PHARMACOLOGICAL ACTIVITIES AND NUTRITIONAL COMPOSITIONS

OF *LEPTOCARPUS DISJUNCTUS*

Mr. Watchara Damjuti

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)

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ฤทธิ์ทางเภสัชวิทยาและองค์ประกอบทางสารอาหารของต้นแส้ม้าฮ่อ

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แส้ม้าฮ่อ มีชื่อวิทยาศาสตร์ว่า *Leptocarpus disjunctus* Mast. เป็นพืชพื้นเมืองทางภาคใต้ของประเทศไทย รับประทานได้แต่หากรับประทานในปริมาณมากจะทำให้มีผลข้างเคียงโดยทำให้เกิดอาการง่วงนอน เนื่องด้วยไม่มีรายงานทางวิทยาศาสตร์ยืนยันฤทธิ์ดังกล่าวรวมทั้งยังไม่มีข้อมูลด้านโภชนาการ ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ก่อให้เกิดการนอนหลับ และข้อมูลทางโภชนาการของต้นแส้ม้าฮ่อ การศึกษาฤทธิ์ต่อการนอนหลับ ใช้สารสกัดเอทานอลและศึกษาในสัตว์ทดลอง (แมลงหวี่ หนูถีบจักร และหนูขาว) ผลการวิเคราะห์ข้อมูลทางโภชนาการ แสดงให้เห็นว่าแส้ม้าฮ่อมีปริมาณวิตามินซีสูง และเป็นแหล่งของกรดไขมันอิ่มตัวหลายชนิด เช่นกรดไลโนเลอิก และกรดไลโนเลนิก ทั้งยังเป็นแหล่งอุดมด้วยแร่ธาตุโพแทสเซียมและฟอสฟอรัส การประเมินฤทธิ์ทำให้นอนหลับ ประกอบด้วย การวิเคราะห์เบื้องต้นของการนอนในแมลงหวี่ การทดสอบพฤติกรรมของหนูทดลองในเขาวงกต การทดสอบภาคสนามเปิด การทดสอบประสิทธิภาพการวิ่งบนแกนหมุน และการทดสอบเวลาการนอนโดยเหนี่ยวนำให้เกิดการนอนหลับด้วยเพนโธบาบิโทนโซเดียม รวมถึงการตรวจสอบโครงสร้างการนอนหลับ การวิเคราะห์สภาวะการนอนหลับ-ตื่น และการวิเคราะห์การเกิดคลื่นเดลตา โดยใช้การบันทึกคลื่นไฟฟ้าสมอง ผลการทดสอบพบว่าสารสกัดเอทานอลของต้นแส้ม้าฮ่อมีฤทธิ์ทำให้นอนหลับ และยังแสดงให้เห็นผลกระทบที่เป็นปฏิปักษ์ในการตัดทอนเวลาการนอนหลับที่เกิดจากฟลูมาซีนิลอย่างมีนัยสำคัญ เพิ่มเวลานอนและการนอนหลับชนิดไม่กลอกตา สารสกัดเอทานอลยังมีผลต่อการลดลงในกิจกรรมการเคลื่อนไหวในสนามทดสอบต่างๆ จึงสามารถสรุปได้ว่าสารสกัดเอทานอลของแส้ม้าฮ่อ มีฤทธิ์ทำให้นอนหลับ โดยทำให้นอนหลับได้นานขึ้น และเพิ่มประสิทธิภาพของการนอนหลับ

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WATCHARA DAMJUTI: PHARMACOLOGICAL ACTIVITIES AND NUTRITIONAL COMPOSITIONS OF *LEPTOCARPUS DISJUNCTUS*. ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., CO-ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., 188 pp.

Leptocarpus disjunctus Mast., the edible plant which has an indigenous warning about its side effects for dizziness and drowsiness symptoms. However, there are no scientific evidences on these activities and also the nutritional compositions report. Hence, this research aimed to investigate hypnotic and anxiolytic activities using animal models, and to provide nutritional compositions. *L. disjunctus* showed high vitamin C content. They mainly contained linoleic acid and linolenic acid. They had a high level of potassium and phosphorus. In addition, anxiolytic activities were evaluated in mice and rats using locomotor determination by evaluated plus maze test, open field test and rotarod performance test. Hypnotic activities were performed using preliminary of sleep analysis in *Drosophila melanogaster* and pentobarbitone sodium induced sleeping time test in mice. The sleep architecture and sleep quality in rats were obtained from sleep-wake analysis and NREM delta activity using electroencephalograph. *L. disjunctus* showed the effective potencies for hypnotic tests, locomotors activities and sleep-wake analysis. They showed a dose relationship in sleeping time of pentobarbitone induced sleeping time test ($p<0.01$) and also showed an antagonistic effect on the shortening in sleep time induced by flumazenil. The consort results in locomotors activities and results in sleeping time and NREM sleep can be summarized that the ethanolic extract of *L. disjunctus* has a tendency to be a hypnotic and anxiolytic plant which possibly has benzodiazepine-like hypnotic activity. Moreover, this study created a nutritional data and provided nourishment of *L. disjunctus*.

Field of Study: Public Health Sciences	Student's Signature ....
Academic Year: 2016	Advisor's Signature ....
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>	=	More than
°C	=	Degree Celsius
µg/100 g	=	Microgram per 100 gram
µg/ml	=	Microgram per millilitre
µl	=	Microliter
µm	=	Micrometre
µV	=	Microvolt
Å	=	Angstrom
AAS	=	Atomic absorption spectroscopy
AASM	=	American Academy of Sleep Medicine
AOAC	=	Association of Official Agricultural Chemists
ATCC	=	American Type Culture Collections
ATP	=	Adenosine triphosphate
cm	=	Centimetre
CNS	=	Central nervous system
DNA	=	Deoxyribonucleic acid
DNPH	=	2,4-Dinitrophenylhydrazine
EEG	=	Electroencephalogram
EMG	=	Electromyogram
EPM	=	Elevated plus maze
g	=	Gram
GABA	=	Gamma-aminobutyric acid

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GC-MS	=	Gas chromatography mass spectrometry
hr	=	Hour
Hz	=	Hertz
IP	=	Intra-peritoneally
Kcal	=	Kilocalories
M	=	Molarity
mg	=	Milligram
mg/kg	=	Milligram per kilogram
mg/ml	=	Milligram per millilitre
min	=	Minute
ml	=	Millilitre
ml/min	=	Millilitre per minute
mm	=	Millimetre
N	=	Normality
NAD	=	Nicotinamide adenine dinucleotide
NADP	=	phosphate
nm	=	Nanometre
NREM N1	=	Non-rapid eye movement 1
NREM N2	=	Non-rapid eye movement 2
NREM N3	=	Non-rapid eye movement 3
NREM sleep	=	Non-rapid eye movement
OFT	=	Open Field Test

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psi	=	Pound per square inch
REM sleep	=	Rapid eye movement
rpm	=	Round per minute
S	=	Second
SD	=	Standard deviation
T3	=	Triiodothyronine
T4	=	Thyroxine
w/v	=	Weight per volume

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CHAPTER I
INTRODUCTION

Background and Rationale

Pharmacology is the study of the relationship between drugs and chemicals on living organisms, focusing on the issues of drugs and chemical reaction. Pharmacology plays an important role in living of the creatures [1]. Since the several centuries, plants have been used for multipurpose such as food, drug, apparel and residence. A large amount of plants have been used as medicine because of their pharmacological effects. Among the changes and social prosperity, herbal remedies have been played a greater emphasis to human being because it has been used for both direct and indirect treatments not only oriental but also western medicines. In addition, medicinal food has been put forward and educated for its effect which affected the organism both pharmacology and toxicity, it is also studied on the biochemistry of the bioactive constituents that tended to promote health and prevent of disease. Medical civilizations scattered in all parts of the world including Thailand, which has a long-standing medical civilization. Due to the variety of ethnicities and diversities, the medical wisdom has developed since glorious past till the present; as a result, the use of herbs as medicine and food spread throughout the country.

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The Thai Drug Act, 1967 suggested that herbal medicine is the medicine derived from flora, animals or minerals which does not mix or transform, it is also part of the

roots, stems, leaves, flowers and not through the process of privatization [2]. The Thai Drug Act, 1979 expanded the meaning of herbal medicine that it is the medicine of the plants, animals, minerals and herbs. It is used as herbal medicine and also useful as food or drink. It is used as a supplement, ingredient in cosmetics, fragrances, dyes, medicines as well as pesticides [3].

Each region of Thailand has a different perspective in eating since the diversities and ethnicities. There are a large number of plants that incessantly consumed as medicinal food and folklore medicine for example, *Boesenbergia pandurata* rhizome is used to treat abdominal cramps, enhance stomach and intestinal movements, improve appetite, and volatile oils have a strong expel effect. Tewtrakul *et al.*, 2009 suggested that *B. pandurata* have been used as food ingredients and in Thai traditional medicine for treatment of several inflammatory related diseases such as gout, allergy, aphthous ulcer and peptic ulcer. Their research study demonstrated that the rhizomes of *B. pandurata* showed the capability in inhibitory activities against nitric oxide production and supported the traditional use. The tuber of *Allium sativum* is used to treat flatulence, anti-ulcers in the stomach and some kind of skin diseases [4]. The research study of Sahbaz *et al.,* 2014 argued that *A. sativum* showed the strength in term of the anti-inflammatory, antibacterial, fibrinolytic, antithrombotic, and wound-healing effects that prevent formation of peritoneal adhesions in a rat model which supported the traditional use of *A. sativum* [5].

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In southern of Thailand, the people continue to consume native plants in a variety of side dish. *Leptocarpus disjunctus* Mast. in Restionaceae family is one of the native plants that still consumed as side dish and ingredient in local food; even though, there is the indigenous warning about its side effect for dizziness and intoxicated symptoms. *L. disjunctus* can be called in Thai as “SAE” or “SAE-MAR-HOR”. It is biennial monocotyledonae plant with taper leaves around 20-30 cm in length. The previous research in 2013 demonstrated that the ethanolic extract of *L. disjunctus* tended to be hypnotic agent by *in vivo* experiment, pentobarbitone induced sleeping time test, and also showed the capability in antioxidant, antityrosinase and antidiabetes by alpha-glucosidase inhibitory assay [6]. In addition, various locomotor activity tests are important for estimation of the effect on the nervous system. This current study aimed to evaluate and assess the anxiolytic potency of *L. disjunctus* on locomotor activities and brain wave effect *in vivo*. As mentioned above, *L. disjunctus* has been consumed as side dish and ingredient in local food, and there are no nutritional composition reports. *L. disjunctus* was evaluated and reported its nutritional composition in this study as well.

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Research Problem

L. disjunctus is one of the native plants that still consumed as side dish and ingredient in local food; even though, there is the indigenous warning about its side effect for dizziness and intoxicated symptoms. There are no nutritional composition reports of this edible plant. The possibility of *L. disjunctus* to induce sleeping time in mice preliminary reported. The anxiolytic-hypnotic effects of *L. disjunctus* on the nervous system especially the locomotor activity and brainwave have never been revealed.

Objectives

1. To evaluate the potential of *L. disjunctus* on anxiolytic-hypnotic activity and brain wave effect.
2. To evaluate *L. disjunctus* nutritional composition profile.

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Expected Benefit

This current research provided the potential on hypnotic and anxiolytic activities and exhibited nutritional composition profile of *L. disjunctus*.

The Scope of the Study

This current research was preclinical behavioural study using fruit fly and rodent models for evaluating *L. disjunctus* anxiolytic-hypnotic activity. Furthermore, the nutritional value of this indigenous food is also investigated.

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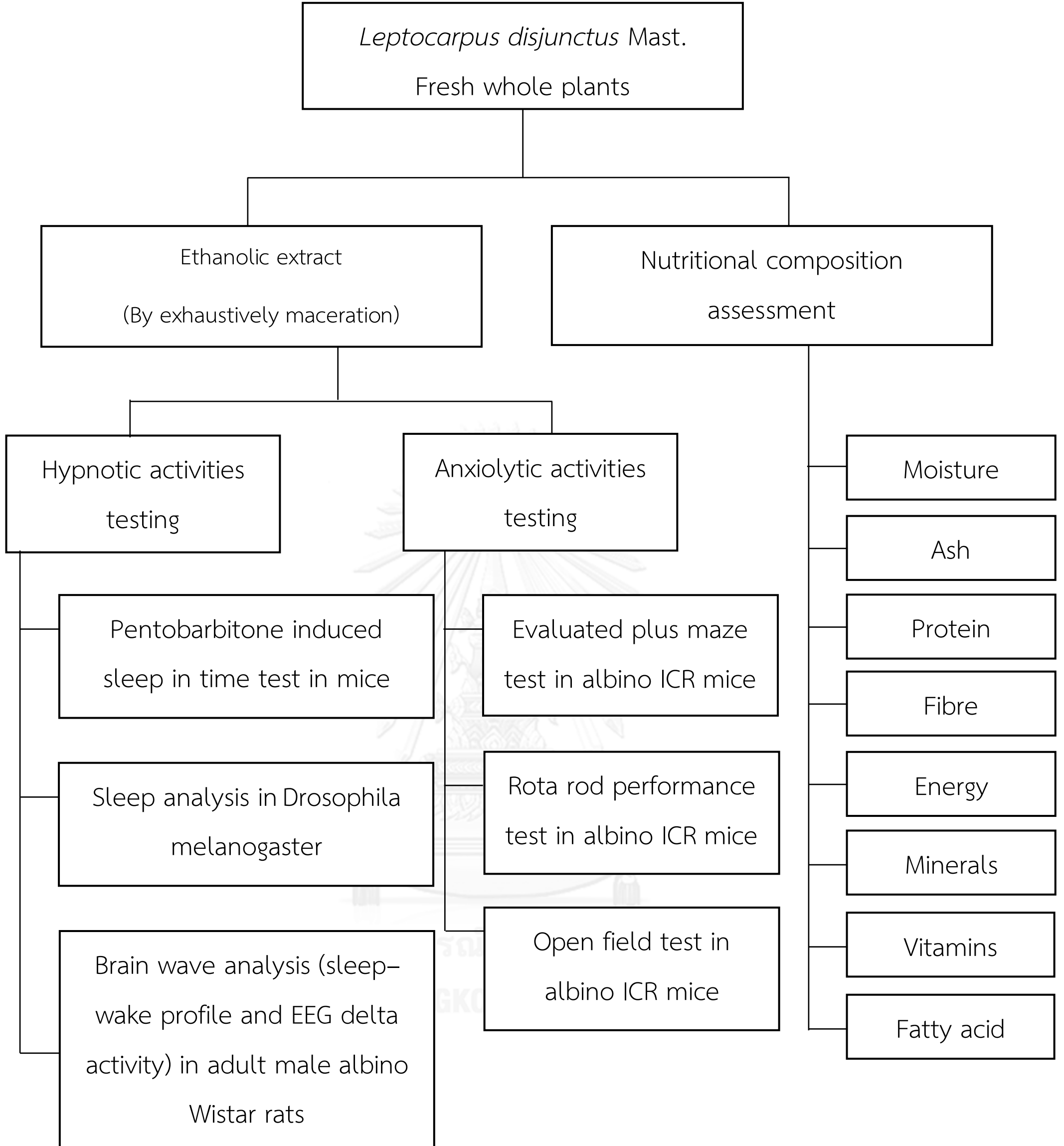
Conceptual Framework



CHAPTER II
LITERATURE REVIEWS

Taxonomy

Kingdom: Plantae

Division: Tracheobionta

Class: Magnoliophyta

Order: Liliopsida

Family: Restionaceae

Genus: *Leptocarpus*

Species: *Leptocarpus disjunctus*

Family Restionaceae

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Restionaceae family is called restiads or restios. There are 30 genera, 550 species in this family, most are perennial and evergreen plants. This family consists of rhizomatous and herbaceous plants. They are classified in the group of monocotyledons that includes several superficially similar families, such as true grasses. They have green, photosynthetic stems and leaves that have been reduced to sheaths. Their flowers are extremely small and in spikelet, which in turn make up the inflorescences [7].

Botanical Description

Perennial herbs with a rush-or sedge-like habit; tufted or with creeping rhizomes, usually covered with closely imbricate scales. Stems (culms) green, terete to angular or flattened, simple or branched, straight or flexuous, solid or hollow. Leaves in adult plants reduced to sheathing scales, sometimes with a small linear or subulate lamina; sheath closely imbricate or loose, margins overlapping, at least at the base. Flowers usually in spikelet with imbricate rigid glumes, some of the outer ones usually empty; in several genera the flowers not in definite spikelet but in branched racemose panicle-like inflorescences with the glumes not or scarcely imbricate; spikelets, when present, 1–many-flowered, either similar or different in the 2 sexes, solitary and terminal, or axillary, or arranged in a racemose inflorescence. Male and female inflorescences either similar or considerably different. Flowers usually actinomorphic, 3- or 2-merous, bisexual or more usually unisexual and plants dioecious, a few species bisexual or monoecious, small, each in the axil of a ± scarious glume; bracteoles 1 or 2, or more commonly absent. Perianth in 2 whorls, rarely absent; tepals 3–6, glume-like or scarious erect. Male stamens 3 (or 2 or 6), opposite the inner tepals; filaments free and filiform or rarely short; anthers 1-locular [rarely 2-locular, dorsifixed, dehiscenceby longitudinal slits; rudimentary ovary sometimes present. Female staminodes 2, 3, or absent; ovary superior, sessile or shortly stipitate, 1–3-locular according to the number of carpels fully developed; styles 1–3; ovule

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solitary in each loculus, pendulous. Fruit a loculicidal capsule, 2- or 3-angled or 1-locular, or a small nut (8).

Distribution

It is native to the Southern Hemisphere with main centre of diversity in southern Africa and southwest Australia, also in east Australia, New Zealand, Malaysia and Chile, 20 genera, 130 species in Australia, single species in China and Thailand [8, 9].

***Leptocarpus disjunctus* Mast.**

Leptocarpus disjunctus Mast. is the native plant in the southern of Thailand which called in the native language as "SAE MAR HOR" or “SAE”. *L. disjunctus* is biennial-monocotyledonae plant, taper leaves with length of about 20- 30 cm. This plant has white and purple bulbs for serving the food. Its flower is white with a long peduncle. Inflorescences like an umbrella with many florets.

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Botanical Description

40–70(–100) cm tall, dioecious or polygamous. Rhizome is creeping, woody, younger parts densely woolly. Stem 1.5–3 mm in diameter, terete, stiff. Sheaths closely apprised to stem, brownish, 1–1.5 cm, leathery, veins longitudinal, primary one straight, apically protruding for 2–5 mm, margin narrow, scarious, apex acute-acuminate. Inflorescences laxly paniculate; spikelets fascicled. Male flowers: bracteoles narrowly ovate, 2–2.5 × ca. 1 mm; perianth 5 segments 4–6, 2 outer ones

opposite each other, dark brown, boat-shaped, folded, 1.7–2 mm, 2–4 inner ones lighter brown, narrowly elliptic, slightly shorter than outer ones; stamens 3; filaments ca. 1.5 mm; anthers 0.7–1 mm, apex brownish, apiculate. Female flowers: segments 6–8, elliptic, 1–1.5 mm, narrowed toward base, apex acute; ovary ellipsoid, 3-angled; style short; stigmas usually 3. Fruit ellipsoid, ca. 1 mm. Seeds ca. 0.5 mm [10].

Figure 1 *Leptocarpus disjunctus* Mast.

Proximate nutrients

Moisture

Moisture content, the value using for indicates the amount of water contained in food, is one of the most important properties of food [11]. Since moisture affects the deterioration of food (food spoilage), the deterioration is due to the microorganisms (microbial spoilage) that affect the shelf life. Food with moisture or high water can be easily spoiled because the conditions are suitable for the growth of microorganism: bacteria yeast and mold [12]. Moisture content affects the food safety because high moisture food is appropriate for pathogen growth and toxin formation that is harmful to consumers [13]. The humidity affects the physical and thermal properties such as melting point, boiling point, thermal conductivity and specific heat. It also affects the sensory food quality such as texture, viscosity and caking. Furthermore, Humidity affects the rate of chemical reactions during storage, such as browning reaction and lipid oxidation [14].

Moisture content in food can be estimated and expressed in two patterns, wet and dry basis. Wet basis generally used and often told as a percentage. Dry basis generally used to analyse the drying process (dehydration) because easy calculation and constant [15].

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Moisture Content Estimation

Water in each food is different in structure attachment to other molecules causing moisture content estimation techniques are different upon the strength of each molecule. It can be categorized into two methods [16].

1. Direct method, direct method is the evaluation of the moisture content such as separation out of the water by physical methods: baking, distillation, chemical reaction (Karl Fischer method) and infrared or microwave radiation method.

    a. Moisture analysis with drying method, drying method is moisture content analysis of the substance comparing the weight before and after annealing at high temperature [17].

    b. Moisture analysis with distillation method, distillation method is moisture content analysis of the substance by sampling the preferred material. The sample will be boiled in water saturated toluene, and water in the sample will evaporate and condense which can be measured and exhibited as volume and weight [18].

    c. Moisture analysis with chemical reaction, Karl Fischer method is the moisture content estimation in food firstly distributed by Karl Fischer, (1935) using titration technique [19]. The plant sample will be titrated with Iodine solution in methanol, sulfur dioxide and pyridine. Iodine will

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react with water when the titration. When water completely reacts, the colour of the solution turns to brown colour. The moisture content can be interpreted by observing the volume of iodine used in the titration. Karl Fischer method is a standard method for moisture quantitative analysis. The value obtained by this method is often used as a benchmark for comparison with other methods. It is appropriate with food samples that contain a little amount of water [20].

d. Moisture analysis with infrared and microwave radiation, infrared and microwave radiation is the moisture analysis by using the radiation to evaporate the water content in plant sample. Infrared and microwave radiation method is the accuracy and convenient but the disadvantage is costly multiple devices and time consuming [21, 22].

2. Indirect method, indirect method is the indirect method to evaluate the moisture content by observing the electrical properties, using electronic devices. Indirectly moisture observation has proven to be fast and easy. It can be done in two ways [23].

   a. The measurement of electrical resistance measured the electrical resistance of the plant by achieving the plant sample into the gap between the electrodes in the container tightly closed. Electrical resistance data can be transformed into the moisture content.

b. The measurement of capacitance, the sample will be contained in a closed container. The container wall will release out high-frequency electrical discharges. This method is more accurate than electrical resistance.

Ash

Ash is the inorganic substances such as calcium, phosphorous and potassium. When the samples are incinerated at high temperature, part of the organic material is burned out, leaving only inorganic substances [24]. The total ash value can tell the quality of the plants by higher ash, indicating contamination inorganic substances in the plant samples [25].

Protein

Protein is high molecular weight biomolecules. The structure of protein depends on the connection of different amino acids. The sequence alignment of the amino acids is important for determining the kind of each protein. Proteins with different structures have different functions as well. Protein structure is complicated, which can be divided into several levels depending on the bonds that cause structural level. If the bonding in the structure of the protein is destroyed, the structure of the protein will lose and unable to work or restore to its original condition.

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Classification of Protein

Protein structure can be divided into groups based on a simple three major groups [26, 27].

1. Simple protein, simple protein is a protein that contains all essential amino acids by no other compounds included in the molecule. It can be divided into two categories based on the outline.
   a. Fibrous protein is a protein with long chain polypeptide structure, and non-soluble in the water but soluble in organic solvents. It serves as the main structure in organism due to its strength and flexibility, such as collagen, elastin, keratin and fibroin.
   b. Globular protein is a protein with coiled polypeptide, and soluble in the water. Globular protein function related to the metabolism of the cells, such as albumin, globumin and cytochrome C.
2. Conjugated protein, the degradation protein that results in amino acids and other substances, non-protein compounds called prosthetic, such as glycoprotein, lipoprotein, nucleoprotein, metal lipoprotein and phosphoprotein.
3. Derived protein, a protein derived from protein degradation of simple and conjugated protein with enzymes or chemical reactions, such as gelatin, a protein derivative from the degradation of collagen by boiling with acid.

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Chemical Properties of Protein

Ninhydrin reaction

Ninhydrin reaction is a reaction to test alpha type amino acid that is a component of proteins by reacting with a ninhydrin solution at high temperatures. The reaction results in aldehyde compound and violet blue formation.

Biuret reaction

Biuret reaction is a reaction to test normal protein compounds by the reaction with a solution of copper sulfate ($CuSO_4$) in alkaline solution that give effect in purple to pink upon molecular dimensions.

Xanthoproteic reaction

Xanthoproteic reaction is a reactive to test protein with an amino acid that has a benzene ring in the molecule, tyrosine and tryptophan by adding nitric acid into the protein solution and results in yellow precipitation.

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Quantitative Analysis of Protein

Kjeldahl method firstly developed by Johan Kjeldahl in 1883 [28], bases on the digestion of proteins and other organic components in sample with acid in the presence of catalyst such as sodium and potassium sulphate to release nitrogen from protein and retain as ammonium salt. Quantitative determination of protein by Kjeldahl method consists of four main steps.

Sample digestion

The sample will be digested with concentrate sulfuric acid and nitrogen in the sample will be converted into ammonium sulphate $(NH_4)_2SO_4$ under high temperature conditions with a catalyst such as $CuSO_4$, Se, $HgSO_4$, HgO and $FeSO_4$.

Ammonia distillation

Ammonia will be distilled using sodium hydroxide by react with ammonium sulphate salt from the sample digestion and results in ammonia gas which can be entrapped with boric solution.

Titration

Titration for finding nitrogen was done by the boric acid solution which entrapped ammonia will be added to titrate with standard solution of sulfuric acid.

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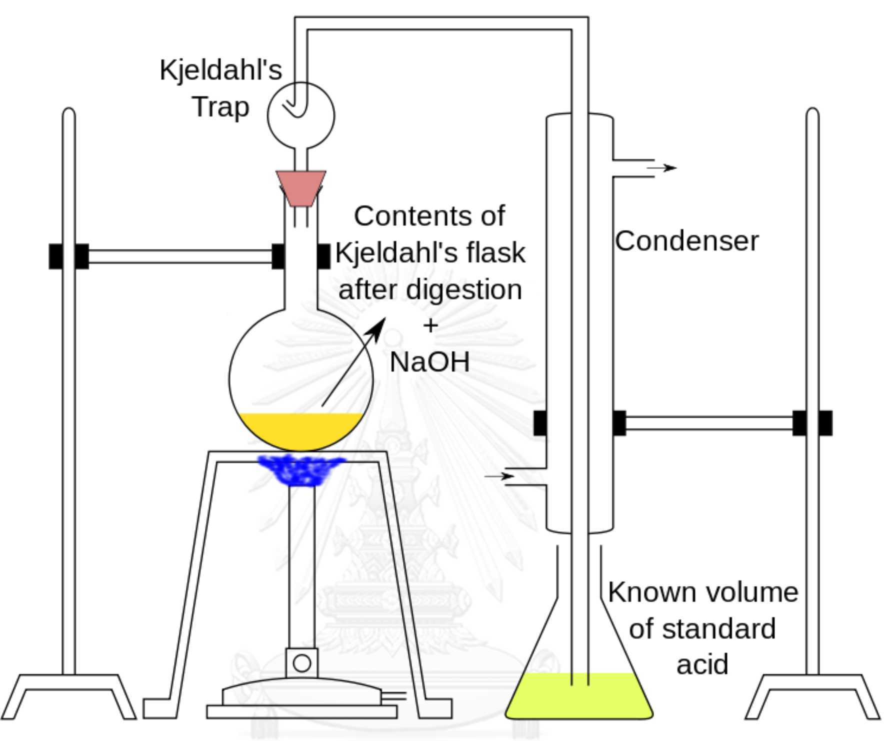
Calculation

The volume of a standard solution of sulfuric acid in the titration will be calculated for the quantity of nitrogen and multiplied with Kjeldahl factor.



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Degradation:

$$\text{Sample} + H_2SO_4 \longrightarrow (NH_4)_2SO_4\,(aq) + CO_2\,(g) + SO_2\,(g) + H_2O\,(g)$$

Liberation of ammonia:

$$(NH_4)_2SO_4\,(aq) + 2NaOH \longrightarrow Na_2SO_4\,(aq) + 2H_2O(l) + 2NH_3\,(g)$$

Capture of ammonia:

$$B(OH)_3 + H_2O + NH_3 \longrightarrow NH_4^+ + B(OH)_4^-$$

Back-titration:

$$B(OH)_3 + H_2O + Na_2CO_3 \longrightarrow NaHCO_3\,(aq) + NaB(OH)_4\,(aq) + CO_2\,(g) + H_2O$$

Figure 2 Kjeldahl method and its chemical reaction equation

Fat

Fat or lipid is an organic compound containing carbon, hydrogen and oxygen. Fat is insoluble in water but soluble in organic solvent such as ether, chloroform, benzene, acetone and ethanol [29]. Lipids from cells or tissues of an organism can be extracted using the organic solvents. Lipids are found in the plant, animals tissue and serves upon the type and chemical structure [30] Fats are compounds of alcohol ester and fatty acid that is a diversified chemical structure but the structure is basically the same. Fats have the basic structure of a hydrocarbon (CH) that is non-polar and hydrophobic properties, insoluble in water but soluble in organic solvents. Some lipids may consist have both hydrophilic and hydrophobic properties in the molecule, amphiphiles property, such as phosphoglycerides and sphingolipids [31]. Fat can be classified into several groups according to the characteristic and chemical properties [32].

1. Simple lipid, simple lipid is the esters of fatty acids with alcohols.
2. Compound lipid, compound lipid is a compound derived from esters of fatty acids with alcohol and other substances included lipid group, such as phospholipid lipid, sphingolipid and glycolipid.
3. Derived lipid, derived lipid is a derivative of the decomposition of lipids and plain lipid, which remains the property of lipids, including fatty acids, glycerol, monoglycerides and triglycerides.
4. Miscellaneous lipids, such as steroid and terpene.

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Fatty Acid

Fatty acid or carboxylic acid is organic acid which the molecule contain the carboxyl group (COOH). Fatty acid can be categorized into two groups by using bonds in the hydrocarbon chain as classification criteria [33].

1. Saturated fatty acid, saturated fatty acid is the fatty acid that has the single bond in each carbon atom and cannot bring hydrogen into its molecule. Saturated fatty acid ($C_nH_{2n}O_2$) is stable molecule, so it cannot react with oxygen (lipid oxidation). Saturated fatty acid has higher melting point than unsaturated fatty acid.
2. Unsaturated fatty acid, unsaturated fatty acid is the fatty acid that has at least one double bond between carbon chains. Unsaturated fatty acid is unstable molecule, so it can react with oxygen. Unsaturated fatty acid can be categorized into two groups.
    a. Monounsaturated fatty acid or monoethenoid, it is unsaturated fatty acid which has one double bond in carbon chains such as oleic acid.
    b. Polyunsaturated fatty acid, it is unsaturated fatty acid that has double bond more than one point in carbon chains. Double bond in unsaturated fatty acid can be subcategorized into two types, Cis and Trans form [34].

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Fatty acid is lipid that rarely found in nature in the form of free fatty acids but found to be the composition of triglycerides in the molecule or triglycerol in oil. Fatty acid plays an importance role in biological metabolism [35]. It distributed the cells to serve as a fuel for muscular contraction and general metabolism. They are consumed by mitochondria to produce adenosine triphosphate (ATP) through beta-oxidation [36].

Table 1 Unsaturated fatty acid of general formula $CH_3(CH_2)_mCH=CH(CH_2)_nCOOH$ [37]

Systematic name	Trivial name	Shorthand designation
Cis-9-dodecenoic	Lauroleic	12:1 (n-3)
Cis-9-tetradecenoic	Myristoleic	14:1 (n-5)
Trans-3-hexadecenoic	-	16:1*
Cis-9-hexadecenoic	Palmitoleic	16:1 (n-7)
Cis-6-octadecenoic	Petroselinic	18:1 (n-12)
Cis-9-octadecenoic	Oleic	18:1 (n-9)
Trans-9-octadecenoic	Elaidic	18:1*
Cis-11-octadecenoic	*Cis*-vaccenic	18:1 (n-7)
Trans-11-octadecenoic	*Trans*-vaccenic	18:1*
Cis-9-eicosenoic	Gadoleic	20:1 (n-11)
Cis-11-eicosenoic	Gondoic	20:1 (n-9)
Cis-13-docosenoic	Erucic	22:1 (n-9)
Cis-15-tetracosenoic	Nervonic	24:1 (n-9)

* The (n-x) no menclature is only used with fatty acids containing *Cis* double bonds.

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Table 2 Saturated fatty acid of general formula $CH_3(CH_2)_nCOOH$ [37]

Systematic name	Trivial name	Shorthand designation
Ethanoic	Acetic	2:0
Propanoic	Propionic	3:0
Butanoic	Butyric	4:0
Pentanoic	Valeric	5:0
Hexanoic	Caproic	6:0
Heptanoic	Enanthic	7:0
Octanoic	Caprylic	8:0
Nonanoic	Pelargonic	9:0
Decanoic	Capric	10:0
Hendecanoic	-	11:0
Dodecanoic	Lauric	12:0
Tridecanoic	-	13:0
Tetradecanoic	Myristic	14:0
Pentadecanoic	-	15:0
Hexadecanoic	Palmitic	16:0
Heptadecanoic	Margaric	17:0
Octadecanoic	Stearic	18:0
Nonadecanoic	-	19:0
Eicosanoic	Arachidic	20:0
Heneicosanoic	-	21:0
Docosanoic	Behenic	22:0
Tetracosanoic	Ligoceric	24:0

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Table 3 Unsaturated fatty acid of general formula $CH_3(CH_2)_m(CH=CH(CH_2)_x(CH2)_nCOOH$ [37]

Systematic name	Trivial name	Shorthand designation
Cis-9-dodecenoic*	Linoleic	18:2 (n-6)
Cis-9-tetradecenoic	Gamma-linolenic	18:3 (n-6)
Trans-3-hexadecenoic	Homo-gamma-linolenic	20:3 (n-6)
Cis-9-hexadecenoic	Arachidonic	20:4 (n-6)
Cis-6-octadecenoic	-	20:5 (n-6)
Cis-9-octadecenoic	Alpha-linolenic	18:3 (n-3)
Trans-9-octadecenoic	-	20:5 (n-3)
Cis-11-octadecenoic	-	22:6 (n-3)
Trans-11-octadecenoic	-	20:3 (n-9)

*The double bond configuration in each instance is *Cis*.

Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry (GC-MS) is an analytical method which using the features of mass spectrometry and gas-liquid chromatography to validate volatile liquid sample substances such as oil and fatty acid after derivatization [38]. GC-MS consists of two major components, gas chromatograph and mass spectrometer. The gas chromatograph utilizes a capillary column depends on length, diameter and thickness of the column as well as the phase properties [39]. Since the different of chemical properties in the sample and relative affinity of stationary phase, the column will separate the molecules. The sample will be eluted from the column at different times. The mass spectrometer downstream, capture and detect the ionized molecules. The mass spectrometer will break the molecule into ionized fragments and detect these fragments by using mass to charge ratio [40].

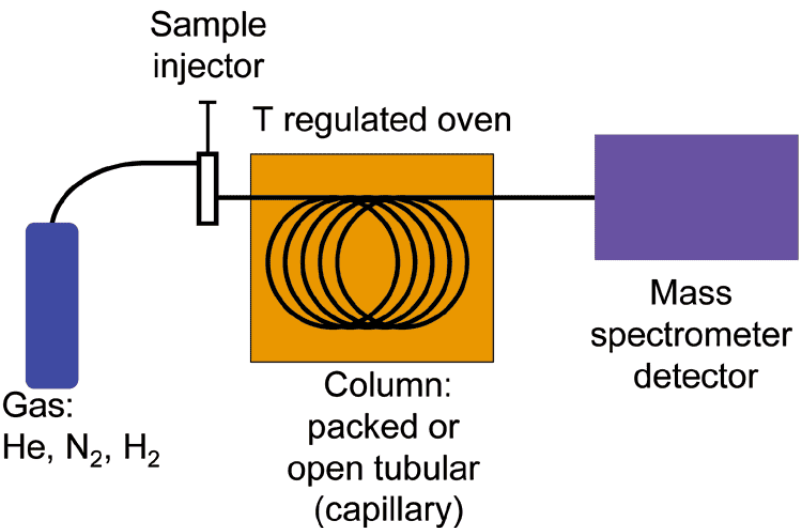

Figure 3 GC-MS schematic [41]

Fibre

Fibre refers to the cell walls of plants such as vegetables, fruits, grains that are not digested in the gastrointestinal tract. Fibre or dietary fibre can be categorized into two groups [42].

1. Insoluble dietary fibre refers to fibre which insoluble in water. Bacteria in intestine cannot digest insoluble dietary fibre, such as cellulose, hemicellulose and lignin.
2. Soluble dietary fibre refers to fibre that can absorb water and swell in the water (gel forming property). Bacteria in intestine can digest soluble dietary fibre, such as resistant starch, oligosaccharide and heteropolysaccharide.

Figure 4 Structure of cellulose (A), hemicellulose (B), oligosaccharide (C) and heteropolysaccharide (D) [43-46]

Micronutrient (vitamins and minerals)

ß-carotene

ß-carotene is a pigment lipid that has orange-yellow colour in the carotenoids group. ß-carotene is a precursor of vitamin A (pro vitamin A) since it can be converted to retinol in the small intestine and liver. ß-carotene plays an important role for human health, it has powerful antioxidant functions and can limit the membranes damaged cell [47, 48]. ß-carotene can decrease the risk of cardio-vascular disease as well as lung cancers [49]. When ß-carotene converted to vitamin A in the intestines, it has effective function such as visual cycle, sperm production, maintenance of epithelial functions, growth and development. In nature, ß-carotene is a precursor to vitamin A *via* the ß-carotene 15,15'-monooxygenase pathway [50]. The ß-carotene separation is based on the polarity, it is a non-polar compound then it can be separated by using non-polar solvent [51].

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Figure 5 The structure of ß-carotene [52]

Vitamin E

Vitamin E is a group of lipid-soluble compounds including tocopherols and tocotrienols [53]. Alpha-tocopherol mostly found in corn oil, wheat germ oil, sunflower, safflower oils and soybean oil [54]. Alpha-tocopherol is an important lipid-soluble antioxidant; it stops the production of reactive oxygen species free radical [55, 56]. It performs functions as antioxidant in the glutathione peroxidase pathway, and protects cell membranes from oxidation by reacted with lipid radicals produced in lipid peroxidation chain reaction [57, 58].

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Figure 6 The structure of Alpha-tocopherol [59]

Vitamin B1

Vitamin B1 or thiamine is a water-soluble vitamin. Thiamine is used in the biosynthesis of the neurotransmitter acetylcholine and gamma-aminobutyric acid (GABA). Thiamine is synthesized only in bacteria, fungi, and plants but it is an essential nutrient in mammals [60]. In mammals, thiamine deficiency can cause optic neuropathy, and peripheral nervous system or cardiovascular system disease. It is also used for digestive problems including poor appetite, ulcerative colitis, ongoing diarrhoea, acquired immunodeficiency syndrome, boosting the immune system and preventing cervical cancer as well [61].

The stable and non-hygroscopic salt thiamine mononitrate is the vitamin used for flour and food fortification. Thiamine is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system [62].

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Figure 7 The structure of thiamine or vitamin B1 [63]

Vitamin B2

Vitamin B2 or riboflavin is a yellow-orange solid substance and water-soluble vitamin same as vitamin B1. Riboflavin plays an important role in the metabolism of nutrients that provide energy such as fat, carbohydrate and protein [64]. It also plays important role for body growth and red blood cell production. Riboflavin can be found in meat, egg, nuts, green leafy vegetables and dairy products. Riboflavin deficiency causes cankers symptoms, chapped lips and light irritated eyes [65].

Figure 8 The structure of riboflavin or vitamin B2 [66]

Vitamin C

Vitamin C or L-ascorbic acid is the water-soluble vitamin. It is an essential nutrient for humans and certain other animal species. Ascorbate and ascorbic acid are both naturally present in the body when either of these is introduced into cells, since the forms interconvert according to pH. In animals, these reactions are especially important in wound healing and in preventing bleeding from capillaries. Ascorbate may also act as an antioxidant against oxidative stress [67]. It can be found in vegetables and fruits. L-ascorbic acid deficiency causes scurvy due to the absorption of iron deficiency, depression, fatigue and anaemia [68].

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Figure 9 The structure of vitamin C [69]

Niacin

Niacin, nicotinic acid or vitamin B3 is an organic compound with colourless and water-solubility solid. Niacin is a derivative of pyridine, with a carboxyl group. Niacin cannot be directly converted to nicotinamide, but it is precursors for the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) [70]. NAD and NADP are coenzymes participating in many hydrogen transfer processes. NAD is important in catabolism of fat, carbohydrate, protein, and alcohol, as well as cell signalling and deoxyribonucleic acid repair; while, NADP mostly in anabolism reactions such as fatty acid and cholesterol synthesis [71]. Insufficient niacin in the diet can cause nausea, skin and mouth lesions, anaemia, headaches, and tiredness. Chronic niacin deficiency leads to a disease called pellagra. The lack of niacin may also be observed in pandemic deficiency disease, which is caused by a lack of five crucial vitamins (niacin, vitamin C, thiamine, vitamin D, and vitamin A) and is usually found in areas of widespread poverty and malnutrition. Niacin has not been found to be useful in decreasing the risk of cardiovascular disease in those already on a statin but appears to be effective in those not taking a statin [72, 73].

O
OH
N

Figure 10 The structure of niacin [74]

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Folic acid

Folic acid, folate or vitamin B9 is water-soluble vitamin that is often used as the food supplement. Folic acid or folate converted by humans to dihydrofolic acid, tetrahydrofolic acid and derivatives. Folic acid plays important role in biological activities, DNA synthesize, DNA reparations, DNA methylation, cofactor as well as red blood cells production and anaemia prevention; furthermore, it is important for pregnancy and infancy for cell division and cell growth [75]. Folates can be found in many foods especially in dark green leafy vegetables [76]. Folic acid deficiency can result in developing embryos, pregnancy complications, diarrhoea, anaemia, nerve damage, mental confusion and behavioural disorders as well.

Figure 11 The structure of folic acid [77]

Sodium

Sodium is a silver-white and highly reactive metal element with symbol Na and atomic number 11. Salts of sodium are highly water-soluble. Sodium is an essential element for animals and plants [78]. In humans, sodium is an essential nutrient for regulation blood volume, blood pressure, neuron function and osmoregulation between cells and extracellular fluid. Sodium chloride is the main source sodium in the diet. Sodium is the most prominent cation in extracellular fluid. Unusually low or high sodium levels in humans are recognized in medicine as hyponatremia and hypernatremia.

Potassium

Potassium is a soft silvery-white alkali metal element with symbol K and atomic number 19. Potassium can be rapidly oxidized in air and reactive with water. Potassium was individually isolated from salts by electrolysis. In nature, potassium occurs only in ionic salts, it is found in seawater. Potassium ions are necessary for the function of all living cells. Potassium ion diffusion is a key mechanism in nerve transmission, and potassium depletion in animals, including humans, results in various cardiac dysfunctions [79]. Potassium accumulates in plant cells, and thus fresh fruits and vegetables are a good dietary source of it.

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Magnesium

Magnesium is a chemical element with symbol Mg and atomic number 12. Magnesium ions are essential to all living cells. Magnesium plays a major role in manipulating important biological polyphosphate compounds, adenosine triphosphate, deoxyribonucleic acid and ribonucleic acid. Magnesium compounds are medicinally used as common laxatives, antacids and nerve excitation stabilizer [80]. It is the metallic ion of chlorophyll, it is a common additive to fertilizers. Magnesium ions are essential to the basic nucleic acid chemistry of life, and essential to all cells of living organisms.

Calcium

Calcium is a soft grey alkaline earth element with symbol Ca and atomic number 20. Calcium is essential mineral for living organisms. Calcium is abundant metal and the most important component of a healthy diet. Calcium deficiency can occur rickets, poor blood clotting and menopause, it can lead to osteoporosis, in which the bone deteriorates. Dairy products, such as milk, are a common source of calcium. Some plants contain calcium, such as seaweeds, almonds, hazelnuts, sesame, and pistachio. In addition, several foods and drinks are often fortified with calcium. Numerous vegetables, notably spinach, chard and rhubarb have high calcium content.

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Iron

Iron is a chemical element with symbol Fe and atomic number 26. Iron is abundant in biology [81]. Iron-proteins are found in all living organisms, ranging from the evolutionarily primitive archaea to humans. The colour of blood is due to the haemoglobin, an iron-containing protein. As illustrated by hemoglobin, iron is often bound to cofactors. The iron-sulphur clusters are pervasive and include nitrogenase, the enzymes responsible for biological nitrogen fixation [82]. Influential theories of evolution have invoked a role for iron sulphides in the iron-sulphur world theory. Iron is a necessary trace element found in nearly all organisms. Iron-containing enzymes and proteins, often containing heme prosthetic groups, participate in many biological oxidations and in transport. Examples of proteins found in higher organisms include haemoglobin, cytochrome and catalase [83].

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Zinc

Zinc is a chemical element with symbol Zn and atomic number 30. Zinc is an essential mineral of human health [84]. In children, zinc deficiency causes growth retardation, delayed sexual maturation, and infection susceptibility. Enzymes with a zinc atom in the reactive centre are widespread in biochemistry, such as alcohol dehydrogenase [85]. Consumption of excess zinc can cause ataxia and lethargy. Zinc is an essential trace element for humans, other animals, plants and microorganisms [86-88]. In proteins, Zn ions are often coordinated to the amino acid side chains of

aspartic acid, glutamic acid, cysteine and histidine. Most zinc is in the brain, muscle, bones, kidney, and liver, with the highest concentrations in the prostate and parts of the eye. Semen is particularly rich in zinc, which is a key factor in prostate gland function and reproductive organ growth. It plays a key role in synaptic plasticity and so in learning [89-91].

Copper

Copper is a soft and malleable element with symbol Cu and atomic number 29. Copper is essential to all living organisms as a trace dietary mineral because it is a key constituent of the respiratory enzyme complex cytochrome c oxidase. In various species of molluscs and crustaceans, copper is a constituent of the blood pigment haemocyanin, which is replaced by the iron-complexed haemoglobin in fish and other vertebrates. The main areas where copper is found in humans are liver, muscle and bone. Copper compounds are used as bacteriostatic substances, fungicides, and wood preservatives.

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Iodine

Iodine is a chemical element with symbol I and has atomic number 53. Iodine and its compounds are primarily used in nutrition. Iodine is an essential trace element for life, the heaviest element commonly needed by living organisms. Iodine's main role in animal biology is as a constituent of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3) [92]. Thyroid hormones are phylogenetically very old molecules

that are synthesized by most multicellular organisms, and that even have some effects on unicellular organisms. Thyroid hormones play a basic role in biology, acting on gene transcription to regulate the basal metabolic rate. Iodine has a nutritional relationship with selenium. Its role in mammary tissue is related to fetal and neonatal development [93].

Phosphorus

Phosphorus is a chemical element with symbol P and atomic number 15. Phosphorus is essential for life. Phosphate is a component of deoxyribonucleic acid, ribonucleic acid, adenosine triphosphate, and also the phospholipids, which form all cell membranes [94]. In medicine, hypophosphatemia is the low condition of soluble phosphate in serum. It caused muscle, neurological dysfunction, and blood cells disruption. Hypophosphatemia can lead to diarrhoea, organ and tissue calcification. Phosphorus can be found in foods containing protein such as meat, grain, dairy and dairy product [95].

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Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) is a spectroanalytical procedure for chemical element quantitative analysis by using the absorption of optical radiation. It is a tool used to analyse the metal elements in the test sample [96]. Atomic absorption spectroscopy is a process in which free atom of particular element absorb a specific wavelength of light. Because each element has a different level of energy, the unique property of the elements makes the electrons transition from the ground state to exited state, then the independent atom will absorbs at specific energy. The higher free atom, the more independent atomic absorption is absorbed. AAS is consist of five major parts; light source, atomizer, monochromator, detector and processing machine [97].

Light source

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Light source of AAS generally is hollow cathode lamp and electrodeless discharge lamp. Hollow cathode lamp contains buffer of inert gas and coats with salts of metals. Hollow cathode lamp can be divided into two main types; single and multi-lamps. Single lamp can release specific wavelengths from the coated salt on the lamp. In addition, multiple lamps is coated by various kind of metal salts, the released energy will be selected by a monochromator.

Atomizer

The components of the thermal energy causing the atoms independently called atomizer. Atoms in the compound will be become the independent atom by the thermal energy absorption. The thermal energy may in the form of heat from the electricity flames. The process that caused independent atom called atomization process, the atomization process that is commonly used including flam atomization, electro thermal atomization or graphite furnace, hydride generation technique and cold vapour technique.

Monochromator

A monochromator is an optical device that transmits a mechanically selectable narrow band of wavelengths from light source (multi lamp).

Detector and Processing Machine

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Line source AAS (LS AAS) that is required for the measurement of atomic absorption is provided by the narrow line emission of the radiation source, and the monochromator simply has to resolve the analytical line from other radiation emitted by the lamp. Photomultiplier tubes are the most frequently used detectors in LS AAS, although solid state detectors might be preferred because of their better signal-to-noise ratio.

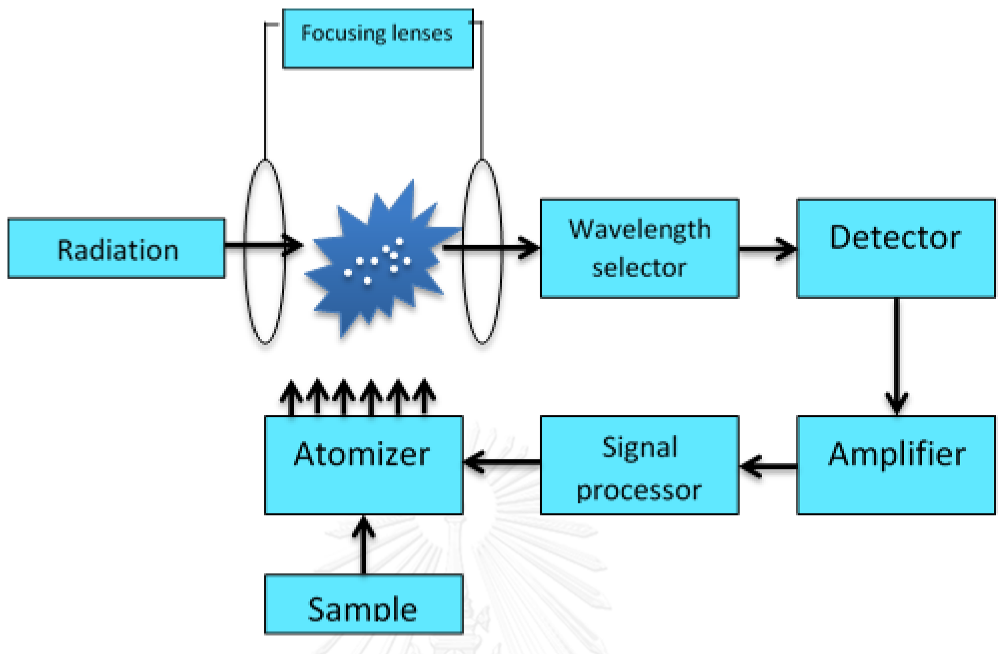

Figure 12 Atomic absorption spectrometer procedure [98]

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Hypnotic and Anxiolytic Evaluation

Anxiolytic agents are a medication or other intervention that inhibits anxiety [99]. This effect is in contrast to anxiogenic agents, which increase anxiety. Together these categories of psychoactive compounds or interventions may be referred to anxiotropic compounds or agents. Some recreational drugs such as ethanol (ethyl alcohol) induce anxiolytic. Anxiolytic medications have been used for the treatment of anxiety and its related psychological and physical symptoms. Anxiolytics have been shown to be useful in the treatment of anxiety disorders. Bright light therapy and other interventions have also been found to have an anxiolytic effect [100]. Beta-receptor blockers such as propranolol and oxprenolol can be used to resist the somatic symptoms of anxiety.

Most hypnotics prescribed today are either benzodiazepines or nonbenzodiazepines. Early classes of drugs, such as barbiturates, have fallen out of use in most practices but are still prescribed for some patients. In children, prescribing hypnotics is not yet acceptable unless used to treat night terrors or somnambulism [101]. Elderly people are more sensitive to potential side effects of daytime fatigue and cognitive impairments, and a meta-analysis found that the risks generally outweigh any marginal benefits of hypnotics in the elderly [102]. A review of the literatures regarding benzodiazepine hypnotics and nonbenzodiazepine (Z-drugs) drugs concluded that these drugs can have adverse effects, such as dependence and

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accidents, and that optimal treatment uses the lowest effective dose for the shortest therapeutic time period, with gradual discontinuation in order to improved health without worsening of sleep [103].

Evaluated Plus Maze Test

The elevated plus maze (EPM) is a rodent model of anxiety that is used as a screening test for putative anxiolytic or anxiogenic compounds. It is a general research tool in neurobiological anxiety research. EPM apparatus consists of two open and two enclosed arms with open roof, elevated 40–70 cm from the floor. The model is based on rodents' aversion of open spaces. This aversion leads to the behaviour termed thigmotaxis, an index of anxiety in mice that involves open areas avoidance by confining movements to enclosed spaces. In EPM this translates into a restriction of movement to the enclosed arms [104]. Anxiety reduction in the plus-maze is indicated by increasing time spent in the open arms and an increase in the proportion of entries into the open arms. Total number of arm entries and number of closed-arm entries are usually employed as measures of general activity. EPM is the most commonly employed behavioural animal anxiety model. There are several issues concerning the validity of the model such as benzodiazepines that reduce measures of anxiety in EPM; in addition, selective serotonin reuptake inhibitors and tricyclic antidepressants are commonly employed in clinical settings to treat anxiety disorders [105]. This raises the possibility that EPM is a suitable model for testing GABA-related compounds, such as

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benzodiazepines or direct $GABA_A$ agonist. EPM is commonly employed model for screening putative anxiolytics and for general research into the brain mechanisms of anxiety [106].

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Figure 13 Evaluated plus maze test apparatus [107]

Rotarod Performance Test

The rotarod performance test (RRP) is a test based on forced motor activity. The test measures parameters such as riding time or endurance. Some of the functions of the test include evaluating balance, grip strength and motor coordination of the subjects in testing the effect of experimental drugs [108]. A rodent is placed on a horizontally oriented, rotating cylinder suspended above a cage floor, which is low enough not to injure the animal, but high enough to induce avoidance of fall. Rodents naturally try to stay on rotarod, and avoid falling to the ground. The length of time that a given animal stays on this rotating rod is a measure of their balance, coordination and physical condition. The speed of the rotarod is mechanically driven, and may either be held constant, or accelerated [109].

A human analogue to RRP might be treadmill running. Hamster, gerbil, and mouse owners can observe the principle in action when an animal climbs on the outside of its wheel, instead of inside of it. However, the rotation of the cylinder in experiments is mechanically driven, the advantage of this test is that it creates a discretely measurable, continuous variable that can be used to quantify the effects of different drugs.

The test may be useful as a sensitive indicator of trauma induced by brain injury to laboratory rats [110, 111]. Alcohol markedly impairs mouse performance in the rotarod test. Research using RRP with various chemical agonists and antagonists

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may help scientists to determine which components of neurons mediate the effects of chemicals. Testing of genetic knockout animals may help determine the genes most responsible for maintaining mammalian balance and coordination. Comparing the performance of different animals with specific brain lesions helps scientists map which structures are critical for maintaining balance [112, 113].

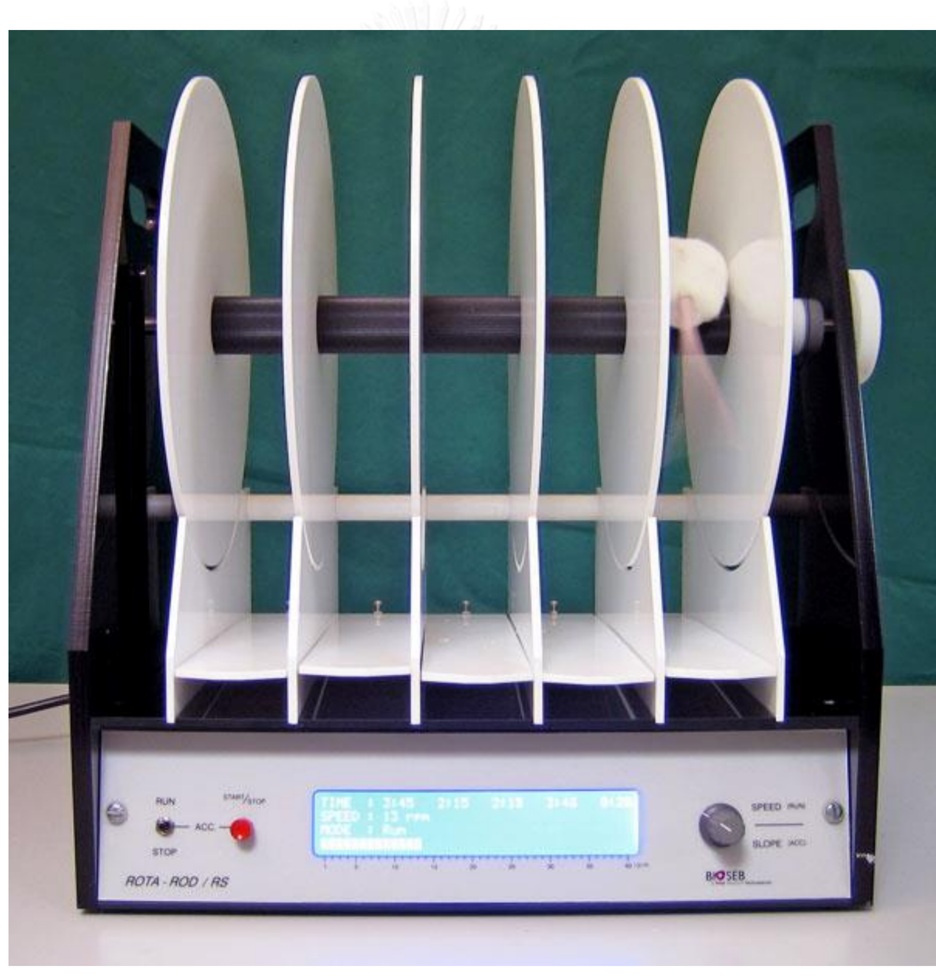

Figure 14 Rota rod performance test apparatus [114]

Open Field Test

The Open Field Test (OF) is an experiment used to assay general locomotor activity levels and anxiety in rodents in scientific research [115]. OF is a commonly used qualitative and quantitative measure of general locomotors activity in rodents [116, 117]. However, the extent to which behaviour in the open field correlates with general locomotors activity in other situations is controversial [118]. The open field is an arena with walls to prevent escape. Commonly, the field is marked with a grid and square crossings. Rearing and time spent moving are used to assess the activity of the rodent. In the modern open field apparatus, infrared beams or video cameras with associated software can be used to automate the assessment process. The OF is also often used to assess anxiety by including additional measures of defecation, time spent in the centre of the field, and the first few minutes of activity [119]. The previous study suggested the relationship between EPM and OF which is popular exploratory activity. Their research study revealed that both EPM and OF can be used for analysing the sedative or stimulant effects of pharmacological agents by using inbred strain mice, BALB/c and C57BL/6J [120].

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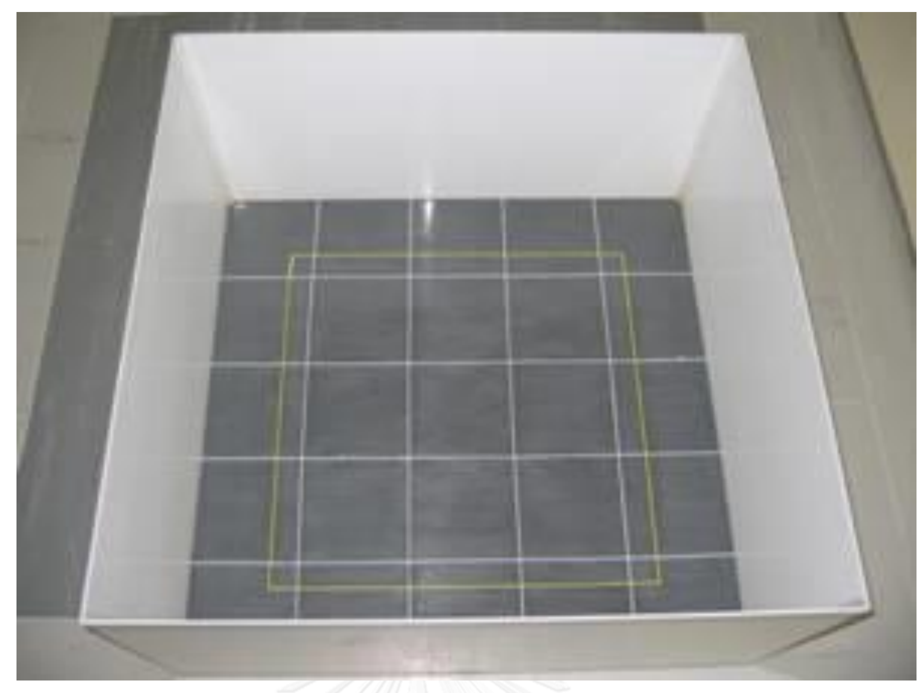

Figure 15 Open field test apparatus [121]

Analysis of Sleep in *Drosophila melanogaster*

Drosophila melanogaster is a species of fly in the family Drosophilidae. The species is known generally as the common fruit fly. *D. melanogaster* is widely used for genetics, physiology, microbial pathogenesis and life history evolution research. Because of easy care, fast breeding (Figure 16), and laying many eggs [122], it is typically used for preliminary research. *D. melanogaster* has four pairs of chromosomes. Wild type fruit flies are yellow-brown, with brick red eyes and transverse black rings across the abdomen.

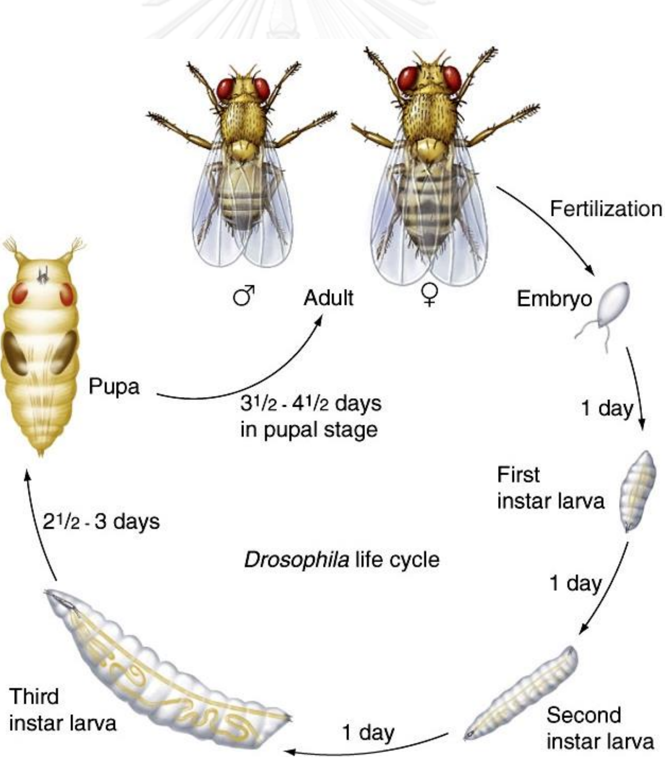

Figure 16 *Drosophila melanogaster* life cycle [123]

They exhibit sexual dimorphism: females are about 2.5 millimetres long; males are slightly smaller with darker backs. Males are easily distinguished from females based on

colour, with a distinct black patch at the abdomen, less noticeable in recently emerged flies (Figure 17), and the sexcombs a row of dark bristles on the tarsus of the first leg. Males D. melanogaster have a cluster of spiky hairs around the reproducing parts used to attach to the female during mating.

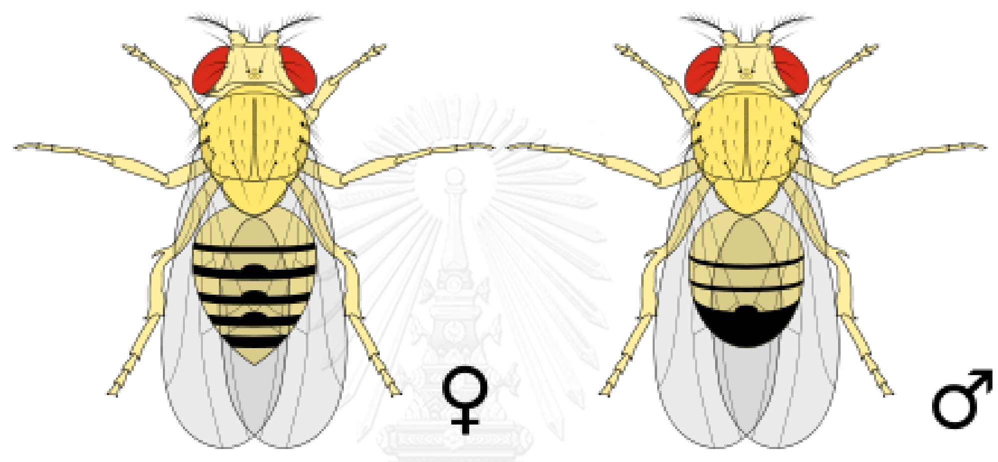

Figure 17 Female (left) and male (right) *D. melanogaster* [124]

D. melanogaster was among the first organisms used for genetic analysis because the comprehend processes such as transcription and replication in fruit flies helps in understanding in other eukaryotes, including humans [125]. *Drosophila* has emerged as an ideal model organism for studying the genetic components of sleep as well as its regulation and functions. In fruit flies, sleep can be conveniently estimated by measuring the locomotors activity of the flies using techniques and instruments adapted from the field of circadian behaviour. The fly is also being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, and cancer, as well as drug abuse.

Sleep–Wake Profile

In animals, sleep is a naturally recurring state characterized by altered consciousness, relatively inhibited sensory activity, and inhibition of nearly all voluntary muscles [126]. It is distinguished from wakefulness by a decreased ability to react to stimuli, and it is more easily reversible than being in hibernation or a coma. During sleep, most systems in an animal are in a heightened anabolic state, accentuating the growth and rejuvenation of the immune, nervous, skeletal, and muscular systems. Sleep in non-human animals is observed in mammals, birds, reptiles, amphibians, and fish, and in some form in insects and even in simpler animals such as nematodes, suggesting that sleep is universal in the animal kingdom. The purposes and mechanisms of sleep are only partially clear and the subject of substantial ongoing research. Sleep is sometimes thought to help conserve energy, though this theory is not fully adequate as it only decreases metabolism by about 5–10%. Additionally it is observed that mammals require sleep even during the hypometabolic state of hibernation, in which circumstance it is actually a net loss of energy as the animal returns from hypothermia to euthermia in order to sleep [127]. In mammals and birds, sleep is divided into two broad types; rapid eye movement (REM sleep) and non-rapid eye movement (NREM or non-REM sleep). Each type has a distinct set of physiological and neurological features associated with species. REM sleep is associated with the capability of dreaming. The American Academy of Sleep Medicine (AASM) divides NREM into three states; N1, N2, and N3 [128].

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NREM sleep

State N1 refers to the transition of the brain from alpha waves which having a frequency of 8-13 Hz (common in the awake state) to theta waves which having a frequency of 4–7 Hz. This state is sometimes referred to as somnolence or drowsy sleep. Sudden twitches and hypnic jerks, also known as positive myoclonus, may be associated with the onset of sleep during N1. Some people may also experience hypnagogic hallucinations during this state. During N1, the subject loses some muscle tone and most conscious awareness of the external environment.

State N2 is characterized by using sleep spindles ranging from 11 to 16 Hz and K-complexes as criterion. During this state, muscular activity as measured by EMG decreases, and conscious awareness of the external environment disappears. This state occupies 45–55% of total sleep in adults.

State N3 and N4 (deep or slow-wave sleep) is characterized by the presence of a minimum of 20% delta waves ranging from 0.5–2 Hz and having a peak-to-peak amplitude >75 μV. EEG standards define delta waves to be from 0 to 4 Hz. This is the state in which parasomnias such as night terrors, nocturnal enuresis, sleepwalking, and somniloquy occur. Many illustrations and descriptions still show a state N3, N4 with 20–50% delta waves.

REM sleep

Rapid eye movement sleep (REM) or paradoxical sleep accounts for 20–25% of total sleep time in most human adults [129]. The criteria for REM sleep include rapid eye movements as well as a rapid low-voltage EEG. During REM sleep, EEG patterns returns to higher frequency saw-tooth waves. Most memorable dreaming occurs in this state.

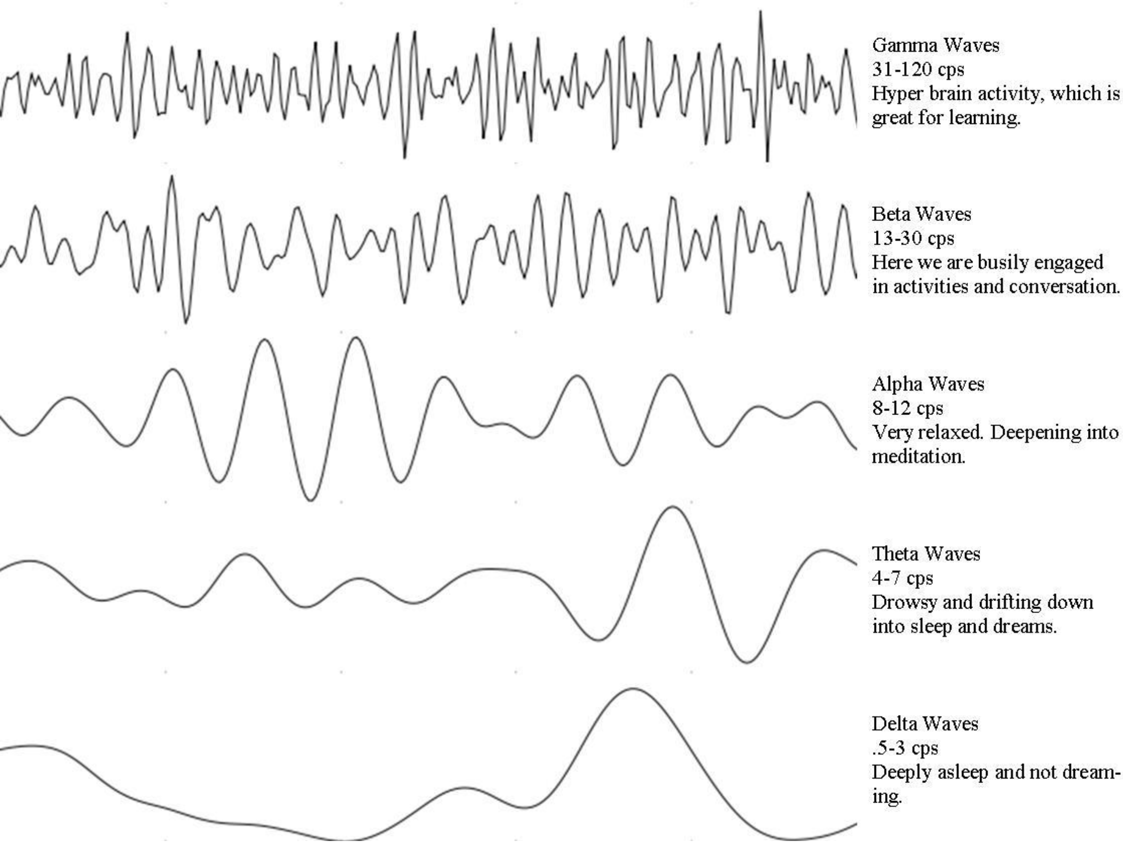

Figure 18 Brainwave pattern [130]

CHAPTER III
MATERIALS AND METHODS

Chemicals and Reagents (HPLC and Analytical grade)

Acetonitrile	Merck, Germany
Alkaline ferricyanide solution	Merck, Germany
Alpha-amylase	Sigma-Aldrich, USA
Alpha-tocopherol acetate	Sigma-Aldrich, USA
Amyloglucosidase	Sigma-Aldrich, USA
Bacto micro assay culture agar	Difco, USA
Bacto micro inoculum broth	Difco, USA
Beta-carotenoid	Sigma-Aldrich, USA
Boric acid	BDH, England
Citric acids	BDH, England
Dehydro-D-isoascorbic	Fluka, USA
Dichloromethane	Merck, Germany
Diethyl ether	Merck, Germany
Dipotassium phosphate	BDH, England
Ethanol	Merck, Germany
Flumazenil	Wuhan Senwayer Century Chemical Co., Ltd, Wuhan, China
Folic acid	Sigma-Aldrich, USA
Hexane	Merck, Germany
Homocysteine	BDH, England
Magnesium carbonate	Sigma-Aldrich, USA
Methanol	Merck, Germany
N-hexane	Merck, Germany

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Nicin	Fluka, USA
Pentobabitone sodium	Sigma-Aldrich, USA
Polysorbate 20	Merck, Germany
Potassium hydroxide	BDH, England
Potassium sulfate	BDH, England
Potassium thiocyanate	BDH, England
Pyrogallol	Sigma-Aldrich, USA
Riboflavin	Sigma-Aldrich, USA
Sodium chloride	BDH, England
Sodium hydroxide	BDH, England
Sodium sulphate	BDH, England
Sulfuric acid	Merck, Germany
Takadiastase	Sigma-Aldrich, USA
Termamyl solution	Sigma-Aldrich, USA
Thiamine	Merck, Germany
Thiourea	Merck, Germany
Vanado-molybdate reagent	Qorpak, USA
Zinc sulphate	Sigma-Aldrich, USA

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Instrumentations

Instrument	Manufacturer
Atomic absorption spectrophotometer	Perkin Elmer, Model 5100PC, USA
Gas chromatography	Hewlett-Packard 6890, Palo Alto, United States
Open field apparatus	-
Rotarod apparatus	Model 519/E3C, Medicraft electro medicals Pvt. Ltd., India
Electroencephalogram	MP 150, Biopac systems, USA
Evaluated plus maze apparatus	-
High perfomance liquid chromatography	Thermo Scientific, Finnigan Surveyor Plus HPLC system, USA
Infrared beamsplit monitors	Drosophila Activity Monitor, Model DAM2, TriKinetics, Inc., USA
Spectrophotometer	Anthos Zenyth 200 RT, Biochrom Ltd., United Kingdom
Stereotaxic apparatus	DW-2000, Chengdu Taimeng Software, China

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Plant Materials

L. disjunctus was collected from Patthalung and Trang provinces of Thailand. Plants material was authenticated by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D., and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Plant Extraction

Fresh whole plants were blended with 95% ethanol by an electric blender. The blended fresh plant was continuously macerated with ethanol until exhaustion. The ethanolic extract was filtered through Whatman number 1 filter paper then concentrated to dryness *in vacuo*. Then the ethanolic fraction was evaporated under rotary evaporator and extract yields were weighed, recorded and stored at -20 °C.

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Nutritional Composition Assessment

Moisture (Drying Method)

The moisture by drying method was done according to the guideline of AOAC official methods of analysis (1984) [131]. Ten grams of homogenously blended *L. disjunctus* whole plants were weighed, homogenized in a pre-weighed crucible, dried in an oven at 103 ± 2 °C for 16 hr, cooled in a desiccator, weighed as quickly as possible and repeated 2 hr heating until constant weight (± < 0.5 µg). The test was done in duplicate.

Ash (Dry Ash Method)

Dry ash method was done according to the AOAC official methods of analysis (1984) [131]. Two grams of homogenously blended *L. disjunctus* whole plants were placed into pre-weighed porcelain crucible and incinerated in a furnace at 600 °C for 2 hr. The crucible was directly transferred to desiccator, cooled, and immediately weighed. The tests were done in duplicate and recorded as percent ash.

Protein (Kjeldahl method) [131]

Frozen *L. disjunctus* was ground until homogenous and set to room temperature. The homogenised samples (10 g) were weighed into the digestion tube of Kjeldahl's flask. It was added with 5 g potassium sulphate (catalyst), glass bead and 10 ml concentrate sulfuric acid, and placed in the digester. The mixture was digested initially at low temperature to prevent frothing and boiled briskly until the solution was clear and free of carbon. If the digest was still yellowish, the digest was cooled, added an additional 5-10 ml sulfuric acid and continue digested until a clear digest was obtained. Heating for several hours after the liquid become clear was performed to complete breakdown of all organic matters. The heated solution was transferred to erlenmeyer flask which was contained 50 ml of 4% boric acid with indicator as receiver on the distillation unit. Water and 50% sodium hydroxide were added to the digests and start the distillation. Sodium hydroxide was added to neutralize sulfuric acid to ensure complete release of ammonia, and distilled until all ammonia released or approximately 150 ml distillate was obtained. Finally, the delivery tube was rinsed with water and allowed the washings to drain into the flask. The distillate was titrated with the standardized 0.1N hydrochloric acid until the first appearance of the pink colour. The volume of acid used was recorded to the nearest 0.05 ml and checked the normality of the standardized 0.1N HCl. For blank, it was contained all reagents that used in nitrogen analysis except the test sample in every batch of analysis to subtract reagent nitrogen from the sample nitrogen. The test was done in duplicate.

Calculation

$$N\ (g\%) = \frac{(A - B) \times 0.0014 \times C}{D} \times 100$$

Where, N = Percent total nitrogen

A = ml 0.1N HCl sample

B = ml 0.1N HCl blank

C = Concentration of HCl (Normality, N)

D = Weight of sample

$$Protein\ (g/100\ g) = A \times B$$

Where, A = % total nitrogen

B = Nitrogen conversion factor (6.25)

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Total dietary fibre (Enzymatic-Gravimetric Method) [132]

Grounded *L. disjunctus* (1 g) was weighed and transferred into 400 ml tall-form beakers and added 50 ml 0.08 M phosphate buffer, pH 6.0. Termamyl solution (0.1 ml) was placed in boiling water bath for 15 min (95-100 ˚C) and shook gently at 5 min intervals. The solution was cooled to room temperature and adjusted to pH 7.5 by adding 10 ml of 0.275 N NaOH solutions. Protease enzyme solution (50 mg/ml in phosphate buffer) was pipetted into sample, and covered beaker with aluminium foil. The mixture was incubated for 30 min at 60 °C with continuous agitation, and added 10 ml of 0.325 N HCI solutions when it became cool. Potential of Hydrogen ion was measured and drupe wise added acid to adjust the final pH (4.0-4.6). Amyloglucosidase (0.3 ml) was added, covered with aluminium foil, and incubated for 30 min at 60 °C with continuous agitation. Preheated 95% ethanol (60 °C) was added and allowed fibre to precipitate at room temperature for 60 min.

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Filtering crucible was weighed and added 0.5 g acid and washed Celite. Celite was wet using stream of 78% ethanol from wash bottle and applied suction to draw it onto fritted glass as even material while suction maintaining the enzyme digested precipitate to crucible was transferred. The residue was washed with three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol, and two 10 ml portions of acetone respectively. The crucible which containing residue was dried overnight in 70 °C vacuum in oven, cooled in desiccator and accurately weighed. The analysed

residue from 1 sample was calculated for protein by using Nx6.25 as conversion factor. The second residue was incinerated for 5 h at 500-600 °C, then was cooled in desiccator and accurately weighed. The test was done in duplicate.

Calculation

$$Blank = Weight\ residue - P_B - A_B$$

Where, Weight residue = Average of residue weight (mg) for duplicate blank

P_B = Weight (mg) of protein in first blank residue

A_B = Weight (mg) of ash in blank residue

$$\%Total\ Dietary\ Fiber = \left(\frac{(Weight\ residue - P - A - B)}{Weight\ of\ sample}\right) \times 100$$

Where, Weight residue = Average of weight (mg) for duplicate sample

P = Weight (mg) of protein in first sample residue

A = Weight (mg) of ash in sample residue

B = Blank

Weight sample = Average of 2 sample weight (mg) taken

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Beta carotene (HPLC Method) [133]

Five grams of homogenously blended *L. disjunctus* whole plants were extracted with acetone in mixer with use of Na_2S0_4 and $MgC0_3$ as desiccants, vacuum-filtered, and concentrated prior to saponification. The samples were extracted at least three times to remove all the carotenoids. The concentrate was saponified overnight at room temperature with 5 ml of 100% (w/v) potassium hydroxide in a mixture of ethanol (50 ml) and water (20 ml). Before solvent extraction, the saponification extract was diluted with 10% sodium chloride in water. Carotenoids were extracted with n-hexane-diethyl ether (70:30) with BHT (0.1% in hexane) as antioxidant.

High Performance Liquid Chromatography

All samples were analysed with the non-aqueous reversed phase system. HPLC was carried out using a Thermo Scientific Finnigan liquid chromatographic system with a final volume loop of 500 µl. The detector is photodiode array detection with C_{18} 5 µm particle size column. The elution mixture is acetonitrile, dichloromethane, and methanol (70:20:10) with 2 ml/min flow rate. The carotenoid was identified by comparing their retention times with those of authentic standards. The quantitation was determined based on an external standard method.

Vitamin E (HPLC Method) [133, 134]

Five grams of homogenously blended *L. disjunctus* whole plants were extracted with 0.5 ml of 1% NaCl, 10 ml of 3% pyrogallol in ethanol and 1 ml of 60% KOH. The concentrate was saponified at 70 °C for 30 min. When cool, added 22.5 ml of 1% NaCl. The sample was extracted with hexane:ethyl acetate (9:1) under shaking for 5 min, then centrifuged at 3000 rpm for 5 min. The lower layer was re-extracted and centrifuged as aforementioned. The supernatant was pooled and vacuumed to yield at the temperature below 40 °C, and adjusted the final volume up to 5 ml by using hexane. Thereafter, 200 μl were transferred to a screw-capped tube, where 600 μl of methanol and 200 μl of the internal standard solution (300 μg/ml of tocopherol acetate in ethanol) were added. After being vortex-mixed and centrifuged (3000 rpm for 5 min), the samples were filtered through a 0.45 pm pore size filter and an aliquot of the overlay was directly injected into the chromatograph.

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High Performance Liquid Chromatography

HPLC was carried out using a Thermo Scientific Finnigan liquid chromatographic system with a final volume loop of 500 µl. The detector is photodiode array detection with C_{18} 5 µm particle size column. Fifty µl of sample were injected. The mobile phase was methanol:water (96:4) and the elution was performed at a flow-rate of 2 ml/min. The analytical column was kept at 45 °C. To determine the compounds in the samples, the working standard solutions were always analyzed together with the samples, and peak area ratios were used for calculations following the internal standard method. Detection was performed at 292 nm and each on lasted 6 min.

Vitamin B1 (Thiochrome Method) [135, 136]

Extraction

Appropriately blended frozen *L. disjunctus* was accurately weighed into a 400 beaker, added with 50 ml of 0.14 N HCI and heated for 30 min in a boiling water bath. The extract was cooled to the room temperature, added 5 ml of freshly prepared thiamine free takadiastase and alpha-amylase in 2.5 M sodium acetate suspension and incubated at 37 °C overnight for the conversion of bound thiamine to its free form. After incubation, the sample was cooled to room temperature, transferred to 100 ml volumetric flask, diluted to 100 ml with water and mixed thoroughly and filtered.

Purification

The chromatographic column was filled with H_2O, transferred the bio rex 70 ion exchange resin to the column, allowing the resin to fall in place by gravity. The column was packed to a height of 7-10 cm and allowed excess H_2O to drain. Twenty five ml of filtrate was pipetted onto the column, and allowed to pass through by gravity flow. The filtrate was discarded and washed the reservoir and column with three successive 10 ml portions of hot H_2O, discarding the washings. KCl (10 ml) was placed on the column, collecting eluate in a 25 ml volumetric flask, and added a second 10 ml portion when the first has totally mitered the resin, collecting eluate in the same 25 ml flask. A third portion of 3 ml was added to the column, again collecting in the 25 ml flask. Diluted contents of the 25-ml flask to volume with acid KCl solution and mixed, then repeated the mentioned steps with a new column, using 25 ml of working thiamine standard containing 0.1 or 0.2 µg/ml of thiamine.

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Conversion to Thiochrome

Acid KCl eluate (3 ml) was pipetted into a reaction tube, 3 ml of alkaline ferricyanide solution was added with gentle swirling, and then 15 ml of isobutyl alcohol immediately added. The reaction tube was shaked vigorously for 90 s. Thiamine was oxidized by potassium ferricyanide to yield as thiochrome. Thiochrome was soluble in isobutyl alcohol, and was very constant under standardized conditions.

Separation of Thiochrome Solution

The reaction tubes were centrifuged for 1-3 minutes, the aqueous lower layers was siphoned out, 2-3 g of anhydrous granular Na_2SO_4 was added to the alchohol solution and centrifuged for 1 min.

Blank Preparation

The second aliquot of the acid KCl eluate was placed into a reaction tube and treated it identically, except that 15% NaOH was added rather than alkaline ferricyanide.

Measurement of Thiochrome

The clear colorless isobutyl alcohol solutions (10 ml) was pipetted into cuvettes, and measured the thiochrome concentration in isobutyl alcohol by spectrophotometry at the excitation and emission wavelength of 358 nm and 480 nm respectively.

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Calculation

The thiamine content was calculated as thiamine hydrochloride.

$$\mu g/g = \frac{S - SB}{ST - STB} \times \frac{C}{A} \times \frac{25}{V} \times \frac{100}{SW}$$

Where, S = % Emission of the sample

SB = % Emission of the sample blank

ST = % Emission of the standard

STB = % Emission of the standard blank

C = Concentration of the standard

A = Aliquot taken

25 = Final volume of evaluate

V = Volume used in purification step

100 = Volume original sample was made up to

SW = Sample weight

Vitamin B2 (Spectrofluorometric Method) [135, 136]

Extraction and Precipitation of Interfering Impurities

Blended frozen *L. disjunctus* was accurately weighed and transferred to 125 ml Erlenmeyer flask. The mixture was added with 50 ml of 0.1 N HCl to the flask, and autoclaved for 30 min at 15 psi. After autoclaving, the sample was cooled and adjusted to pH 6.0 with NaOH to precipitate protein and other interfering substances. Since riboflavin was unstable in alkaline solution, the extract was swirled constantly during the addition of alkali to prevent local areas of high pH. Immediately, 1 N HCl was added to bring the pH to 4.5 for further protein precipitating, and the solution was diluted to 100 ml with H_2O and filtered. One N HCl drop wise was added to 50 ml aliquot of the filtrate until no more precipitation and followed by an approximately equal number of drops of 1 N NaOH with constant shaking, and made up this sample extraction to 100 ml with water and filtered again.

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Analysis

Sample solution (10 ml) was mixed and 1 ml H_2O or 1 ml of riboflavin working standard (0.5 µg/ml). Glacial acetic acid (1 ml) was added and mixed, then added 0.5 ml of 3% $KMnO_4$ to each tube, mixed, and allowed standing for exactly 2 min. Finally, 3% H_2O_2 (0.5 ml) was added and mixed thoroughly.

Fluorometry

The fluorescence of the sample solution containing H_2O (A) or riboflavin (B). It was measured afyer mixing within 10 sec with 20 mg of $Na_2S_2O_4$ (C). The excitation and emission wavelengths were set of 380 and 520 nm respectively.

Calculation

The riboflavin content of the sample was calculated from the following formula:

$$\frac{A-C}{B-A} \times \frac{riboflavin\ increment}{10} \times dilution\ factor \times \frac{1}{sample\ weight} = \mu g/g$$

Where, A = Emission intensity of test sample

B = Emission intensity of test standard

C = Emission intensity of blank

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Vitamin C (2,4 Dinitro-Phenylhydrazine Method) [135]

Extraction

Ten grams of homogenously blended *L. disjunctus* were extracted and diluted a representative portion with a sufficient amount of 17% HPO_3 in water H_2O.

Preparation of Tests for Total Vitamin C

The test tubes were prepared for 2 sets (total test and blank test). Aliquots of the following solutions: 0.85% HPO_3 (Reference) were placed, first series of standards (2, 4, 6, 8, and 10 μg/ml), reference, second series of standards standard, reference, standard of 8 μg/ml and samples in series of 15 each being followed by a reference and a standard of 8 μg/ml. The reagents were added in the prescribed sequences and within the time limits specified in Table 5. The mixture was mixed by a Vortex after each addition except for the solutions containing nitric acid that should be mixed by bubbling nitrogen.

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Table 4 Sequences of Addition of Reagents for Total Vitamin C

Reagent	Sequence	Blank Test	Total Test
Homocysteine in 45% K_2HPO_4 in water	1	0.5 ml	-
0.1% 2,6-dichloroindophenol in 5% boric acid in water	2	-	0.5 ml
Homocysteine in 45% K_2HPO_4 in water	3	-	0.5 ml
3.33 % boric acid in water	4	0.5 ml	-
1.2% 2,4-dinitrophenylhydrazine and 3% thiourea in 9 N H_2SO_4	5	1.0 ml	1.0 ml
50% citric acids in methanol and concentrate Nitric Mixture	6	2.0 ml	2.0 ml

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Sequence 1. Boric acid solution (3.33 %) was added to all blank test tubes and note time to proceed with Sequence 4 in about 1.5 hr.

Sequence 2. Proceeded without delay after Sequence 1 by adding 2,6-dichloroindophenol and boric acid solution to all total test tubes that can be handled in about 2 min and follow immediately with Sequence 3.

Sequence 3. Boric acid solution (3.33 %) was added about 2 min after the addition of the previous reagent in the same test tube and let stand about 1.5 hr before proceeding with Sequence 5.

Sequence 4.Homocysteine and K_2HPO_4 mixture was added only to blank test tubes about 1.5 hr after the first addition in Sequence 1.

Sequence 5. DNPH (1.2 %) and thiourea mixture (3 %) was added to all tubes starting with blank tests and hold tubes in a water bath at 32 °C for 80 min and another set of 52 °C.

Sequence 6. Reagent nitric and acetic acids mixture were added to all tubes, which must be cooled to 15 °C, and mix gently by bubbling nitrogen. All tubes were moved to room temperature for the period of time required for the spectrophotometric measurements.

After the linearity of the standard curves was established, only the 8 μg levels need to be determined. Each standard and sample was done in duplicate.

Preparations of Tests For Determination of Ascorbic Acid

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The same way was proceeded as in the above with the following sequnces, then proceeded with total test as in Table 4, and prepared a dehydro-D-isoascorbic acid blank by following the total test sequence by replacing 2,6-dichloroindophenol and boric acid solution by 3.33 % boric acid solution in Sequence 2.

Spectrophotometric Measurement

The absorbance at 520 nm was read, 30-90 min after completion of Sequence 6. Adjusted spectrophotometer reading to 0 absorbance with one reagent reference tube treated in same way as all other tubes. Reading 75 min following the addition of sulfuric acid was allowed the turbidity due to sugar osazones to disappear and renders the absorbance contribution was due to fructose and glucose equivalent in the blanks and total tests.

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Calculations

The concentrations of ascorbic acid and D-isoascorbic acid were calculated according to the following equations:

$$\mu g\ AA/mL\ sample\ = 8\left(\frac{f\left(\frac{c}{d}\right)-e}{b\left(\frac{c}{d}\right)-a}\right)\cdots 1$$

$$\mu g\ IAA/mL\ sample\ = 8\left(\frac{e-\left(\frac{aq}{8}\right)}{c}\right)\cdots 2$$

Where, a = Absorbance of 8 μg ascorbic acid at 52℃

b = Absorbance of 8 μg ascorbic acid at 32℃

c = Absorbance of 8 μg D-isoascorbic acid at 52℃

d = Absorbance of 8 μg D-isoascorbic acid at 32℃

e = Absorbance of sample at 52℃

f = Absorbance of sample at 32℃

q = result obtained by equation 1.

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Niacin (Microbiological Method) [137, 138]

Inoculum

Stock cultures of *Lactobacillus plantarum* (ATCC 8014) were prepared by inoculation in Bacto micro assay culture agar for 24 hr at 37 °C. Inoculum was prepared for subculture from a stock culture of microorganism to 10 ml of Bacto micro inoculum broth. After 24 hr at 37 °C, the cell was centrifuged under aseptic conditions and the supernatant liquid was decanted. The cell was re-suspended in 10 ml sterile 0.85% NaCl solution, re-centrifuged and finally diluted 1:100 with 0.85% sterile NaCl. This bacterial suspension was used to inoculate to each of the assay tubes.

Calibration Curve

Fifty microliter of the bacterial suspension was used to inoculate to each of the assay tubes with 0.0, 0.025, 0.05, 0.1, 0.15, 0.2 and 0.25 μg niacin per tube in 10 ml of Bacto micro assay culture medium. Incubated mixture for 24 hr at 37 °C and after incubating, centrifuge and wash cell with 50 ml portion 0.85% NaCl solution, then re-suspended cells in the NaCl solution to a final volume of 25 ml. Evaporated 10 ml aliquot of cell suspension on steam bath and dry until constant weight at 100 °C *in vacuo*. Added 0.85% NaCl solution to final volume of 5.0 ml, added 5.0 ml basal medium to each test tube, mixed and observed the light transmission at 550 nm. The standard curve was obtained by relationship between amount of niacin and % transmittance.

Niacin Extraction and Analysis

Weight 10 g of homogenously blended *L. disjunctus* whole plants, transfered to a 250 ml beaker and add 100 ml of 1 N H_2SO_4 for dispersing the sample evenly in the liquid. Autoclaved the mixture for 30 min at 121 °C and cool, then adjusted the mixture to pH 6.0 with NaOH under vigorous stirring. Filtered and added diluted HCI until no further precipitation, adjusted the clear filtrate to pH 6.8 with NaOH and diluted with water to the final measured volume. Fifty microliter of the bacterial suspension was used to inoculate to 1 ml of the filtrate in 9 ml in basal medium. Incubated mixture for 24 hr at 37 °C and after incubating, centrifuged and washed cells same as aforementioned process. Finally, added 0.85% NaCl solution to final volume of 5.0 ml, added 5.0 ml basal medium to each test tube, mixed and observed the light transmission at 550 nm. The amount of niacin was calculated by using the calibration curve equation. The test was done in duplicate.

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Folic acid (Microbiological Method) [137, 138]

Inoculum and Procedure

The test organism, *Streptococcus faecalis* (ATCC 8043) was maintained through weekly transfers on folic acid free double strength basal medium. Dilute a measured volume of basal medium with an equal volume of water containing 2 ng folic acid/ml. Added 10 ml portions of diluted medium to two screw cap test tubes, autoclaved 15 min at 121 °C, cool tubes rapidly and store in refrigerator. Cells from stock culture of *S. faecalis* was transferred to two sterile tubes containing the liquid culture medium and incubated in a constant temperature bath for 24 hr at 37 °C. Under aseptic condition, the culture and decant the supernatant were centrifuged. the cell was washed with 10 ml 0.85% NaCl solution and repeated procedure two more times. After third washing with 0.85% NaCl solution, re-suspended the cells. The cells suspension was obtained is the inoculum.

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Calibration Curve

Inoculum (1 m) was added to 300 ml sterile basal medium containing 1.0 ml standard stock solution (100 µg folic acid/ml). The mixture was incubated for 24 hr at 37 °C and after incubating, centrifuged and washed cell with 50 ml portion 0.85% NaCl solution, then re-suspends cells in the NaCl solution to a final volume of 25 ml. Aliquot of cell suspension (10 ml) was evaporated on steam bath, dried to constant weight at 100 °C *in vacuo*. NaCl solution (0.85 %) was added to final volume of 5.0 ml, added

5.0 ml basal medium to each test tube, mixed and observed the light transmission at 550 nm. The standard curve was obtained by relationship between amount of niacin and % transmittance.

Folic Extraction and Analysis

Homogenously blended *L. disjunctus* whole plants (10 g) was transferred to a 250 ml beaker and added 100 ml of 1 N H_2SO_4 for dispersing the sample evenly in the liquid. The mixture was autoclaved for 30 min at 121 °C and cooled, then adjusted the mixture to pH 6.0 with NaOH under vigorous stirring. The portion was filtered and added diluted HCI until no further precipitation, adjusted the clear filtrate to pH 6.8 with NaOH and dilute with water to the final measured volume. Fifty microliter of the bacterial suspension was used to inoculate to 1 ml of the filtrate in 9 ml in basal medium. The mixture was incubated for 24 hr at 37 °C and after incubating, centrifuged and washed cells same as aforementioned process. Finally, the portion was added 0.85% NaCl solution to final volume of 5.0 ml, add 5.0 ml basal medium to each test tube, mixed and observed the light transmission at 550 nm. The amount of niacin was calculated by using the calibration curve equation. The test was done in duplicate.

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Sodium, Potassium, Magnesium, Calcium, Iron, Zinc and Copper (Atomic Absorption Spectrophotometric Method) [131, 132]

Extraction

One gram of homogenously blended dried *L. disjunctus* was placed into glazed and contained in high-form porcelain crucible. Incineration was performed at 500 °C for 2 hr and cooled. The ash was wetted with 10 drops H_2O, and carefully added 4 ml HNO_3, Excess HNO_3 was evaporated on hot plate set at 100-120 °C. The crucible was placed into the furnace and incinerated additional 1 hr at 500 °C. The crucible was cooled, dissolved ash in 10 ml HCl and transferred to 50 ml volumetric flask.

Instrumentation

The instrument was set up as in Table 5. The standard solutions were read within analysis range before and after each group of 6-12 samples. The burner was flushed with H_2O between samples and reestablished absorption points each time, prepared calibration curve from average of each standard before and after sample group of element.

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Table 5 Condition of instrument

Element	Wavelength, Å	Flame
Ca	422.7	Rich Air-C_2H_2
Cu	322.7	Air-C_2H_2
Fe	248.3	Rich Air-C_2H_2
Mg	285.2	Rich Air-C_2H_2
Zn	213.8	Air-C_2H_2
Na	589.0	Air-C_2H_2
K	766.5	Air-C_2H_2

Standard Solution

Calcium stock solution, stock solution was prepared by weigh 1.249 g $CaCO_3$ and dissolved in minimum amount of 3N HCl and diluted to 1 liter.

Copper stock solution, stock solution was prepared by dissolve 1 g pure Cu metal in minimum amount HNO_3 and add 5 ml HCl. Evaporate stock solution until almost to dryness and diluted to 1 liter with 0.1 N HCL.

Iron stock solution, stock solution was prepared by dissolve 1 g pure Fe wire in 30 ml 6 N HCl with boiling and diluted to 1 liter.

Magnesium stock solution, stock solution was prepared by dissolve 1 g pure Mg metal in 50 ml H_2O and slowly added 10 ml HCl and diluted to 1 liter.

Zinc stock solution, stock solution was prepared by dissolve 1 g pure Zn metal in 10 ml 6 N HCl and diluted to 1 liter.

Sodium stock solution, stock solution was prepared by weigh 3.089 g Na_2SO_4 and dissolved in water and diluted to 1 liter.

Potassium stock solution, stock solution was prepared by weigh 2.228 g K_2SO_4 and dissolved in water and diluted to 1 liter.

Working standard solutions were further prepared by dilution the stock solution into 0, 5, 10, 15 and 20 μg/ml.

Calculation

$$\% \, Element = (\mu g/ml) \times \left(\frac{F}{Sample\ weight}\right) \times 10^{-4}$$

Where, F= ml original dilution × ml final dilution/ml aliquot, if original 100 ml volume is diluted

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Iodine (Spectrophotometric Kinetic Assay) [139]

Sample Preparation and Procedure

Ten grams of homogenously blended *L. disjunctus* were placed in porcelain crucible. One ml of 1 M zinc sulphate solution was added with 1 ml of 30 % m/V potassium carbonate solution. The mixture was completely dried in a drying cabinet at 95 °C, then a porcelain lid on the crucible was placed and heated at 550 °C approximately 1.5 hr and maintained this temperature for 1 hour. The crucible was cooled at the room temperature, the residue was dampened with a few drops of water, added a further 1 ml of 30% m/V potassium carbonate solution and 1 ml of 1 M zinc sulphate solution then repeats the ashing procedure was repeated for 1.5 hr again. The cooled ash was transferred to a centrifuge tube with 50 ml of distilled water and the tube was spin at 50 Hz for 5 min. A half of the solution was decanted and stored it in a clean polyethylene container before analyzing it by the Auto Analyzer.

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The standards, samples, and blanks were placed into respectively sample cups and analysed them at a rate of 20 per hr in the room maintained at a constant temperature. Each standard, sample and reagent blank solution were transferred into test tube. Each tube was added with 1 ml of distilled water, 1 ml of potassium thiocyanate solution and 2 ml of ammonium iron (III) sulphate reagent. The orange solutions were mixed in the tubes well on a vortex mixer. At exactly 90-s intervals, they were added with 1 ml of sodium nitrate solution and again mixed on a vortex mixer, and measured the colour at a wavelength of 450 nm on a spectrophotometer after 20 min, stilled at exactly 90-s intervals.

Calculation

From the absorbance values of the sample and blank solutions, the iodine content of the solutions was determined in μg/ml from the calibration graph. The iodine of the sample was calculated in μg/100 g using the following equation:

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$$Iodine\ content\ (\mu g/100g) = \frac{[(A-B)\times 5]}{W}$$

Where, A = the iodine content of the sample solution (μg/ml)

B = the mean iodine content of the blank solution (μg/ml)

W= the amount of sample in gram

Phosphorus (Vanado-Molybdate Colorimetric Method) [140]

Preparation of standard curve

To a series of 100 ml volumetric flasks 0, 2.5, 5, 10, 20, 30, 40 and 50 ml of the standard phosphate solution (0.003 M KH_2PO_4) were added and diluted each to 50-60 ml with distilled water. Ammonia solution (0.1 ml) was added and adjusted into acid with HNO_3 (1:2). The Vanado-molybdate reagent (25 ml) was added, mixed, allowed standing for 10 minutes and measured the optical density at 470 nm.

Procedure

The ash was boiled with 10 ml of 5 M HCl and washed the solution into the 100 ml flask with water. Addition of 0.1 ml ammonia solution was performed to neutralize and then adjusted with HNO_3 (1:2). Vanado-molybdate reagent (25 ml) was added and measured the optical density after allowed to standing for 10 minutes.

Calculation

The absorbance values of the sample and blank solutions were prepared a calibration graph by using range of standards. The phosphate content of the solutions was determined in µg/ml from the calibration graph.

Fatty Acid (Gas Liquid Chromatography Method) [141, 142]

Ten grams of homogenously blended *L. disjunctus* was extracted twice with chloroform-methanol (2:1). The chloroform layer was evaporated to dryness under nitrogen and stored at -15 °C until analysis.

The extract was transesterified by adding 2 ml of petroleum ether and 100 µl of 2 N sodium methoxide. The mixture was immediately shaken and allowed to react for 10 min, and then added 250 µl of water. The mixture was shaken and the upper layer was removed into auto sampler vial containing 50 mg of anhydrous sodium sulphate as drying agent. Sample was tested within 12 h after transesterification.

Gas Liquid Chromatography (GLC)

The fatty acid composition was determined by gas chromatography using a Hewlett Packard 6890 gas chromatograph equipped with auto sampler. The chromatograph was fitted with capillary column (DB-WAX, 30m, 0.25 µm) and temperature was programmed from 125 to 225°C at 4 °C/min with nitrogen carrier gas that set at a flow rate of 18 ml/min. The injection port was maintained at 180 °C and the flame ionization detector at 300 °C. Peaks were identified by comparison with known fatty acid methyl ester standards.

Calculation

$$\% \, Area \, percent \, fatty \, acid_x = \left[\frac{A_x}{(A_T - A_{IS})}\right] \times 100$$

Where, A_x = Area counts of methyl or ethyl ester X

A_T = Total area counts for chromatogram

A_{IS} = Area counts of internal standard

Hypnotic and Anxiolytic Evaluation

Experimental Animals

Adult male albino ICR mice (35-45 g) were obtained from National Laboratory Animal Center, Mahidol University, Thailand. All mice were housed in Faculty of Sciences, Rangsit University, Thailand, under standard environmental conditions of temperature, relative humidity and light (24±1 °C, 60-70% humidity, 12 h light: 12 h dark cycle). Food and water were given *ad libitum* until 2 h before the experimental procedures.

Adult male albino Wistar rats (250–300 g) were obtained and bred form Laboratory Animal Center, Heilongjiang University of Chinese Medicine, Harbin, China, the rats were randomly housed under standard environmental conditions of temperature, relative humidity and light (24±1 °C, 60-70% humidity, 12 h light: 12 h dark cycle). Rats were allowed access to food and water *ad libitum*. Food and water were given *ad libitum* until 4 h before the experimental procedures.

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Drosophila melanogaster were cultured, randomized and used as experimental animals, housed in Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, Harbin, China under standard environmental conditions of temperature, relative humidity and light (25±1 °C, 60-70% humidity, 12 h light: 12 h dark cycle). *D. melanogaster* were cultured in nutrient broth until breeding and spawning. Eggs of *D. melanogaster* were continuously alimented until become larva, pupa and adult respectively. Adults were sex sorted out for

preventing undesirable pregnancy within 12 hr and sub-cultured in broth. Sex of adult fly were distinguished by observe abdomen's tip.

Pentobarbitone induced sleeping time test

Pentobarbitone induced sleeping time in mice was tested according to Carvalho-Freitas & Costa, 2002 [143]. Male ICR mice were divided into 5 groups of ten each. Mice in group I were received 5% polysorbate 20 at same volume of treatment groups. Mice in group II, III, IV, V, VI and VII were respectively orally received 10, 50, 100, 200 mg/kg ethanolic extract, flumazenil with 200 mg/kg ethanolic extract and flumazenil with 5% polysorbate 20. Food and water were starved for two hours before the experimental procedures. The control group was received only vehicle at the same volume as the treated groups. Pentobarbitone sodium (35 mg/kg; Merck, Germany) was injected to induce animal sleep by intra-peritoneally after 30 minutes of administration of the extract. The sleeping time was recorded by observing time of righting reflex [144].

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Evaluated Plus Maze Test in Mice

Evaluated plus maze test was done according to Hosseinzadeh & Noraei (2009) [145]. The apparatus was consisting of two open arms (15×5 cm) and two closed arms (15×5×12 cm), with the open pair perpendicular to the closed one. The maze was made of clear acrylic and located 30 cm above a black floor. Ethanolic extract in sterile saline for injection (10, 50 and 100 mg/kg) and vehicle was administered intra-perritoneally (IP), and the mice was individually placed at the center of the plus maze and observed for 5 min at 30 minute later. The time (in seconds) spent by animals in the open and closed arms were registered. Anxiolytic compounds reduced the animal's natural aversion to the open arms and promoted exploration. Therefore, increasing time spend in the open arms was considered to reflect an anxiolytic effect, in comparison with the control group.

Table 6 Experimental group of evaluated plus maze test

Group	Number	Receive
1		Ethanolic extract (10 mg/kg)
2		Ethanolic extract (50 mg/kg)
3		Ethanolic extract (100 mg/kg)
4		Vehicle (saline)

Rota Rod Performance Test in Mice

Motor coordination and balance was tested using the Rotarod apparatus according to Hosseinzadeh & Noraei (2009) [145]. Ethanolic extract in sterile saline for injection (10, 50 and 100 mg/kg) and vehicle was administered intra-perritoneally (IP). Mice were placed on a horizontal metal rod coated with rubber (3 cm diameter) rotating at an initial speed of 10 rpm/min. Terminal speed of the rod was 20 rpm in accelerated studies and the rotational velocity of the rod was increased linearly from 10 to 20 rpm within 20 s. The time each animal is able to maintain its balance walking on top of the rod was measured. Mice were given two trials with a maximum time of 300 s and a 30–60 min intertrial rest interval. Before the beginning of all experiments, the riding ability of the animals in the Rotarod was checked. Thus, the mice were initially put on a rotating rod, and mice that immediately dropped off (within 30 s) were removed from the experiment.

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Table 7 Experimental group of rota rod performance test

Group	Number	Receive
1		Ethanolic extract (10 mg/kg)
2		Ethanolic extract (50 mg/kg)
3		Ethanolic extract (100 mg/kg)
4		Vehicle (saline)

Open Field Test in Mice

Open field test was done according to Hosseinzadeh & Noraei (2009) [145]. The apparatus has floor of 100 × 100 cm which was divided by red lines into 25 squares of 20 × 20 cm. The walls, 50 cm high, were painted in white. The test room was illuminated at the same intensity at the colony room. Ethanolic extract in sterile saline for injection (10, 50 and 100 mg/kg) and vehicle was administered intra-perritoneally (IP). Each mouse was placed in the center of the open field, and its behavior were observed for 5 min. The number of leanings (one or two paws in contact with the wall), rearing, grooming (face cleaning, paw licking, fur licking, head scraping and rubbing) were considered. At the end of each test, the whole area was cleaned with non-odor wet napkin and a dry paper towel.

Table 8 Experimental group of open field test

Group	Number	Receive
1		Ethanolic extract (10 mg/kg)
2		Ethanolic extract (50 mg/kg)
3		Ethanolic extract (100 mg/kg)
4		Vehicle (saline)

Analysis of Sleep in *Drosophila melanogaster*

Analysis of sleep in *D. melanogaster* was done according to Gilestro (2012) [146] with some modifications. The weighing cottons were put into the test tube and soaked by 20 µl of *L. disjunctus* ethanolic extract in 5% sucrose (5, 10 and 20 mg/ml). The test tubes were closed with cap at soaked cotton side. One *D. melanogaster* was put into each test tube then, the tube entrance was closed with dry cotton. Each test tube was inserted into the experiment arena and observed the sleeping time under experiment environment by using infrared beam-split monitors. The sleeping times was recognized by observing the movement of *D. melanogaster* for 24 hr. The locomotors rest from sleep was measured by arousal threshold. When *D. melanogaster* fell asleep the arousal thresholds was decrease. Sleep in *D. melanogaster* is a period of inactivity at least five minutes (136). The treated groups were compared to negative control group (Vehicle, 5% Sucrose) by using statistical analysis. Thirty-two male and female *D. melanogaster* was investigated for each dose of *L. disjunctus* and vehicle.

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Sleep–Wake Profile and EEG Delta Activity [147, 148]

Surgery and sleep recording

The rats were anaesthetized by intraperitoneal injection with pentobarbitone sodium (60 mg/kg) and placed in a stereotaxic apparatus. Rats were implanted with electrodes for recording of electroencephalogram (EEG) and electromyogram (EMG) in freely moving condition. Subdural EEG was recorded from visual cortex and frontal cortex according to guidelines of a standard brain atlas [149]. EMG was recorded from dorsal neck muscle. Rats were allowed to recover for two weeks with proper post-operative care. Rats were fed with the sample and positioned on a receiver plate within their home cages to record the signals from transmitter. Signals were collected from receiver. Total recording time for each experimental condition in each rat was 6 hr. The digital data of EEG and EMG were converted to an analogue signal and analysed by Sleepsign 2.0.

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Sleep–wake profile and EEG delta activity were studied by divided rats in to 4 groups, 8 each. Each group will receive different dose of testing compound orally by gavage consist of ethanolic extract dissolved in polysorbate 20 (50, 100, 200 mg/kg body weight). Polysorbate 20 (2 ml/kg body weight) and diazepam (5 mg/kg body weight) will be served as negative and positive control respectively.

Table 9 Experimental group of sleep–wake profile and EEG delta activity

Group	Number	Receive
1		Ethanolic extract (50 mg/kg)
2		Ethanolic extract (100 mg/kg)
3		Ethanolic extract (200 mg/kg)
4		Polysorbate 20 (2 ml/kg)

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Data analysis

1. Data of hypnotic and anxiolytic activities in rodent model was expressed as mean ± SD. Statistical analysis was perform using one-way ANOVA and follow by the Dunnett's test, *post-hoc* analysis, for multiple comparisons. Values of $p<0.05$ were considered to be statistically significant.

2. Data of hypnotic in *Drosophila melanogaster* was expressed as mean ± SD. Statistical analysis was performed using *t*-test. Values of $p<0.05$ were considered to be statistically significant.

Ethics

This dissertation was approved for ethical considerations from Research Institute of Rangsit University and carried out in accordance with Research Institute of Rangsit University, Ethical Committee Acts (RSEC 01/2556, RSEC 08/2557).

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CHAPTER IV

RESULTS

Nutritional Composition

The common nutrients of *L. disjunctus* showed in Table 10, *L. disjunctus* expressed the total energy as 63 Kcal/100 g, 4 Kcal come from fat. While, the moisture tended to be plenty amount with the number 84.3 g/100 g, these can be occurred because the procedure based on wet basis.

Table 10 Proximate composition of *L. disjunctus* (% wet basis)

Proximate composition		per 100 gram
Energy [a]	Kcal	63
Energy from fat [a]	Kcal	4
Moisture	g	84.3
Ash	g	0.6
Protein [b]	g	1.8
Total fat	g	0.5
Fibre	g	5

a Calculated value

b Nitrogen-to-protein conversion factors are: 6.25 for food substance.

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In addition, the fatty acid profile of *L. disjunctus* was shown in Table 11. Linoleic acid tended to be a major fatty in *L. disjunctus* (129.7 mg/100g). Linolenic acid, palmitic acid and oleic acid were plenty found, while lauric acid and margaric acid were slightly found.

Table 11 Fatty acid composition of *L. disjunctus* (% wet basis)

Fatty acid		% Fatty acid	mg/100 g
C12:0	Lauric acid	0.5	1.9
C14:0	Myristic acid	0.7	2.2
C15:0	Pentadecanoic acid	0.5	1.8
C16:0	Palmitic acid	18.1	61.6
C16:1	Palmitoleic acid	3.5	10.3
C17:0	Margaric acid	0.3	1.1
C18:0	Stearic acid	1.6	5.3
C18:1	Oleic acid	7.9	26.2
C18:2	Linoleic acid	38.7	129.7
C18:3	Linolenic acid	20.8	69.5
C20:0	Arachidic acid	0.8	2.2
C22:0	Behenic acid	0.9	2.8
C22:5	Docosaenoic acid	0.8	2.5

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L. disjunctus is the common source of phosphorus and potassium which is dependently found in total minerals. While, iron and sodium can be slightly found, iodine is not found in this plant. *L. disjunctus* is the common source of vitamin C which is dependently found as showed in Table 12; whereas, beta-carotene, vitamin E and folic acid can be slightly found in this plant.

Table 12 Minerals and vitamins (% wet basis) of *L. disjunctus*

Minerals and vitamins		**per 100 gram**
Iodine	mg	-
Sodium	mg	5
Potassium	mg	237
Magnesium	mg	13.3
Calcium	mg	17.8
Phosphorus	mg	44.6
Iron	mg	0.8
Beta-carotene	mg	0.708
Vitamin E	mg	0.531
Vitamin B1	mg	0.05
Vitamin B2	mg	0.09
Vitamin C	mg	19.2
Niacin	mg	1.25
Folic acid	mg	0.042

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Pentobarbitone Induced Sleeping Time Test

The sleep prolongation of *L. disjunctus* was investigated. Righting reflexes of male mice which were given a single dose of the vehicles control, ethanolic extract (10, 50, 100 and 200 mg/kg), flumazenil with ethanolic extract (200 mg/kg) and flumazenil with vehicle control 30 minute before pentobarbitone sodium injection to induce sleep were recorded. It was found that the ethanolic extract (50, 100 and 200 mg/kg) significantly prolonged sleeping time, compared to vehicle control ($p<0.01$); in addition, the sleeping time in flumazenil induced group shown to be non-statistical different against the vehicle control (Figure 19).

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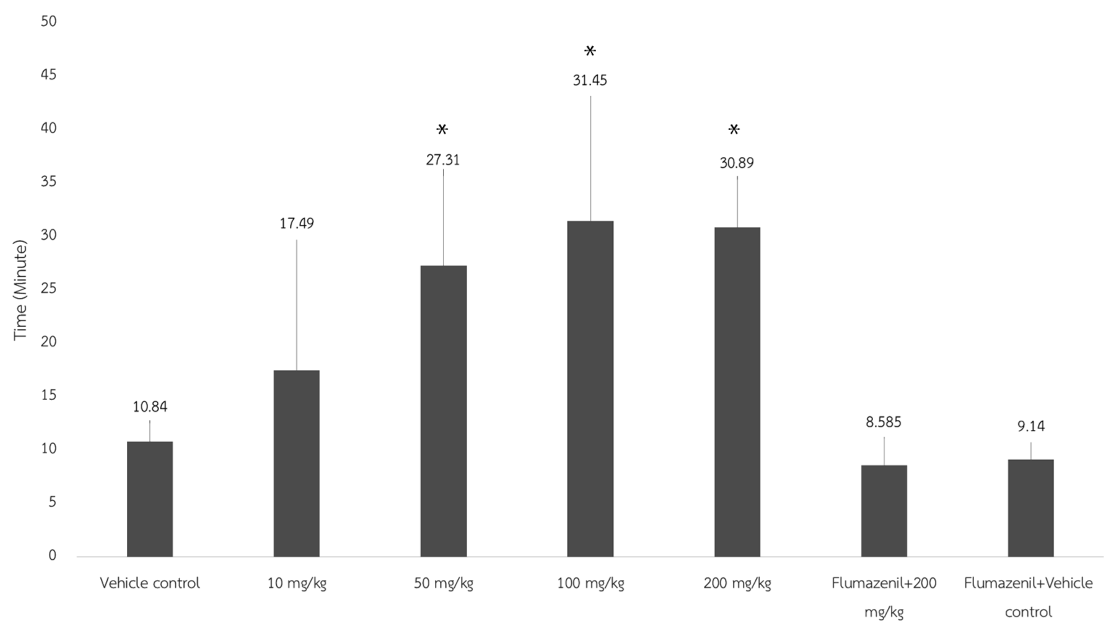

Figure 19 Effects of *Leptocarpus disjunctus* ethanolic extract on pentobarbitone induced sleeping time in male mice. Data were expressed as mean ± SD (n=70) of time (min), *p < 0.01 compared to vehicle control by Dunnett’s test.

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Evaluated Plus Maze Test

In order to determine the anxiety effect of ethanolic extract, the elevated plus maze (EPM) was used as a screening test for anxiogenic compounds. The mice were given a single dose of the vehicles control and ethanolic extract (10, 50 and 100 mg/kg). After 30 min, the mice were placed onto the centre of plus maze apparatus, and the time spent in the open arms and close arms of the apparatus were recorded. The ethanolic extract (10, 50 and 100 mg/kg) significantly increased time spent in open arms, and decreased time spent in close arms (Table 13). The results also demonstrated dose-response relationship in open arm increasing time and close arm decreasing time.

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Rota Rod Performance Test

Rota rod performance test (RT) was conducted for a screening of motor response. The mice were given a single dose of the vehicles control and ethanolic extract (10, 50 and 100 mg/kg). After 30 min, the mice were placed onto the treadmill of the apparatus. The time that mice spent the apparatus was recorded. The ethanolic extract (10, 50 and 100 mg/kg) significantly decreased time spent on the treadmill when compared to vehicle control (Table 13). The results also showed dose relationship by decreasing time spent on the treadmill.

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Open Field Test

The anxiogenic effect were conducted using open field test (OF) which was used to assay general locomotor activity levels and anxiety in rodents was attempted. The mice were given a single dose of the vehicles control and ethanolic extract (10, 50 and 100 mg/kg). After 30 min, the mice were placed onto the centre of apparatus. The behaviour of mice were counted and recorded. The behaviour was classified into three types (grooming, leaning and rearing). The ethanolic extract (10, 50 and 100 mg/kg) significantly decreased number of grooming, leaning and rearing when compared to vehicle control (Table 13). The results shown the dose relationship in anxiogenic effect decreasingly.

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Table 13 Effects of *L. disjunctus* ethanolic extract on time in evaluated plus maze test, rotarod performance test and open field test. Data are expressed as mean ± SD. *$p < 0.01$, compared with vehicle control, Dunnet test.

	Time (Second)			Behaviour (Time)		
	Evaluated plus maze test		Rotarod performance test	Open field test		
	Open arm	Close arm		Grooming	Leaning	Rearing
Vehicle	13.31±6.83	286.69±6.83	79.03±18.26	10.40±1.90	43.80±6.18	8.60±3.27
10 mg/kg	46.16±10.78*	253.84±12.79*	30.57±9.97*	6.10±2.38*	25.60±8.83*	3.0±2.45*
50 mg/kg	56.60±8.09*	244.4±8.09*	21.30±6.24*	5.40±3.30*	4.60±1.17*	1.90±0.99*
100 mg/kg	60.5±7.74*	239.50±8.84*	16.00±4.27*	2.60±1.78*	2.30±1.57*	1.80±1.32*

Analysis of Sleep in *Drosophila melanogaster*

Analysis of sleep in *D. melanogaster* was conducted for observation the potential in hypnotic compound. *D. melanogaster* were given a single dose of ethanolic extract in 5 % sucrose (5 and 10 mg/ml) and vehicle control (5 % sucrose). The sleeping time under experiment environment was recorded using infrared beam-split monitorsa in female fruit fly shown to be more potent than male fruit fly. The ethanolic extract at 5 mg/ml affected the sleeping time in all day, day and night time of female fruit fly. Whereas, the ethanolic extract at 10 mg/ml only shown effectiveness on all day and night time. Despite, the ethanolic extract only at 5 mg/ml potentiated among all day, day time and night time sleeping time of male fruit fly, but less potent in higher dose (Table 14).

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Table 14 Effects of *L. disjunctus* ethanolic extract on sleeping time in preliminary sleep analysis of *Drosophila melanogaster*. Data was expressed as mean ± SD.

	Sleeping time (minute)		
	All day	**Day**	**Night**
Female			
5 % Sucrose	707.031±137.43	238.281±97.41	468.750±78.09
5 mg/ml	802.031±197.59	289.375±104.28	513.438±111.42
10 mg/ml	721.250±168.96	236.719±129.05	485.000±106.52
Male			
5% Sucrose	859.219±168.79	366.875±92.74	492.969±100.03
5 mg/ml	915.156±110.31	401.719±54.86	513.438±73.06
10 mg/ml	782.188±186.93	330.313±90.00	452.031±106.03

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Sleep–Wake Profile and EEG Delta Activity

The sleep-wake profile and EEG Delta activity of *L. disjunctus* was conducted. Rats were received a single dose of ethanolic extract dissolved in polysorbate 20 (50, 100, 200 mg/kg body weight). It was found that the ethanolic extract (50, 100 and 200 mg/kg) significantly increased sleeping time of experimental animals (Figure 20). Furthermore, the data from electroencephalograph was recorded and classified into sleep state; wake, REM and NREM sleep. It was found that the NREM sleep extremely increased, whereas, REM sleep slightly increased (Figure 21).

The EEG signal of NREM sleep was quantified in term of Delta wave quantification. The percent of delta wave was calculated and presented in table 15. It was found that the ethanolic extract can maintain delta wave in NREM state of sleep in all six hours when compared to negative control group (Figure 22).

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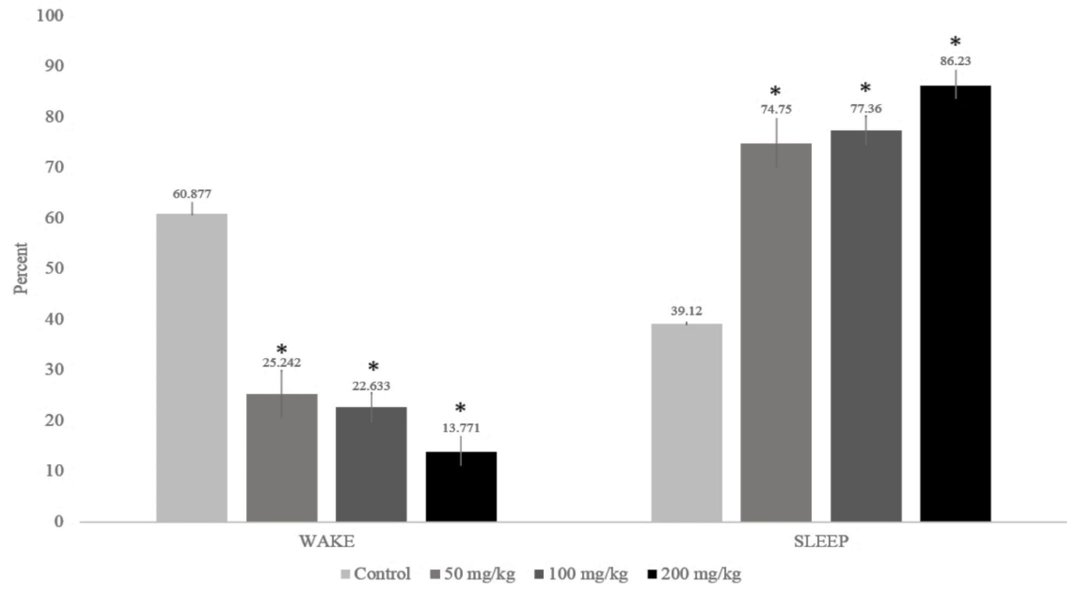

Figure 20 Effects of *L. disjunctus* ethanolic extract on total waking and sleep times in electroencephalogram sleep-wake analysis. Data are expressed as percent mean ± SD of time in male rats, compared with vehicle control by Dunnett's test (*p < 0.01).

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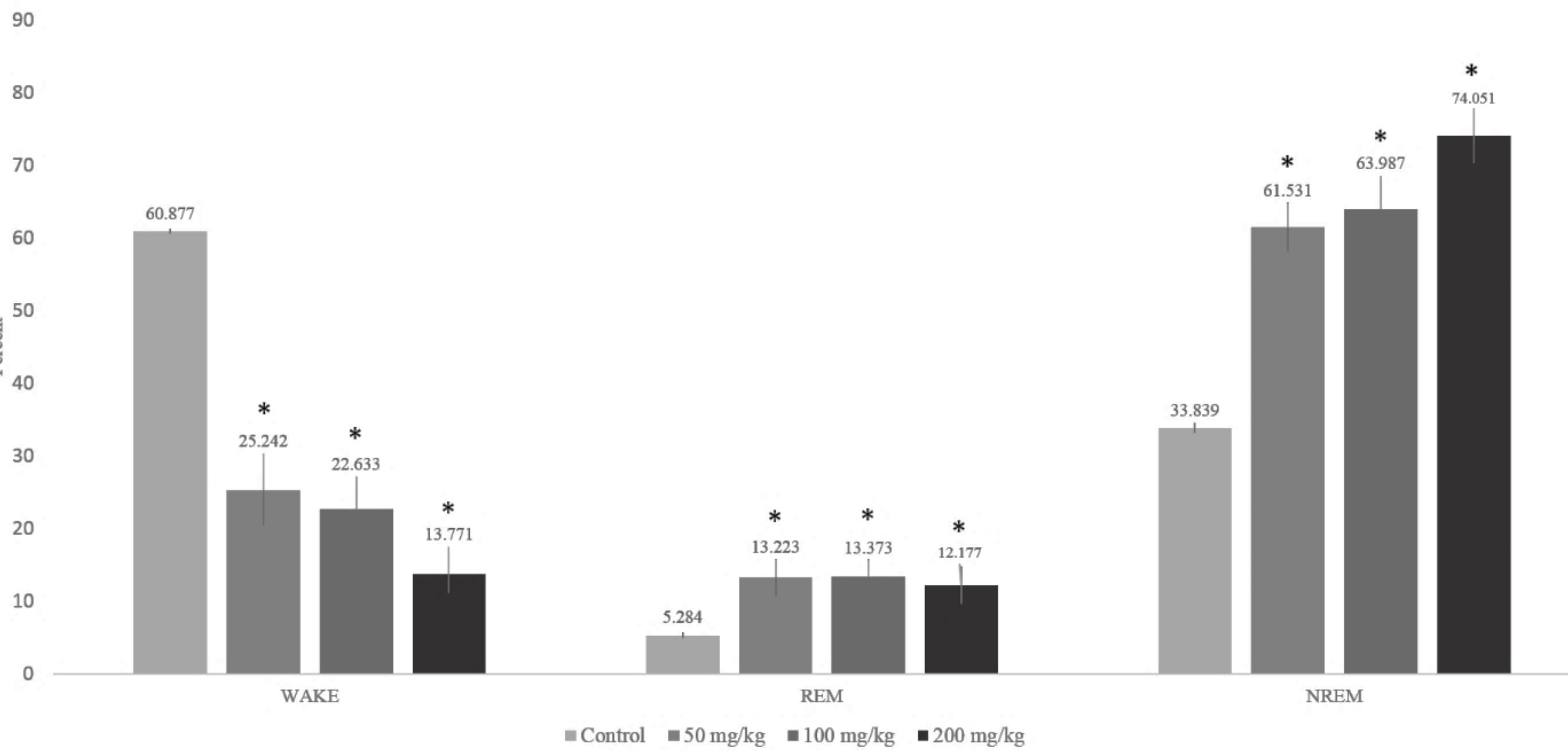

Figure 21 Effects of *L. disjunctus* ethanolic extract on percent time of wake, rapid eye movement sleep (REM) and non-rapid eye movement sleep (NREM). Data are expressed as percent mean ± SD of time in male rat, compared with vehicle control by Dunnett's test (*p<0.01).

Table 15 Effects of *L. disjunctus* ethanolic extract on percent delta activity during NREM sleep. Data are expressed as percent delta power ± SD of time in male mice, compared with vehicle control by Dunnett's test (*p < 0.01).

Dose (mg/kg, P.O.)	% EEG Activity					
	0-1 h	**1-2 h**	**2-3 h**	**3-4 h**	**4-5 h**	**5-6 h**
Control	107.391±8.478	112.463±8.948	112.783±8.300	115.053±12.201	116.326±9.576	118.444±9.859
50 mg/kg	109.289±5.343	112.531±10.668	114.474±6.940	108.745±8.398	111.941±7.350	106.759±5.840
100 mg/kg	103.403±5.652	109.698±4.007	105.838±5.831	106.289±7.942	114.399±8.558	110.382±8.020
200 mg/kg	100.692±3.949	107.239±3.123	105.242±4.948	100.127±7.041	104.284±5.205	100.905±5.230

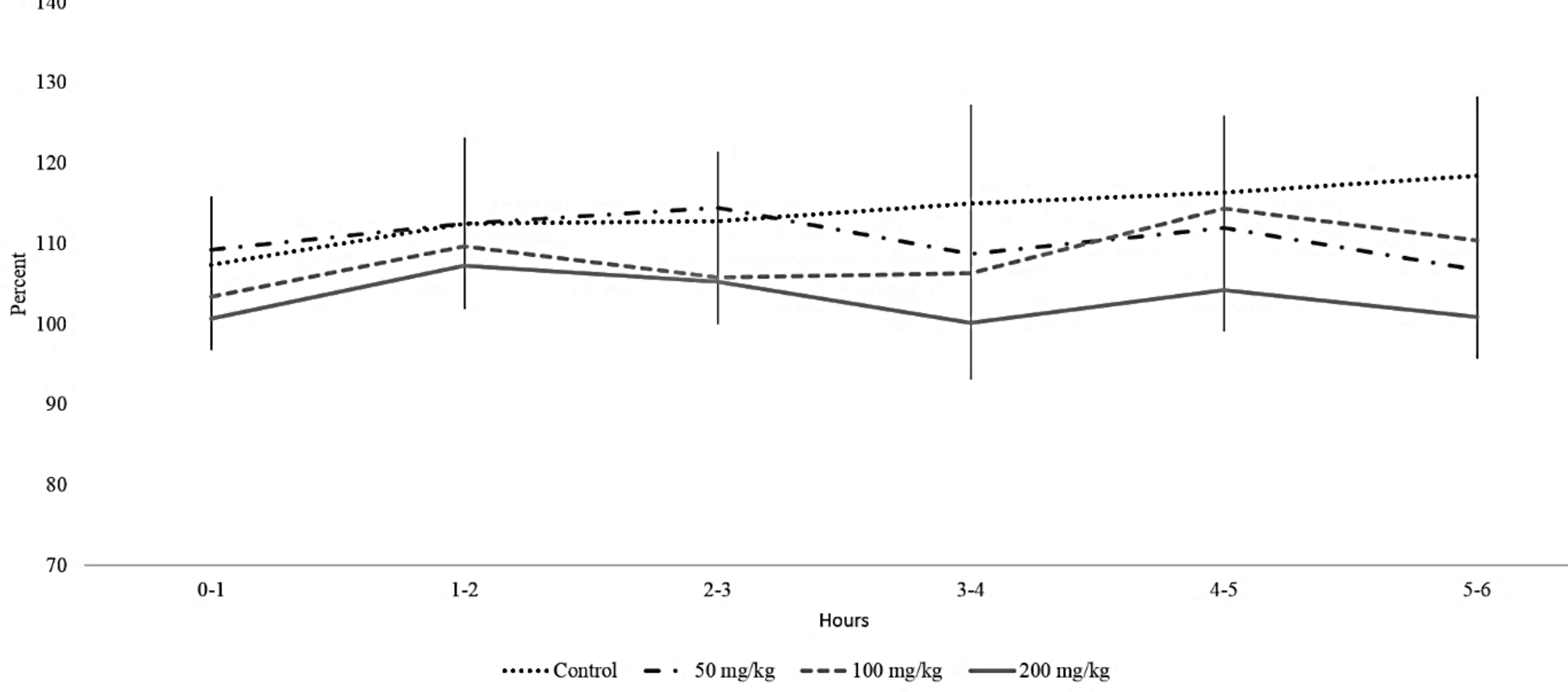

Figure 22 The relationship between time and dose response on delta activity during NREM sleep. Data are expressed as percent delta power ± SD, compared with vehicle control by Dunnett's test (*p < 0.01).

CHAPTER V
DISCUSSIONS AND CONCLUSION

Leptocarpus disjunctus Mast., is an edible plant which commonly consumed indigenously in Southern of Thailand, even though, there is the side effect warning for dizziness and intoxicate symptom. Furthermore, the nutritional compositions of this plant have never been reported. This dissertation aimed to evaluate the anxiolytic and hypnotic effect of *L. disjunctus* using several animal models as well as to present the data on the nutrients composition of *L. disjunctus.*

For nutritional analysis, *L. disjunctus* was evaluated on proximate nutrients, vitamins, and minerals basis. In term of nutrients, they are chemical from food which are necessary for nourishing and thriving living organism. Nutrients can be divided into two categories, macronutrients, and micronutrients. Macronutrients are important nutrients that living organism needs in large amounts for providing energy. Macronutrients can be subcategorized into primary nutrients: carbohydrate, protein and fat [150]. Micronutrients are nutrients that do not provide energy but the body needs in small amounts including vitamins and minerals. It has an important role in the functioning of the body, such as helping to release energy, supporting metabolism, contributing enzymatic and hormonal function. In *L. disjunctus*, fibre was found to be 5 g/100 gram fresh plant. In addition, protein was found to be 1.8 g/100 gram fresh plant. The main fatty acid which was found in *L. disjunctus* was linoleic acid, linolenic

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acid and palmitic acid, whereas, lauric acid, pentadecanoic acid and margaric acid can be slightly found. In term of vitamin, vitamin C which accounted for 19.2 mg/100 gram fresh plant was found to be the main vitamin in this plant. In addition, Vitamin B2, B1 and B9 can also be slightly found from this plant. Potassium which was dependently found to be 237 mg/100 gram fresh plant whereas, sodium and iron can be slightly found.

Drosophila melanogaster is a species of insect in the family Drosophilidae. The species is generally known as the common fruit fly. Because of easy care, fast breeding, and laying many eggs [122], it is typically used for preliminary research prior to higher animal models. *D. melanogaster* is widely used for genetics, physiology, microbial pathogenesis and life history evolution research. *D. melanogaster* was among the first organisms used for genetic analysis because the comprehend processes such as transcription and replication, and also helped in understanding of other eukaryotes [125]. *D. melanogaster* has emerged as an ideal model organism for studying the genetic components of sleep as well as its regulation and functions. In this present research, the infrared beam-split movement monitors was used for instrumentation. The individual flies were placed into glass chambers, where their confined motion were detected by motion detection (flies walk back and forth from end to end), and were counted by infrared light beams and uploaded from the monitors to a host computer for storage and analysis at periodic intervals (Figure 23). It was found that 5 mg/ml was

the most effective dose in both male and female *D. melanogaster* (Table 14). The sleeping time of male and female increased, but there was non-statistical different compared to vehicle control group. However, the sleeping time of both male and female *D. melanogaster* was slightly decreased in the highest dose among all day, daytime and night-time sleep. Normally, the average sleeping time of male *D. melanogaster* is longer than female [151, 152]. The non-significant effect might be resulted from the test that was restricted to directly feed the *D. melanogaster* , and the plant extract was so pungent odour and bitter that the fruit flies did not like [153]. While the lowest dose has less pungent, so *D. melanogaster* might be regularly consumed the plant extract.

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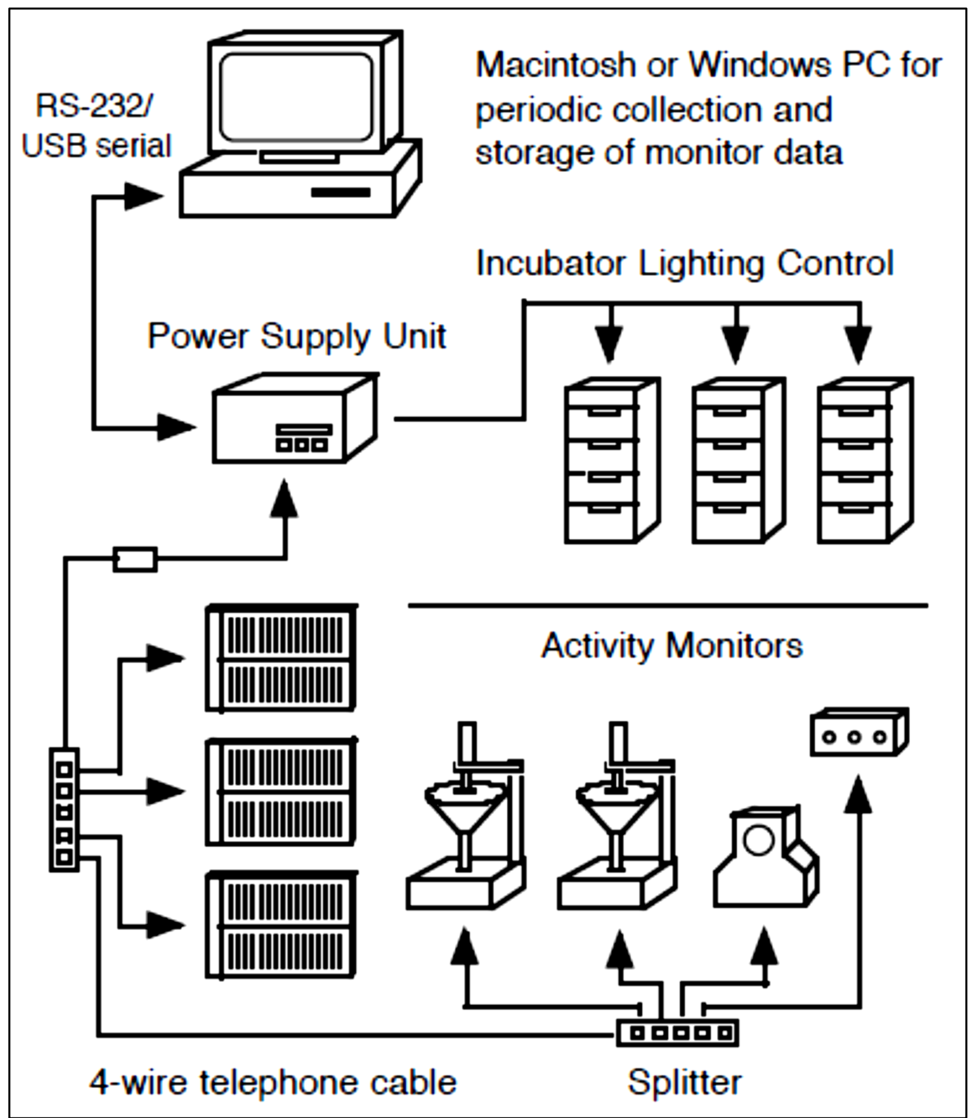

Figure 23 Drosophila activity monitoring system [154]

In the potentiation of pentobarbitone induced sleeping time test, the ethanolic extract at 50, 100, 200 mg/kg significantly increased the sleeping time in mice compared to vehicle control using righting reflex as an indicators duration of sleeping time (Figure 24).

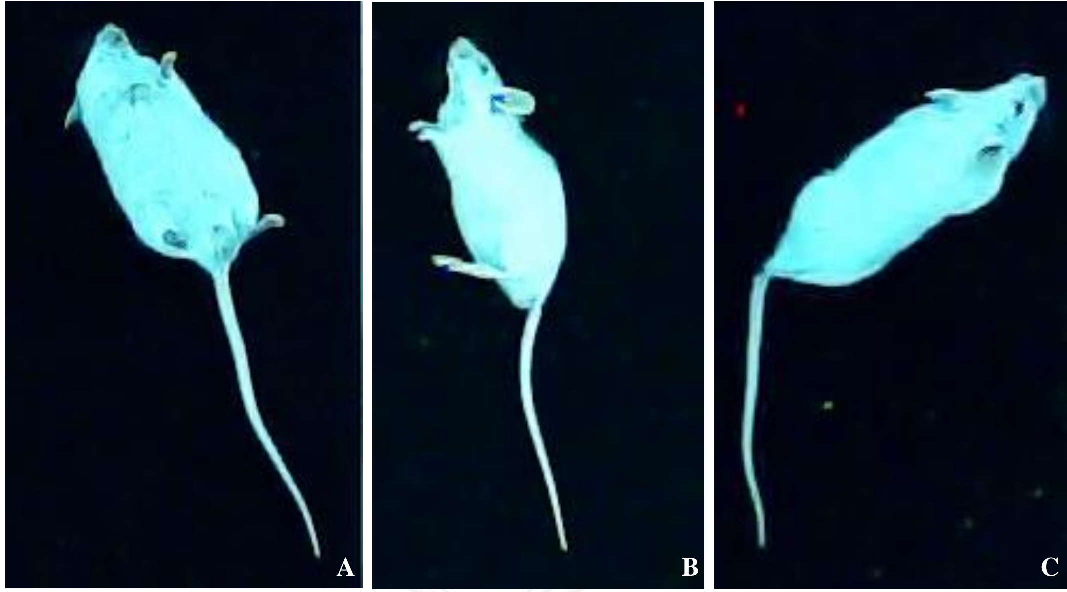

Figure 24 Unconscious ICR mice with loss of righting reflex (A), Subconscious ICR mice with loss of righting reflex (B) and consciousness ICR mice without righting reflex (C)

To investigate the detailed mechanisms involved in hypnotic potencies, 3.5 mg/kg flumazenil suspended in 10% dimethyl sulfoxide was orally pre-treated for 30 minutes before treated 200 mg/kg ethanolic extract. Flumazenil is one of several benzodiazepine derivatives with a high affinity for the benzodiazepine binding site on the gamma-aminobutyric acid or GABA receptor. Flumazenil act as a competitive antagonist. It blocks many of the actions of benzodiazepines, zolpidem, zaleplon, and eszopiclone unless the central nervous system effects of other sedative-hypnotics such as ethanol and opioids. Flumazenil is a $GABA_A$ receptor antagonist of the CNS effects of benzodiazepines, and well known that benzodiazepine receptor agonists caused hypnotic effects in both human and animal. GABA appears to interact at two sites between alpha and beta subunits, triggering chloride channel opening with

resulting membrane hyperpolarization. The benzodiazepine antagonist flumazenil also binds at this site and can reverse the hypnotic effects of zolpidem. These binding sites are distinct from those of the barbiturates (Figure 25). From the results, the sleeping time showed no obvious effect when compared to vehicle control which was pre-treated with flumazenil. It possibly summarised that the ethanolic extract could not trigger the chloride channel opening and resulting membrane hypo-polarization. These findings strongly suggested that ethanolic extract possibly expressed hypnotic activity *via* $GABA_A$ receptor.

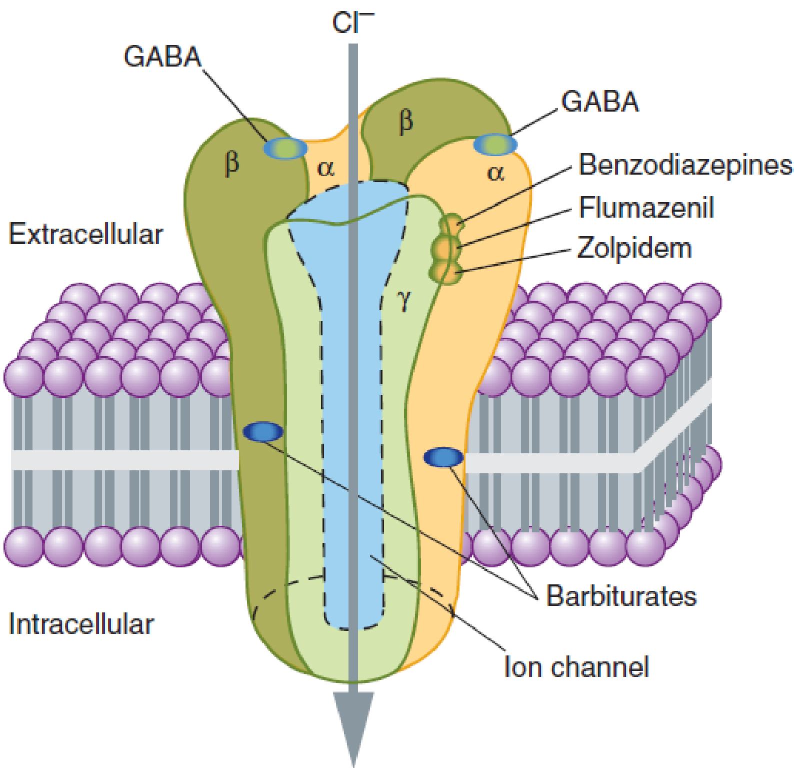

Figure 25 A model of the GABA receptor with Cl^- channel macromolecular complex [155].

In addition, the ethanolic extract also showed a significant increase in average of time spent in the open arms of maze among three doses, indicating anxiolytic effect of the extract [156]. The elevated plus maze has been described as a simple method for assessing anxiety responses of rodents. The first elevated plus maze was Y-shaped apparatus. Y-shaped apparatus included an elevated open alley which produced a strong approach–avoidance conflict [157]. It was modified into an elevated maze with four arms (two open and two enclosed) that were arranged to form a plus shape as described by Handley and Mithani [158]. The anxiety behaviour of rodents was assessed using the ratio of time spent on the open arms to the time spent on the closed arms. The elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance). There is great diversity in possible applications of the elevated plus maze. The anxiolytic and anxiogenic effects of pharmacological agents, drugs of abuse and hormones can be investigated. Furthermore, beyond its utility as a model to detect anxiolytic effects of benzodiazepine-related compounds, the elevated plus maze can be used as a behavioural assay to study the brain sites and mechanisms such as GABA glutamate and serotonin mechanism underlying anxiety behaviour. The ethanolic extract is consistent with the previous reports, some anxiolytic agent such as diazepam can increase the percentage of the time spent in the open arms [159].

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The treated animals showed habituation behaviour after successive open field test exposure, which was less evident in the exploratory behaviours (Table 13). The previous research study suggested that open-field could be used for assessing anxiety-like behaviours in mammal, and the antianxiety agents also showed the less exploratory behaviours [160, 161].

In rota rod performance test, animals treated with ethanolic extract, decreased locomotors activity. The decreasing of locomotors activity referred to the damages of motor coordination [162]. This evidence supported that the ethanolic extract affected on motor coordination, induced muscle relaxation and motor imbalance. Results obtained in open field and rota rod performance tests conformed to the effect which was detected in evaluated plus maze and pentobarbitone induced sleeping time test (Table 13), and also accommodated to the preliminary of sleep analysis in *D. melanogaster* and pentobarbitone induced sleeping time test. Anti-anxiety, sedative and muscle relaxant agent such as diazepam, lorazepam and alprazolam can affect the neurotransmitter production such as GABAergic expression [163]. The ethanolic extract showed the effectiveness similar to anti-anxiety agent by impact on the locomotors activity.

From the previous study, red ginseng extract provided antianxiety properties. It was conducted to investigate the anxiolytic effects using several methods such as evaluated plus maze test, electroshock test, rota rod performance test and forced

swimming test, and it was shown to be effective potency among all tests [164, 165]. Furthermore, it also decreased gamma aminobutyric acid $GABA_A$ receptor in the hypothalamus which increased NREM sleep *via* GABAergic systems [166]. *Cirsium japonicum* Fisch. ex DC. ethanolic extract also showed to possess antidepressant-like properties by behavioural changes which were evaluated using forced swimming test and open field test [167], and it showed the anxiolytic effects by increased chloride ion influx and blocked co-administration of the $GABA_A$ receptor [168]. *Valerian officinalis* L. is another medicinal herb that has been used for treating sleep disorder and nervous tension. The previous research study evaluated the anxiolytic and sedative effect using behavioural paradigms, and it had a tendency to be antianxiety agent by observing locomotors activity (elevated plus maze test). It tended to be antidepressant and muscle relaxant agents by showing effect on forced swimming and horizontal wire test [169]. Moreover, it conformed to another study that *V. officinalis* extract showed the anxiolytic and sedative effects, their results suggested that its extract could bind with GABA receptors *via* GABAergic mechanisms [170].

Sleep-wake analysis was performed by electroencephalogram signal observation from frontal cortex and visual cortex. Waking time significantly decreased by *L. disjunctus* ethanolic treatment. Sleeping and waking state of human was controlled by chemical substance and circadian rhythm in the brain and body which caused sleep-wake cycle and resulted in alternating succession [171].

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Polysomnography is a useful equipment to observe physical changes of the body. The transition from waking state to NREM state associated with neurotransmitters in thalamus which locates between cerebral cortex and mid brain [172]. NREM sleep is classified using two criterions (1) Spindle or Sigma band, 7–14 Hz is wave from thalamic reticular neurons which scatter on ventral thalamus [173]. Their functions were controlled by thalamo-cortico-thalamic circuits which comprise of connective neuron between thalamus and cerebral cortex. (2) Delta activity is processed in the brain to monitor the functioning of the brain and mind which is created by thalamocortical neurons and neocortex [174]. Delta activity can be subdivided into two class clock-like waves (1–4 Hz) and cortical waves (1–4 Hz) which is the characteristic of depolarization wave from neurons and neuroglia [175].

NREM sleep can be divided into 4 states according to the evaluation sleep criteria of Rechtschaffen and Kales in 1968; and 3 states according to the evaluation sleep criteria of The American Academy of Sleep Medicine (AASM), 2007 [176]

NREM1 occurs at the onset of sleep by changing from the waking state into sleep. In this manner, EEG can detect the significant increase of theta wave, 4-7 Hz and the significant decrease of alpha wave, 8-13 Hz, EMG can detect the signal from hypnagogic jerk [177].

NREM2 has been classified using spindle wave as criterion. Spindle wave is the rapidly change of theta wave from NREM1 to brainwave at the frequency around 7-14

Hz with low amplitude within <0.5 seconds and the K-Complex which span of high frequency and positive sharp waves followed by sharp negative, while EMG can be found only a little until undetected [178].

NREM3-4 have been classified by the presence of a delta, 0.5-2 Hz, high amplitude wave, spindle and K-complex which still occurs but less than NREM2, while EMG can be found only a little until undetected. In addition, mechanism of REM sleep associated with neurotransmitters in the brain between pons and midbrain or mesencephalon [171], the signal from REM sleep can be detected from rostral structure. While, the rostral region has been linked to medulla and spinal cord, thus resulting in a state of lack of muscle tone (Muscle atonia) while entering REM sleep [179]. REM sleep can divided into two phases; tonic and phasic according to the function of neuroanatomical loci [180, 181].

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Tonic phase can be classified by desynchronized EEG, lacked muscle tone, reduced function of monosynaptic reflexes and polysynaptic reflexes [182]. In this phase, the muscle will automatically reduce muscular tone and go into a temporary paralysis state whereas, diaphragm, sphincter, cardiac muscle and some of the gastrointestinal tract still work properly. The temporary paralysis is associated with glycine discharging. Glycine is an amino acid in motor neurons. Glycine acts as Postsynaptic inhibition which inhibits the signal at presynaptic terminal and resulting in the inhibition of the neurotransmitters to postsynaptic cell causing inhibitory

postsynaptic potential (IPSP) [183, 184] In phasic phase, it can classified using electromyograph by myoclonias observation and using electroencephalograph by sawtooth waves with additional theta wave observation [185, 186]. Multidirectional movement of eye ball can occur by the function of the middle ear bones. Furthermore, in phasic state, it was also found changes in blood pressure, heart rate, sleep apnea, tongue movement and muscle twitching [187-189].

Sleep mechanism is believed to occur in anterior hypothalamus. Neurophysiology clarifies the sleep mechanism by one of the most impotent mechanisms causing of sleep and tend to be a key mechanisms occurred in ventrolateral preoptic (VLPO) nucleus, which consists of GABAergic neuron. GABA acts to inhibit neurons in the brain by acting on receptors in the neuronal membrane. It binds both pre and postsynaptic process which leads to the opening of the receptor which results in either chloride ion goes into cell or potassium ion goes out of cell causing hyperpolarization (Figure 26) [190].

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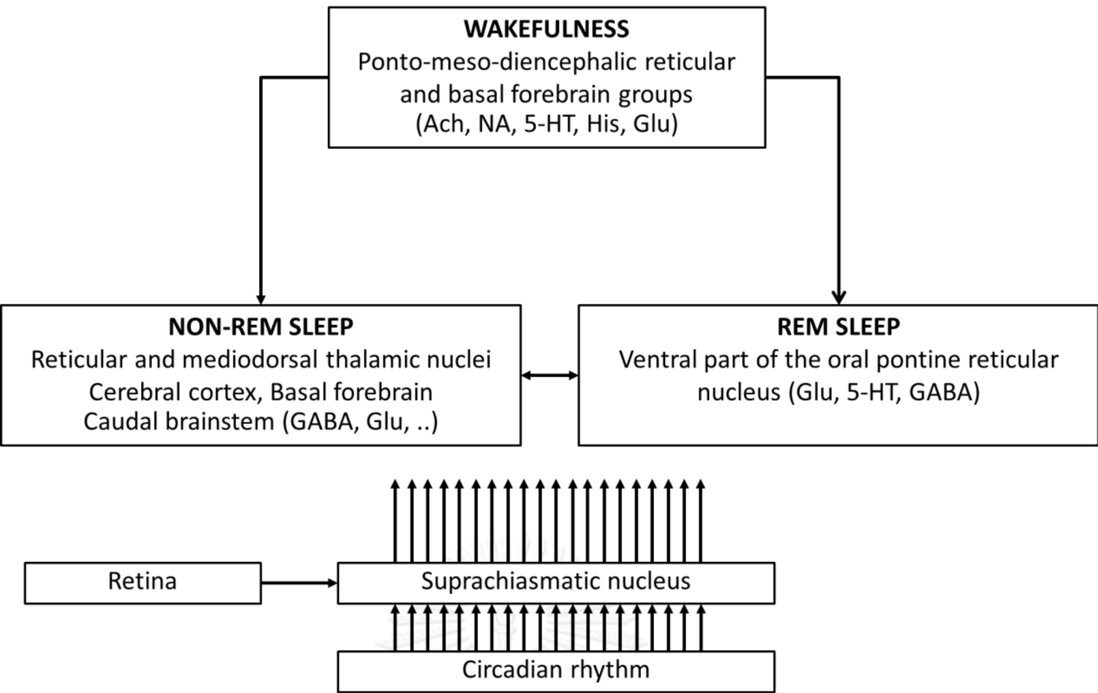

Figure 26 The mechanism of awakening and sleeping centre [191]

VLPO nucleus is associated with the function of neuron which works for awakening such as histaminergic neurons, orexinergic neurons, locus coeruleus, dorsal raphe and cholinergic neurons in spinal cord [192-194]. VLPO nucleus is an intermediary between waking and sleeping centre. Therefore, GABAergic is an important part in this mechanism, the evidence indicates that NREM sleep caused by GABAergic which serves to build neurotransmitters GABA to the neocortex (Figure 27). It also inhibits cholinergic neurons and produces cortical activity which respectively serves as awaking control centre and NREM sleep production [194].

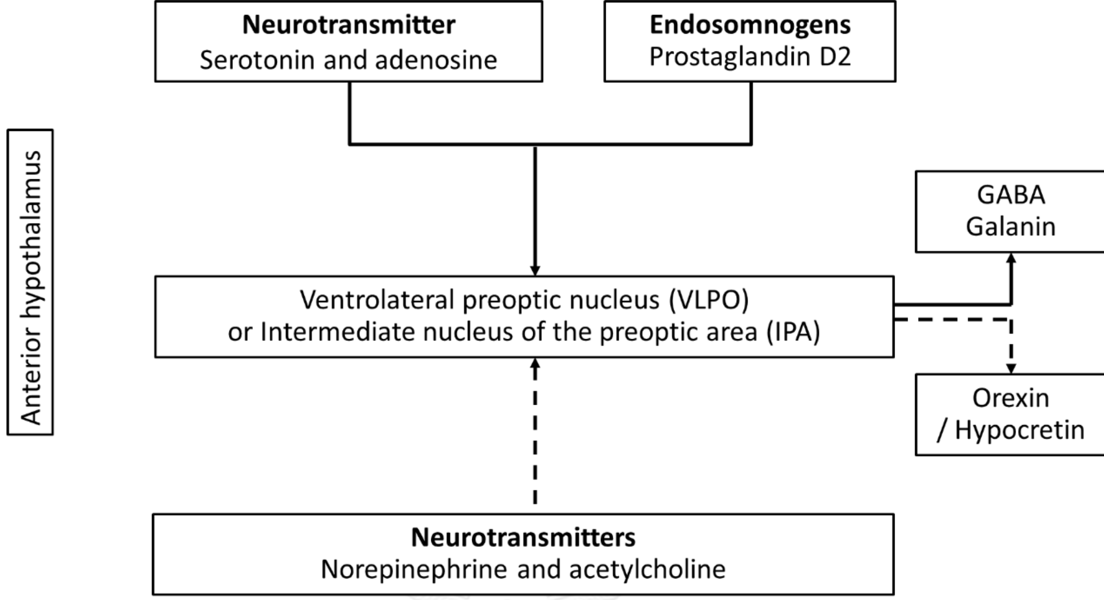

Figure 27 The mechanism of ventrolateral preoptic (VLPO) nucleus [191]

L. disjunctus ethanolic extract at the doses of 50, 100 and 200 mg/kg significantly decreased the duration of waking state, whereas duration of REM sleep (rapid eye movement) and total duration of NREM sleep (non-rapid eye movement) significantly increased, compared to vehicle control group (p<0.01). Although, there was no significant difference observed between the two effective doses (50 and 100 mg/kg), but significantly change in 200 mg/kg (Figure 19). From this finding, the highest dose tended to be the dependent dose. Normally, delta waves began to appear in state 3 and dominated spectral activity in state 4 of NREM. Delta EEG power density or EEG delta power was used to describe quality of sleep as a measurement of intensity of NREM sleep. Present research study examined delta power density in NREM sleep for 6 hours (Figure 20). From the results, there is no significant change in percent EEG activity among 4 experimental groups, and the relationship between time and dose

response on delta activity during NREM sleep which was found that both ethanolic extract and vehicle control group showed no statistical different throughout the six hours during the test (Figure 21). It revealed that the quality of sleep was not reduced whereas NREM state statistical increased (Figure 22).

Some herbs tended to possess anti-anxiety which also showed effective potency by decreased waking state. On the other hand, they increased sleeping time. For example, Kava-kava extract also showed significant decrease in waking state and increased in sleeping state. Kava-kava extract showed a significant increase in delta activity during NREM state in sleep-disturbed rats [195]. *Valeriana wallichi* L. aqueous root extract was performed to investigate the effects on sleep-wake profile. Both NREM sleep and duration of total sleep was significantly increased after treatment [196]. In addition, chamomile was another herb that demonstrated a benzodiazepine-like hypnotic activity by investigating hypnotic activities in sleep-disturbed rat model [197, 198]. It could shorten sleep latency and also prolong sleeping time, so hypnotic effect could be affected by benzodiazepine receptor agonists [199]. It seemed likely that *L. disjunctus* ethanolic extract might have a component which effect through benzodiazepine receptors.

In conclusion, *L. disjunctus* showed the effectiveness in anxiolytic, hypnotic effects and increased time in NREM sleep which tended to be anxiolytic plant. In further study, its active compound and mechanism on neurotransmitter expression should be studied. Nutritional compositions of *L. disjunctus* have been scientifically documented. They were recognized to be a good source of several active components and health promoters.

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APPENDIX

แบบรายงานผลการพิจารณาจริยธรรมการวิจัยในสัตว์
มหาวิทยาลัยรังสิต

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1. ชื่อโครงการวิจัย :

ภาษาไทย : ฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากต้นแส้ม้าฮ่อ

ภาษาอังกฤษ : *In vitro* and *In vivo* Bioactivities of *Lebtocarpus Disjunctus* Ethanolic Extract

2. ชื่อหัวหน้าโครงการวิจัย : รองศาสตราจารย์ ดร.นิจศิริ เรืองรังษี

3. หน่วยงานที่สังกัด : คณะเภสัชศาสตร์ มหาวิทยาลัยรังสิต

4. สถานที่ดำเนินการเลี้ยงและใช้สัตว์

ศูนย์สัตว์ทดลองแห่งชาติ มหาวิทยาลัยมหิดล ศาลายา จังหวัดนครปฐม

ข้อเสนอโครงการวิจัยนี้ได้ผ่านการพิจารณาจากคณะกรรมการพิจารณาจริยธรรมการใช้สัตว์แล้ว เห็นว่ามีความสอดคล้องกับจรรยาบรรณการใช้สัตว์ สภาวิจัยแห่งชาติ จึงเห็นสมควรให้ดำเนินการเลี้ยงและใช้สัตว์ ตามข้อเสนอการวิจัยนี้ได้

คณะกรรมการจริยธรรมการวิจัยในสัตว์ มีมติเห็นชอบ ดังนี้

(√) รับรองโครงร่างการวิจัย

( ) ไม่รับรอง

5. หมายเลขที่ให้การรับรอง : RSEC 01/2556

6. วันที่ให้การรับรองและวันที่สิ้นสุด : 1 เมษายน 2556 – 31 ตุลาคม 2557

ลงนาม ....

(ผู้ช่วยศาสตราจารย์ ดร.พงษ์จันทร์ อยู่แพทย์)

ประธานคณะกรรมการพิจารณาจริยธรรมการวิจัยในสัตว์

มหาวิทยาลัยรังสิต

มหาวิทยาลัยรังสิต
RANGSIT UNIVERSITY

Figure 28 Ethical consideration approval letter (RSEC 01/2556)

แบบรายงานผลการพิจารณาจริยธรรมการวิจัยในสัตว์ทดลอง
มหาวิทยาลัยรังสิต

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1. ชื่อโครงการวิจัย :

ภาษาไทย : ฤทธิ์ทางเภสัชวิทยาและองค์ประกอบทางสารอาหารของต้นแซ่ม้าฮ่อ

ภาษาอังกฤษ : Pharmacological activities and nutritional compositions of *Leptocarpus disjunctus*

2. ชื่อหัวหน้าโครงการวิจัย : รองศาสตราจารย์ เภสัชกร ดร.นิจศิริ เรืองรังษี

3. **หน่วยงานที่สังกัด :** คณะเภสัชศาสตร์ มหาวิทยาลัยรังสิต

4. **สถานที่ดำเนินการเลี้ยงและใช้สัตว์**

คณะเภสัชศาสตร์ มหาวิทยาลัยการแพทย์แผนจีนเฮยหลงเจียง และคณะวิทยาศาสตร์ มหาวิทยาลัยรังสิต

ข้อเสนอโครงการวิจัยนี้ได้ผ่านการพิจารณาจากคณะกรรมการพิจารณาจริยธรรมการใช้สัตว์ทดลองแล้ว เห็นว่ามีความสอดคล้องกับจรรยาบรรณการใช้สัตว์ สภาวิจัยแห่งชาติ จึงเห็นสมควรให้ดำเนินการเลี้ยงและใช้สัตว์ทดลอง ตามข้อเสนอการวิจัยนี้ได้

คณะกรรมการจริยธรรมการวิจัยในสัตว์ทดลอง มีมติเห็นชอบ ดังนี้

(√) รับรองโครงร่างการวิจัย

( ) ไม่รับรอง

5. หมายเลขที่ให้การรับรอง : RSEC 02/2557

6. วันที่ให้การรับรองและวันที่สิ้นสุด : 1 พฤษภาคม 2558 – 31 พฤษภาคม 2559

ลงนาม ....

(รองศาสตราจารย์ ศรีพงษ์จันทร์ อยู่แพทย์)

ประธานคณะกรรมการพิจารณาจริยธรรมการวิจัยในสัตว์ทดลอง

มหาวิทยาลัยรังสิต

มหาวิทยาลัยรังสิต
RANGSIT UNIVERSITY

Figure 29 Ethical consideration approval letter (RSEC 02/2557)

Table 16 Nutritional composition of *L. disjunctus* (per 100 g based on wet basis)

Nutrient		per 100 gram		
		Exp1	Exp2	AVG
Energy	Kcal			63
Energy from fat	Kcal			4
Moisture	g	84.3	84.3	84.3
Ash	g	0.6	0.6	0.6
Protein	g	1.8	1.8	1.8
Total fat	g	0.9	0.5	0.5
Fibre	g	5.0		5.0
Iodine	μg	0	0	0
Sodium	mg	4.5	4.7	5.0
Potassium	mg	241	232	237
Magnesium	mg	12.9	13.7	13.3
Calcium	mg	17.9	17.6	17.8
Phosphorus	mg	44.9	44.3	44.6
Iron	mg	0.8	0.8	0.8
Zinc	mg	0.8	0.8	0.8
Copper	mg	0	0	0
β-carotene	μg	708	708	708
Vitamin E	μg	520	542	531
Vitamin B1	mg	0.05	0.05	0.05
Vitamin B2	mg	0.09	0.09	0.09
Vitamin C	mg	19.7	19.4	19.2
Niacin	mg	1.24	1.26	1.25
Folic acid	μg	39.7	44.2	41.9

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Table 17 Fatty acid content in *L. disjunctus* (based on wet basis)

Fatty acid		% Fatty acid			mg/100g
		Exp1	Exp1	AVG	
C12:0	Lauric acid	0.5	0.6	0.5	1.9
C14:0	Myristic acid	0.6	0.7	0.7	2.2
C15:0	Pentadecanoic acid	0.6	0.5	0.5	1.8
C16:0	Palmitic acid	17.7	18.5	18.1	61.6
C16:1	Palmitoleic acid	3.8	3.1	3.5	10.3
C17:0	Margaric acid	0.3	0.3	0.3	1.1
C18:0	Stearic acid	1.6	1.6	1.6	5.3
C18:1	Oleic acid	8.0	7.9	7.9	26.2
C18:2	Linoleic acid	38.5	38.9	38.7	129.7
C18:3	Linolenic acid	20.7	20.9	20.8	69.5
C20:0	Arachidic acid	0.8	0.7	0.8	2.2
C22:0	Behenic acid	1.0	0.8	0.9	2.8
C22:5	Docosaenoic acid	0.9	0.7	0.8	2.5

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Table 18 Pentobarbital induced sleeping time test of *L. disjunctus* ethanolic extract

	Vehicle control	10 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	Flu+200 mg/kg	Flu+Vehicle control
Exp1	8.233	19.350	0.000	48.890	30.633	3.567	9.250
Exp2	11.133	6.740	37.050	37.840	31.900	5.833	8.200
Exp3	11.567	9.590	33.670	18.100	32.367	10.667	7.400
Exp4	10.400	22.700	30.010	0.000	28.500	8.833	10.567
Exp5	9.133	9.800	19.190	30.500	28.000	11.183	9.700
Exp6	14.767	34.940	21.780	37.630	20.667	9.833	10.883
Exp7	9.433	31.000	9.830	17.950	33.900	7.867	8.033
Exp8	10.900	45.840	38.530	40.630	39.333	12.467	11.233
Exp9	9.767	31.910	24.920	20.060	32.367	7.567	7.467
Exp10	13.033	8.830	13.350	14.760	31.233	8.033	8.683
AVG	10.837	22.070	22.833	26.636	30.890	8.585	9.142
SD	1.940	13.464	12.546	14.887	4.767	2.633	1.411

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Table 19 Evaluated plus maze test of *L. disjunctus* ethanolic extract

	Vehicle control		10 mg/kg		50 mg/kg		100 mg/kg	
	Open arm	Close arm	Open arm	Close arm	Open arm	Close arm	Open arm	Close arm
Exp1	10.4	289.6	28.6	271.4	55.7	244.3	53.2	246.8
Exp2	25.1	274.9	63.4	236.6	44.4	255.6	61.3	238.7
Exp3	17.2	282.8	54.0	246	53.8	246.2	52.2	247.8
Exp4	4.5	295.5	61.2	238.8	64.0	236	68.8	231.2
Exp5	10.9	289.1	53.5	246.5	67.2	232.8	70.4	229.6
Exp6	15.8	284.2	46.0	254	47.4	252.6	51.4	248.6
Exp7	4.8	295.2	52.6	247.4	47.1	252.9	46.51	253.49
Exp8	14.9	285.1	28.7	271.3	51.3	248.7	66.9	233.1
Exp9	n/a	n/a	38.3	261.7	62.9	237.1	65.5	234.5
Exp10	n/a	n/a	35.3	264.7	62.2	237.8	68.8	231.2
AVG	13.31	286.69	46.16	253.84	55.60	244.40	60.50	239.50
SD	6.83	6.83	10.78	12.79	8.09	8.09	7.74	8.84

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Table 20 Rota rod performance test of *L. disjunctus* ethanolic extract

	Saline	10 mg/kg	50 mg/kg	100 mg/kg
Exp1	72.4	28.5	16.8	16.5
Exp2	67.3	17.8	23.6	8.9
Exp3	99.2	19.2	14.5	17
Exp4	58.6	26.6	12.3	15.2
Exp5	65.2	45.5	26.6	19.4
Exp6	91	34.8	22.5	16.2
Exp7	69.2	38.6	21.7	18.8
Exp8	60.3	19.5	16.5	16
Exp9	101.1	42.9	26.2	9.2
Exp10	106	32.3	32.3	22.8
AVG	79.03	30.57	21.3	16
SD	18.26	9.97	6.24	4.27

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Table 21 Open field test of *L. disjunctus* ethanolic extract

	Grooming (Time)				Leaning (Time)				Rearing (Time)			
	Saline	10 mg/kg	50 mg/kg	100 mg/kg	Saline	10 mg/kg	50 mg/kg	100 mg/kg	Saline	10 mg/kg	50 mg/kg	100 mg/kg
Exp1	15	8	6	5	32	43	3	1	10	5	2	0
Exp2	11	10	8	4	44	36	6	2	14	1	1	0
Exp3	9	2	6	2	45	23	4	5	13	0	3	1
Exp4	11	5	0	5	41	30	5	4	8	0	3	2
Exp5	9	6	8	3	45	26	6	4	5	2	0	3
Exp6	10	4	0	2	50	15	6	2	5	1	2	2
Exp7	10	5	6	0	49	18	3	1	6	4	3	4
Exp8	10	8	7	3	52	25	4	0	11	6	2	3
Exp9	8	5	5	0	36	24	5	2	6	6	1	1
Exp10	11	8	8	2	44	16	4	2	8	5	2	2
AVG	10.4	6.1	5.4	2.6	43.80	25.60	4.60	2.30	8.6	3	1.9	1.8
SD	1.90	2.38	3.03	1.78	6.18	8.83	1.17	1.57	3.27	2.45	0.99	1.32

Table 22 Analysis of sleep in *Drosophila melanogaster* of *L. disjunctus* ethanolic extract

Female						
	All day	**SD**	**Day**	**SD**	**Night**	**SD**
5 % Sucrose	707.031	137.43	238.281	97.4068	468.75	78.0922
5 mg/ml	802.031	197.587	289.375	104.277	513.438	111.416
10 mg/ml	721.25	168.958	236.719	129.045	485	106.521

Male						
	All day	**SD**	**Day**	**SD**	**Night**	**SD**
5% Sucrose	859.219	168.791	366.875	92.7427	492.969	100.032
5mg	915.156	110.311	401.719	54.8548	513.438	73.0603
10mg	782.188	186.931	330.313	89.9994	452.031	106.033

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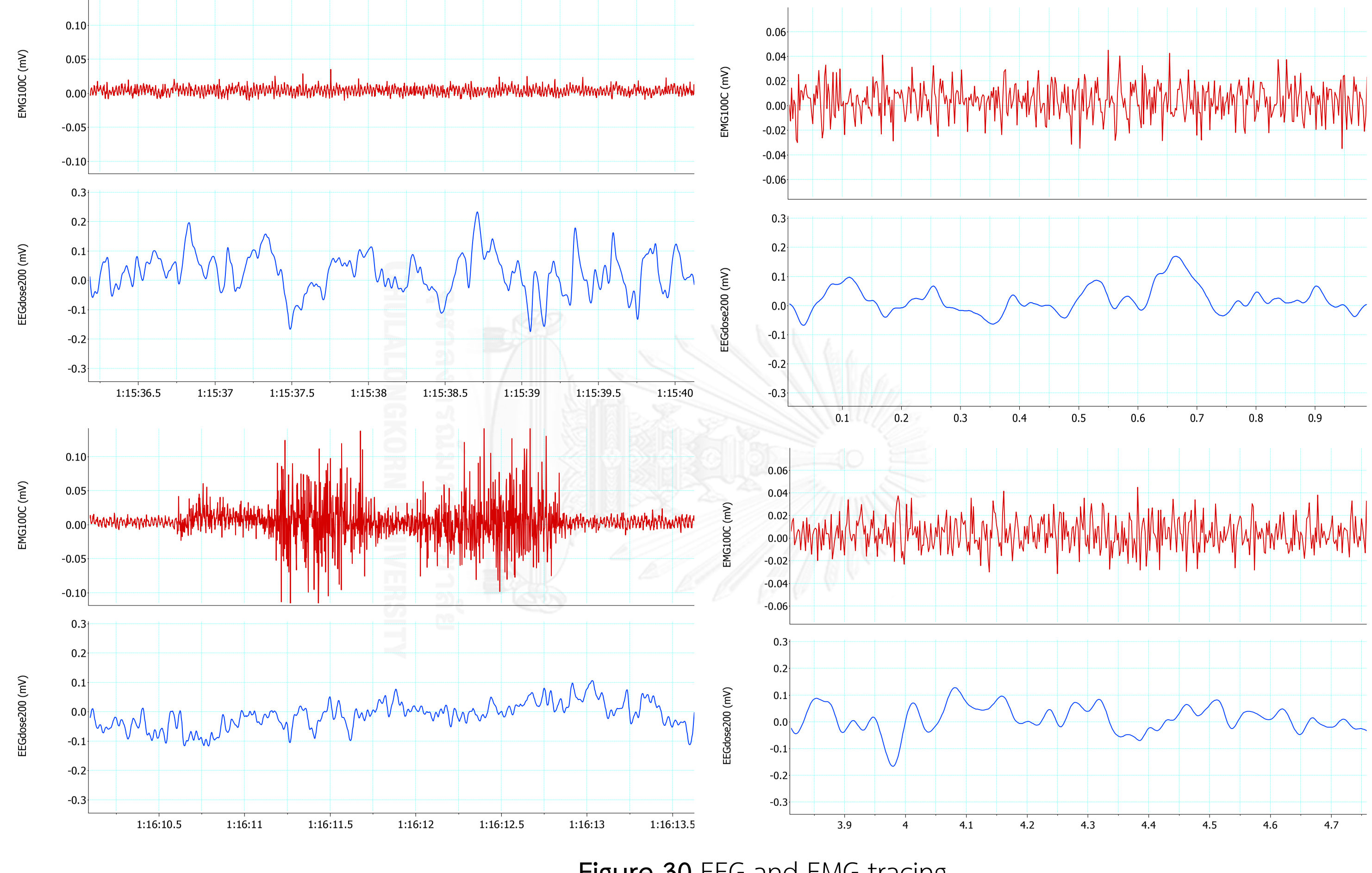

Figure 30 EEG and EMG tracing

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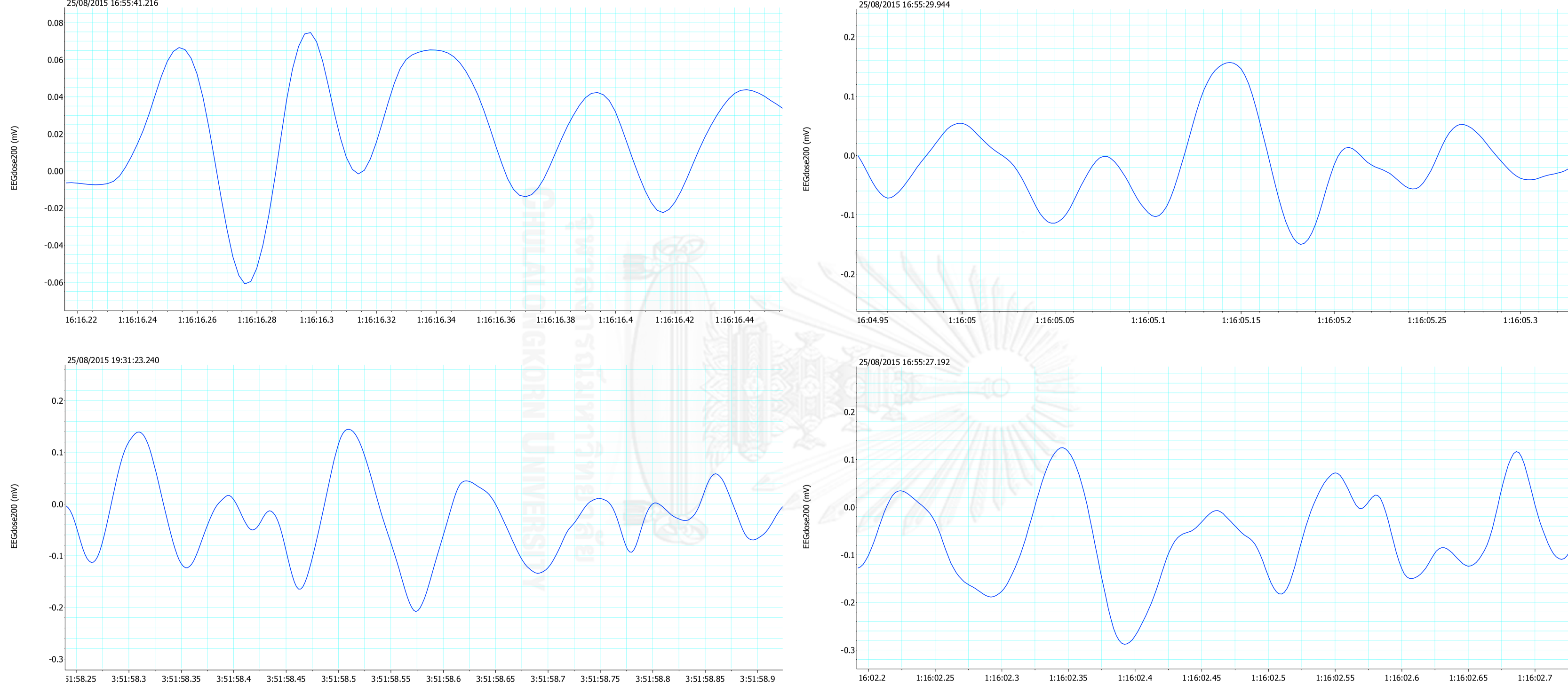

Figure 31 Beta wave pattern

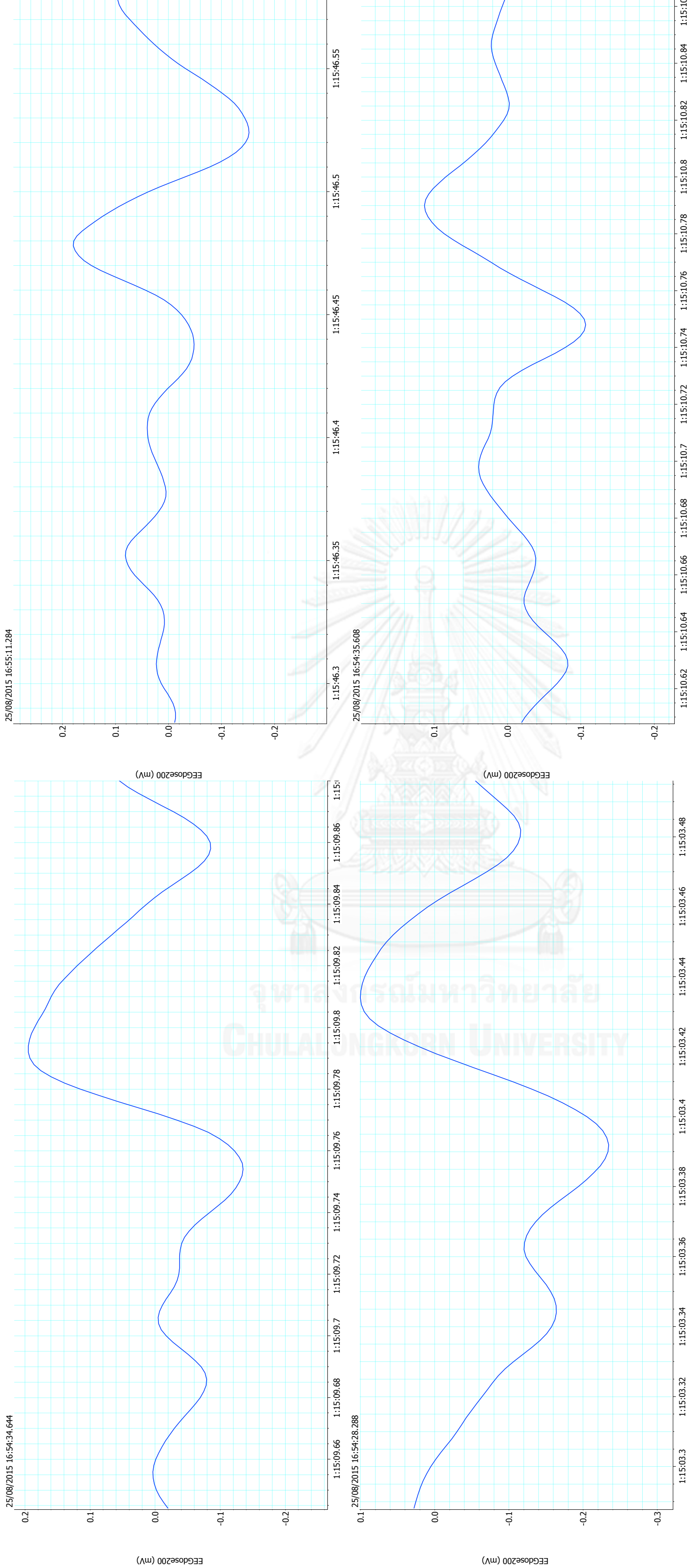

Figure 32 Theta wave pattern

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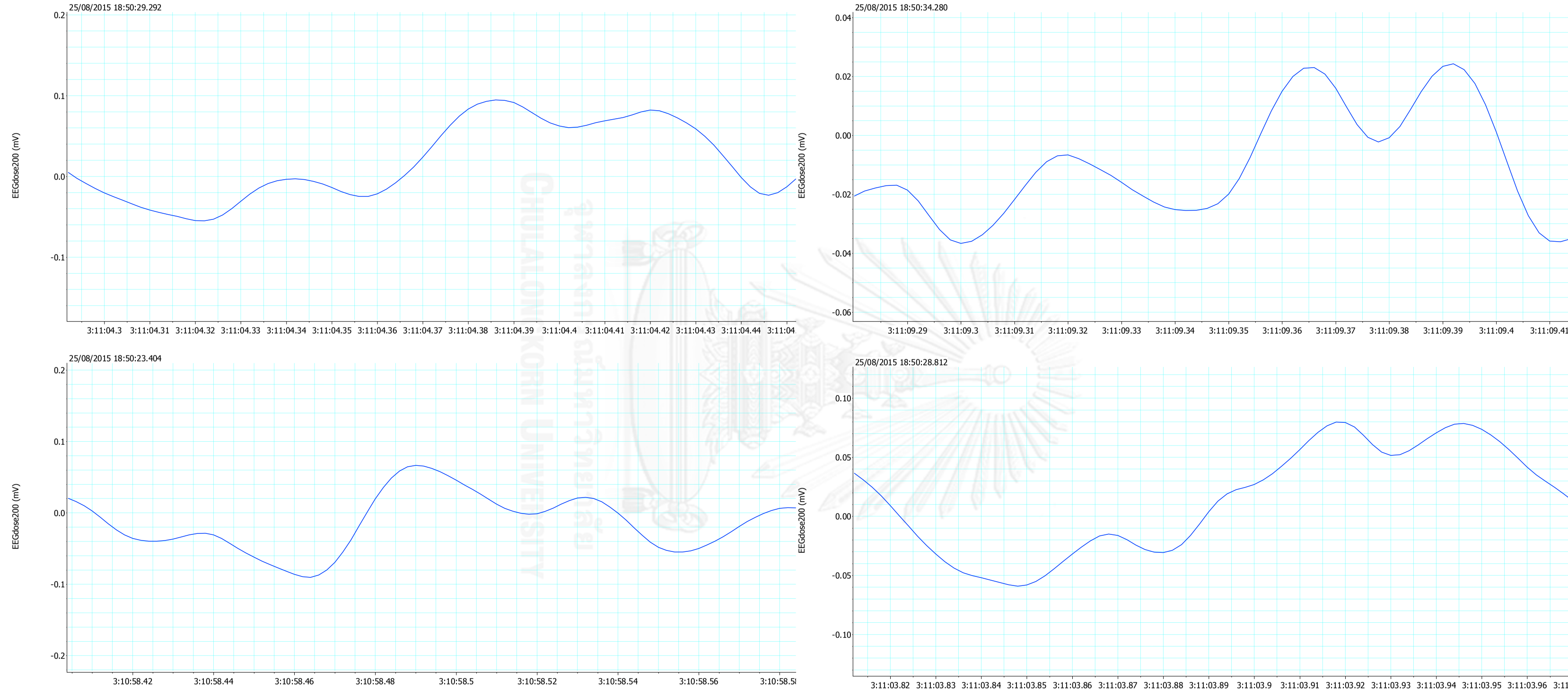

Figure 33 Delta wave pattern

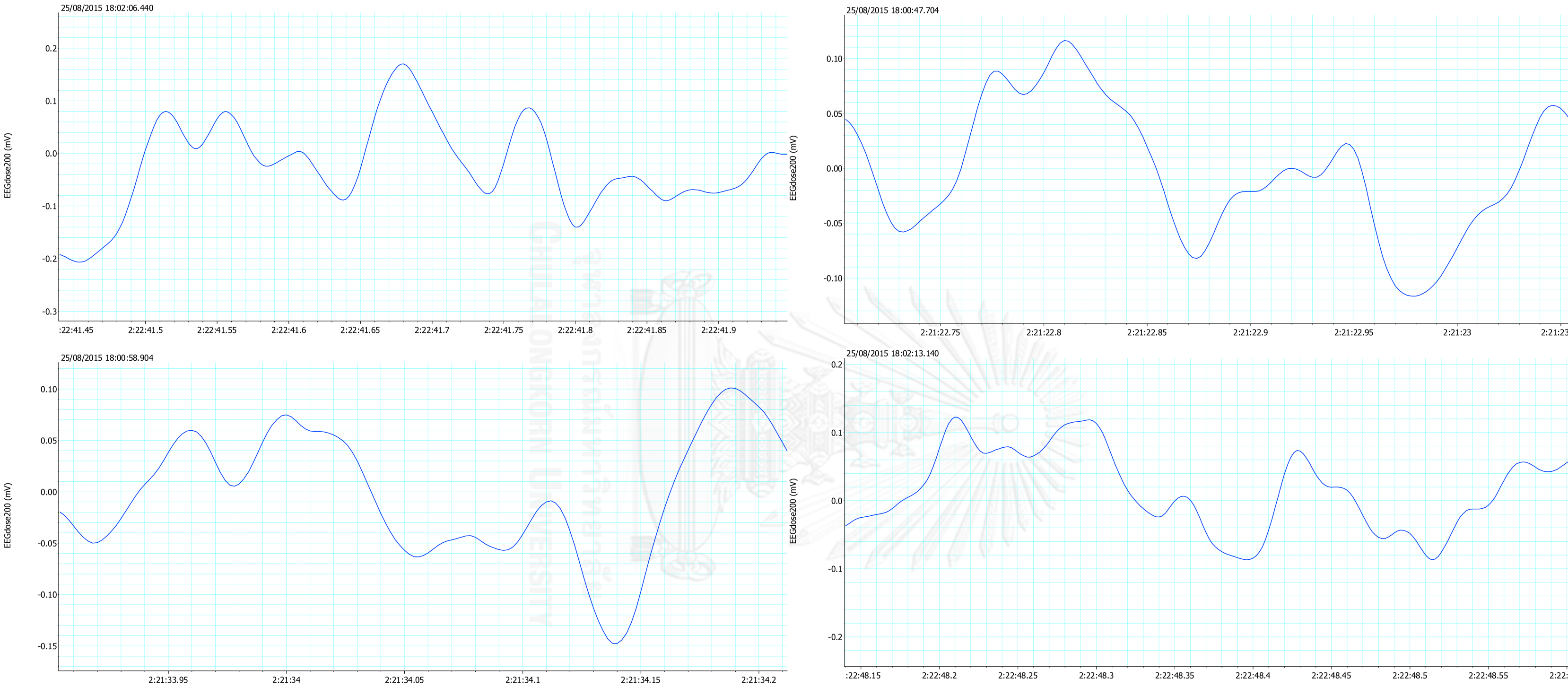

Figure 34 Sleep spindle pattern

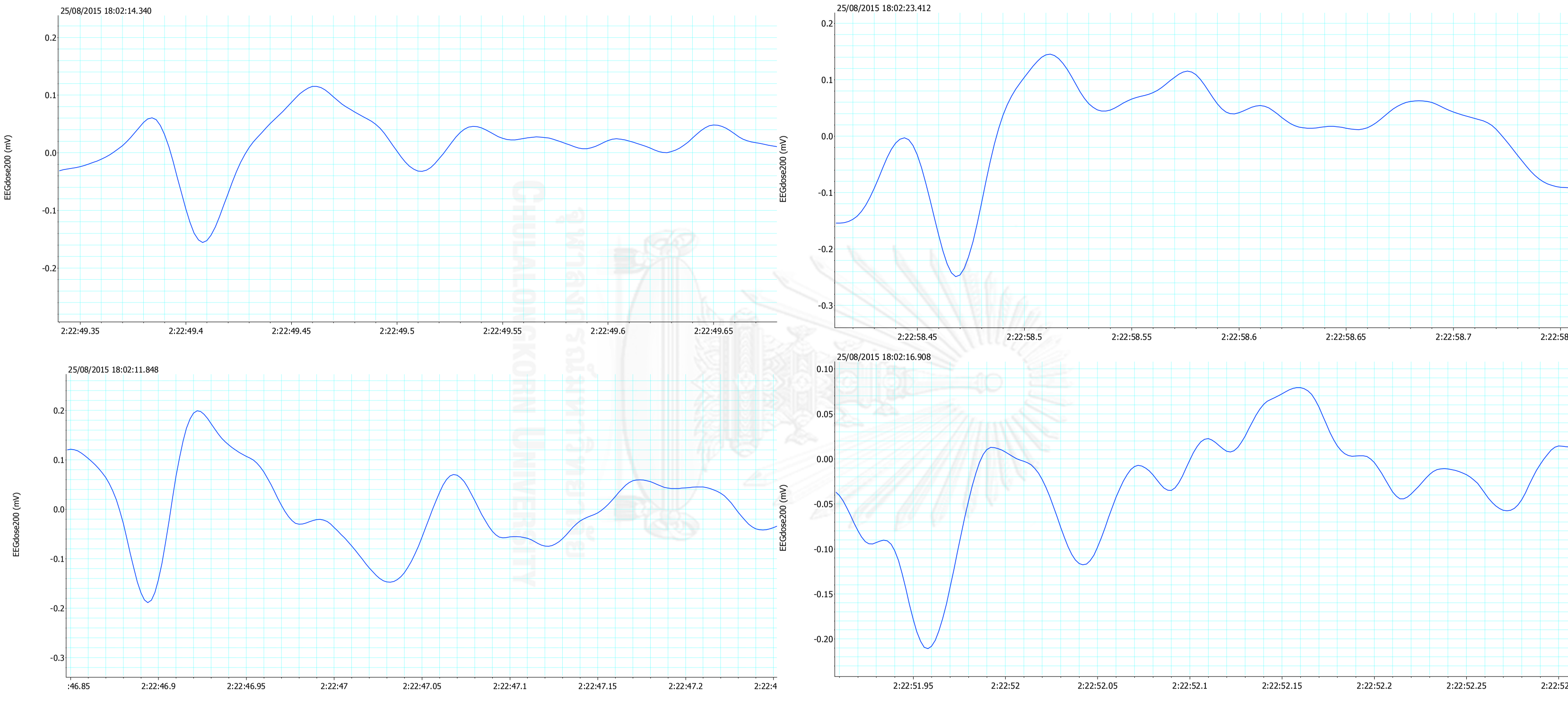

Figure 35 K complex pattern

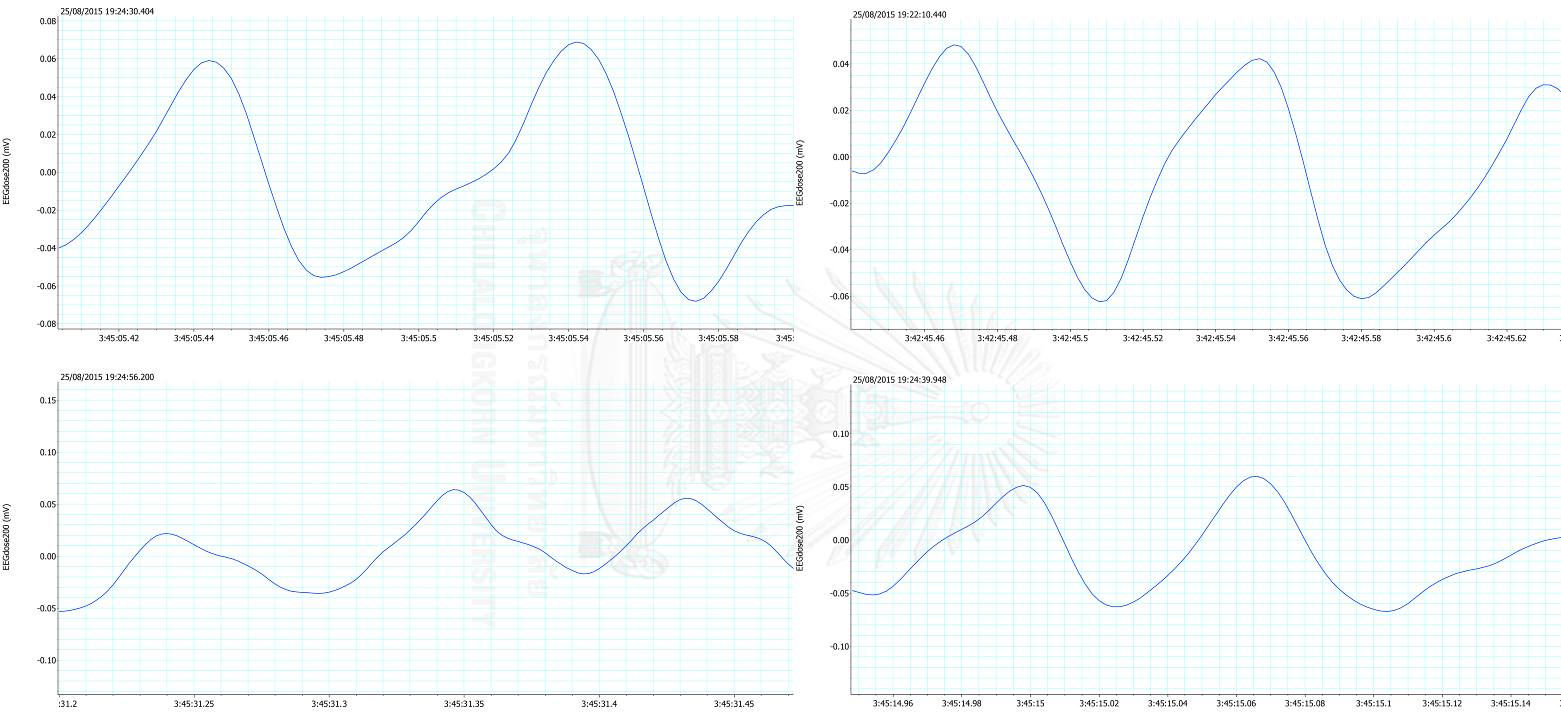

Figure 36 Saw tooth wave in tonic stage of REM sleep

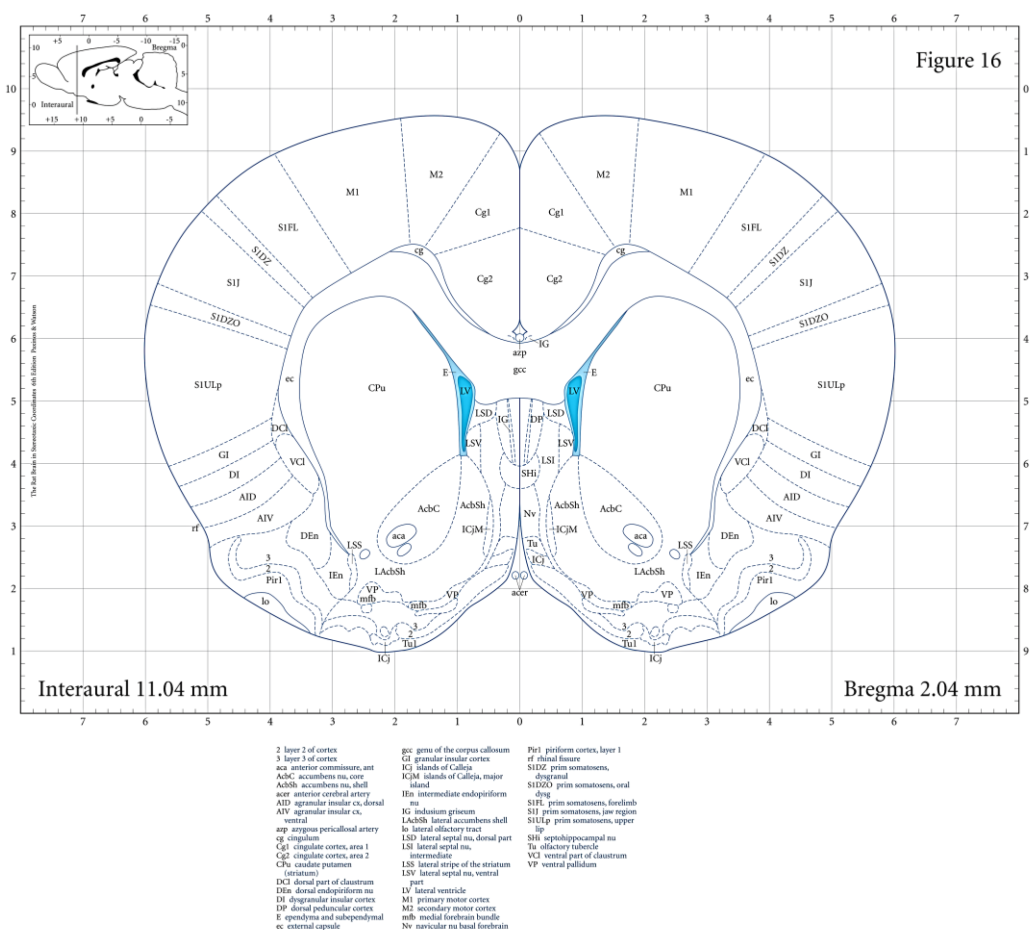

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Figure 37 Primary (M1) and secondary motor (M2) cortex landmark (Coronal view)

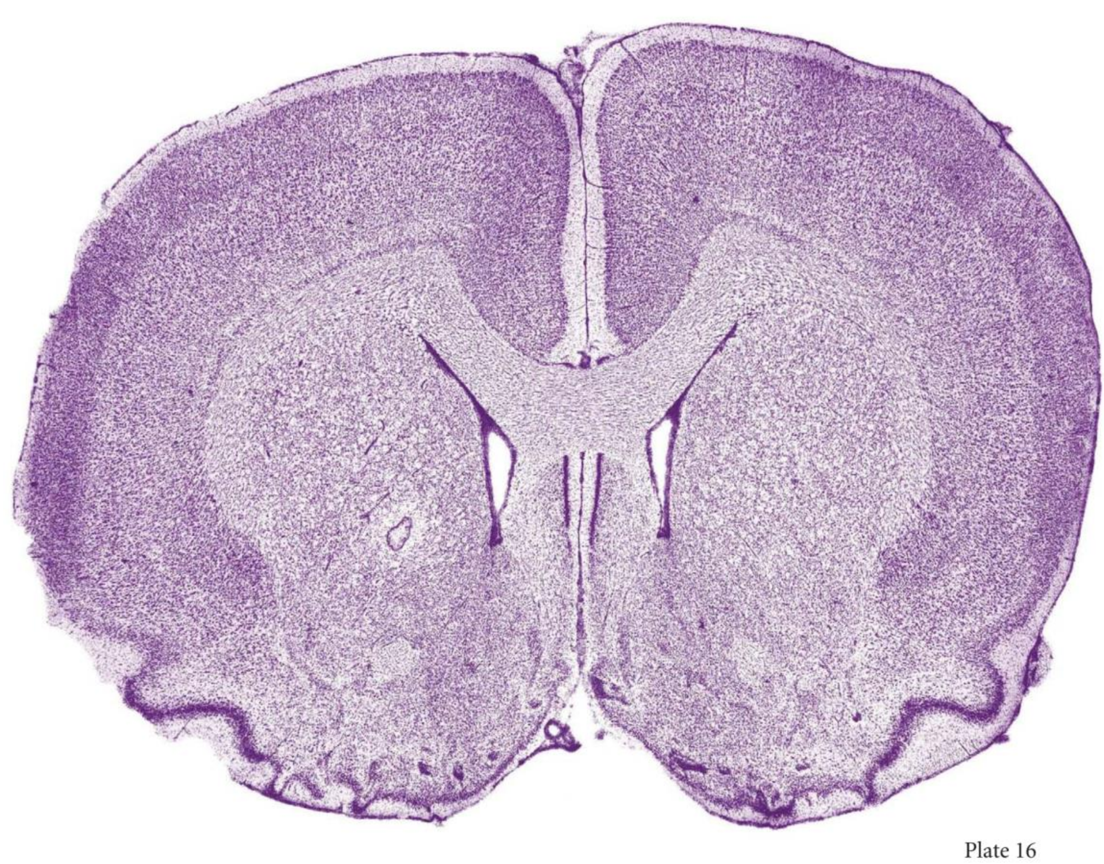

Figure 38 Primary and secondary motor cortex landmark (Coronal view)

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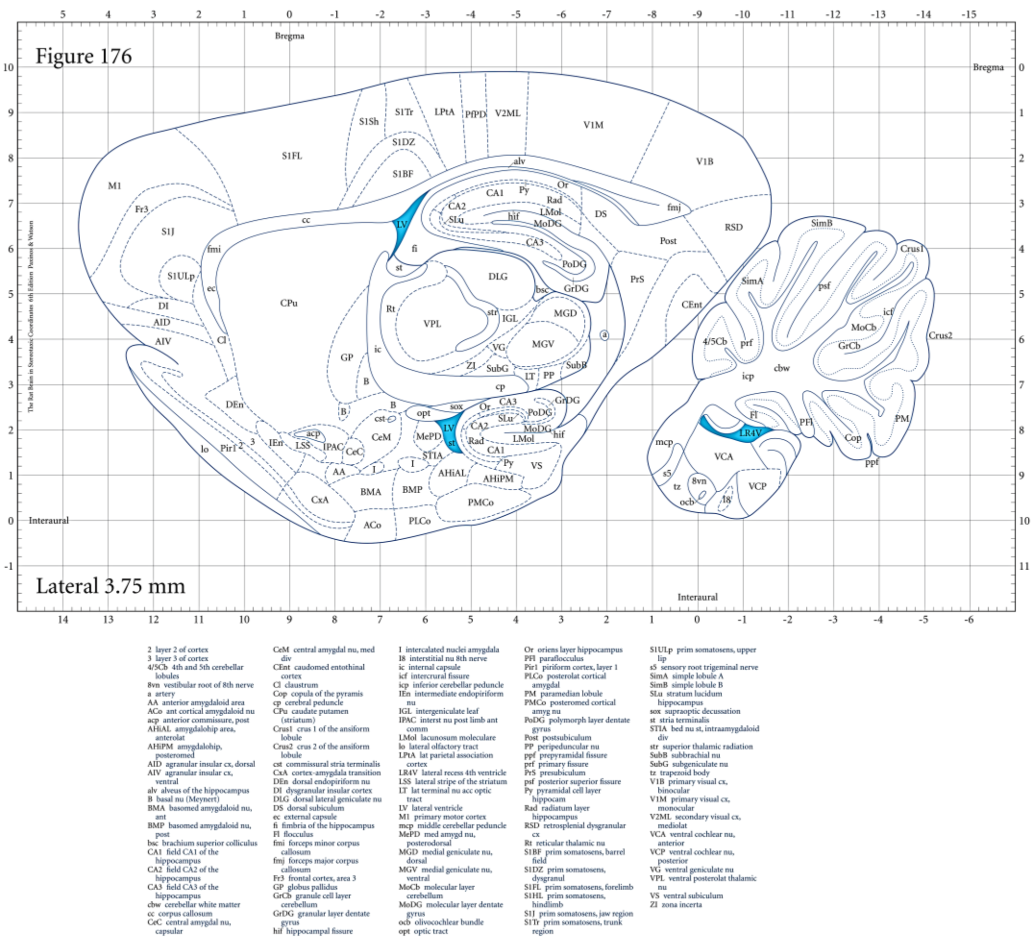

Figure 39 Primary (M1) and secondary motor (M2) cortex landmark (Sagittal view)

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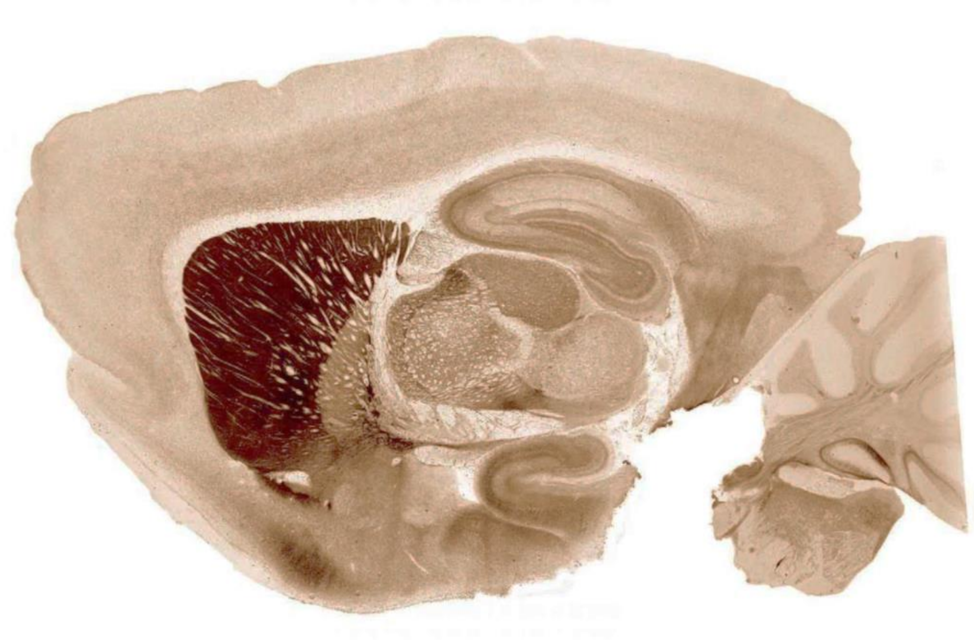

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Figure 40 Primary and secondary motor cortex landmark (Sagittal view)

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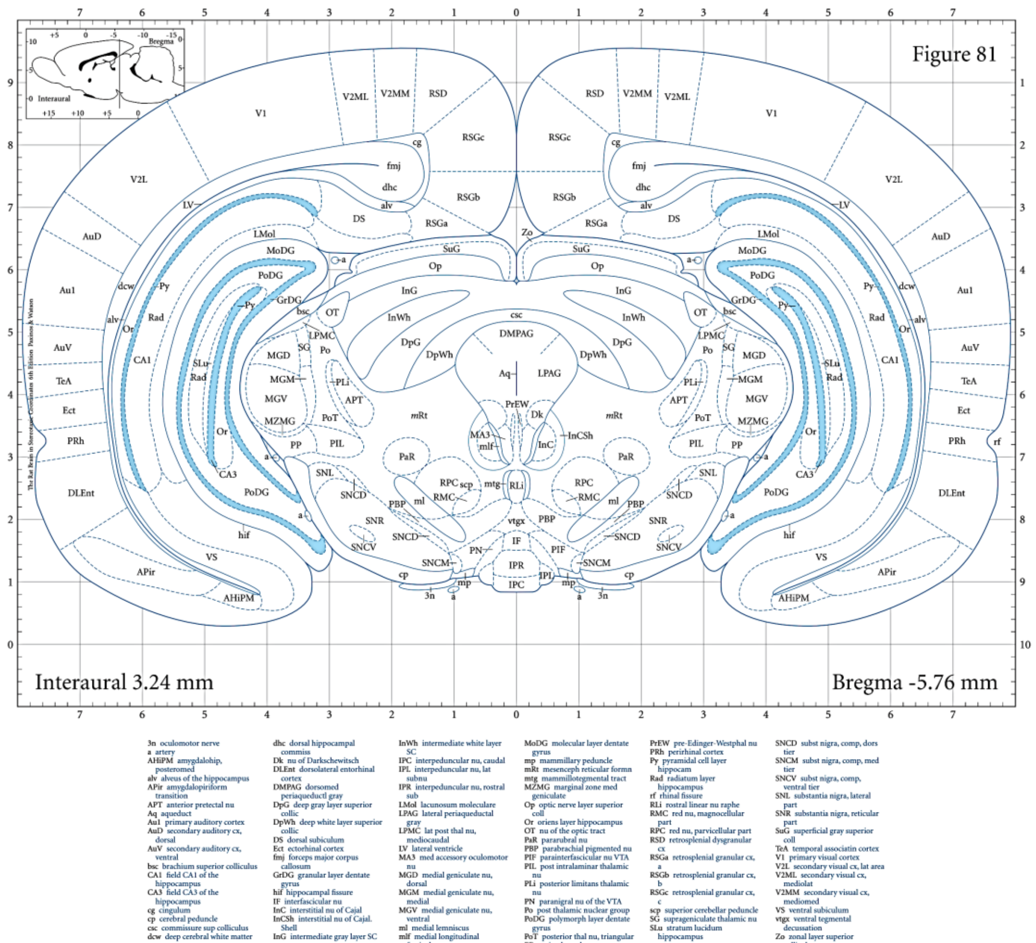

Figure 41 Visual cortex (V1) landmark (Coronal view)

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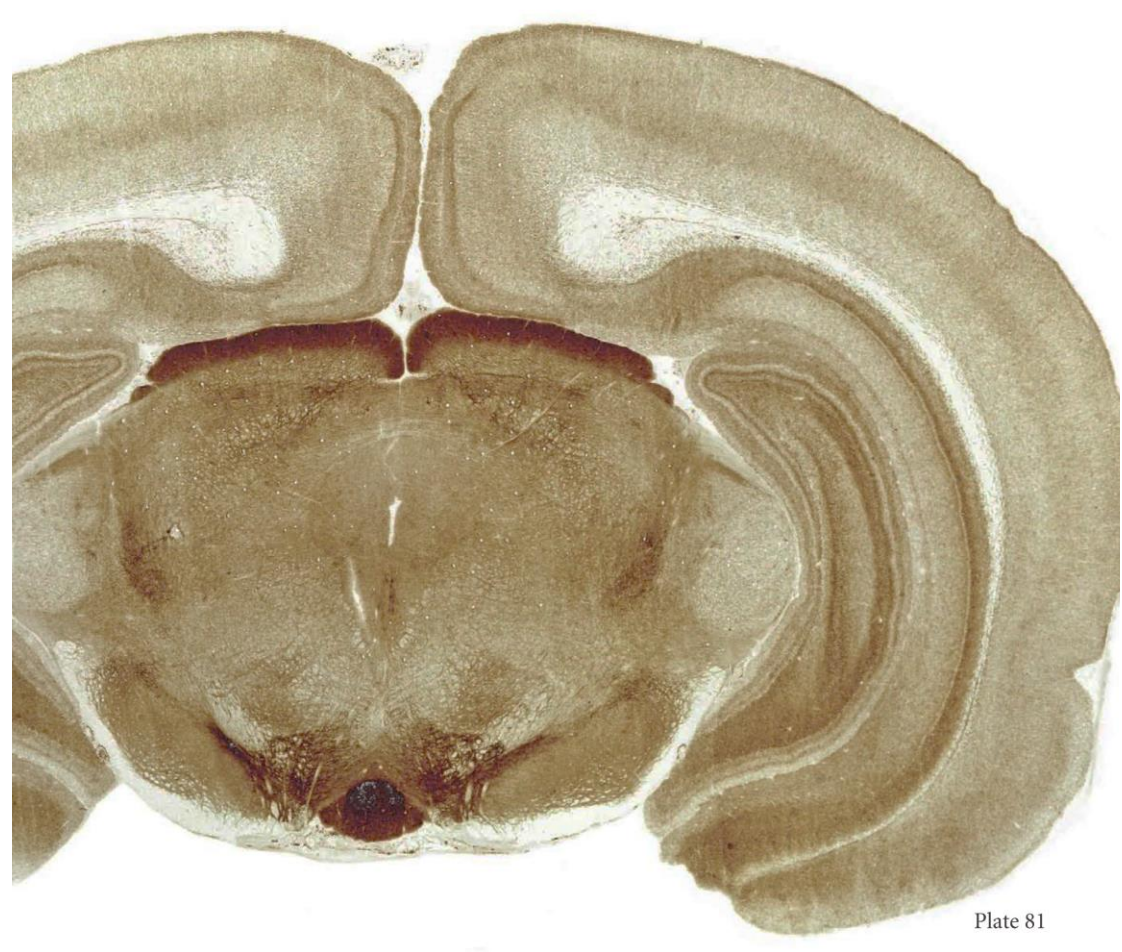

Figure 42 Visual cortex landmark (Coronal view)

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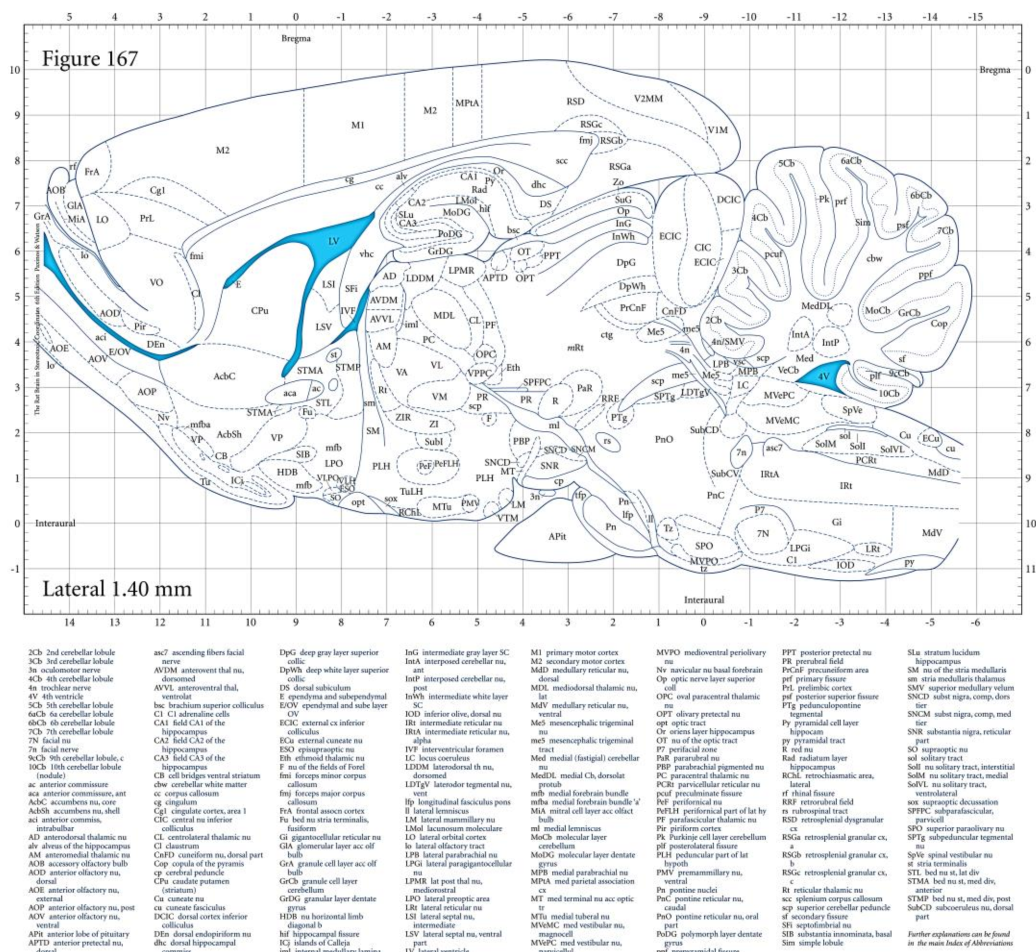

Figure 43 Visual cortex (V1) landmark (Sagittal view)

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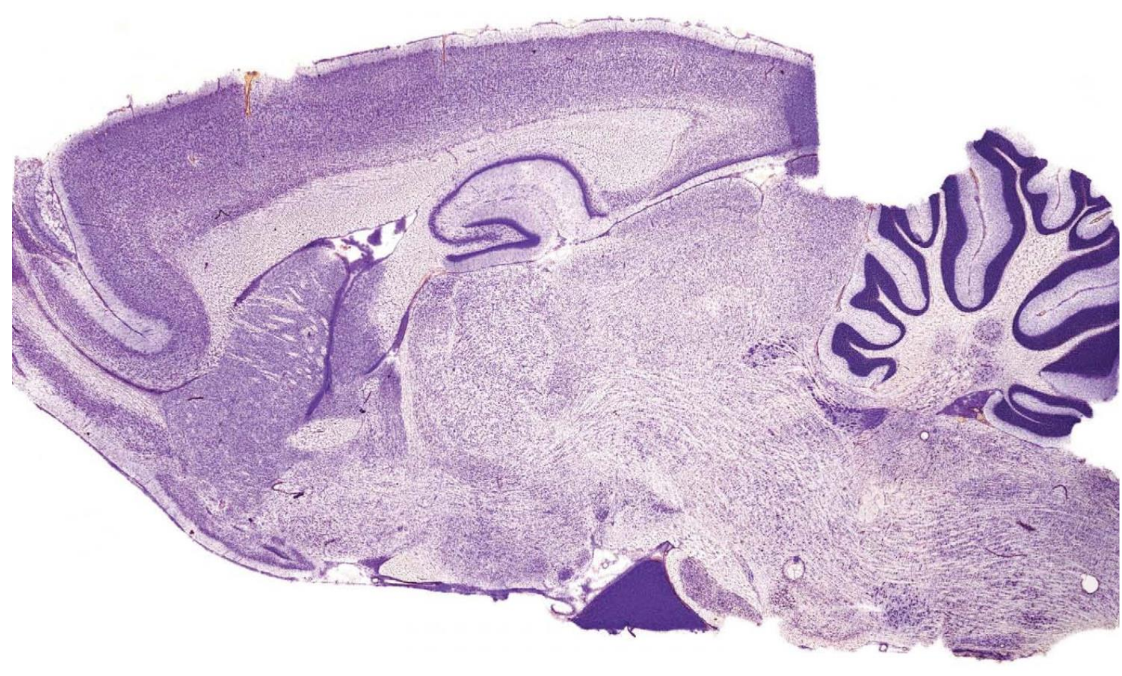

Figure 44 Visual cortex landmark (Sagittal view)

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VITA

Mr. Watchara Damjuti was born on October 31, 1989 in Trang, Thailand. He got Bachelor's degree of Applied Thai Traditional Medicine (Applied Thai Traditional Medicine) from Thai Traditional Medicine College, Rajamangala University of Technology Thunyaburi, Thailand in 2012, and got Master's degree of Public Health Sciences (Traditional Thai and alternative medicines) from College of Public Health Sciences, Chulalongkorn University, Thailand in 2013. He attended to study Doctor of Philosophy in Public Health Sciences (Traditional Thai and alternative medicines), Chulalongkorn University, Thailand. During the study, he received The 90th Anniversary of Chulalongkorn University, Rachadapiseksomphot Endowment Fund from Graduate School and Overseas Research Experience Scholarship for Graduate Students from Graduate School and College of Public Health Sciences, Chulalongkorn University for carrying his experiment at Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, Harbin, People Republic of China.

Proceedings and Awards

He participated and won winner award oral presentation at the 3rd International Conference on Advanced Pharmaceutical Research, Rangsit University, Thailand. He participated and presented poster presentation at The 13th Asia Pacific Federation of Pharmacologist (APFP), which hosted by The pharmacological and therapeutic society of Thailand. He also participated and won oral presentation at the 8th Thailand-Japan International Academic (TJIA) Conference which is organized with the collaboration of the Royal Thai Embassy in Japan (Tokyo) and universities from both Thailand and Japan.