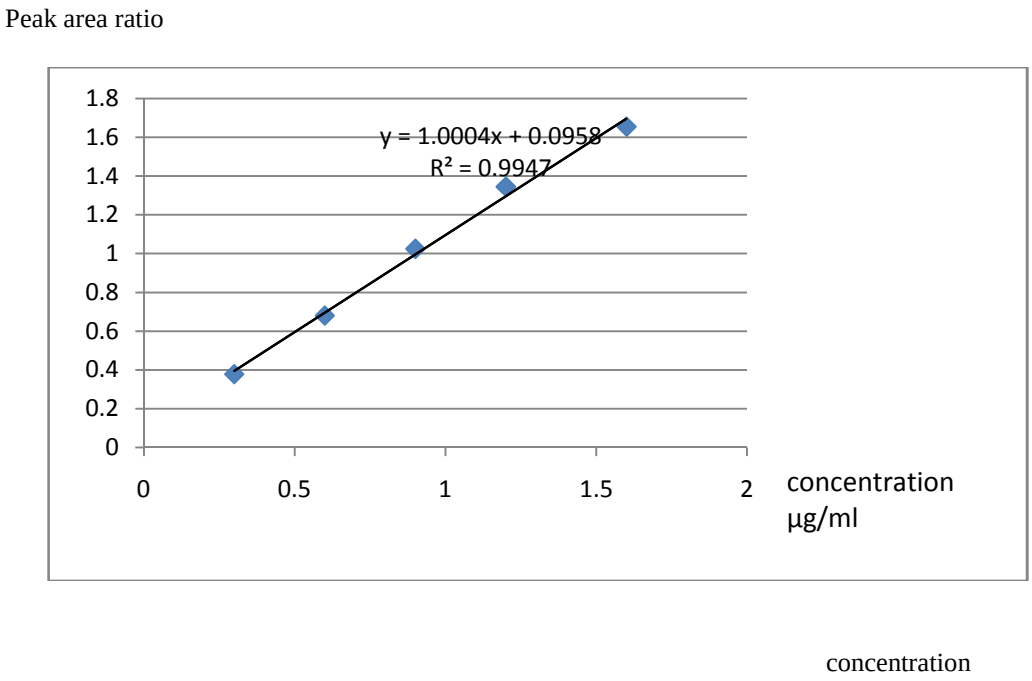


Figure 4.6 Calibration curve for analytical rutin in HPLC.

4.6.3 Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true for accuracy assessment can be obtained in several ways. One alternative is to compare results of the method with results from an established reference method. Secondly, accuracy can be assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material. If such certified reference material is not available, no blank-sample matrix of interest can be spiked with a known concentration by weight or volume, which is selected in this study. After extraction of the analysis from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent.

Accuracy was tested in term of percentage recovery by addition of standard solution in various concentrations. Percent recovery of rutin was in the range of 99.30-101.98 and average of percent recovery was 100.62. As presented in table 4.11. The accuracy test of this method was in the acceptance criteria (96-104 %R). Therefore the method could be used to determine the analytical compounds in longan plant sample.

Table 4.11 Result of accuracy and intra-day precision test of HPLC analytical method

Add concentration (µg/ml)	Items	Sample					
		1	2	3	4	5	6
E1(0.32)	Area(E1-E0)	1328340	1349618	1324248	1370759	1390536	1364075
	% recovery	97.38	98.94	97.08	100.48	101.98	99.99
	%R Average	99.30					
	%RSD	1.89					
E2(0.65)	Area(E2-E0)	2505299	2498926	2521967	2494269	2448923	2475150
	% recovery	102.18	101.92	102.86	101.73	99.88	100.95
	%R Average	101.59					
	%RSD	1.03					
E3(0.93)	Area(E3-E0)	3639595	3765544	3729347	3641441	3726392	3763328
	% recovery	98.51	101.92	100.94	98.56	100.86	101.86
	%R Average	100.44					
	%RSD	1.54					
E4(1.20)	Area(E4-E0)	4848885	4841413	4852440	4782425	4849629	4851340
	% recovery	100.00	99.85	100.07	98.63	100.02	100.05
	%R Average	99.77					
	%RSD	0.57					
E5(1.53)	Area(E5-E0)	6080629	6083538	6072420	6082437	6082245	6092437
	% recovery	101.94	101.99	101.81	101.98	101.98	102.15
	%R Average	101.98					
	%RSD	0.10					
Average of %R		100.62					
Average of %RSD		1.03					

4.6.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision is usually expressed as the variance, relative standard deviation (%RSD) or coefficient of variation of a series of measurements. Precision was considered at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision).

4.6.4.1 Intra-day precision

Relative standard deviation was calculated from six replications of sample preparation in one day at various concentrations. It found that %RSD of rutin was in the range of 0.10-1.89%. Mean %RSD of rutin was 1.03%. As presented in Table 4.11. These relative standard deviations of intra-day precision were in the range of acceptance criteria (%RSD $\leq$ 2). Therefore the method could be used to determine the analytical compounds in longan seed sample.

4.6.4.2 Inter-day precision

Relative standard deviation was calculated from three replications of sample preparation for three days at various concentrations. It found that %RSD of rutin was in range of 0.02-0.40%. Mean %RSD of rutin was 0.13%. As presented in Table 4.12 These relative standard deviations of inter-day precision were in the range of acceptance criteria (%RSD $\leq$ 2). Therefore the method could be used to determine the analytical compounds in longan seed sample.

Added concentration (µg/ml)	Items	Day		
		1	2	3
E1(0.3)	%R	100.00 100.49 98.94	99.99 99.89 99.89	99.89 99.93 99.88
	Average	99.88%		
	%RSD	0.40%		
E2(0.6)	%R	102.19 101.93 101.74	101.90 101.84 102.03	101.91 101.91 101.94
	Average	101.93%		
	%RSD	0.12%		
E3(0.9)	%R	100.94 100.86 101.86	100.91 101.02 100.93	100.99 101.03 100.99
	Average	101.06%		
	%RSD	0.09%		
E4(1.2)	%R	100.00 100.02 100.05	100.01 100.08 100.02	100.04 100.03 100.03
	Average	100.03%		
	%RSD	0.02%		
E5(1.5)	%R	101.95 101.98 101.98	101.98 101.96 101.96	101.95 101.94 101.98
	Average	101.96%		
	%RSD	0.02		
Average of %R		100.97%		
Average of %RSD		0.13%		

Table 4.12 Inter-day precision test of HPLC analytical method

4.6.5 Detection Limit

Detection limit (DL) was determined during the evaluation of the detection of analyze. DL was defined as the lowest concentration that can be detected. The concentration of DL was the same concentration or less than QL. DL of rutin standard solution was resulted 0.13 µg/ml. This concentration can be detected.

4.6.6 Quantitation Limit

Quantitation limit (QL) was determined during the evaluation of the linear range of calibration curve. QL of rutin was resulted 0.38 µg/ml. The result was calculated by parameter that shown in table 4.13.

Table 4.13 Quantitation limit of rutin by HPLC analytical method.

Concentration(µg/ml)	Peak area average		Peak area ratio
	Rutin	hesperidin	
0.23	909777	3605972	0.2523
0.35	1400149	3606773	0.3882
0.43	1854976	3606799	0.5143
0.53	2244784	3606659	0.6224
0.62	2725276	3606771	0.7556
Intercept SD,(σ)	0.050		
Slope (S)	1.289		
r^2	0.997		

4.7 Development of TLC- densitometric analytical methods

4.7.1 Development of TLC-densitometric condition

The anthrone in ethanol was used as spray reagent. Because of the color intensity of spot chromatogram was stable when kept longer than 30 min at room temperature after heat at 120 ^{o}C for 20 min. While color intensity of spot that developed by 20% sulfuric acid in methanol was unstable.

TLC combined with densitometer was selected to determine rutin in the longan seed sample by using butanol-glacial acetic acid (9:3) as developing solvent. Figure 4.13 TLC densitograms of standard rutin, IS and sample of longan seed extract in the TLC-densitometric analytical method.

4.7.2 Selection of internal standard

Of all compounds tested for the internal standard, only the hesperidin showed a spot appearance in between the spot of rutin and the other spot without interference the other compound, as shown in figure4.7 Therefore, rutin was chosen as internal standard.

4.8 Validation of TLC analytical method.

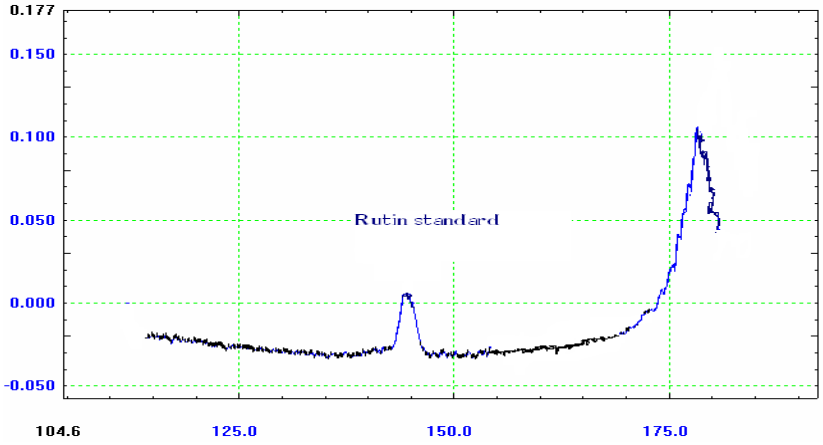
Specificity, linearity and range, accuracy, precision detection limit, quantitation limit were considered in the method validation study.

4.8.1 Specificity

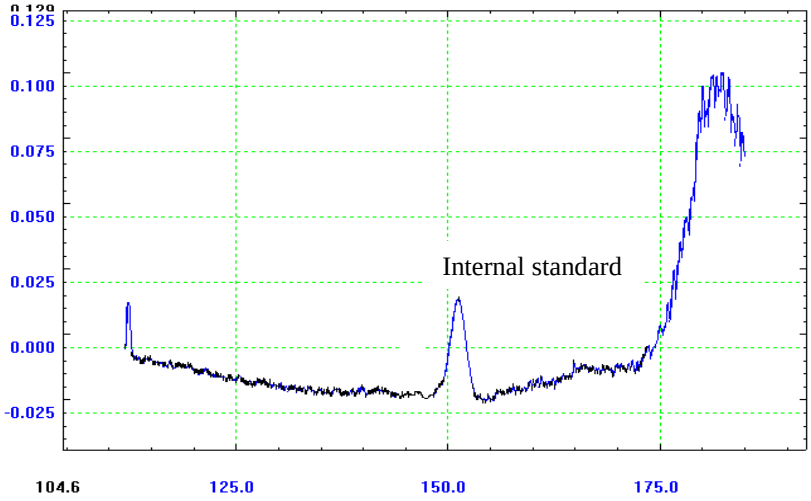
Specificity is the ability to assess unequivocally the analysis in the presence of components which may be expected to be present.

Figure 4.7 represented the TLC densitogram from TLC-densitometer of each compound in standard solution and sample solution. Densitogram of each compound in sample solution was identical to chromatogram in standard solutions.

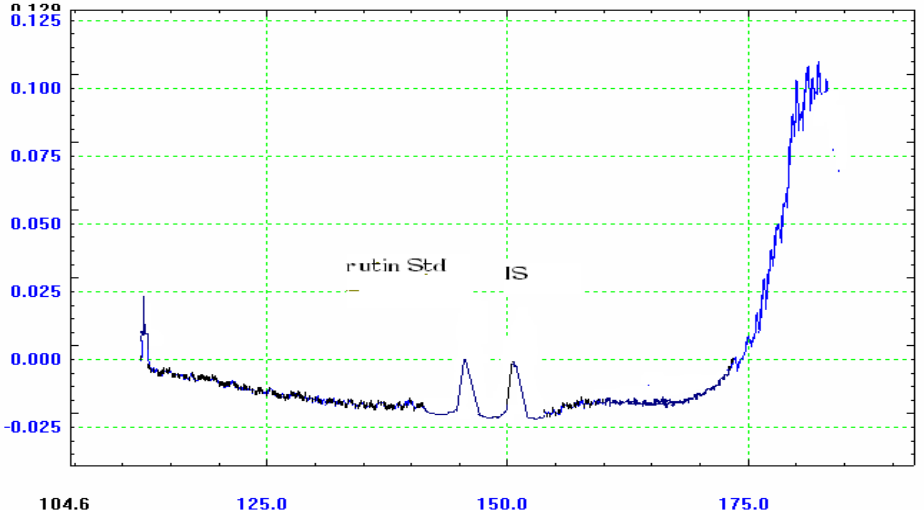
Figure 4.7 TLC densitogram of rutin standard **(a)**, hesperidin internal standard (IS) **(b)**, rutin+ IS **(c)**, longan seed extract **(d)** and longan seed extract + IS (**e)** detected by TLC- densitometer.



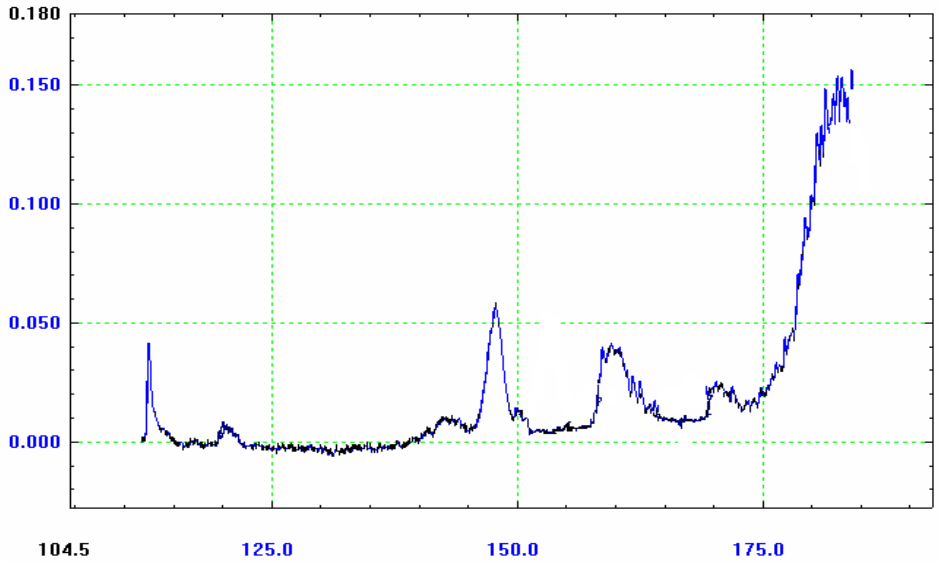
a



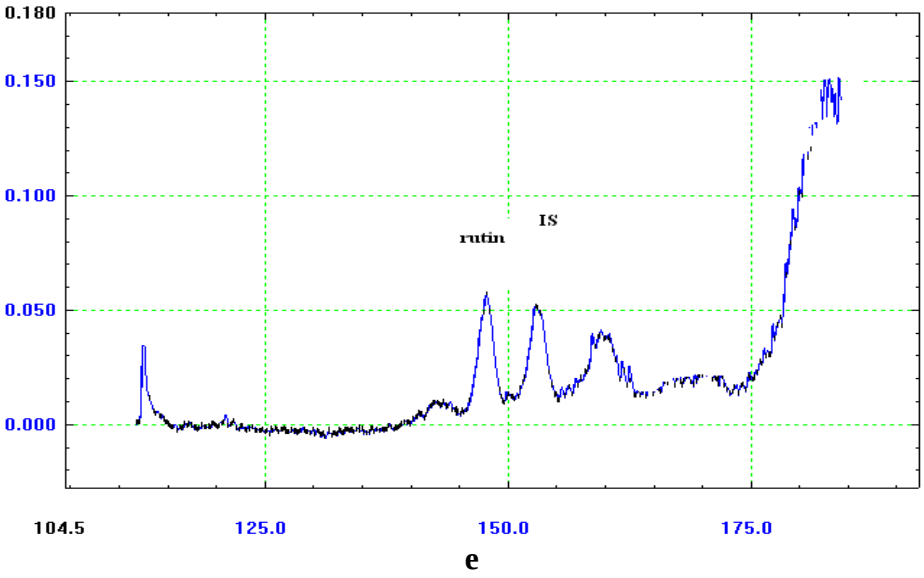
b


0.125
0.100
0.075
0.050
0.025
0.000
-0.025
rutin Std
IS
104.6
125.0
150.0
175.0

c


0.180
0.150
0.100
0.050
0.000
104.5
125.0
150.0
175.0

d



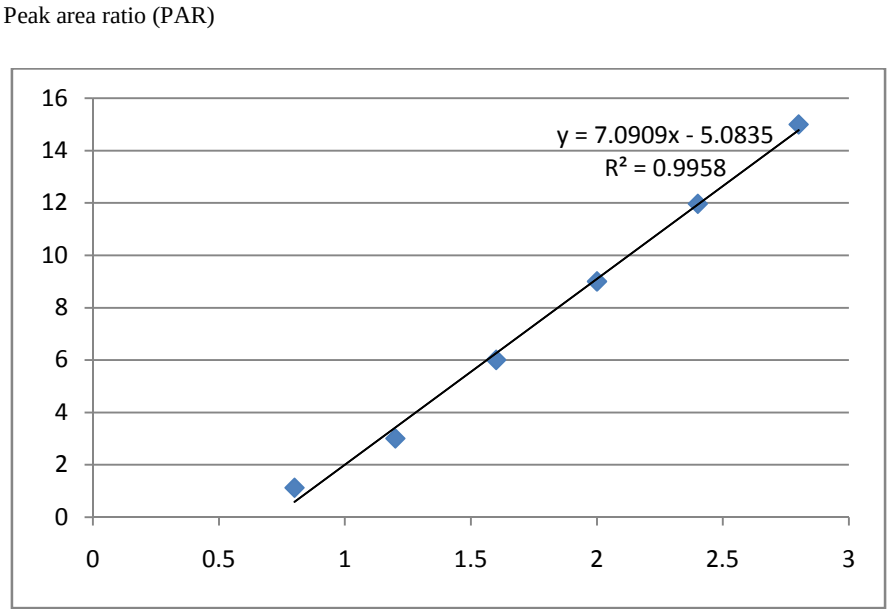
4.8.2 Linearity and range

The standard calibration curve of rutin standard was plot between peak area ratio (rutin/IS) and concentration of standard rutin solutions (Data of table 4.14). The equation coefficient of determination was y = 7.090X-5.083. The equation coefficient of determination (r^2) was 0.995, as shown in Figure4.8.The concentration of standard calibration curve was in the range 0.82-2.83 mg/ml.

Table 4.14 Data of linearity and range test of TLC- densitometric analytical method of rutin.

Concentration of rutin (mg/ml) ,CSs_1-CSs_6	Peak area of Rutin (n=3)	Peak area of internal std (n=3)	Ratio of Peak area of rutin and Is	
0.8	4.801	4.288	1.120	1.120
	4.803	4.281	1.122	
	4.800	4.290	1.119	
1.2	12.849	4.284	2.999	2.999
	12.840	4.278	3.001	
	12.851	4.289	2.996	
1.6	25.689	4.283	5.998	6.002
	25.695	4.281	6.002	
	25.678	4.275	6.007	
2.0	38.520	4.279	9.002	8.999
	38.540	4.284	8.996	
	38.533	4.282	8.999	
2.4	51.372	4.288	11.980	11.964
	51.390	4.281	12.004	
	51.375	4.282	11.998	
2.8	64.215	4.285	14.986	14.996
	64.235	4.280	15.008	
	64.218	4.283	14.994	

Figure 4.8 Calibration curve for analytical rutin in TLC-densitometric analytical method



4.8.3 Accuracy

Accuracy was tested in term of percentage recovery by addition of standard solution in various concentrations. Percent recovery of rutin was in the range of 98.41-100.46 and average of percent recovery was 99.45. As presented in table 4.15. The accuracy test of this method was in the acceptance criteria (96-104 %R). Therefore the method could be used to determine the analytical compounds in longan sample

Table 4.15 Result of accuracy and intra-day precision test of TLC-densitometric analytical method of rutin

Conc (mg/ml)	Items	Number of sample					
		1	2	3	4	5	6
0.814	Peak area (SE_1-SE_0)	4.790	4.691	4.781	4.697	4.699	4.693
	%recovery (%R)	99.77	97.71	99.58	97.83	97.87	97.75
	Average	98.41					
	%RSD	0.76					
1.211	Peak area (SE_2-SE_0)	13.010	12.909	12.899	12.867	12.847	12.900
	%recovery	101.28	100.49	100.41	100.16	100.01	100.42
	Average	100.46					
	%RSD	0.44					
1.620	Peak area(SE_3-SE_0)	25.599	25.610	25.907	25.688	25.790	25.799
	%recovery	99.66	99.700	100.86	100.00	100.40	100.44
	Average	100.18					
	%RSD	0.47					
2.014	Peak area(SE_4-SE_0)	38.454	37.990	37.540	38.499	37.491	38.473
	%recovery	99.800	98.60	97.43	99.91	97.30	99.85
	Average	98.82					
	%RSD	1.24					
2.406	Peak area(SE_5-SE_0)	50.899	50.801	51.019	51.299	51.389	51.018
	%recovery	99.07	98.88	99.3	99.84	100.02	99.3
	Average	99.40					
	%RSD	0.44					
Average of %recovery		99.45					
Average of % RSD		0.67					

\

4.8.4 Precision

4.8.4.1 Intra-day precision

Relative standard deviation was calculated from six replications of sample preparation in one day at various concentrations. It found that %RSD of rutin was in the range of 0.44-1.24%. Mean %RSD of rutin was 0.67%. As presented in table 4.15. These relative standard deviations of intra-day precision were in the range of acceptance criteria (%RSD $\leq$ 2). Therefore the method could be used to determine the analytical compounds in longan seed sample.

4.8.4.2 Inter-day precision

Relative standard deviation was calculated from three replications of sample preparation for three days at various concentrations. It found that %RSD of rutin was in range of 0.12-0.57%. Mean %RSD of rutin was 0.30%. As presented in table 4.16. These relative standard deviations of inter-day precision were in the range of acceptance criteria (%RSD $\leq$ 2). Therefore the method could be used to determine the analytical compounds in longan seed sample.

Table 4.16 Inter-day precision test of TLC-densitometric analytical method of rutin.

Added concentration (mg/ml)	Items	Day		
		1	2	3
SE_1 (0.814)	Peak area	4.790	4.617	4.965
	%Recovery	99.77	96.17	103.42
	Average	99.79		
	%RSD	0.18		
SE_2 (1.211)	Peak area	13.010	13.814	12.786
	%Recovery	101.28	107.54	99.53
	Average	102.78		
	%RSD	0.53		
SE_3 (1.620)	Peak area	25.599	25.376	25.509
	%Recovery	99.66	98.79	99.31
	Average	99.25		
	%RSD	0.12		
SE_4 (2.014)	Peak area	38.454	38.219	38.398
	%Recovery	100.04	99.19	99.65
	Average	99.63		
	%RSD	0.12		
SE_5 (2.406)	Peak area	50.899	49.998	51.003
	%Recovery	99.07	97.31	99.27
	Average	98.55		
	%RSD	0.57		
Average of %recovery		100.00		
Average of %RSD		0.304		

4.8.5 Detection Limit

Detection limit (DL) was determined during the evaluation of the detection of analyze. DL was defined as the lowest concentration that can be detected. The concentration of DL was the same concentration or less than QL. DL of rutin standard solution was resulted 0.21mg/ml. This concentration can be detected.

4.8.6 Quantitation Limit

Quantitation limit (QL) was determined during the evaluation of the linear range of calibration curve. QL of rutin was resulted 0.62mg/ml. The result was calculated by parameter that shown in table 4.17.

Table 4.17 Quantitation limit of rutin by TLC analytical method.

Concentration(µg/ml)	Peak area average		Peak area ratio
	Rutin	hesperidin	
0.52	3.000	4.301	0.6975
1.1	5.991	4.298	1.3939
1.56	8.898	4.298	2.0700
2.0	12.111	4.300	2.8165
2.53	14.917	4.295	3.4707
Intercept SD,(σ)	0.088		
Slope (S)	1.412		
r^2	0.996		

4.9 Determination of rutin contents in various longan seed sample

Three longan seed samples (LG1, LG2 and LG3) were collected on june,2008, Chiangmai province. Each sample was quantitative determined for rutin by HPLC. The analytical system as in content 3.6 and percentage contents of this compound was shown in table 4.18

Table4.18 The percentage contents of longan seed sample

Longan sample	Data of extraction content, %w/w of rutin	Mean±SD
LG1	0.1762, 0.1739, 0.1776	0.1759±0.002
LG2	0.1866, 0.1901, 0.1883	0.1883±0.002
LG3	0.1801, 0.1789, 0.1802	0.1797±0.001
Average of percentage content	0.1813±0.002	

From the data obtained, rutin in each longan seed sample was found in the range of 0.1759-0.1883%w/w with average at 0.1813±0.002%w/w. The maximum content was observed in LG2. The results show percentage contents was not significantly from those of the extraction group.

4.10 Determination of rutin content in standardized extract from fraction B-4 (scheme 3.2)

The percentage contents of this compound was shown in table 4.19

Table4.19 The percentage contents of standardized extract sample

Longan sample	Data of extraction content, %w/w of rutin	Mean±SD
B-4 No.1	0.2201, 0.2198, 0.2200	0.2199±0.000
B-4 No.2	0.2199, 0.2201, 0.2198	0.2199±0.000
B-4 No.3	0.2200, 0.2202, 0.2200	0.2201±0.000
Average of percentage content	0.2200±0.000	

From the data obtained, rutin in each modified longan seed sample was found in the range of 0.2199-0.2201%w/w with average at 0.2200±0.000%w/w. The maximum content was observed in B-4 No.3. The results show percentage contents was not significantly from those of the extraction group.

CHAPTER V

CONCLUSION

5.1 Extraction, isolation and preparation the standardized herbal extract from longan seed.

Several studies shown longan seed extract would be a new source of anti-amnesic. However, there is limited information the pharmacologically chemical marker compound and the processes employed to prepare the standardized herbal extracts. The purpose of the standardization of herb extract is to reproduce the chemical consistency from batch to batch which is essential for further pre-clinical and clinical studies as well as product development.

Four compounds quercetin, gallic acid, propyl gallate and rutin were obtained from longan seed. Base on the anti-amnesic activities using passive avoidance task and marris water maze test in 2-VO mice model, rutin was the one of the active principles at dose 100mg/kg. Additionally, the previous study confirm that rutin may potential prove to have a neuroprotective effect and revealed protective the memory impairment**.** Kishore and Singh reported that rutin, a bioflavonoid and a potent antioxidant or free radical scavenging is found to improve learning and memory of normal mice with no cognitive deficits in the doses of 30mg and 40mg **[**61**].** Rutin (50mg/kg, single injection, intra-peritoneal) prevented the scopolamine-induced memory impairment in zebrafish and no affected general locomotor activity **[**62**]**. Rutin has a protective effect on trimethyltin-induced memory dysfunction in rats and rutin could be attributable to

inhibitory effect against microglial activation and its role in synapse formation via neurotropic factors in hippocampus [46]. Both rutin and quercetin improved spatial memory impairment in the 8-arm radial maze task and neuronal death in the hippocampal CA1 area [47].

The previous studies have shown that longan seed contains many tannin compounds such as gallic acid, ellagic acid and corilagin . The photo-degradation of the most representative phenolic compound such as gallic acid[63]. Generally, tannin develops the oxidative browning reactions during processing and storage [64]. Rutin is a more stable against heat and oxidation than tannin compounds [65]. Moreover, screening HPLC analysis of the longan seed extracts showed that rutin present in high quantity. All these studies suggest that rutin possess the anti-amnesic compounds and suitable used as a marker compound for standardization in this study.

Therefore, this work shows a possible treatment to remove the probletic compounds fraction of sample extraction. Acid-base extraction is applied to separate unwanted entities (e.g., gallic acid and propyl gallate) from the standardized herbal extract.

5.2 High performance liquid chromatography analytical method (HPLC analytical method).

HPLC method was used to determine of rutin in longan seed. The optimum conditions were comprised of C-18 reverse phase (Alltima® column 5µm, 4.6×150 mm i.d.) isocratic elution with 0.5%formic acid and acetonitrile (30:70%v/v) as the mobile phase and photodiode array detection at 365 nm. The retention time of rutin was at 2.97 min while that of hesperidin used as internal standard was at 7.09 min.

Method validation was studied with, specificity, linearity and range, accuracy, precision, detection limit and quantitation limit (according to ICH guideline). The accuracy presented as percent recovery was found in the range of 99.30-101.98%. The intra-plate precisions measured as %RSD was found less than 2. The linearity was determined that amount range of 0.31-1.54 µg/ml. The good linearity was measured as linear regression (r^2) ≥ 0.99 with insignificant intercept from origin. The QL of rutin was determined 0.38µg/ml and DL was 0.13µg/ml in this condition.

The data of HPLC analytical method validation was shown in table 4.19**a** all parameters were found in the acceptable limit ranges.

5.3 Thin layer chromatographic densitometric analytical method (TLC densitometric analytical method)

TLC method combined with densitometer was used to determinate of rutin in longan seed by using butanol and glacial acetic acid (9:3) as developing solvent. The developing TLC plate was sprayed with anthrone in ethanol and heated at 120°C for 20 min. The plate was kept at room temperature for 10 min and detected with densitometer for wavelength 365 nm.

Method validation was studied with, specificity, linearity and range, accuracy, precision, detection limit and quantitation limit (according to ICH guideline). The accuracy presented as percent recovery was found in the range of 98.41-100.46%. The intra-plate precisions measured as %RSD was found less than 2. The linearity was determined by spotting the solution, that amount range of 0.81-2.83 mg/ml. The good linearity was measured as linear regression (r^2) ≥ 0.99 with insignificant intercept from origin. The QL of rutin was determined 0.62mg/ml and DL was 0.21mg/ml in this condition.

The data of TLC analytical method validation was shown in table 4.19**b** all parameters were found in the acceptable limit ranges.

5.4 Determination of rutin in various longan seed sample

The present study has revealed the average of percentage content of active principle compound in dried ground plant of various samples was 0.1813±0.002%w/w. The maximum contents rutin that obtained from longan sample was 0.1883±0.002%w/w and minimum was 0.1759±0.002%w/w. the percentage contents was not significantly from those of the extracted group.

Rutin in each standardized extract sample was found in the range of 0.2199-0.2201%w/w with average at 0.2200±0.000%w/w. The maximum content was observed in B-4 No.3. The results show percentage contents was not significantly from those of the extraction group.

The study was to developed the standardized herbal extract and rutin posses the anti-amnesic compounds and suit used as a marker compound. Further work is needed to completely determine the concentration of chemical marker in different plant samples (e.g., cultivars, collection and storage) and the information on chemical constituents be further used as the criteria for plant variety selection.

REFERENCES

[1] Wang, Y.W., Zhou, H.D., and Zhou, X.F. 2006. Clearance of amyloid-beta in Alzheimer's disease: progress, problems and perspectives. **Drug discovery today**.11: 931-938.

[2] Alzheimer's disease facts and figures. 2007. **Alzheimer's association**: 2-30.

[3] FDA-Approved treatments for Alzheimer's disease. 2007. **Alzheimer's association**: 3.

[4] Col, S.L., and Vassar, R. 2007. The Alzheimer's disease β-secretase enzyme, BACEI, **Molecular Neurodegeneration**: 1-25.

[5] Alzheimer's disease Facts and Figures. 2009. **Alzheimer's association**: 6-80.

[6] Delagarza, V.W. 2003. Pharmacologic Treatment of Alzheimer's disease. An Update. **American Family Physician** 68: 1365-1372.

[7] Horne, S. 2010. Using herb that enhance acethylcholine is a "bright" idea. **Holistic Health Journal**.

[8] Limmutavapirat, C. 2005. Longan *Dimocarpus longan* Lour. .**Thai journal of pharmacy**:12-31.

[9] Massieu, L., Moran, J., and Christen, Y. 2004. Effect of *Ginkgo biloba* (EGb 761) on staurosporine induced neuronal death and caspase activity in cortical cultured neuron. **Brain Res**. 1002:76-85.

[10] Morton, J. 1987. Longan. **Fruits of warm climates**: 259-262.

[11] *Dimocarpus longan* (Sapindaceae) [online]. 2007. Available from: http://www.montosogardens.com/dimocarpus_longan.htm [2011, April 8]

[12] Rangkadilok, N., *et al*. 2005. Polyphenolic compounds in longan fruit and their pharmacological activities. **31st Congress on science and technology of Thailand**: 1-4.

[13] Oh, M.S., Hus, Y., and Bae, H. 2005. The multi-herbal formula guibitang enhances memory and increases cell proliferation in the rat hippocampus. **Neuroscience Letters** 379, 105-208.

[14] Hsu, F.L., Lu, F.H., and Cheng, J.T. 1994. Influence of acetonylgeraniin, a hydrolysable tannin from *Euphoria longana*, on orthostatic hypotension in a rat model. **Planta Medica** 60, 297-300.

[16] Cheng, J.T., Lin, T.C., and Hsu, F.L. 1995. Antihypertensive effect of corilagin in the rat. **Canadian Journal of Physiology and Pharmacology** 73, 1425-1429.

[17] China Pharmacopoeia Committee, 2005. Pharmacopoeia of the People's Republic of China, eighth ed. **People's Medical Publishing House**, Beijing.

[18] Rangkadilok, N., *et al*. 2007. Evaluation of free radical scavenging and antityrosinase activities of standardized longan fruit extract. **Food and Chemical Toxicology** 45: 328-336.

[19] Jaitrong, S., Rattanapanone, N., and Manthey, J.A. 2006. Analysis of the phenolic compounds in longan (*Dimocarpus longan* Lour.) peel. **Proc. Fla. State Hort. Soc** 119:317-375.

[20] Hsieh, M.C., *et al*. 2008. Antioxidative activity and active components of longan (*Dimocarpus longan* Lour) flower extracts. **J. Agric. Food. Chem**. 56:7010-7016**.**

[21] Lapsongphol, S., and Mahayothee, B. 2007. Characterization of volatile compounds of fresh and dried longan using headspace-solid phase microextraction combined with GC-MS. **The 9th Agro-Industrial Conference: Food Innovation Asia 2007: "Q" Food for Good Life**: 1-8.

[22] Kleiman, R., Earle, F.R., and wolff, I.A. 1968. Dihydrosterculic acid, a major fatty acid component of *Euphoria longana* seed oil.**The AOCS-AACC Joint Meeting Washington**: 317.

[23] Losuwanarak, N., *et al*. 2009. Effect of longan seed on scopolamine-induced learning and memory deficit in mice.**Thai J. Pharm. Sci 33:31-38.**

[24] Park, S.J., *et al*. 2010. The memory-enhancing effects of Euphoria longan fruit extract in mice. Journal of **Ethno pharmacology** 128: 160-165.

[25]Yang, B., Zhao, M., and Jiang, Y. 2009. Anti-glycated activity of polysaccharides of longan (*Dimocarpus longan* Lour.) fruit pericarp treated by ultrasonic wave. **Food chemistry** 114: 629-633.

[26] Rangkadilok, N., *et al*. 2005. Identification and quantification of polyphenolic compounds in longan (*Euphoria longana* Lam.) fruit. **J. Agric. Food Chem** 53: 1387-1392.

[27] Albury, A.The development of pharmacological treatments for the neurodegenerative disorder Alzheimer's disease [Online].2009.Available from: http://www.scribd.com/doc/24609353/Alzheimer-s-Disease [2011, April,7].

[28] Springer, K. and Criscuolo, C.L. Effects of Alzheimer's Disease on the human brain [Online].2009. Available form: http: http://review-of-current-research.stsd.wikispaces.net/file/view/Effects+of+Alzheimer%E2%80%99s+Disease+on+the+Human+Brain.pdf [2011, April,9].

[29] Basics of Alzheimer's disease What it is and What you can do.2010. **Alzheimer's association**: 5-32.

[30] Simon, H. Alzheimer's disease [Online].2003. Available from: http:// www.alzheimers.org/trials/index.html[2011, April,8].

[31] Markesberg, W.R. 1996. Oxidative Stress Hypothesis in Alzheimer's disease. **Free Radical Biology & Medicine** 23(1): 134-147.

[32] Shor, T.J., *et al*. 2001. Neurogenesis in the adult is involved in the formation of trace memories. **Nature** 410: 372-376.

[33] Dirnagl, U., Ladecola, C., and Moskowitz, M.A.1999. Pathology of ischeamic stroke: an integrated view. **Trends Neuroscience** 22:391-397.

[34] White, B.C., *et al*. 2000. Brain ischemia and referfusion : molecular mechanisms of neuronal injury. **Journal of the Neurological Sciences** 179:1-33.

[35] Moro, M.A., *et al*. 2004. Role of nitric oxide after brain ischemia. **Cell Calcium** 36:265-275.

[36] Adibhatla, R.M., and Hatcher, J.F. 2006. Phospholipase A_2, reactive oxygen species and lipid peroxidation in cerebral ischemia. **Free radical Biology and Medicine** 40 : 376-387.

[37] Ladecola, C. 1997. Bring and Dark sides of nitric oxide in ischemic brain injury. **Trends Neurosciences** 20: 132-139.

[38] Chan, S.A., *et al*. 1998. Fosphenytoin reduce hippocampus neuronal damage in rat following transient global ischemia. **Acta Neurochirurgica** 140: 175-180.

[39] De Keyser, J., Sulter, G.and Luiten, P.G. 1999. Clinical trials with neuroprotective drug in acute-ischemic stroke: are we doing the night thing? . **Trends in Neuroscience** 22: 535-540.

[40] Traystman, R.J. 2003. Animal models of focal and global cerebral ischemia. **National Research Council Institute of Laboratory Animal Resources Journal** 44: 85-95.

[41] Block, F. 1999. Global ischemia and behavioral deficits. **Progress in neurobiology** 38: 279-295

[42] Block, F. and Schwarz, M. 1998. Global neuronal damage relates to behavioral deficit. **A pharmacological approach Neuroscience** 82:781-803.

[43] Rossato, J.I., *et al*. 2006. A link between the hippocampus and the striatal memory systems of the brain. **Annals of the Brazillian academy of sciences** 78: 515-523.

[44] D' Hooge, R. and De Deyn, P.P. 2001. Applications of the morris water maze in the study of learning and memory. **Brain Research Review** 36: 60-90.

[45] Vohora, D. and Pillai, K.K. 2000. Effect of locomotor activity on the passive avoidance that for the evaluation of cognitive function. **Indian Journal of Pharmacology** 32: 242-245.

[46] Koda, T., Kuroda, Y. and Imai, H. 2009. Rutin supplementation in the diet has protective effects against toxicant-induced hippocampal injury by suppression of microglial activation and pro-inflammatory cytokines. **Cell Mol Neurobiol** 29: 523-531.

[47] Pu, F., *et al* . 2007. Neuroprotective effects of quercetin and rutin on spatial memory impairment in an 8-Arm Radial Maze Task and neuronal death induced by repeated cerebral ischemia in rats. **J Pharmacol science** 104: 329-334.

[48] Lee, M.H., *et al*. 2001. Monoamine Oxidase B and Free Radical Scavenging Activities of Natural Flavonoids in *Melastoma candidum* D. Dun. **J. Agric Food Chem** 49: 5551-5555.

[49] Torel, J., Cillard, J. and Cillard, P. 1986. Antioxidant activity of flavonoids and reactivity with peroxy radical. **Phytochemistry** 25(2): 383-385.

[50]Yoting, C., *et al*. 1990. Flavonoids as superoxide scavengers and antioxidants. **Free radical biology and medicine** 9: 11-21.

[51] Sánchez-Moreno, C., Larrauri, J.A. and Sauracalixto. 1999. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape

juices and related polyphenolic constituents. **Food research international** 32: 407-412.

[52] Nayeem, N. and MD, K. 2010. Isolation of phenolic compounds from the methanolic extract of *Tectona grandis.* kanataka, India. Research Journal of pharmaceutical, **Biological and Chemistry** 1(2): 221-225.

[53] Han, X.H., *et al*.2007. Monoamine Oxidase Inhibitory Compounds from *Cayratia japonica*. **Arch Pharm Res** 30(1) : 13-17.

[54] Oh, S.O., *et al*. 2008. Antifungal Activity of Eucalyptus-derived phenolic against postharvest pathogens of Kiwifruits. **Plant Pathol.J.** 24(3): 322-327.

[55] Duangpen Pattamadilok.,2007.**Phytochemical study Canavalia rosea and Elateriospermum tapos**. Master ‘s thesis, Faculty of Pharmaceutical Science, Chulalongkorn University.

[56] Oraphan Wanakhachonkrai.,2006. **Effects of N-(2-propylpentanoyl) urea on impairment of learning memory and neuronal cell death after bilateral common carotid arteries occlusion in mice**. Master ‘s thesis, Faculty of Pharmaceutical Science, Chulalongkorn University.

[57] Sharma, A. and Gupta, H. 2010. Quercetin-A Flavonoid. **Chron Young Sci** 1:10-15.

[58] Gallic acid [Online].2003.Available from: http://www.reagent.com[2011, April,7]

[59] Propyl gallate.2005. **European Pharmacopoeia** 5:2325.

[60] Ma, B., *et al*. 2009. Flavonol glycosides and triterpenes from the leave of *Uncaria rhynchophylla* (Miq) Jack. **Asian Journal of Traditional Medicines** 4: 85-91.

[61] Kishore, K. and Singh, M. 2005. Rutin (a bioflavonoid antioxidant) attenuates age-ralated memory deficits in mice. **IndMedica** 16:6-10.

[62] Richetti, S.K., *et al*. 2011. Quercetin and Rutin prevent scopolamine-induced memory impairment in zebrafish. **Behav Brain Res** 217:10-15.

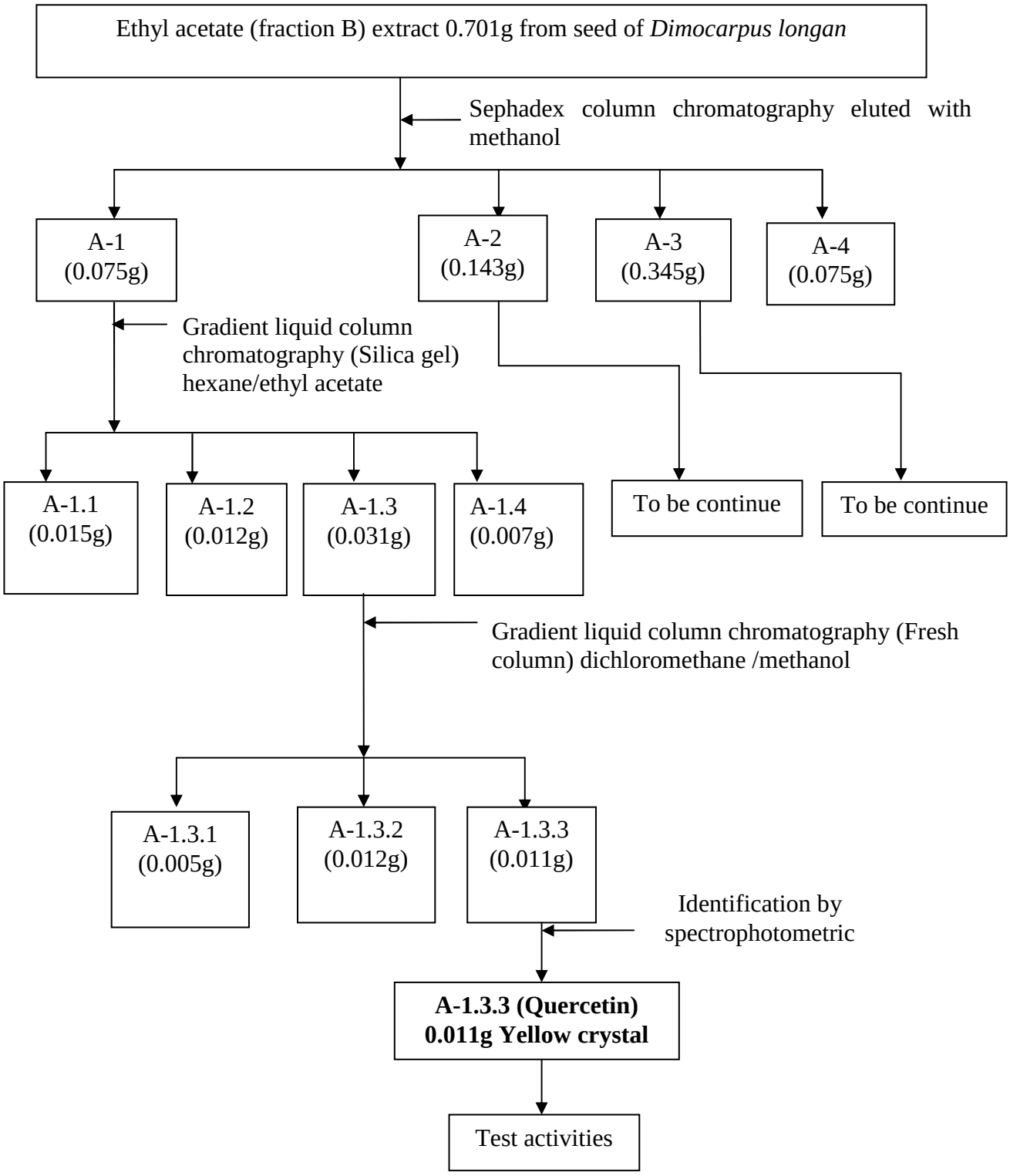
[63] Lucas M.S., *et al*. 2008.Gallic acid photochemical oxidation as a model compound of winery wastewaters. **J Environ Sci Health** 43: 1288-1295.

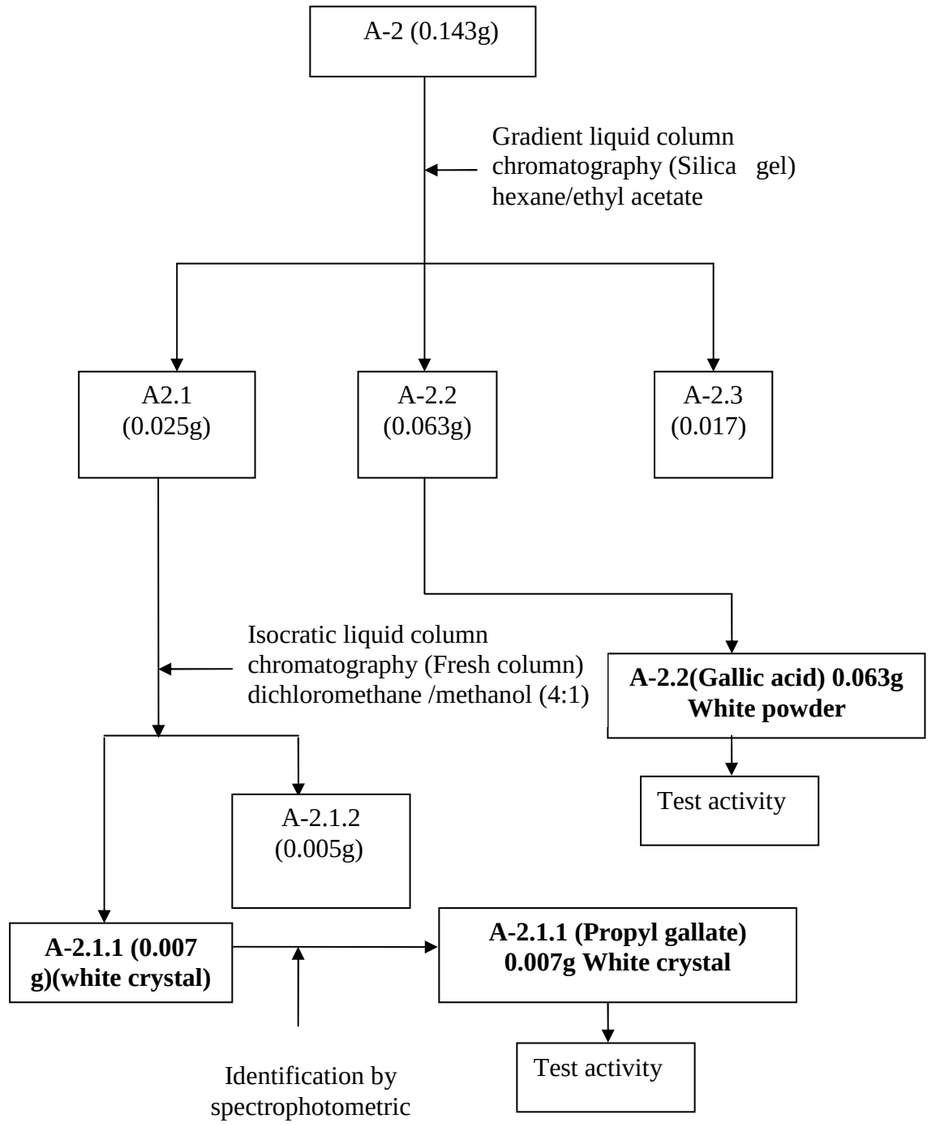
[64] Hui Y.H., *et al*. 2006.Tannins. **Handbook of fruit and fruit processing**: 400.

[65] Dharmananda S**.** EMBLIC MYROBALANS: AMLA Key herb of Ayurvedic medicine [online]. Available from: http://www.itmonline.org/arts/amla.htm. [2011, April,7]

APPENDIX

Scheme 3.4 Isolation scheme for the positive effect on memory impairment and neuronal cell death after bilateral carotid arteries occlusion from longan seed




A-2 (0.143g)
Gradient liquid column chromatography (Silica gel) hexane/ethyl acetate
A2.1 (0.025g)
A-2.2 (0.063g)
A-2.3 (0.017)
Isocratic liquid column chromatography (Fresh column) dichloromethane /methanol (4:1)
A-2.2(Gallic acid) 0.063g White powder
Test activity
A-2.1.2 (0.005g)
A-2.1.1 (0.007 g)(white crystal)
A-2.1.1 (Propyl gallate) 0.007g White crystal
Identification by spectrophotometric
Test activity

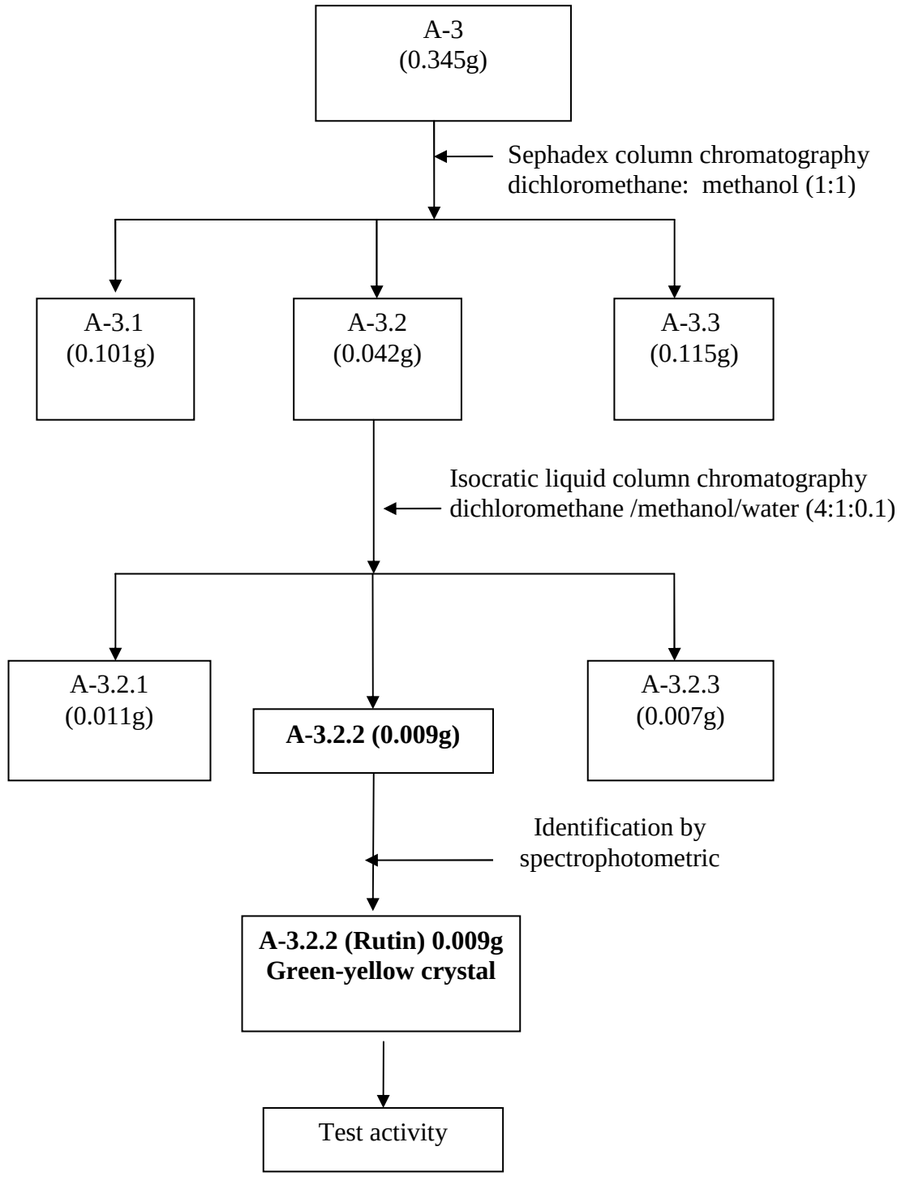

A-3
(0.345g)
Sephadex column chromatography
dichloromethane: methanol (1:1)
A-3.1
(0.101g)
A-3.2
(0.042g)
A-3.3
(0.115g)
Isocratic liquid column chromatography
dichloromethane /methanol/water (4:1:0.1)
A-3.2.1
(0.011g)
A-3.2.2 (0.009g)
A-3.2.3
(0.007g)
Identification by
spectrophotometric
A-3.2.2 (Rutin) 0.009g
Green-yellow crystal
Test activity

Table 4.20 Method validation test of HPLC analytical method **(a)** and TLC – densitometer analytical method **(b)**

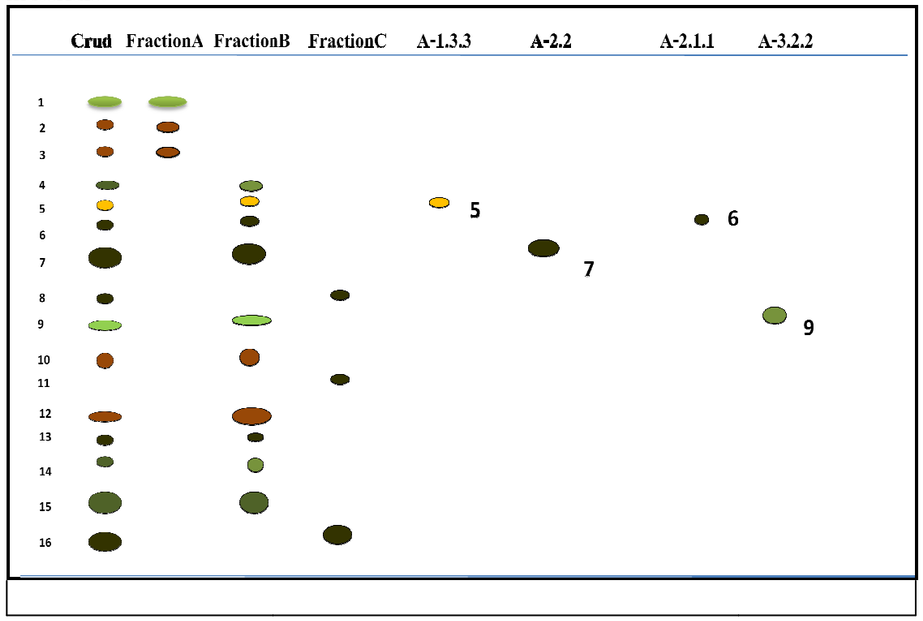
Method	Rutin
Accuracy (%R)	
Mean	100.62
Range	99.30-101.98
Precision (%RSD)	
intra-day	1.03
inter-day	0.13
Linearity	
R^2	0.9940
Slope	1.000
Intercept	0.095
QL (µg/ml)	0.38
DL(µg/ml)	0.13

a (HPLC)

Method	Rutin
Accuracy (%R) Mean Range	 99.45 98.41-100.46
Precision (%RSD) intra-day inter-day	 0.67 0.30
Linearity R^2 Slope Intercept	 0.995 7.090 5.083
QL (mg/ml)	0.62
DL(mg/ml)	0.21

b (TLC)

Figure 4.9 Spots on thin layer chromatograms of the solvent extracts sample of plant materials.



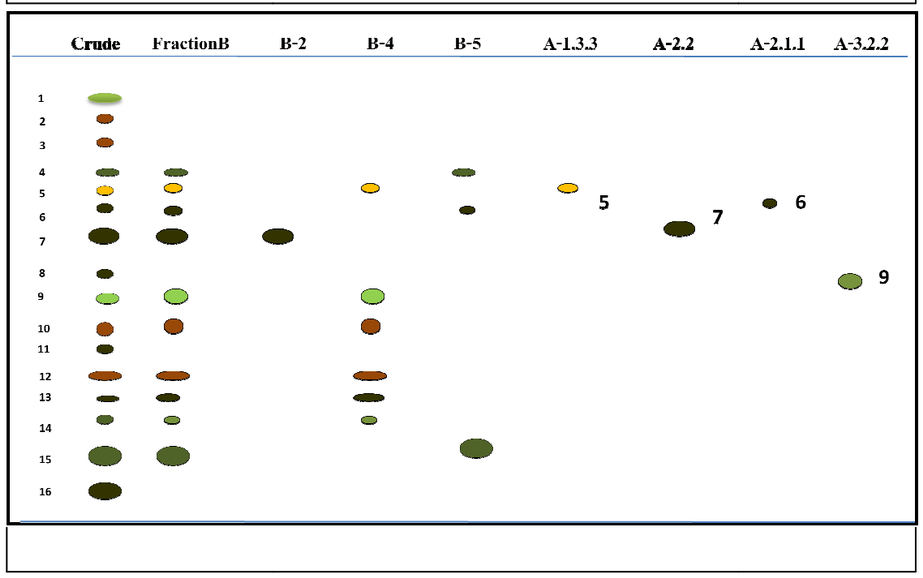


Table 4.21 R_f values and characteristic of spots on TLC-plate of longan seed extracts and isolated compounds.

Spot No.	R_f values	Detector,Spray reagent and Characteristic of spots		
		Anthrone	$FeCl_3$	UV_{365}
1	0.97	√ (green)	-	√
2	0.89	-	-	√
3	0.85	-	-	√
4	0.79	-	√ (green-black)	-
5	0.71	√ (yellow)	-	√
6	0.62	-	√ (green-black)	√
7	0.55	-	√ (green-black)	√
8	0.51	-	√ (green-black)	√
9	0.42	√ (green)	-	√
10	0.39	-	-	√
11	0.35	-	√ (black)	-
12	0.33	-	-	√
13	0.31	-	√ (black)	√
14	0.27	√ (green)	-	-
15	0.23	√ (green)	-	√
16	0.17	√ (green)	-	-

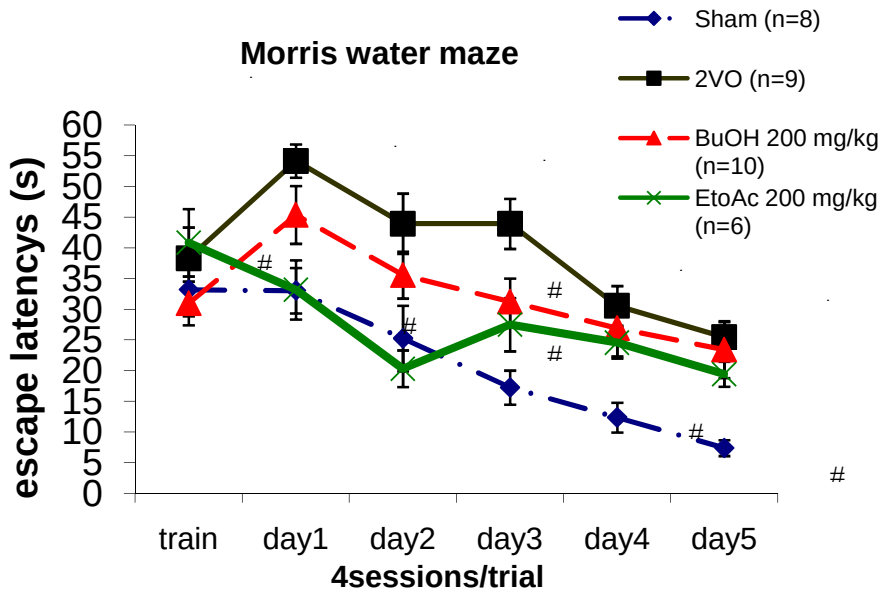


Figure 4.10 Activity of the several solvent extract from longan seed on 2-VO-induced of memory in the MWM performance. The escaping latency onto the platform was measured is 5 consecutive days.

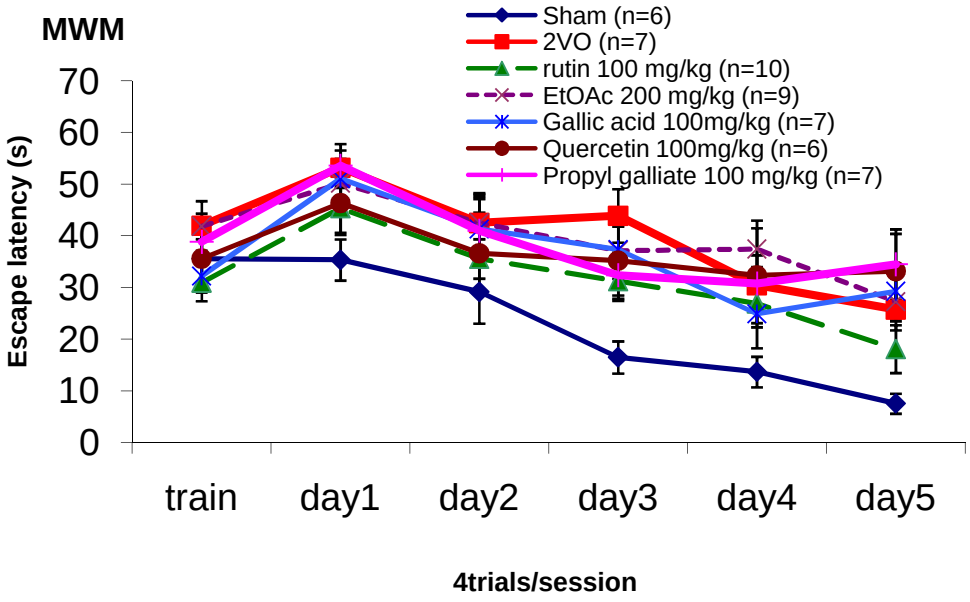


Figure 4.11 Effect of the isolated compound (100mg/kg) from longan seed on 2-VO-induced of memory in the MWM performance. The escaping latency onto the platform was measured is 5 consecutive days.

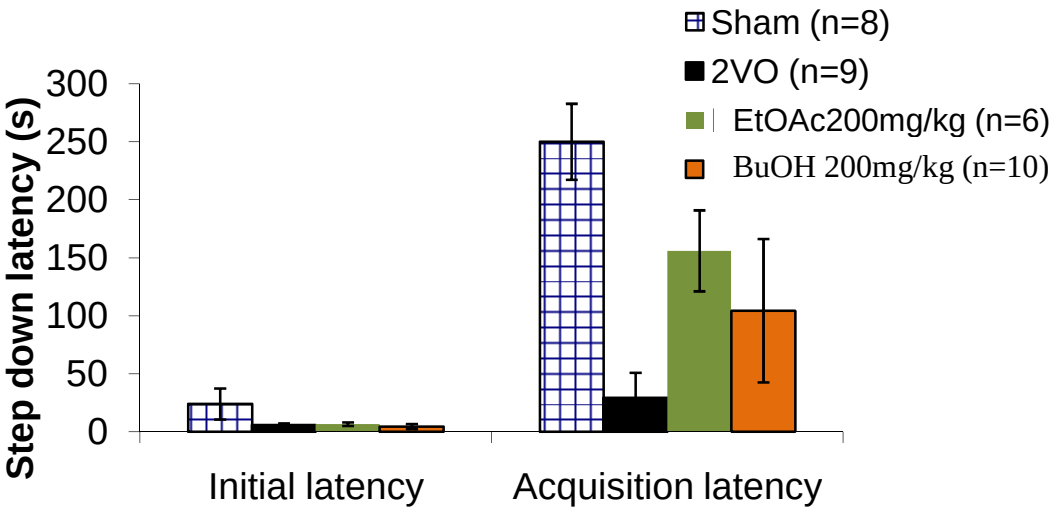


Figure 4.12 Effect of solvent from longan seed extracts (ethyl acetate and butanol) on 2-VO-induced disruption of memory in step-down test.

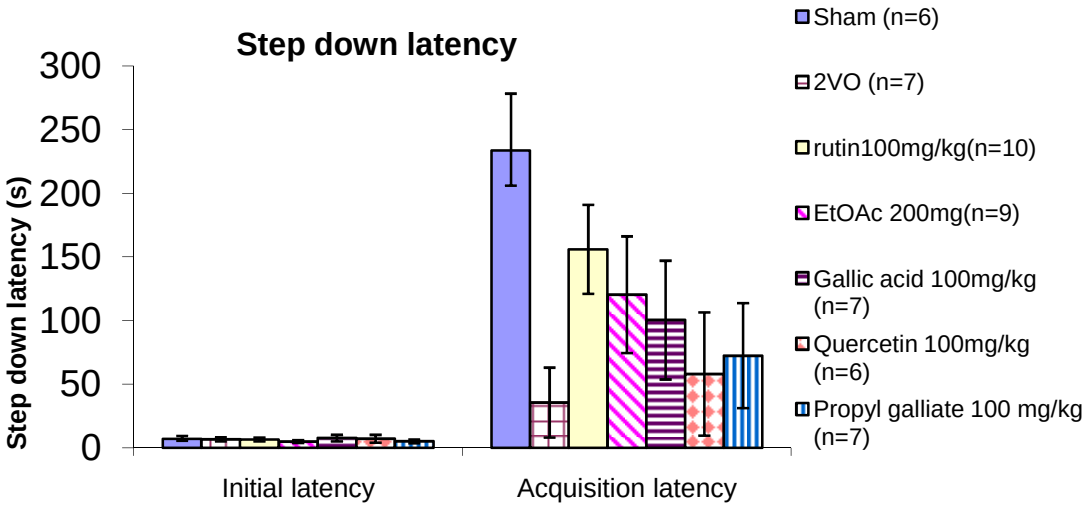


Figure 4.13 Effect of isolated compound from longan seed extract on 2VO-induced disruption of memory in step-down test.

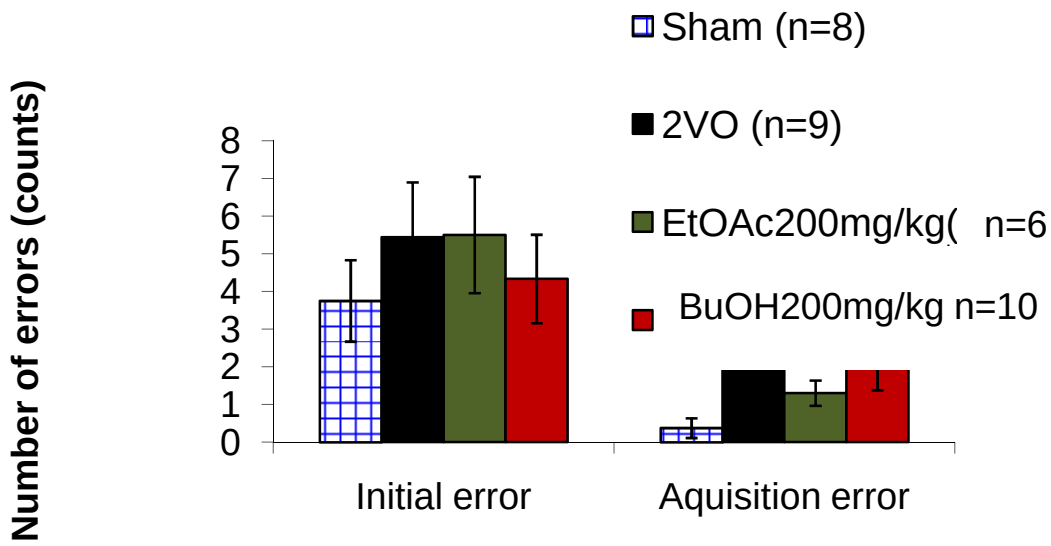


Figure 4.14 Effect of solvent from longan seed extracts (ethyl acetate and butanol) on number of error

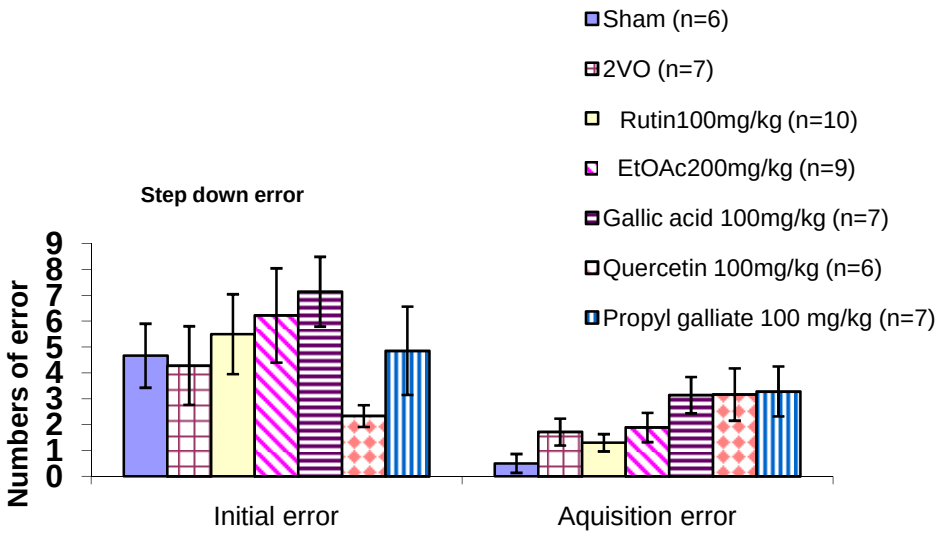
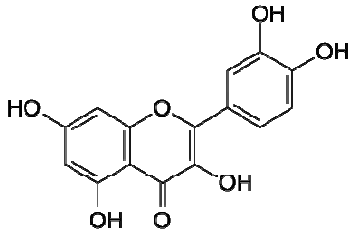


Figure 4.15 Number of error of rutin, gallic acid, quercetin and propyl gallate.



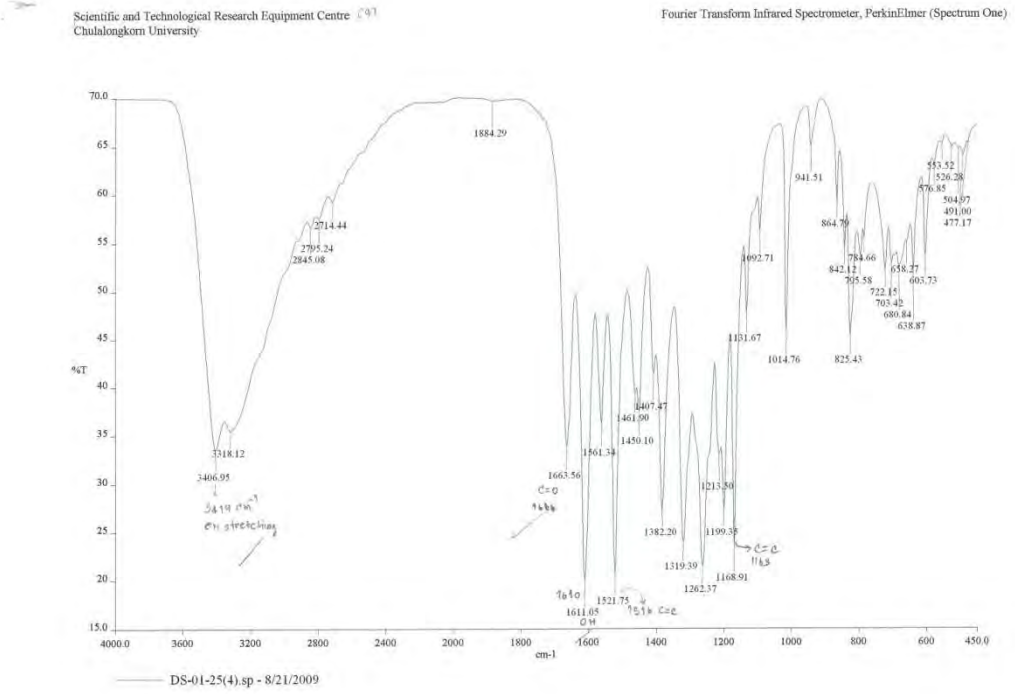
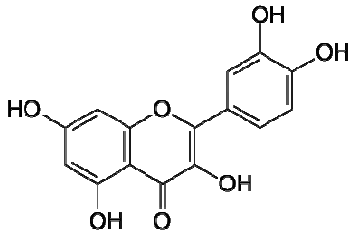


Figure 4.16 The IR spectrum of quercetin (A-1.3.3 ,KBr-disc).



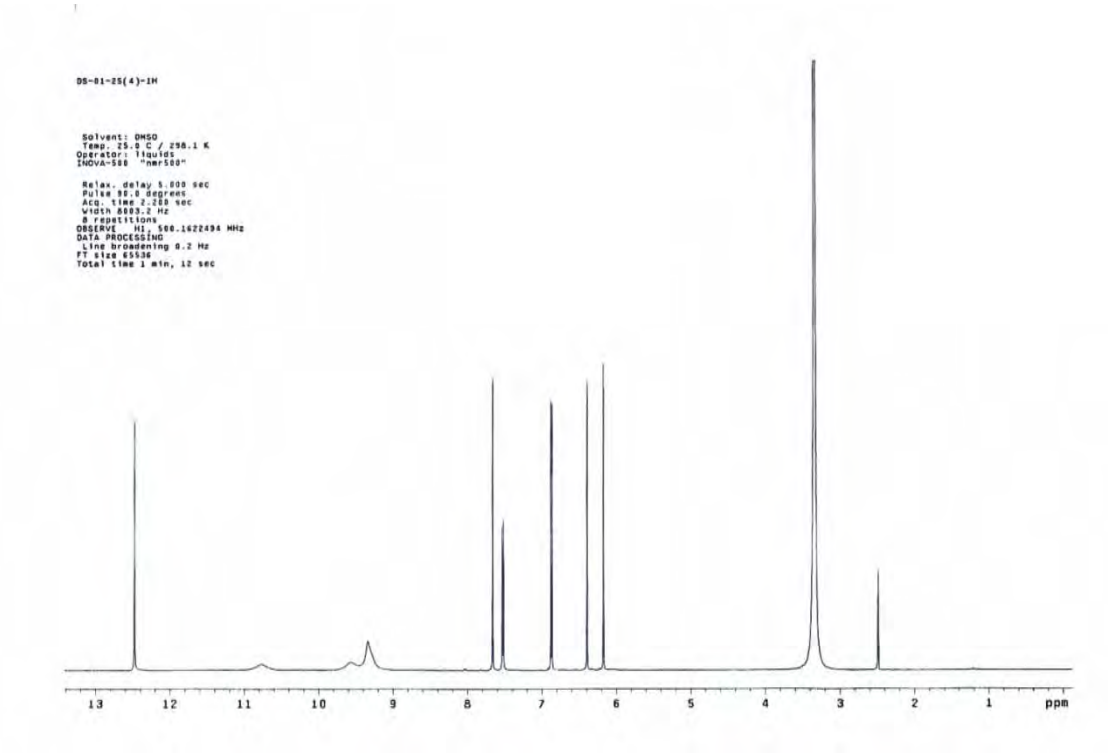
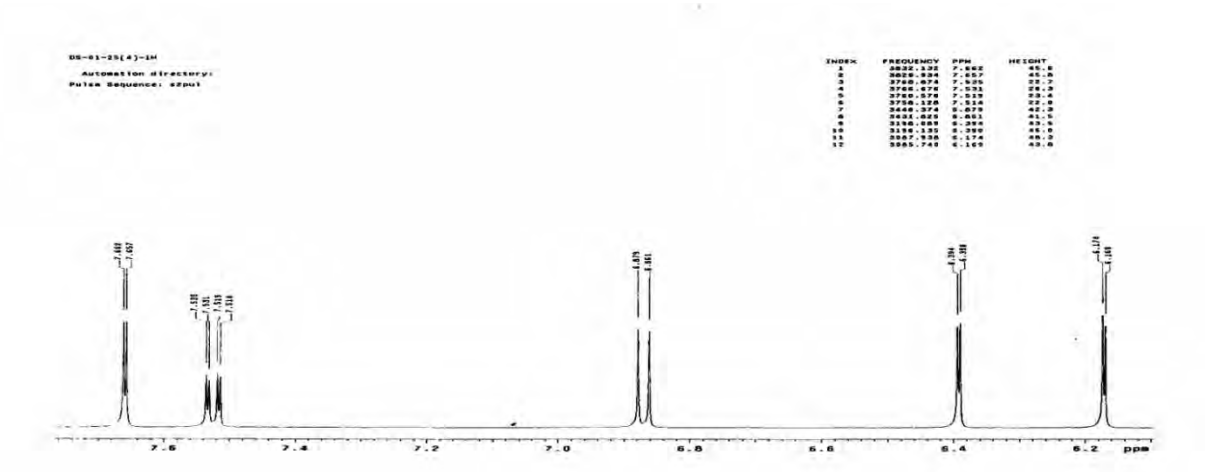
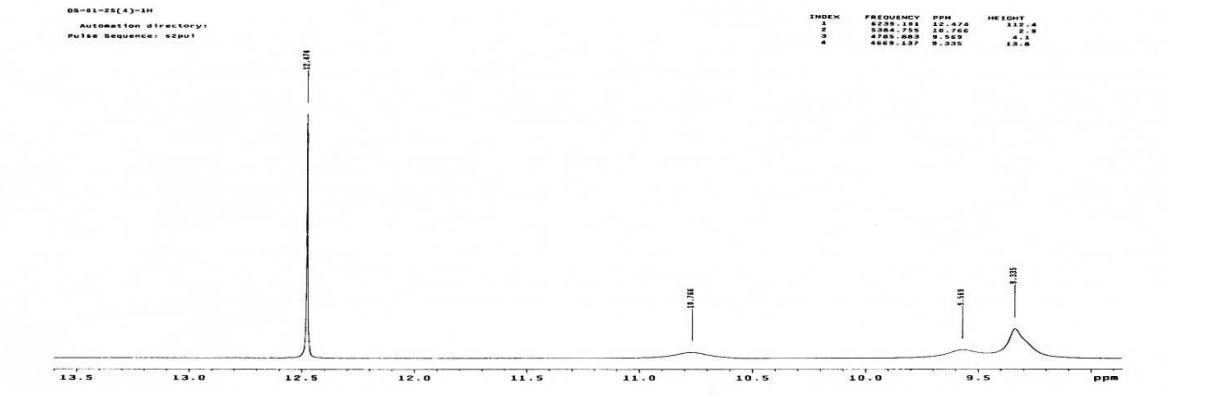


Figure 4.17 The 300 MHz ^{1}H NMR spectrum of quercetin
(A -1.3.3 ,full spectrum)



(a)



(b)

Figure 4.18 The 300 MHz ^{1}H NMR spectrum of quercetin (A- 1.3.3).

a) expend from 0.2-7.7ppm
b) expend from 9.3-12.5 ppm

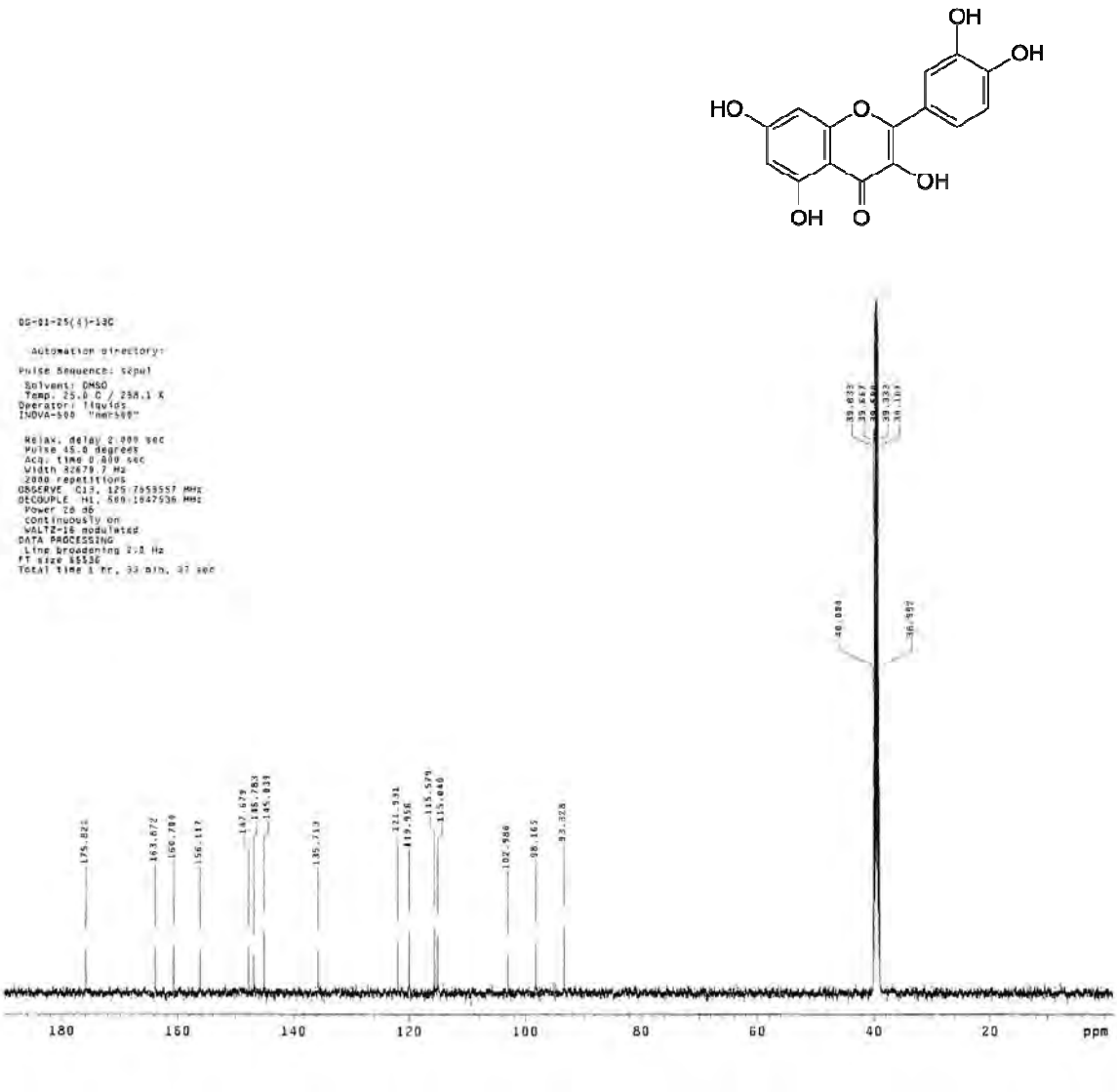
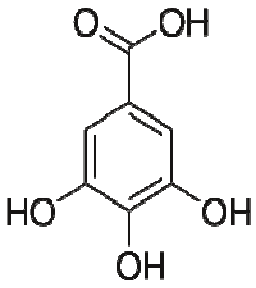


Figure 4.19 The 125 MHz ^{13}C NMR spectrum of quercetin (A- 1.3.3 in DMSO -d_6).



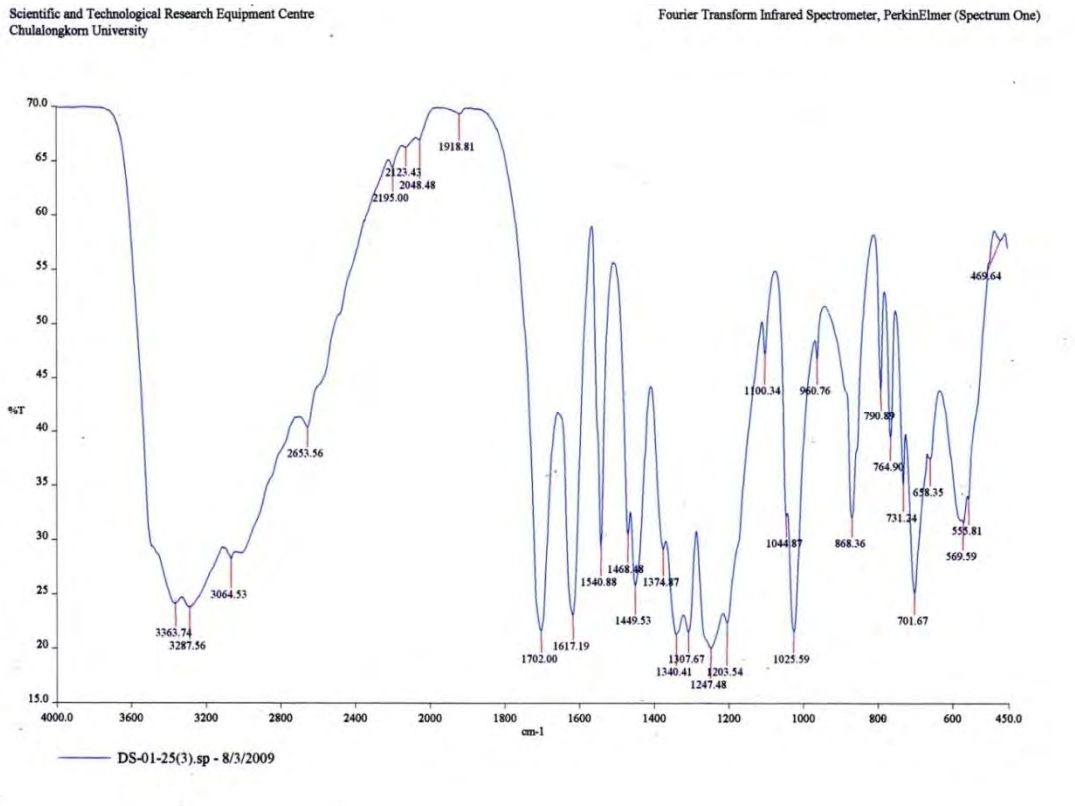
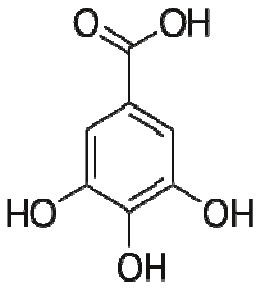


Figure4.20 IR spectrum of gallic acid (A-2.2,KBr-disc).



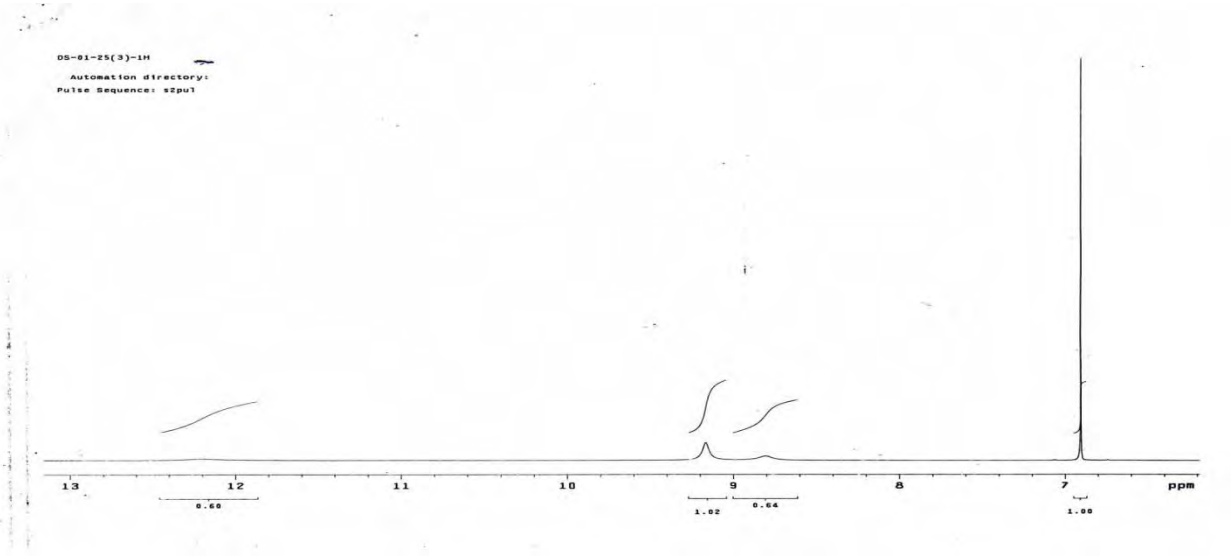


Figure 4.21 The 300 MHz ^{1}H NMR spectrum of gallic acid
(A -2.2 ,DMSO-d_6 ,Full spectrum)

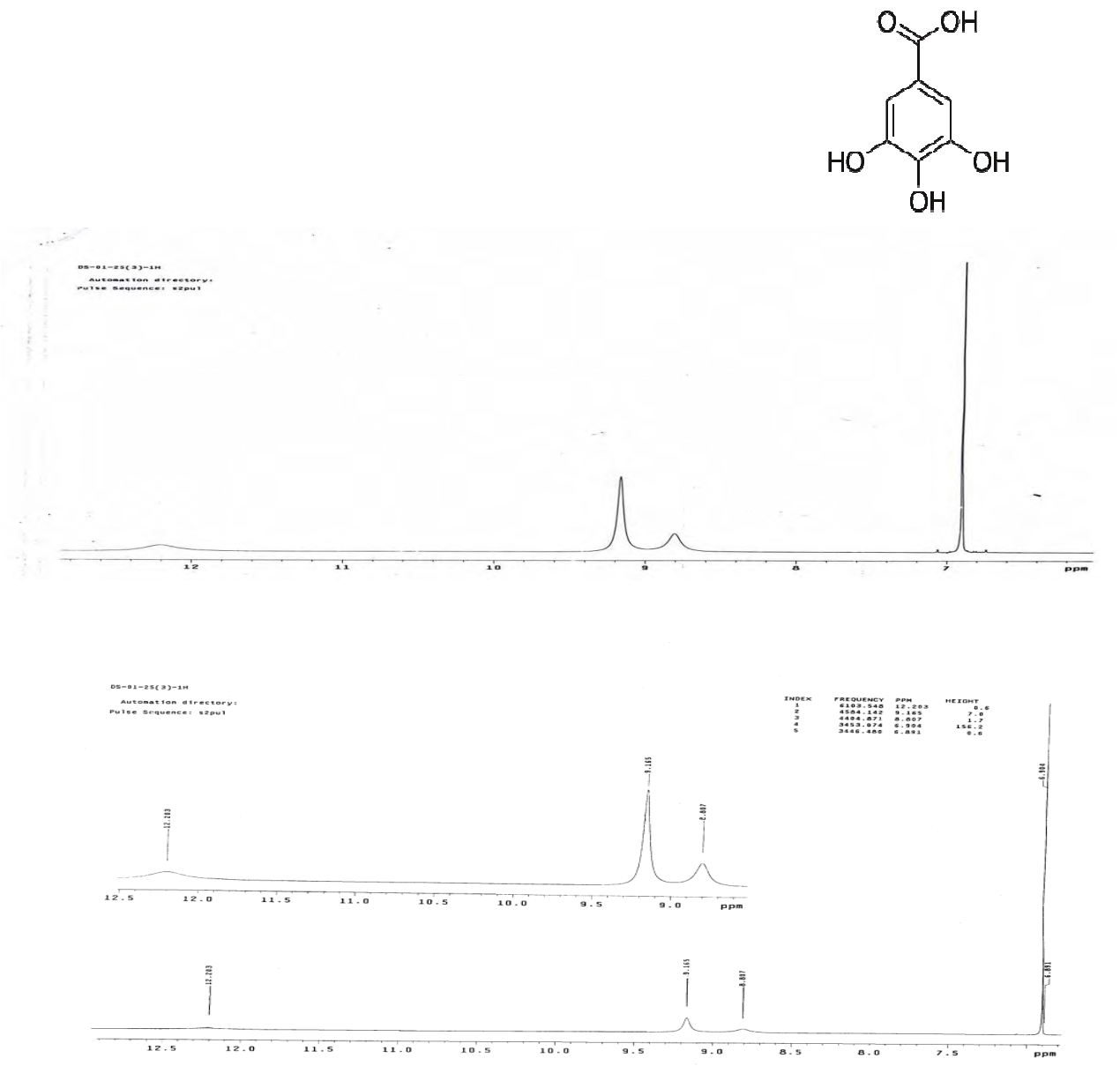


Figure 4.22 The 300 MHz ^{1}H NMR spectrum of gallic aid(A -2.2 ,DMSO-d_6).
(expend from 6.5-12.5 ppm).

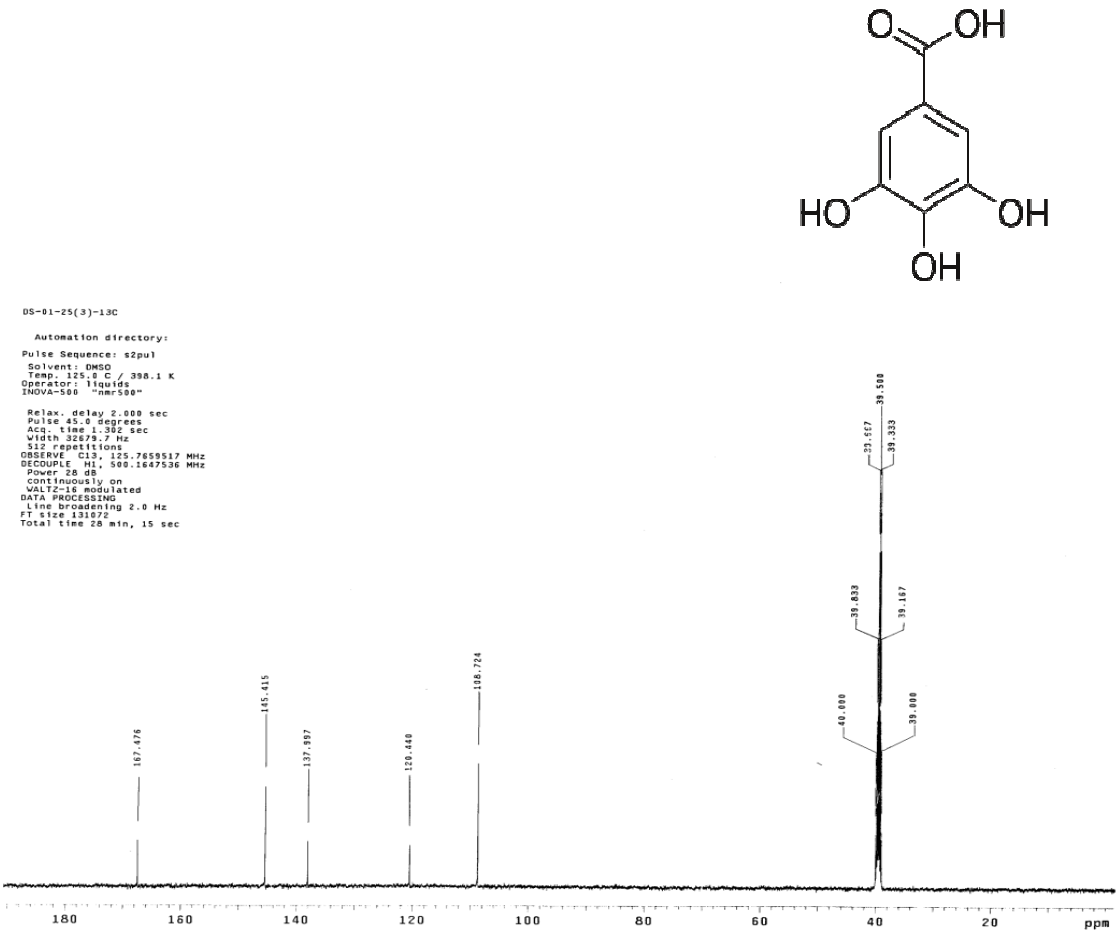
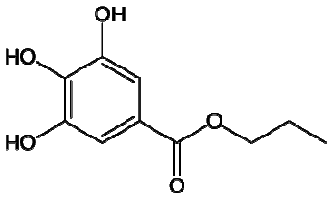


Figure 4.23 The 125 MHz ^{13}C NMR spectrum of gallic acid (A -2.2 ,DMSO-d_6).



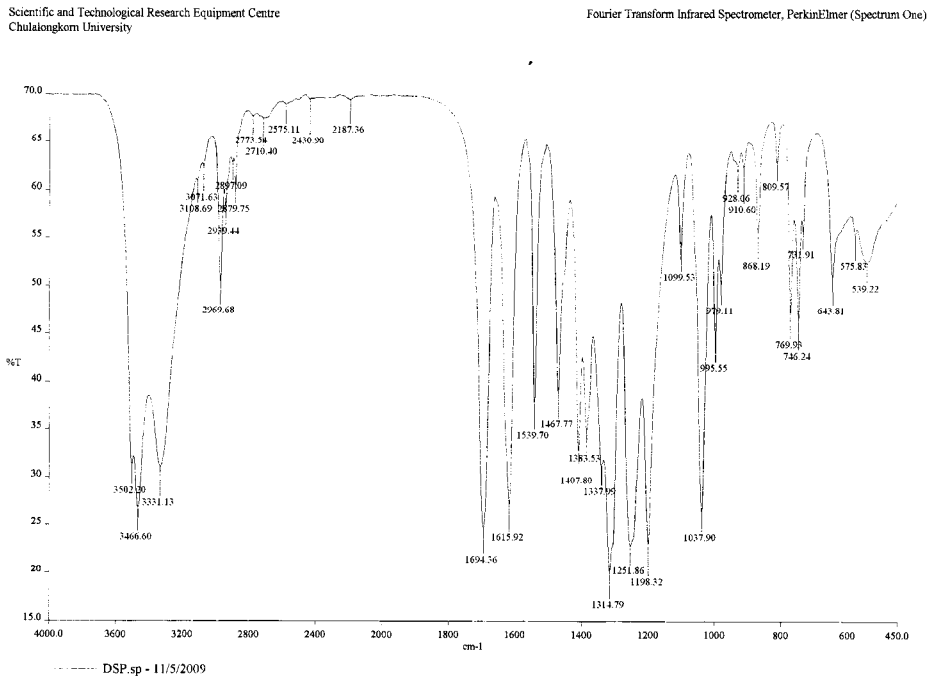
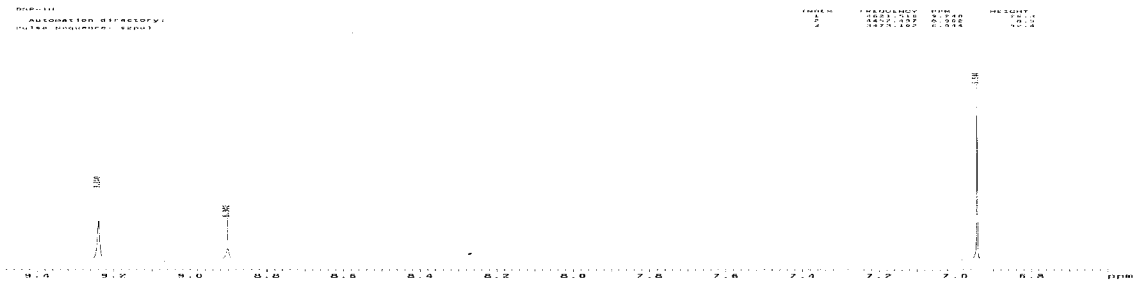


Figure 4.24 The IR spectrum of propyl gallate (A-2.1.1 ,KBr-disc)

Figure 4.25 The 300 MHz ^{1}H NMR spectrum of propyl gallate (A -2.1.1 ,DMSO-d_6) (full screen)

(a)



(b)

Figure 4.26 The 300 MHz ^{1}H NMR spectrum of propyl gallate (A- 2.1.1 ,DMSO-d_6).
a)expend from 0.00-4.50 ppm
b)expend from 6.80-9.50 ppm

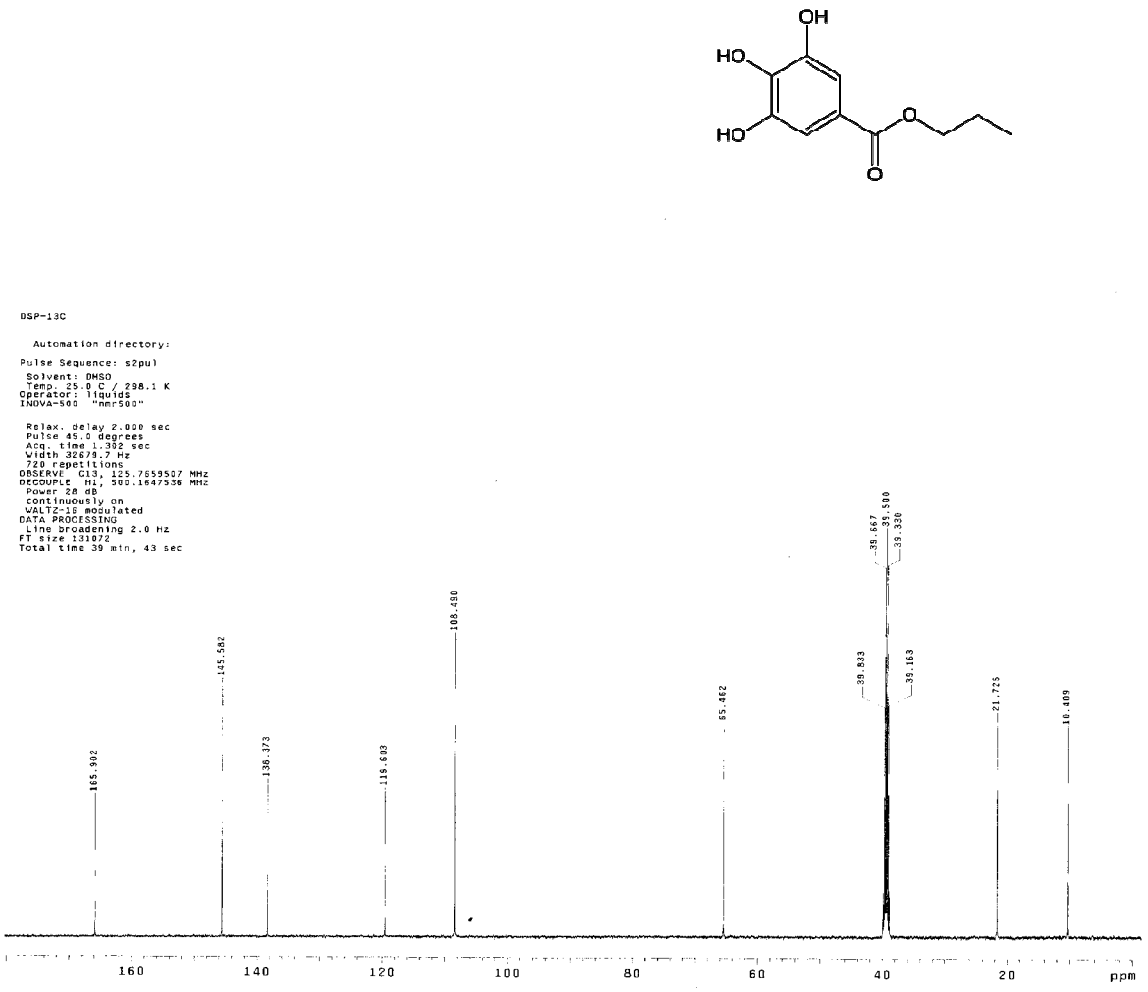


Figure 4.27 The 125 MHz ^{13}C NMR spectrum of propyl gallat (A- 2.1.1 in DMSO-d_6).

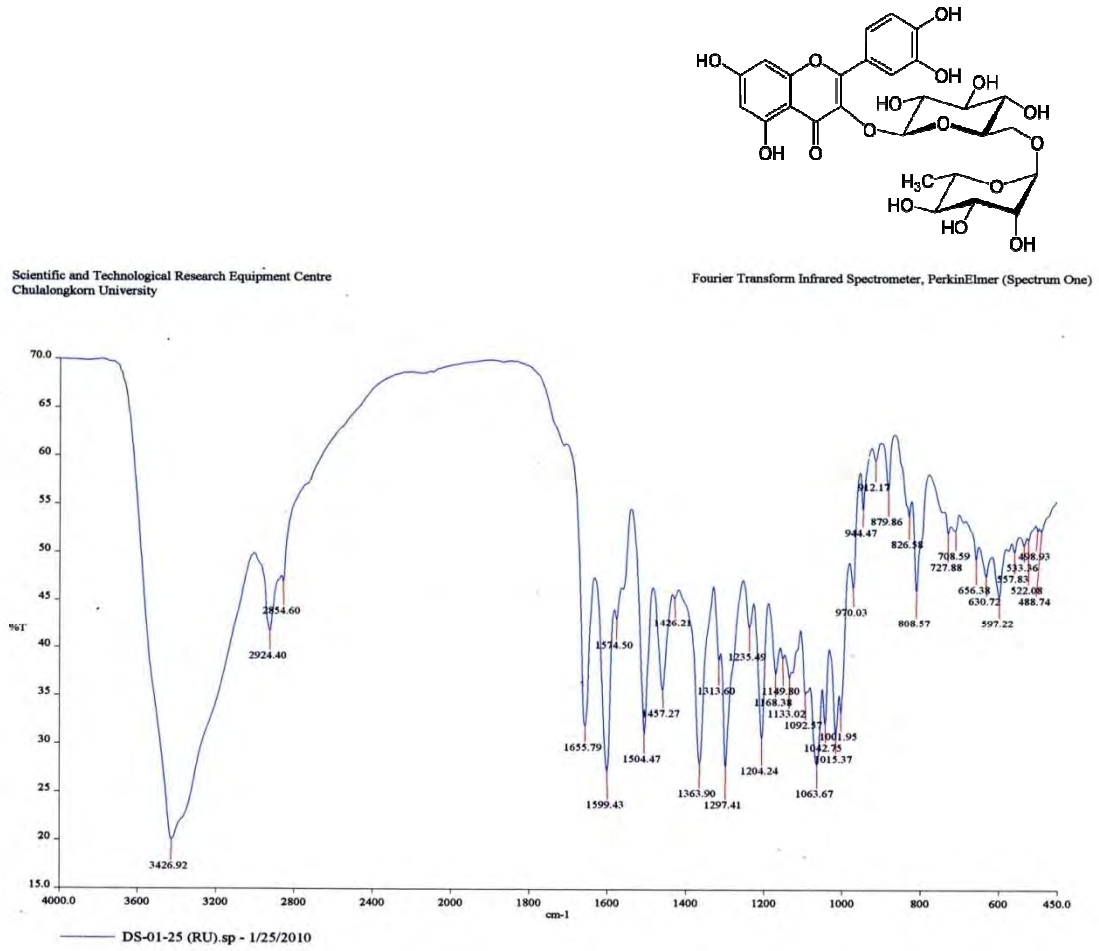


Figure 4.28 The IR spectrum of rutin (A-3.2.2 ,KBr-disc).

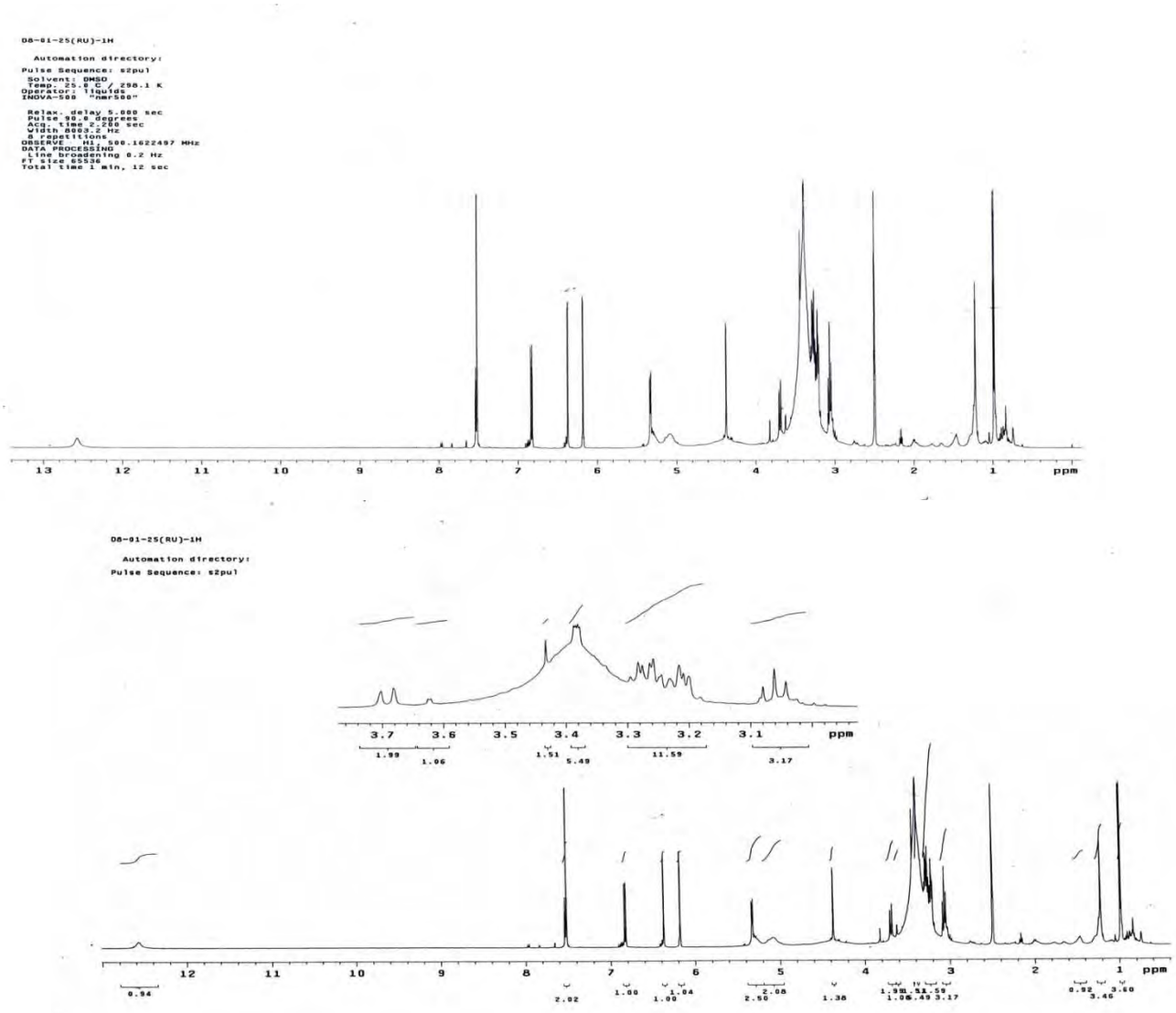
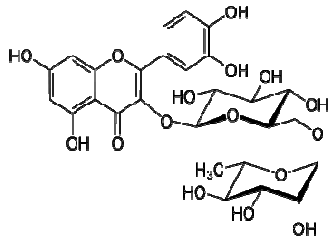
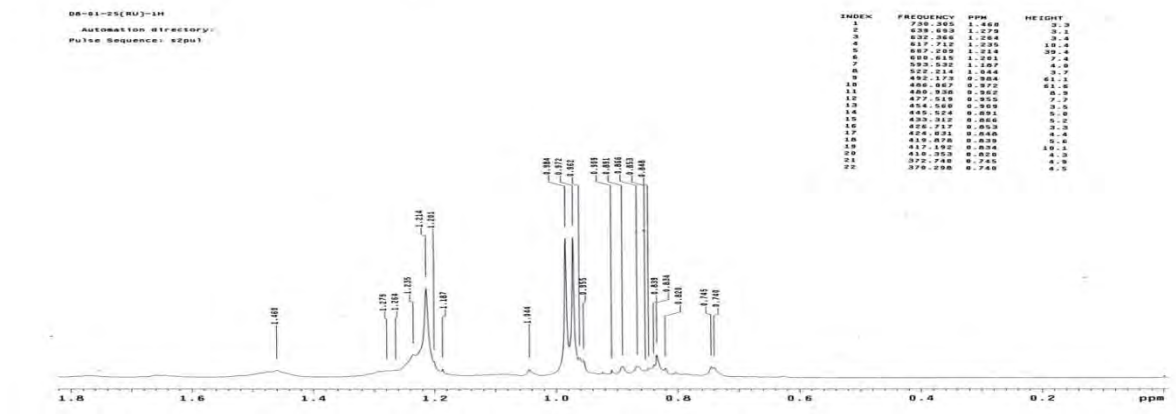
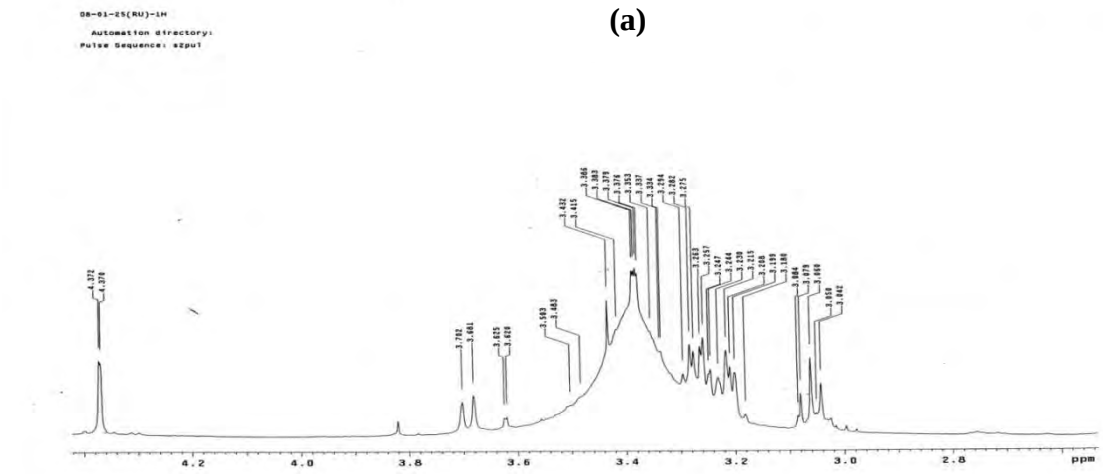


Figure 4.29 The 300 MHz ^{1}H NMR spectrum of rutin (A -3.2).
(full screen)





(a)



(b)

Figure 4.30 The 300 MHz ^{1}H NMR spectrum of rutin (A -3.2.2).

a) expend from 0.7-1.5 ppm
b) expend from 2.9-4.5 ppm

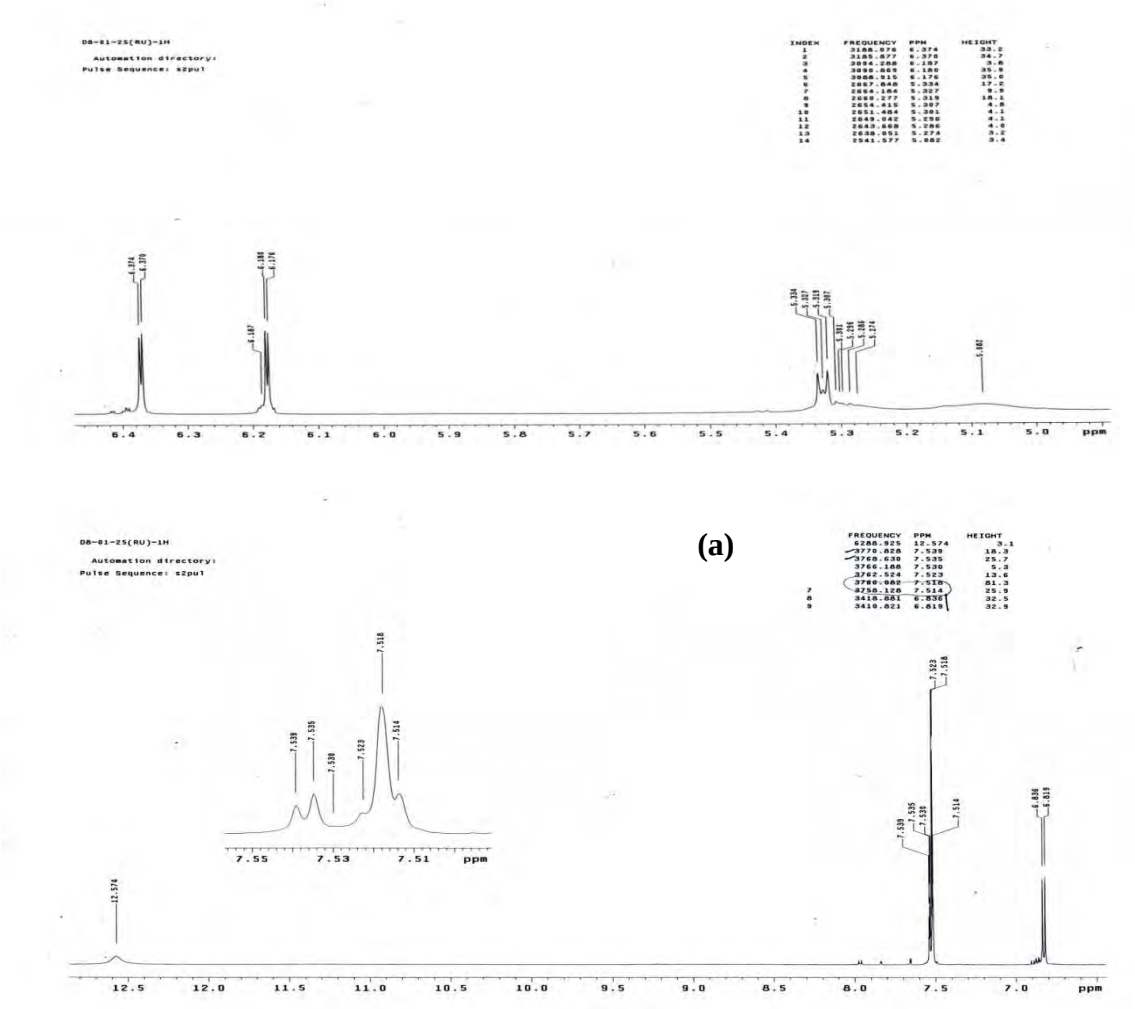


Figure 4.31 The 300 MHz ^{1}H NMR spectrum of rutin (A -3.2.2).

b) expend from 5.0-6.4 ppm

c) expend from 6.8-12.6ppm

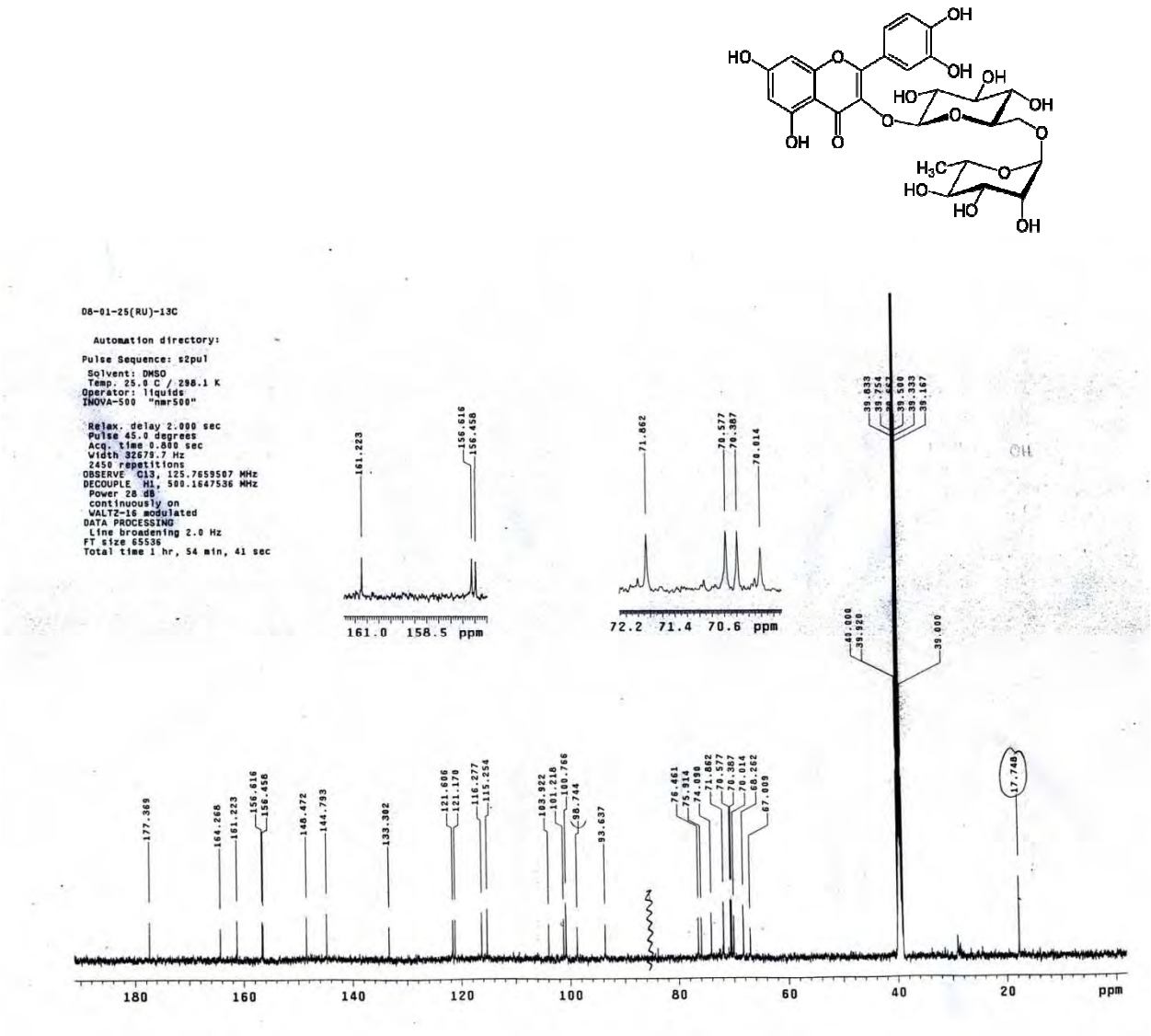


Figure 4.32 The 125 MHz ^{13}C NMR spectrum of A-3.2.2 in DMSO-d_6

VITAE

Miss Chutipan Nusuk was born on February 13, 1981 in Songkhla province, Thailand. She receive her bachelor degree of Science in Pharmacy in 2004 from faculty of Pharmaceutical sciences, Prince of Songkhla University.