IN VITRO AND *IN VIVO* BIOACTIVITIES OF *LEPTOCARPUS DISJUNCTUS* ETHANOLIC EXTRACT

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ฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากต้นแส้ม้าฮ่อ

นายวัชระ ดำจุติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Thesis Title	*IN VITRO* AND *IN VIVO* BIOACTIVITIES OF *LEPTOCARPUS DISJUNCTUS* ETHANOLIC EXTRACT
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วัชระ ดำจุติ: ฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากต้นแส้ม้าฮ่อ (*IN VITRO* AND *IN VIVO* BIOACTIVITIES OF *LEPTOCARPUS DISJUNCTUS* ETHANOLIC EXTRACT) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ชนิดา พลานุเวช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. นิจศิริ เรืองรังษี, 86 หน้า

แส้ม้าฮ่อ มีชื่อทางวิทยาศาสตร์ว่า *Leptocarpus disjunctus* Mast. แส้ม้าฮ่อเป็นพืชและอาหารพื้นเมืองทางภาคใต้ของไทย หากรับประทานในปริมาณมากทำให้มีอาการง่วงนอน และเวียนศีรษะ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาสารทุติยภูมิ และฤทธิ์ทางชีวภาพ จากสารสกัดเอธานอลจากต้นแส้ม้าฮ่ อ การตรวจคัดกรองสารทุติยภูมิพบว่ามีสารคูมารินกลัยโคไซด์ แทนนินกลัยโคไซด์ สเตอรอยด์ และน้ำตาล การศึกษาฤทธิ์ทางชีวภาพในหลอดทดลองพบว่าสารสกัดเอธานอลจากต้นแส้ม้าฮ่อ ไม่มีฤทธิ์ต้านจุลชีพ ศึกษาฤทธิ์ต้านอนุมูลอิสระพบว่าสามารถต้านอนุมูลอิสระดีพีพีเอชโดยมีค่า EC_{50} เท่ากับ 0.8 มิลลิกรัม/มิลลิลิตร สารบีเอชทีที่ใช้เป็นสารควบคุมบวก มีค่า EC_{50} เท่ากับ 5.96 มิลลิกรัม/มิลลิลิตร ผลการวิเคราะห์ฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นด้วยวิธี Ferric reducing antioxidant power (FRAP) assay มีค่า FRAP เทียบเท่า เฟอรัสซัลเฟต 6 ไมโครโมล/มิลลิลิตร ที่ความเข้มข้น 25 มิลลิกรัม/มิลลิลิตร วิตามินซี ซึ่งใช้เป็นสารควบคุมบวกแสดงค่า FRAP เทียบเท่า เฟอรัสซัลเฟต 3 ไมโครโมล/มิลลิลิตร ที่ความเข้มข้น 0.031 มิลลิกรัม/มิลลิลิตร การศึกษาฤทธ์ต้านไนตริกออกไซด์พบว่ามีค่า EC_{50} เท่ากับ 2.32 มิลลิกรัม/มิลลิลิตร เมื่อเทียบกับสาร เควอซิตินซึ่งมีค่า EC_{50} เท่ากับ 0.29 มิลลิกรัม/มิลลิลิตร การทดสอบ ฤทธิ์ต้านออกซิเดชัน โดยการวัดสีของเบต้าแคโรทีน พบว่าสารสกัดเอธานอล ของแส้ม้าฮ่อ และสารมาตรฐานเควอซิติน ยับยั้งออกซิเดชันได้เท่ากับ ร้อยละ 4 และ 74 ตามลำดับ ไม่พบความเป็นพิษทั้งในการศึกษาความเป็นพิษต่อไรทะเล และฤทธิ์ที่ทำให้เซลล์ไวต่อแสง การศึกษาฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส พบค่า EC_{50} เท่ากับ 15.15 มิลลิกรัม/มิลลิลิตร เมื่อเทียบกับสารมาตรฐานกลูตาไทโอนซึ่งมีค่า EC_{50} เท่ากับ 0.02 มิลลิกรัม/มิลลิลิตรและ ฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสโดยมีค่า EC_{50} เท่ากับ 6.13 มิลลิกรัม/มิลลิลิตร เมื่อเทียบกับสารมาตรฐาน ดีออกซีนอจิริไมซิน โดยมีค่า EC_{50} เท่ากับ 0.0124 มิลลิกรัม/มิลลิลิตร การศึกษาในหนูทดลองที่ได้รับยาเพนโทบาบิโทน พบว่าแส้ม้าฮ่อมีฤทธิ์เพิ่มการนอนหลับของหนู โดยฤทธิ์ที่สัมพันธ์กับขนาดของสารสกัดที่ได้รับ ผลการศึกษาฤทธิ์ทางชีวภาพของแส้ม้าฮ่อแสดงให้เห็นว่าพืชชนิดนี้มีศักยภาพในการต้านออกซิเดชัน ยับยั้งเอนไซม์ไทโรซิเนส และเอนไซม์แอลฟากลูโคซิเดสในหลอดทดลอง รวมทั้งมีศักยภาพในการเพิ่มการนอนหลับในหนูทดลอง

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต.....

ปีการศึกษา 2556 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5578956853: MAJOR PUBLIC HEALTH SCIENCES

KEYWORD: ANTIOXIDANT/ ANTIMICROBIAL/ TOXICITY/ ENZYME INHIBITION/HYPNOTIC TEST

WATCHARA DAMJUTI: *IN VITRO AND IN VIVO*BIOACTIVITIES OF *LEPTOCARPUS DISJUNCTUS* ETHANOLIC EXTRACT. ADVISOR: CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 86 pp.

Leptocarpus disjunctus Mast. is edible plant in the Southern region of Thailand. Although there is the indigenous warning about its side effects for dizziness and intoxicated symptoms. It has been eaten as side dish of the local food for a long time. This study aimed to investigate the secondary metabolites and biological activities of *L. disjunctus*. Determination of the secondary metabolites presented coumarin glycoside, tannin, steroid and sugar. Whole fresh plant was extracted with ethanol and performed *in vitro* and *in vivo* tests. This study found that *L. disjunctus* ethanolic extract had no antimicrobial property against*Micrococcus luteus, Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli, Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhimurium*,*Shigella* sp., *Candida albicans* and *Saccharomyces cerevisiae*.Furthermore the ethanolic extract showed antioxidant propertieson various types of radicals. DPPH radical scavenging assay showed radical scavenging activity with EC_{50} of 0.8 mg/ml and positive control (BHT) showed EC_{50} of 5.96 mg/ml. Ferric reducing antioxidant power (FRAP) assay indicated that the ethanolic extract at the concentration of 25 mg/ml had a reducing power value equivalent to ferrous sulphate 6μmol/ml and ascorbic acid at the concentration of 0.031 mg/ml had a reducing power value equivalent to ferrous sulphate 3 μmol/ml. Nitric oxide radical inhibitory assay showed scavenging activity with EC_{50} of 2.32 mg/ml and quercetin activity was more pronounced with EC_{50} of 0.29 mg/ml. Beta-carotene bleaching assay indicated that the ethanolic extract presented antioxidant activity only 4% compared to the positive control (BHT) which showed antioxidant capacity of 74%. Brine shrimp lethality and phototoxic activity tests indicated thatthe ethanolic extract of *L. disjunctus* had no cytotoxicity and genotoxicity against brine shrimp (LD_{50}>500 mg/ml) and microorganisms respectively. Tyrosinase inhibitory assay indicated *L. disjunctus* was able to inhibit the enzyme with EC_{50} of 15.15 mg/ml while positive control (glutathione) had EC_{50} of 0.02 mg/ml. Alpha-glucosidase inhibitory assay indicated that ethanolic extract was able to inhibit enzyme with the EC_{50} of 6.13 mg/ml.The positive control (1-deoxynojirimycin) had EC_{50} of 0.0124 mg/ml. Furthermore, the ethanolic extracts of *L. disjunctus* whole fresh plant exhibited dose relationship of hypnotic effect in pentobarbitone induced sleeping time in mice. This study revealed the bioactive potentials of *L. disjunctus*for antioxidation, anti tyrosinase, anti glucosidase and hypnotic agents.

Field of study Public Health Science Student's signature.....

Acedemic Year 2013 Advisor's signature.....

Co-advisor's signature.....

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LIST OF ABBREVATIONS

ANOVA	=	Analysis of variance
ATCC	=	American type culture collection
BCB	=	β -carotene Bleaching
BHT	=	Butylated hydroxyl toluene
CFU/ml	=	Colony-forming units per milliliter
cm	=	Centimeter
°C	=	Degree Celsius
CNS	=	Central nervous system
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyridoneucleic acid
DOPA	=	L-3,4-dihydroxyphenylalanine
DPPH	=	2,2diphenyl-1-picrylhydrazyl
EC_{50}	=	Fifty percent effective concentration
FRAP	=	Ferric reducing antioxidant power
$GABA_A$	=	Gamma aminobutyric acid A
hr	=	Hour

I.P.	=	Intra peritoneally
LD_{50}	=	Fifty percent lethal dose
M	=	Molar
MBC	=	Minimum Bactericidal Concentration
MFC	=	Minimum Fungicidal Concentration
mg/ml	=	Milligram per milliliter
MHA	=	Mueller Hinton agar
MIC	=	Minimum Inhibitory Concentration
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mMol/ml		Millimol per milliliter
N	=	Normality
NA	=	No activity
nm	=	Nanometer
NO	=	Nitric oxide
P.O.	=	Per oral

pH	=	Potential of Hydrogen ion
RONS	=	Reactive oxygen and nitrogen species
ROS	=	Reactive oxygen species
SDA	=	Sabourauddextose agar
SOD	=	Superoxide dismutase
TPTZ	=	Tripyridyl Triazine
U/ml	=	Unit per milliliter
UV	=	Ultraviolet
UVA	=	Ultraviolet A
v/v	=	Volume by volume
W/m^2	=	Watts per square meter
w/v	=	Weight by volume
w/w	=	Weight by weight
µl	=	Microliter
µM	=	Micro molar

CHAPTER I

INTRODUCTION

Background and Significance of the Study

All living organisms were controlled through biochemical processes such as metabolism [1]. It can be said that biochemistry is the basic education, leading to the other study especially study to find the effects of substances in particular natural substances. Thus causing the education, which known as “Pharmacognosy”, the study of the physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources [2]. Thailand has long history of plants as medicine and food. Thailand is a diverse country because it is one of the rich countries in the region recognizes the rich resources [3]. Each region has a different perspective in the eating because of the wisdom, which is passed along from generation to generation. Social development and the state of present society, the rapid consumption of a western diet increases. These lifestyle changes affect the dietary habits of Thais. However, the consumption of native still not much affected. There are some plants that are edible plants that can be found in the region. In southern of Thailand, people continue to consume native plants in a variety of side dishes such as *Ficus fistulosa* Reinw., *Muntingla calabura* Linn., *Clausena cambodiana* Guill., *Coleus parvifolius* Benth. and *Leptocarpus disjunctus* Mast.*L. disjunctus* (Family Restionaceae) is the native plant in the southern of Thailand, which is called in the native language as “SAE” or “SAE-MA-HOR” *L. disjunctus* has been eaten as side dishes of the local food for a long time although there is the indigenous warning about its side effects for dizziness and intoxicate symptoms. However, there has been no scientific evidence for *L. disjunctus* biological activities.

Leptocarpus disjunctus Mast. is the native plant in the southern of Thailand, which called in the native language as "SAE". *L. disjunctus* is biennial-monocotyledonae plant, taper leaves length of about 20-30 cm, which similar as *Allium wakegi* [4] same family as garlic small annual crops. White and purple

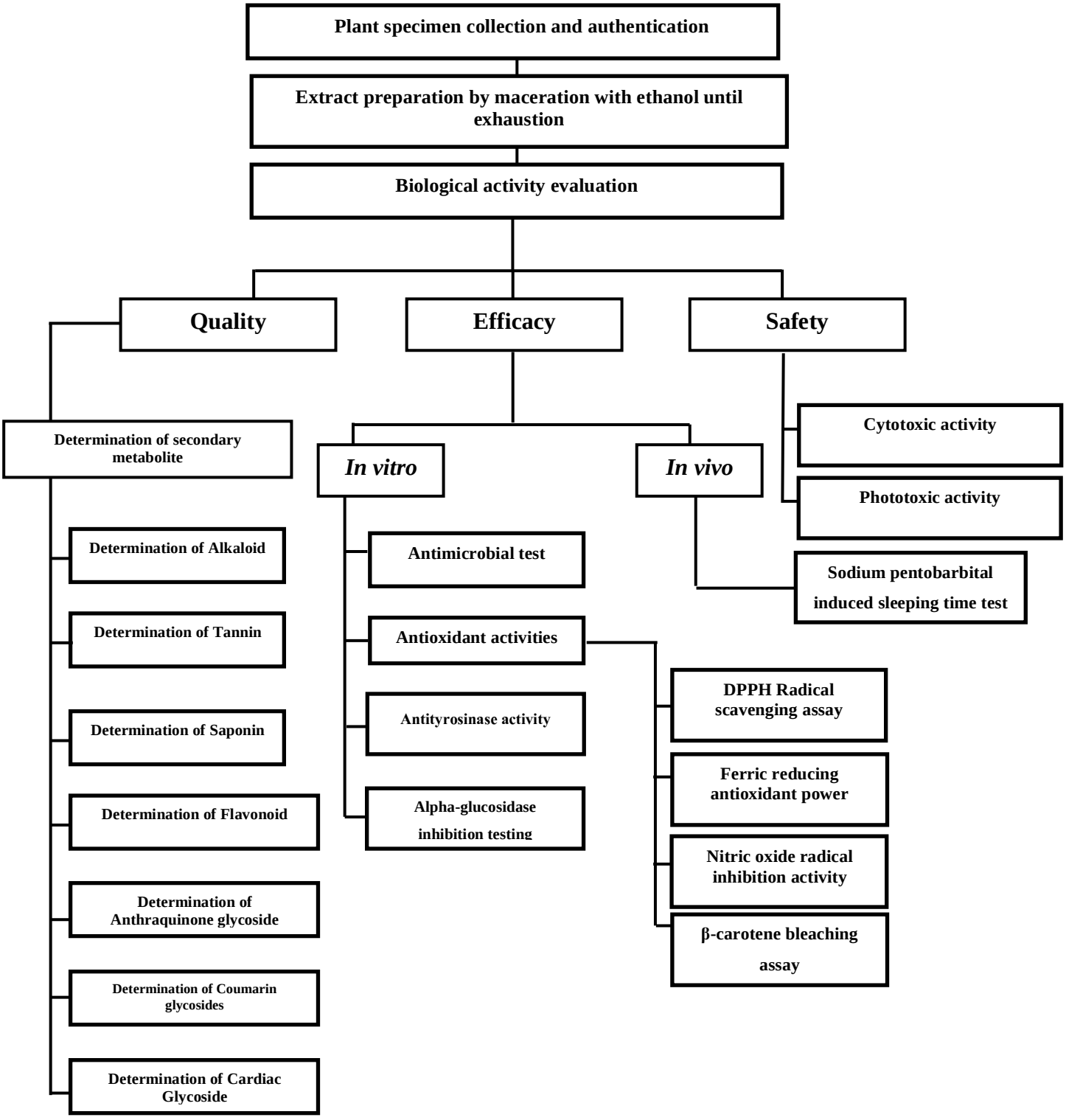
heads are underground for serving the food and there are long hollow leaves. Its bouquet is white with a long peduncle. Inflorescences like an umbrella with many florets. The Restionaceae family is the name of botanical family, which is flowering as the native plants of southern hemisphere and belong to monocotyledons' group, which consist of various similar families instance the rushes and sedges and they have green leaves that have been reduced to sheaths. The Restionaceae likely originated during the Cretaceous period, based on evidence from fossil pollen [5]. The family consists of rhizomatous plants and they belong to a group of monocotyledonae plant that includes superficially similar families. Plants in this family are found in southern Africa Australia and Southeast Asia [6].

The purpose of this study is to investigate the secondary metabolite profile and the bioactivity of ethanolic extract from whole plant of *L. disjunctus*. The efficacy activities consist of antimicrobial activity, DPPH radical scavenging activity, ferric reducing antioxidant power, nitric oxide radical inhibition activity, β-carotene bleaching activity, antityrosinase activity and alpha glucosidase inhibition testing. The safety evaluates with brine shrimp lethality activity and photoxic activity and hypnotic activity in experimental animals by using pentobarbital induced sleeping time.

Objectives of the Study

1. To evaluate selected *in vitro* biological activities of ethanolic extract of *Leptocarpus disjunctus* whole plants.
2. To evaluate hypnotic activity of ethanolic extract of *Leptocarpus disjunctus* whole plants by pentobarbital induced sleeping time assay in mice.
3. To screen the secondary metabolites of ethanolic extract of *Leptocarpus disjunctus* whole plants by color test.

Conceptual framework



CHAPTER II

LITERTURE REVIEWS

Taxonomy

Kingdom: Plantae

Division: Tracheobionta

Class: Magnoliophyta

Order: Liliopsida

Family: Restionaceae

Genus: Leptocarpus

Species: *Leptocarpus disjunctus*

Family Restionaceae

Restionaceae family is called restiads or restios. There are 30 genera, 550 species in this family, almost are perennial and evergreen plants. This family consists of rhizomatous and herbaceous plants. They were 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 spikelets, which in turn make up the inflorescences [5].

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 solitary in each loculus, pendulous. Fruit a loculicidal capsule, 2- or 3-angled or 1-locular, or a small nut [6]

Distribution It is native to the southern hemisphere with main center of diversity in southern Africa and southwestern Australia, also in eastern Australia, New Zealand, Malaysia and Chile, 20 genera, 130 species in Australia, 1 specie in China and Thailand [7].

***Leptocarpus disjunctus* Mast.**

Leptocarpus disjunctus Mast. is the native plant in the southern of Thailand, which call 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, which similar as *Allium wakegi* [4] same family as garlic small annual crops. 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 as shown in Figure 1.

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 × about 1 mm; perianth 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 about 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, about 1 mm. Seeds about 0.5 mm. [8]

Figure 1. *Leptocarpus disjunctus*

Secondary Metabolites

Secondary metabolites are groups of chemicals produced by plants for interactions with other plants and other living organisms. Secondary metabolites such as color or aroma compounds involve in pollination by insect. Various toxins from plants always show as the clue of plant defense against herbivore. These compounds from plants are known as natural products, which exhibit pharmacological activities on human health [9].

Glycosides

Glycosides are molecules in which a sugar group is bonded through their anomeric carbon to non-sugar molecule. Type of glycosidic bonds, which can link glycosides are o-glycoside, glycosylamine, thioglycoside, or c-glycoside. The sugar group is called glycone and the non-sugar group is aglycone or genin.

Classification of Glycosides [10]

Glycosides are classified by structure type of aglycone.

Anthraquinone Glycosides

Anthraquinones are quinone derivative compounds found in several types such as free form, glycoside form and reduced form such as oxanthones and anthrannols. Anthraquinone glycosides contain derivatives of anthraquinone as aglycone or genin group. They are mainly found in various dicot plants and have a laxative effect. Anthraquinones have the simple structure containing 3-ring anthracene system and are red to orange or yellow to brown color. They can be soluble in alkaline solution and nonpolar organic solvents such as ether, benzene and chloroform. In natural, anthraquinones are often found with glucose, primeverose and small number of rhamnose glycone [11].

Coumarin Glycosides

Coumarin glycosides or lactones contain the coumaric acid as aglycone or genin group. Coumarin glycosides are shikimate-derived metabolites. An example is apterin, which is reported to dilate the coronary arteries as well as block calcium channels. Coumarin glycosides contain o-hydroxycinnamic acid (o-coumaric acid) as aglycone group. They can be found in Graminae, Orchidaceae, Leguminoceae, Umbelliferae, Rutaceae and Labiatae [12, 13].

Cardiac Glycosides

Cardiac glycosides contain a steroidal nucleus as aglycone part. These glycosides are found in the plant genera Digitalis and Strophanthus [14]. They act on the myocardial muscle and exhibit both beneficial and toxic activities on the heart. Cardiac glycosides can be used as poison and heart medication. It has been used for healing heart diseases because cardiac glycosides are mainly in the treatment of heart failure due to their antiarrhythmic effects. These effects are caused by the ability to increase cardiac output by increasing the contraction by prolonging the plateau phase of cardiac depolarization, thus slowing ventricular contraction and allowing more time for ventricular filling [15].

Type of Cardiac Glycoside [11]

Cardiac glycosides can be classified into 2 groups using kind of unsaturated lactone ring at seventeenth carbon atom as criterion.

Cardenoline type: When lactone ring at seventeenth carbon atom is 5 membered unsaturated lactone ring such as digitoxigenin aglycone.

Bufadenolide type: When lactone ring at seventeenth carbon atom is 6 membered unsaturated lactone ring such as scillarenin aglycone.

The sugar that found in cardiac glycoside can be hexose sugar such as glucose or pentose sugar such as rhamnose and it can be monosaccharide, disaccharide, trisaccharide and tetrasaccharide. In addition, deoxy sugars such as antirose (deoxyglucose), digitalose (methyl ether of antiarose), digitoxose (deoxyhexoses) and cymarose (methyl ether of digitoxose) can also be found.

Flavonoid Glycosides

Flavonoid glycosides or flavonoids can be found as pigment in any parts of plants especially flowers. These compounds are water soluble polyphenolic molecules with promising antioxidation activity. Flavonoid glycosides are usually found in cell sap of flower, fruit, soluble pigments in leaf and aglycone usually found in pulp.

Types of Flavonoid Glycosides

Flavonoid glycosides can be divided in to 9 groups according to their structures.

Flavones, Isoflavones and Flavonols are the yellow pigment that always found in Restionaceae such as luteolin, one of the most common flavones; gossypetin, one of the flavone that give anxiolytic effect; glossypitin, found in *Restio tetraphyllus*; quercetin was found in one species, *Restio tetraphyllus*; and hypolaetin was found in *Hypolaena* species [16] as shown in Figure 2.

Flavonones are pale yellow substance rarely found in plant such as hesperidine and naringin.

Flavononols are colorless substance rarely found in plant.

Leucoanthocyanins are colorless substance rarely found in glycoside form. Leucoanthocyanin based on cyanidin were found specifically in the three *Restio* species, *R. pallens , R. tenuiculmis* and *R. tetraphyllus* [17].

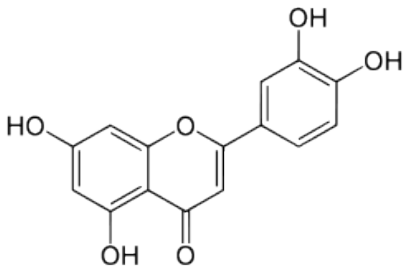
Anthocyanins are the pink to purple pigment that found in petal, leaf and fruit.

Catechins are colorless substance always found in general plant especially in woody plant. Catechin is the substrate of condense tannin.

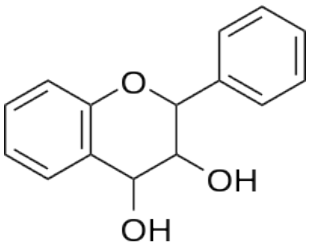
Chalcones and dihydrochalcones are deep yellow pigment that found in plant.

Aurones are the yellow pigment that found in free and glycoside form and have 2-benzylidene coumaranone structures.

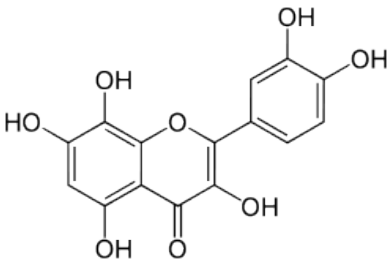
Xanthones are the yellow pigment, which have derivative structure of benzophenone.



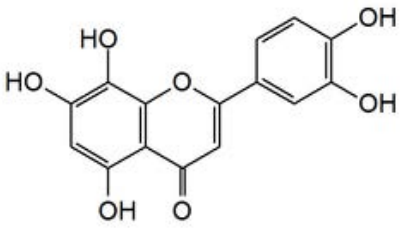
Luteolin



Leucoanthocyanin



Gossypetin



Hypolaetin

Figure 2. Flavonoid aglycone found in Restionaceae family

Saponins

Saponins are secondary metabolites that found in natural sources. Saponins can be found in various plant species. They are amphipathic glycosides containing one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative [19, 20] and perform the foaming like soap when shaken in aqueous solutions [18]. Saponins have sapogenin as aglycone and almost are steroids or triterpenoids [21]. This part will combine with the third carbon atom, O-glycoside. Most of the sugar that found in this glycoside is oligosaccharide were 1-5 unit.

Types of Saponins

Steroidal Saponins

Mostly steroidal saponins have spiroketal steroid nucleus ring structure. This saponin can be found a few in natural and it can be found in monocoteleadonae plant [22]. Almost steroidal saponins in natural are glycoside form.

Triterpenoid Saponins [10]

Mostly triterpenoid saponins are pentacyclic rings structure and in natural they can be found in glycoside and free sapogenin form. They are always found in dicoteledonae plant.

Tannins

Tannins are biomolecule found in bitter plant. Tannins are widely applied to large any polyphenol compound containing sufficient hydroxyls groups capable to bind and precipitate proteins and various other organic compounds.

Tannin compounds are widely found in various plants species and play a role in protection from predation and in plant growth regulation [23].

Types of Tannins [10]

Tannins can be classified into two large types: true tannins and pseudo tannins.

True tannins can be classified into two groups. Hydrolysable tannins or pyrogallol tannins occurred by polyhydric compounds such as gallic acid or ellagic acid bonding to a large molecule. These are substances that can be hydrolysed by acid or enzyme tannase. These substances are amorphous and colloidal dispersions. Condensed tannins or phobatannins are the polymer of phenolic compounds, which have associated structures with flavonoid and catechins and flavan-3,4,diols. These substances are not digested but when hydrolysed with acid or enzyme tannase, they be broken up to water insoluble red substance.

Pseudo tannins are substances that have lighter molecular than true tannins and they always show similar property to true tannin such as gallic acid, catechin and chlorogenic acid.

Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly nitrogen atoms. This group also includes some related compounds with neutral [24] and even weakly acidic properties [25]. In addition to hydrogen, carbon and nitrogen, alkaloids may also contain oxygen and other elements such as phosphorus.

Alkaloids can be purified from extracts by acid-base extraction. Their pharmacological effects are always used for medications and can be found as recreational drugs. Alkaloids act on a diversity of metabolic systems in humans. They almost have a bitter taste [21].

The boundary between alkaloids and other nitrogen-containing natural compounds is not clear-cut [26]. Compounds like amino acid, peptides, proteins and nucleotide are usually not called alkaloids. Natural compounds containing nitrogen in the exocyclic position are usually accredited to amines rather than alkaloids [27].

Determination of Secondary Metabolites

Glycoside Examination [28]

Anthraquinone Glycosides

Anthraquinone-o-glycosides are hydrolysed in acidic solution to free anthraquinone aglycone, which can be extracted in nonpolar organic solvent. When back extraction in diluted alkali solution, anthraquinone color of red can be observed.

Coumarin Glycosides

Coumarin glycosides can be examined by heating the compound and coumarin glycoside can sublimate and the vapour adsorbed on alkali filtered paper. Under alkali condition, coumarins turn to cis-cinnamic acid, which change to trans-cinnamin acid that exposed to ultraviolet at 365 nm and give fluorescent green color.

Cardiac Glycosides

There are no reagents that can specify cardiac glycoside then it needs to detect cardiac glycoside by chemical structure detection. The examination starts with the extraction using alcohol then remove interfere foreign substances by adding lead hydroxide to precipitate material before testing as the followings.

Steroid Nucleus Examination: Liebermann-Burchard test consists of acetic anhydride and sulphuric acid, which give blue or purple color but some steroid can give positive as yellow color.

Unsaturated Lactone Ring Examination: Kedde reagent consists of 3,5-dinitrodenzoic acid and alkali, which give purple color or use Raymond reagent, which consists of meta-dinitrobenzene and alkali, which give blue color because nitro group reagent can interact with methylene of unsaturated lactone and give color substance.

Deoxy Sugar Examination: Keller-Kiliani reagent consists of ferric chloride, sulphuric acid, glacial acetic acid, which gives purple color at boundaries between sulphuric acid and testing substance.

Flavonoids

Cyanidine reaction is the reduction reaction for detection of 2-phenylbenzopyrone in molecule. It can be operated by adding magnesium ribbon or zinc dust in the alcoholic solution of sample combined with concentrate hydrochloric acid. 2-Phenylbenzopyrone in each molecule give different color such as flavone will give orange to red color, flavonol will give red to crimson color and flavonone will give crimson to magenta color.

Saponins

When shake saponin with the water, it will be occurred honeycomb froth. This froth can be stable about thirty minutes. However the froth can be occurred by protein and acid in some plants. Therefore, the trial is required to achieve a drop of alkaline solution reacts with the acid of plant. The salt will occur, which does not cause foaming when shaken. Saponins can be boiled with acid for hydrolysis reaction and free aglycone, which does not foaming when shaken.

Tannins

Tannins can be found in free or glycoside form. The detection can be done using ferric chloride as reagent that gives blue green or dark green color.

Alkaloids Examination [11]

The basic principle of alkaloids examination is that all alkaloids can precipitate, turbid or present color when react with some testing solution. Chemical reaction's mechanism of alkaloid and testing solution depend on the chemical nature of the testing solution. The mechanism of the reaction can be classified as follows.

Wagner's reagent or iodine-potassium iodide reagent containing iodine and potassium iodide and gives brown sediment of loose complex compound when interact with alkaloid.

Mayer's reagent or potassium mercuric iodide reagent, which contain mercuric chloride and potassium iodide, gives white sediment when interact with all alkaloids.

Marme’s reagent or potassium cadmium iodine, which contains potassium iodine and cadmium iodide, it gives white or yellow sediment as the result when interact with alkaloid.

Dragendorff’s reagent contains bismuth nitrate in nitric acid and potassium iodide. When interact with alkaloid, it will gives orange to red sediment.

Hager’s reagent, which contains picric acid in water, give yellow sediment when react with alkaloid.

Oxidants (Free radicals)

Free radicals are unpaired electron atoms or molecule that are very unstable and reactive. Therefore they have to bond with other molecules and are responsible for aging, tissue damage, and possibly some diseases. Free radicals are found in biological systems, which are often associated with oxygen and other substances. They are often referred to reactive oxygen and nitrogen species (RONS) [30].

Free Radicals in Oxygen Atom’s Group [31]

Superoxide group: Superoxide free radical group is the primary reactive oxygen species (ROS) that occurred in electron transfer of cellular system [32]. Superoxide is the oxygen molecule that receive single oxygen atom ($O_2^{\bullet}$) and also has ability to destroy itself. It can decompose to be secondary ROS such as hydrogen peroxide. Superoxide reacts with hydrogen peroxide to be hydroxyl radical [33]. Superoxide is the first free radical that produced when oxygen molecule receive single electron. Superoxide radical will be produced when hydrogen peroxide receives ultraviolet ray or oxidation reaction of flavin polyphenol and quinone as shown in Figure 3.

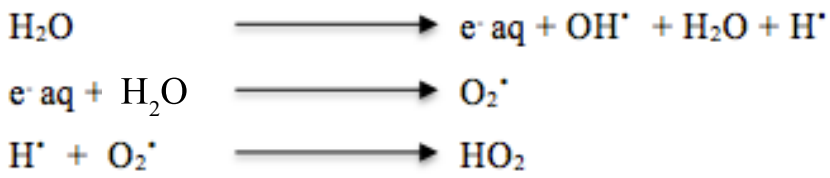


Figure 3. Superoxide free radical reaction

Hydroxyl group: Hydroxyl radical is the most aggressive radical because it structure consists of oxygen and hydrogen atom (OH•). Hydroxyl radical has the ability to pull electrons from other molecules. This radical can cause lipid encapsulated cells, enzyme, vitamin c and doxyriboneucleic acid damage. In living organism, hydroxyl radical is generated from disintegration of hydrogen peroxide by heat or radiation. Hydroxyl radical can react with organic compound (RH) such as nucleic acid, lipid, carbohydrate and protein.

Hydrogen peroxide group: In living organism, occurring peroxide will be created as hydrogen peroxide, H_2O_2. Normally hydrogen peroxide in organism is the active and stable substance. It can be transferred through the cell membrane from outside to destroy molecule inside the cells. Hydrogen peroxide with low concentrations do not cause cell death but when combines with superoxide or heavy metal, it will cause free radical. Among all radicals, hydrogen peroxide is the only one stable compound that can be measured easily. However hydrogen peroxide is not active when prepared *in vitro.* Anaerobic organism has mechanism to destroy hydrogen peroxide over aerobic cells by using catalase and peroxidase enzyme.

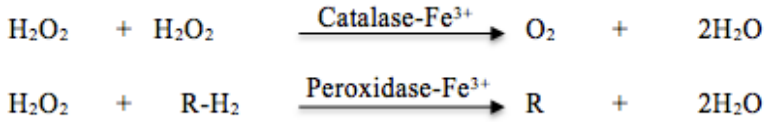


Figure 4. Hydrogen peroxide deactivation by catalase and peroxidase enzyme

Peroxide group: Peroxide is formed in human and animal organisms as a short-lived product in biochemical processes and is toxic to cells. The toxicity is due to oxidation of proteins, membrane lipids and DNA by the peroxide ions. The class of biological enzymes called SOD (superoxide dismutase) is developed in nearly all-living cells as an important antioxidant agent. They promote the disproportionation of superoxide into oxygen and hydrogen peroxide, which is then rapidly decomposed by the enzyme catalase to oxygen and water.

Peroxyl group: Peroxyl radical ($ROO^{\bullet}$) is produced from living organism tissue, which is the result of the production of hydroxyl radicals ($OH^{\bullet}$). There is evidence both direct and indirect indicate that hydroxyl radical can initiate lipid peroxidation reaction resulting hydroperoxyl or perhydroxyl radicals ($HOO^{\bullet}$) [35].

Hypochlorus acid: Hypochlorus acid (HOCl) is produced in autoimmune system for destroy bacteria and virus. It occurs by integration of hydrogen peroxide and chloride, which affect on destruction of protein, amino and deoxyriboneucleic acid.

Free Radicals in Nitrogen Atom's Group

Free radical in nitrogen atom's group such as nitric oxide (NO) consist of antibonding electron in orbital. Nitric oxide is found in both atmosphere and living organism and serves as neurotransmitter or vasodilator, protection of cell adhesion and host defense from parasite, officiating in immunosuppression and killing cancer cells [34].

Nitric oxide synthase is the related enzyme in producing nitric oxide. In macrophage, nitric oxide producing is for destroying bacteria, virus and cancer cell. Moreover nitric oxide synthase that produced in neuron, endothelial cell and platelet cell serves as a producing nitric oxide agent to be vasodilator and neurotransmission. Apart from the benefits mentioned above, nitric oxide radical still has disadvantage, it is free radical that has oxidative stress and destroys antioxidant system in body.

Antioxidants

Antioxidants are a group of natural elements that can be found in the food or through supplementation. Antioxidants help protect the body from the formation of free radicals. Free radicals cause damage to the cells that lead to poor functioning of the immune system. This then opens the door for infection and degenerative disease to attack the body. Because free radicals so effectively join with other compounds, they can cause great damage very rapidly. Sometimes the damage can be irreversible [35, 36].

Antioxidants are the compounds that can destroy oxidants or free radicals. Important characteristics of antioxidant agents are the ability to donate electron to oxidant or reactive oxygen substances and inhibit oxidative stress reaction [37].

Antioxidant agents serve as protection agent for biological substances in living organism such as genetic material and regulatory enzymes or cell's structure protection of organelles or epithelial cell. Furthermore antioxidant agents have ability to destroy free radical, which occurred by acting at various points of the free radicals producing [38]. Biomolecular substance can act as antioxidant agent in living organism such as albumin, glucose, carnosine, taurine, uric acid, estrogen, dihydrolipoic acid, polyamine etc. Furthermore antioxidant can be from natural sources or secondary metabolites such as flavonoid and polyphenol.

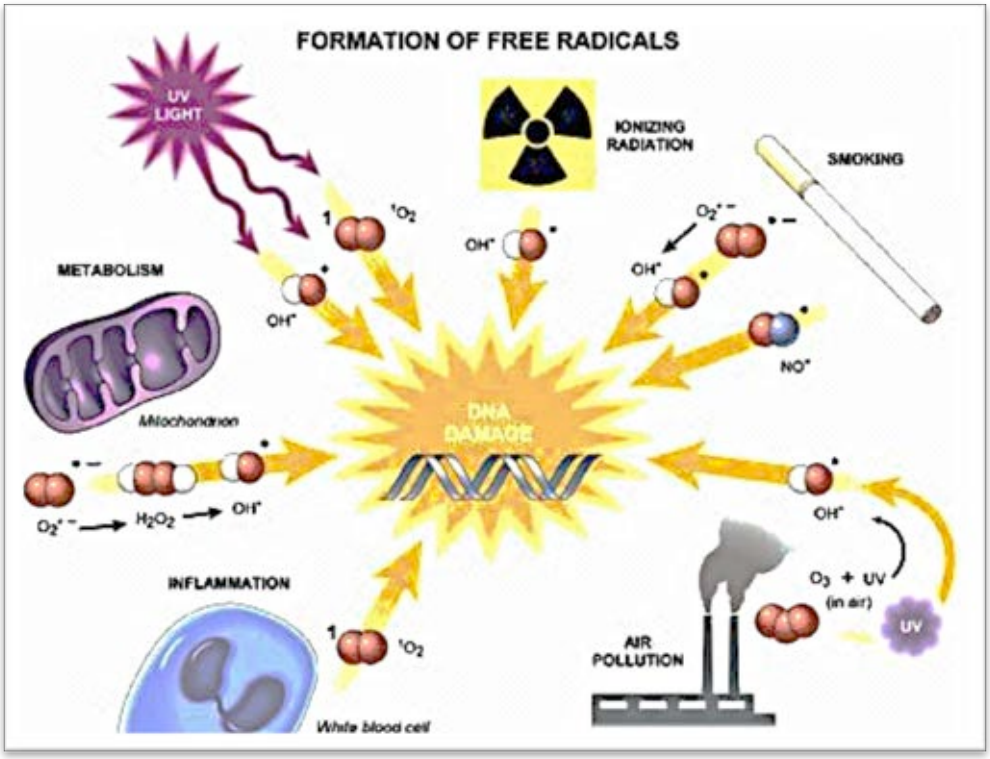


Figure 5. The formation of free radicals

Antioxidation Activity Testing

DPPH Radical Scavenging Assay

A simple method, which has been developed to determine the antioxidant activity of foods, utilizes the stable 2,2diphenyl-1-picrylhydrazyl (DPPH) radical. The delocalization of the spare electron within DPPH molecule causes the stability of this free radical and gives characteristics of deep violet color with a maximum absorbance of 517 nm. The color turns from purple to yellow when receives hydrogen atom from antioxidant as shown in Figure 6 [39].

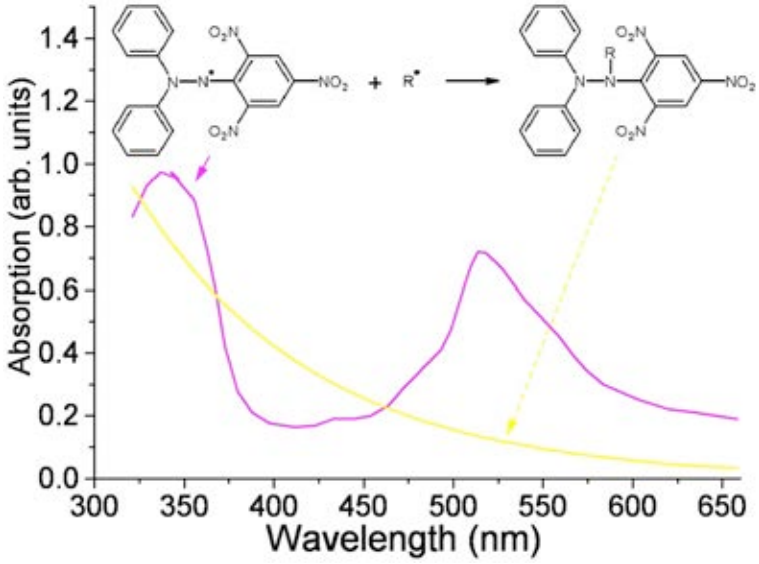


Figure 6. DPPH and antioxidant reaction

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power is an assay often used to measure the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols. This method uses ferric tripyridyl triazine, complex compound of iron as the tested compounds. Iron atom in this compound is reduced by the antioxidant and produces a complex compound of ferrous Fe^{2+}- TPTZ that have the blue color with the maximum absorbance at a wavelength of 593 nm as shown in Figure 7 [40].

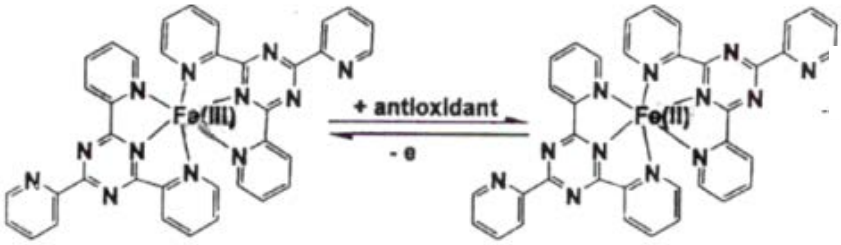


Figure 7. The reaction between ferric to ferrous ion

β -carotene Bleaching (BCB) Test

The β-carotene bleaching assay is the determination of the antioxidant ability to delay the bleaching of β-carotene in a water or linoleic acid emulsion. Bleaching is based on the loss of the yellow color of β-carotene due to its reaction with radicals, which are formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants [41, 42] as shown in Figure 8.

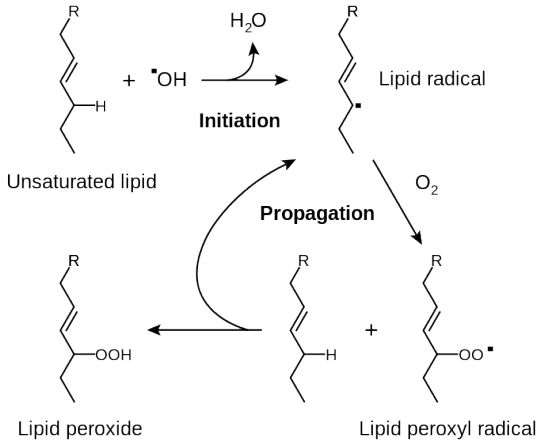


Figure 8. The free radical mechanism of lipid peroxidation

Nitric Oxide Radical Inhibition Activity

Nitric oxide, also known as nitrogen monoxide, is a molecule with chemical formula NO. It is a free radical. The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduce production of nitrite ions as shown in Figure 9. The absorbance was measured by observing blue color with spectrophotometry at wavelength of 540 nm. [42, 43].

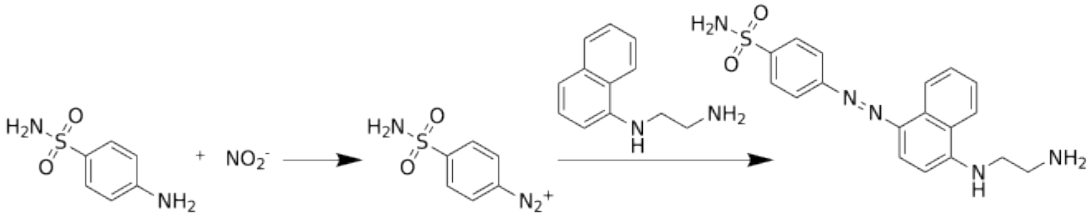


Figure 9. The Griess test reaction

Enzyme Inhibition Tests

Antityrosinase Activity

Tyrosinase is an important enzyme in the synthesis of the pigment, melanin. Tyrosinase is the first enzyme, which stimulates more melanin, thereby inhibiting the Tyrosinase enzyme can reduce the formation of melanin pigment. Melanin can be divided into two types, pheomelanin and eumelanin. The disorder causes excessive melanin production can be the cause of freckles or dark spots and skin cancer. In the creating process of two types of melanin, tyrosine is used as a precursor in the synthesis of melanin as shown in Figure 10.

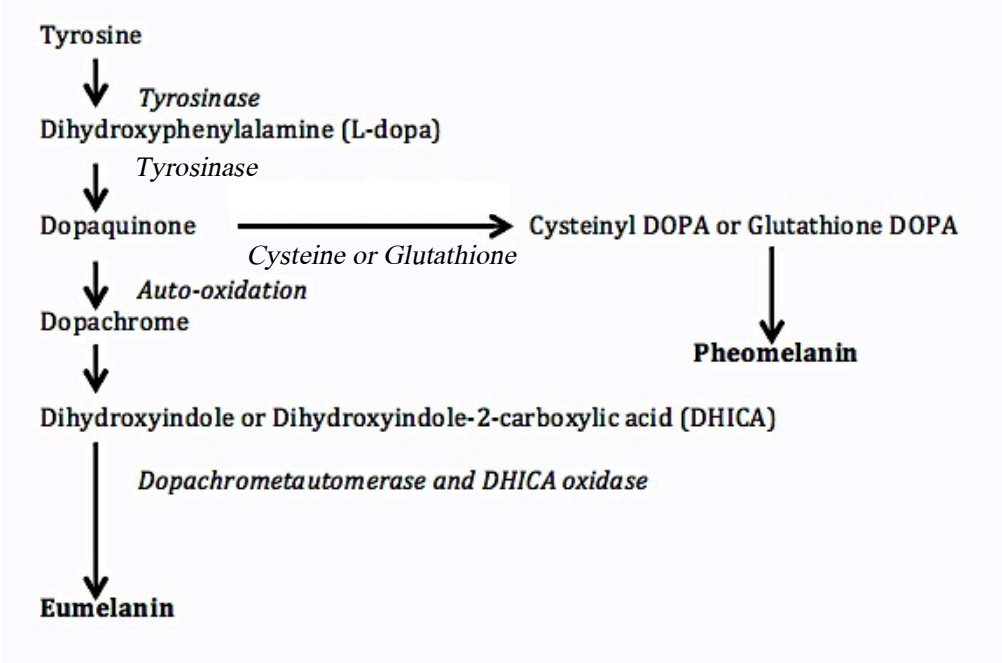


Figure 10. The biosynthesis of melanin

There are natural effectively substances affect on the inhibition of tyrosinase such as tranexamic acid, kojic acid, dipalmitate, alpha-arbutin, ascorbic acid and ascorbyl glucoside. *In vitro* assay of tyrosinase activity can use tyrosine or dihydroxyphenylalamine (L-dopa) as substrate of tyrosinase enzyme. Dopaquinone can chemically change to dopachrome, which can be quantitated by spectrophotometry at 475 nm.

Alpha-glucosidase Inhibition Testing

Hyperglycemia (high blood glucose) happens when the body has too little insulin or when the body can't use insulin properly [46]. Postprandial hyperglycemia occurs after meal digestion. Starch is digested by salivary and pancreatic α-amylase to form oligosaccharide-dextrins. The α-glucosidases are two complex enzymes in small intestine that cleave dextrins to absorbable glucose. The synthetic or natural α-glucosidase inhibitors are interested as therapeutics to delay postprandial hyperglycemia [45].

Antimicrobial Test

Antimicrobial test is the experiment for study about the substance, which can kill or inhibits the microorganism such as bacteria and fungi [46].

Agar Diffusion Testing

The Agar diffusion testing (zone of inhibition test or the Kirby Bauer disk diffusion test) is the method, which measures the effect between an antimicrobial agent and microorganisms that grow in culture. A known quantity of bacteria will grow on specific agar plates. The bacteria will be swabbed uniformly or using two-agar technique across a culture plate that fills with specific agar to the type of microorganism. A filter-paper disk will be impregnated and placed on the surface of the agar then the compound will diffuses from the filter paper into the agar or the substance will be dropped into the hole that pierce by using sterile cork borer. If the compound is effective against microorganisms at a certain concentration, there are no colonies of microorganisms grow around the hole (zone of inhibition) or the filter-paper disk. The zone of inhibition is a measurement of the compound that effectiveness through the size.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of the test substance that capable to inhibit microorganism growth. MIC can be determined by agar dilution method, broth dilution method and broth micro dilution method.

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

MBC is the lowest concentration of the test substance that capable to kill a particular bacterium. MFC is the lowest concentration of the test substance that capable to kill a particular fungus. MBC and MFC can be determined by inoculating the broth from MIC test to agar media and observed the growth of bacteria or fungi.

Toxicity Tests

Brine Shrimp Lethality Method

Brine shrimp (*Artemia salina*) is indispensable organisms in ecosystem. *A. salina* is a aquatic species. The anatomy of brine shrimp can be divided into 3 parts: head, middle (thorax) and tail (abdomen). Brine shrimps move on its back, upside down with their leafy-legs upper most [47] and always uses for toxicological activity test of the natural products because it is a sensitive indicator species [48, 49].

The cytotoxic activity of medicinal plant extracts can be screened by extract concentration and brine shrimp lethality relationship. In various studies, brine shrimp lethality assay has been an authentic assay to estimate toxicity assay [50, 51].

Figure 11. *Artemia salina*

Phototoxic Activity Test

In human, exposure with the potent photosensitizing agents can increase sensitivity to sunlight especially UVA wavelength (>315 nm) which causes phototoxic dermatitis of variable intensity. Photosensitivity reaction of the human skin after contact with photosensitizing plants is well known as phytophotodermatitis. It is a classical example of phototoxic reaction which is defined as imflammatory skin reaction caused by exposure to sunlight and contact with some plants containing furocoumarins, frequently the psoralen. Phototoxic reactions resemble hyperpigmentation or sunburn and might also present with irritant, urticarial and allergic, as well as erythema, oedema, blistering and sometime vesiculation. Phototoxicity assay using microorganisms are basically similar to those used for testing antimicrobial activity and further coupled with UV 360 nm irradiation. So this technique is able to quickly screen the possibly phototoxic compounds in plant extracts from inhibition zone [63].

Hypnotic Activity

Pentobarbital Induced Sleeping Time Assay

Pentobarbital is a compound in a group of barbiturates, which are effective in the short and long hypnotic effects by binding to the $GABA_A$ receptor at the wall of neurons in the brain. Pentobarbital causes the chloride channel to open up longer and get the very severe chloride ion to cells, causing hyper polarization. As a result, the nervous system in the brain is pressed (CNS depression). Sodium pentobarbital is used as an anesthetic in veterinary medicine and also used in combination with complementary agents in animal euthanasia injectable solutions commercial grade [56]. Hypnotic agents can prolong sodium pentobarbital induced sleeping time, which is observed the mice for the latency period (time between sodium pentobarbital administration and onset of sleep determined by losing of righting reflex) and duration of sleep (time between the loss and recovery of righting reflex as shown in Figure 12) [52-54] .

Figure 12. The loss of righting reflex

CHAPTER III

MATERIAL AND METHODOLOGY

Chemicals

1. 1-Deoxynojirimycin (Sigma-Aldrich, USA)
2. 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St. Louis, U.S.A.)
3. 2,4,6-Tripyridyl-s-Triazine (Sigma-Aldrich, St. Louis, U.S.A.)
4. 3,5-Di-*tert*-4-butylhydroxytoluene (Sigma-Aldrich, St. Louis, U.S.A.)
5. Amikacin sulfate (T.P. Drug Laboratories (1969) co., LTD, Thailand)
6. Ampicillin sodium (T.P. Drug Laboratories (1969) co., LTD, Thailand)
7. Ascorbic acid (Sigma-Aldrich, St. Louis, U.S.A.)
8. Benzene (Merck, Germany)
9. Bismuth (III) nitrate (Sigma-Aldrich, St. Louis, U.S.A.)
10. Cadmium iodine (BDH Chemical Ltd, Poole, England)
11. Chloroform (Merck, Germany)
12. Di- Sodium hydrogen orthophospateanhydrous (Ajax Finechem Pty Ltd, Auckland, New zealand)
13. Di-Sodium sulphate anhydrous (Ajax Finechem Pty Ltd, Auckland, New zealand)
14. Dimethyl sulfoxide (Merck, Germany)
15. Ethanol (Merck, Germany)
16. Furrous sulphate (Sigma-Aldrich, St. Louis, U.S.A.)
17. Glacial acetic acid (Merck, Germany)
18. Hydrochloric acid (Merck, Germany)
19. Iodine (Suksapan, Bangkok, Thailand)
20. Iron (III) chloridehexahydrate (Ajax Finechem Pty Ltd, Auckland, New Zealand)
21. L-3,4-dihydroxyphenylalanine (Sigma-Aldrich, St. Louis, U.S.A.)
22. Lead acetate (Sigma-Aldrich, St. Louis, U.S.A.)
23. Linoleic acid (Sigma-Aldrich, St. Louis, U.S.A.)
24. Magnesium metal (Sigma-Aldrich, St. Louis, U.S.A.)

25. Mercuric chloride (Mallinckrodt, INC., St Luis, USA)
26. Muller-Hinton agar (Merck, Germany)
27. Muller-Hinton broth (Merck, Germany)
28. Mushroom tyrosinase (Sigma-Aldrich, St. Louis, U.S.A.)
29. Naphthylethylenediaminedihydrochloride (Sigma-Aldrich, St. Louis, U.S.A.)
30. p-nitrophenyl-α-D-glucopyranosiade (Sigma-Aldrich, St. Louis, U.S.A.)
31. Phosphoric acid (CARLO ERBA, Farmitaliacarloerba, Italy)
32. Polyoxyethylenesorbitanmonooleate (Merck-Schuchardt, Muchen, Germany)
33. Potassium hydroxide (BDH Chemical Ltd, Poole, England)
34. Potassium iodine (BDH Chemical Ltd, Poole, England)
35. Potassium phosphate monobasic (Ajax Finechem Pty Ltd, Auckland, New zealand)
36. Qurcetin (Sigma-Aldrich, St. Louis, U.S.A.)
37. Sabouraud Dextrose Agar (Merck, Germany)
38. Sabouraud Dextrose Broth(Merck, Germany)
39. Sodium acetate (III) hydrate (QReC, Auckland, New Zealand)
40. Sodium carbonate (Ajax Finechem Pty Ltd, Auckland, New Zealand)
41. Sodium chloride (QReC, Auckland, New Zealand)
42. Sodium hydroxide (J.T. Baker Inc., Sweden)
43. Sodium nitroprusside (Sigma-Chemical Co., U.S.A.)
44. Sodium pentobarbital (OVATION Pharmaceuticals, Inc., U.S.A.)
45. Sulphanilamide(Sigma-Aldrich, St. Louis, U.S.A.)
46. α-glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich, U.S.A.)
47. β-carotene (Sigma-Aldrich, St. Louis, U.S.A.)

Materials

1. Cork borer (6 mm.)
2. Filter paper No.4 (Whatman, England)
3. Microtiter plates with 96 wells (Costar, U.S.A.)
4. Cuvette (Barloworld Scientific Ltd., Staffordshrine, United kingdom)

Instruments and Equipments

1. Autoclave (ALP Co., Ltd, Japan)
2. Auto pipette (Biohitproline, Sartorius AG, Germany)
3. Centrifuge (Scientific industries INC., Bohemia, New york, U.S.A.)
4. Digital balance (SI-234, Denver instruments, Bohemia, New york, U.S.A.)
5. Lamina hood (Astec SC 1200 AC, Bioquell UK Ltd., United kingdom)
6. Microplate reader (Asys UVM340, Biochrom Ltd., United kingdom)
7. Microplate reader (Anthos zenith 200 rt, Biochrom Ltd., United kingdom)
8. Rotary evaporation (Buchi R210, Switzerland)
9. Spectrophotometer (Shimadzu-w 1800, Shimadzu Corp., Japan)
10. Ultrasonic sonicator (Analytical Lab Science Co. Ltd., Thailand)
11. Water bath

Materials and Methods

Plant Materials

L. disjunctus was collected from Pattalung province of Thailand. Whole plant was authenticated by Ruangrungsi N. and voucher specimen was deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Whole plants of *L. disjunctus* were air dried by airflow oven at 45 °C for 6 hours before determination of the secondary metabolites.

Determination of Secondary Metabolites

Determination of Anthraquinone Glycoside

Anthraquinone glycoside was tested by Brontrager test. Five grams of dried whole plants were boiled in potassium hydroxide (0.5 N) for 2-3 minutes to hydrolyse glycoside to aglycone form and oxidiseanthrones and anthranols to free anthraquinone form. The hydrolysate was filtered, cooled, acidified by adding glacial acetic acid and extracted with benzene. The anthraquinone was present if benzene layer changed the color from yellow to pink or red.

Determination of Coumarin Glycosides

Two grams of blended, dried whole plants were added to the test tube, added a few drop of pure water, placed the filter paper that was soaked with 10 % sodium hydroxide at the internal side of test tube and closed the test tube with cap. Incubated in water bath for 5 minutes and observed the filtered paper under ultraviolet light. Coumarin glycoside was present if filtered paper glew under ultraviolet light.

Determination of Cardiac Glycoside

Ten grams of blended dried whole plants were shaked with 50 ml of 10 percent warmed lead acetate and filtered. The filtrate was extracted with ethanol. The chloroform extract was dried on sodium sulphate anhydrous and evaporated to dryness. The chloroform extract was tested with the different reagents as follows:

Legal test: small quantity of extract was dissolved in pyridine and afew drop of 2 percent sodium nitroprusside together with a few drop of 20 percent sodium hydroxide, a deep red color that faded to a brownish yellow indicates the presence of cardenoloides.

Liebermann-Burchard test: the steroidal nucleus by small quantity of extract was treated with petroleum ether for washed the color out. Then bring the residue dissolved in chloroform and added few drops of acetic anhydride, added a few drops of concentrate sulfuric acid and observed the cardiac glycoside was present if the color turned to blue green.

Keller-Killianitest: the deoxy sugar detection by small quantity of extract was dissolved in 5 ml of ethanol (95 % v/v), treated with 1 ml of concentrate glacial acetic acid and few drops of 10 percent ferric chloride, rinsed with concentrate sulfuric acid and observed cardiac glycoside was present if the color turned to purple at the joint between sample extract and sulfuric acid.

Determination of Flavonoid

Flavonoid was tested by cyanidin or Shinoda test. Two grams of blended dried whole plants were dissolved in 5 ml of ethanol (95 % v/v) and treated with a few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. If flavonoid was present, the pink crimson or magenta color was developed.

Determination of Saponin

Two grams of blended dried whole plants were taken in a test tube and shaken vigorously with water. If stable characteristic honeycomb like froth was obtained, saponins was present.

Determination of Tannin

Two grams of blended dried whole plants were warmed with water and filtered. Tests were carried out with the filtrate using ferric chloride test. Five percent solution of ferric chloride in 90 % alcohol was prepared before test. A few drops of this solution was added to a little of the above filtrate. Tannin was present if dark green or deep blue color to black was obtained.

Determination of Alkaloid

Five grams of blended dried whole plants were dissolved in 5 percent hydrochloric acid and filtered. The filtrate was treated with the following reagents and the precipitate was observed.

Wagner’s test: positive with brown precipitate

Mayer’s test: positive with white precipitate

Marme’s test: positive with white to yellow precipitate

Dragendorff’s test: positive with orange to red precipitate

Hager’s test: positive with yellow precipitate

Ethanolic Extraction of *L. disjunctus* Whole Plants

Whole fresh plants were blended with 95% ethanol by an electric blender. The blended fresh plants were continuously macerated with 95% ethanol until exhaustion. The ethanol extract was filtered through Whatman number 1 filter paper then evaporated to dryness *in vacuo*. The extract yield was weighed and stored at -20 °C.

Antimicrobial Test

Microorganisms

The pathogenic and non pathogenic microorganisms including five gram positive, six gram negative bacteria and two fungal strains were described in the table 1.

Table 1. Tested microorganisms

Tested microorganism	
Gram positive bacteria	
Non-spore forming bacteria	*Micrococcus luteus* ATCC 9341[3]
	Staphylococcus aureus ATCC 6538P[1]
	Straphylococcus epidermidis (Isolates)[2]
Spore forming bacteria	*Bacillus subtilis* ATCC 6633[1]
	Bacillus cereus ATCC 11778[3]
Gram negative bacteria	
Non-spore forming bacteria	*Escherichia coli* ATCC 25922[1]
	Enterobacter aerogenes ATCC 13048[3]
	Pseudomonas aeruginosa ATCC 9027[1]
	Salmonella typhi (Isolates)[2]
	Salmonella typhimurium (Isolates)[2]
	Shigella sp. (Isolates)[2]
Fungi	*Candida albicans* ATCC 10230[1]
	Saccharomyces cerevisiae ATCC 9763[1]

[1] From Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

[2] From Department of Microbiology, Faculty of Sciences, Chulalongkorn University

[3] From Department of Microbiology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University

Agar Diffusion Metho

The modified method was done by two-layer agar technique according to Tadega *et al.,* 2005 [60]. The bacterial and fungal strains were grown on Mueller Hinton agar (MHA) and Sabouraud Dextose agar (SDA) respectively. After incubated at 37°C for 24 hours, the culture were suspended in sterile 0.85% NaCl to obtain the turbidity equivalent to 0.5 McFarland standard (1×10^8 CFU/ml). Mixed 100 µl of the suspension with 3 ml of sterile seeds agar and pour to sterile base agar. The plates were allowed drying at room temperature. Agar's wells were performed by cork borer (6 mm). Twenty microliters of plant extract (200 mg/ml in dimethyl sulfoxide (DMSO) was added to each well. Ampicillin and amikacin (1 mg/ml, 20 µl) were used as positive control and DMSO (20 µl) was used as a negative control. Then, incubated the plates at 37°C for 24 hours and measured the diameters of inhibition zone (in millimeters). Each extract was tested in triplicate. The antimicrobial activity was interpreted according to Alves *et al.,* 2000 [61] as follows in table 2.

Table 2. Antimicrobial activity potential

Zone inhibition	Classified strength
< 9 mm	Inactive
9-12 mm	Less active
13-18 mm	Active
> 18 mm	Very active

Antioxidant Activity

DPPH Radical Scavenging Assay

The antioxidant activity was assessed by observing the ability to scavenger DPPH free radical as described by Brand-William *et al.,* 1995 [41]. DPPH radical scavenging assay was performed in 96 well microplate. One hundred microliter of various concentrations of the extract and positive control (BHT) in ethanol were added to 100 µl of DPPH radical ethanolic solution (120 µM). Incubated the plate at room temperature for 30 minutes in the dark and the absorbance was measured at the wavelength of 517 nm. Each sample was done in triplicate. The radical scavenging activity was evaluated from the inhibition of decreasing in absorbance at 517 nm as below equation.

$$\%\ Inhibition = \frac{[Absorbance\ control - Absorbance\ sample]}{Absorbance\ control} \times 100$$

The scavenging activity was expressed as EC_{50} value that indicated the concentration of sample required to scavenge 50% of DPPH free radical.

Ferric Reducing Antioxidant Power (FRAP)

The antioxidant power was assessed by observing the reducing power as described by Benzie and Strain, 1996 [42]. The various concentrations of extract, positive control (ascorbic acid) and ferrous sulphate were prepared in ethanol. FRAP working reagent was freshly prepared by mixing acetate buffer (300 mM pH 3.6), TPTZ (2,4,6-tripyridyl-*s*-triazine) (10 mM in 40 mM HCl) and $FeCl_3.H_2O$ (20 mM) in ratio of 10:1:1. Mixed 5 µl of each concentration of the extract, positive control and ferrous sulfate and 150 µl of FRAP working solution in a 96 well plate. Incubated the plate at room temperature for 30 minutes. The absorbance was measured at 593 nm with a 96 well microplate reader. Each sample was done in triplicate. The reducing antioxidant power was evaluated as the amount of ferrous sulphate obtained from the calibration curve.

Nitric Oxide Radical Inhibitory Activity

Nitric oxide radical inhibitory activity was evaluated according to the method of Rawiwan *et al.*, 2012 and Tsai *et al.* [44, 45]. Two hundred microliter of various concentrations of extract and positive control (quercetin) in cuvette were mixed with 200 µl of sodium nitroprusside (5 mM) in phosphate buffer, pH 7.5 and incubated at 25 °C for 120 min. Griess reagent (400 µl) was added and the absorbance was measured by spectrophotometry at wavelength of 540 nm. Each sample was done in triplicate. The percentage of nitric oxide radical inhibitory activitywas calculated as equation below and EC_{50} that indicated the concentration of sample required to scavenge 50% of nitric oxide radical inhibition activity was calculated.

$$\%\ Inhibition = \frac{[Absorbance\ control - Absorbance\ sample]}{Absorbance\ control} \times 100$$

β-carotene Bleaching Assay

β-carotene bleaching assay was evaluated according to the method of Jayaprakasha *et al.*, 2002 [43]. One milliliter of β-carotene solution (0.2 mg/ml in chloroform) was added with 20 µl of linoleic acid and 200 µl of Tween 20. The mixture was evaporated *in vacuo* at 40 °C for 10 minutes to remove chloroform solvent, added with oxygenated distilled water (50 ml) and shaken to form a liposome solution. Aliquots (4 ml) of the emulsion were transferred into the test tubes containing 0.2 ml of the various concentrations of extract and positive control (BHT), subjected to thermal at 50 °C and monitored the absorbance at wavelength of 470 nm by taking measurements at 30 min intervals for 120 min and the rate of bleaching of β-carotene was recorded. Each sample was done in triplicate. The dose response relationship was expressed as kinetic graph, which indicated between the absorbance of sample and times and the antioxidant activity of the extract was evaluated in the bleaching of the β-carotene using the following formula.

$$\%\ Antioxdant\ acitivity = \left[1 - \frac{A0 - A180}{C0 - C180}\right] \times 100$$

Where, A0 = the absorbance values measured at zero time

A180 = the absorbance values measured at end time

C0 = the absorbance values measured at zero time of control

C180 = the absorbance values measured at end time of control

Toxicity Tests

Brine Shrimp Lethality Assay

Brine shrimp lethality assay was evaluated according to the procedure described by Mayer *et al.,* 1982 [62]. The study was started by hatching of *A. salina.* Artificial sea water at the concentration of 36.66 % (w/v) was firstly prepared and oxygenated for 24 hours in brine shrimp hatching box (Figure 13) under illuminator only one side of the hatching box. Two mg of brine shrimp cysts were added and incubated at room temperature (25-29°C), After 24 hours, newly hatched nauplii swam from the dark side to the light side of the hatching box. Then transferred 10 nauplii by using pasture pipette to each vials filled with 5 ml of saline water. One hundred microliter of *L. disjunctus* extracts at various concentrations in ethanol were pipetted into a small filter paper and left until ethanol was exhaustively evaporated. Put the prepared filter paper into each brine shrimp's vials. Five replicate were done for each concentration. Observed the percent death of nauplii at 6, 12, 18, 24 and 48 hours and calculated for the LD_{50}.

Figure 13. Brine shrimp hatching box

Phototoxic Activity

Phototoxic activity was evaluated according to Apirach *et al.*, 2010 [63]. The bacterial and fungal strains were grown on Mueller Hinton agar (MHA) and Sabouraud Dextose agar (SDA) respectively. After incubated at 37°C for 24 hours, the cultures were suspended in sterile 0.85% NaCl to obtain the turbidity equivalent to 0.5 McFarland standards (1 x 10CFU/ml), mixed 100 µl of the suspension with 3 ml of sterile seeds agar and pour to sterile base agar. The plates were allowed drying at room temperature. Agar's wells were performed by cork borer (6 mm). Twenty microliters of plant extract (200 mg/ml in dimethyl sulfoxide (DMSO) was added to each well. DMSO (20 µl) was used as a negative control. Incubated the plates at 37°C for 24 hours under ultraviolet light at wavelength of 360 nm from 2 UV lamps with the shine power of 15W/m^2 in the chamber size 60×50×30 cm (Figure 14). The diameters of inhibition zone were measured in millimeters. Each extract was tested in triplicate. The inhibition zones were determined according to Alves *et al.*, 2000 [61]

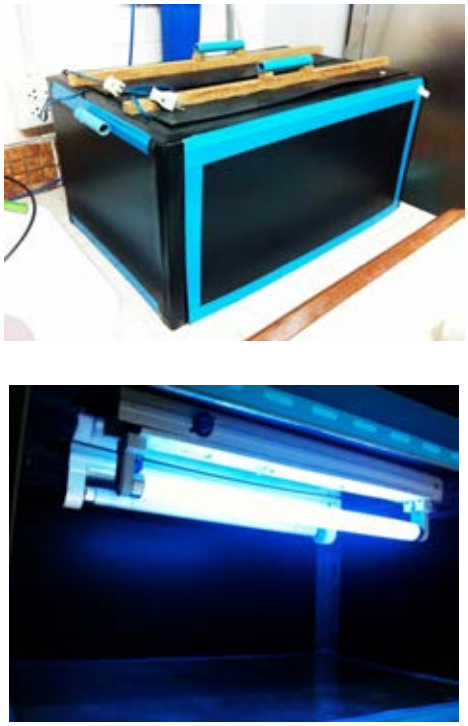


Figure 14. UV chamber (Top) and UV light (Bottom)

Enzymes Inhibition Tests

Antityrosinase Activity

Antityrosinase activity was determined using the modified dopachrome method with L-DOPA as substrate in 96 well microplate according to Masuda *et al.*, 2005 and Chan *et al.*, 2008 [64, 65]. The extracts and positive control (L-glutathione) were dissolved in 1 ml of DMSO (50 percent DMSO in distilled water) and then diluted to different concentrations. Each sample (40 µl) was mixed with 80 µl of 0.1 M sodium phosphate (pH 6.8) and 40 µl of L-DOPA solution (19.7 mg in 0.1 M sodium phosphate, pH 6.8). Forty µl of mushroom tyrosinase solution (31 U/ml) was added in the reaction. The dopachrome formation was measured at the wavelength of 475 nm. The percentage of tyrosinase inhibition activity was calculated as equation below and EC_{50} was calculated.

$$\%\, Tyrosinase\ inhibition\ = \left[1 - \left(\frac{A1 - A2}{B}\right)\right] \times 100$$

Where, A1 = absorbance intensity of extract

A2 = absorbance intensity of extract without enzyme

B = absorbance intensity of negative control

Alpha-glucosidase Inhibition Testing

The activity of alpha-glucosidase was done using 1 mM p-nitrophenyl-α-D-glucopyranosiade as substrate according to Dewi *et al.*, 2007 and Elya *et al.*,2012 [66, 67]. The extract and positive control (1-Deoxynojirimycin) were prepared in 10% DMSO in distilled water and diluted by two fold dilution. In 96 well plates, 50 µl of the extracts and positive control were mixed with 50 µl of 0.1M sodium phosphate buffer, pH 6.8 and 50µl of 0.5 U/ml α-glucosidase. The plate was incubated at 37°c for 10 minutes. Fifty microliter of substrate were added and incubated again at 37°c for 20 minutes. The reaction was terminated by adding 100 µl of 0.2 µM Na_2CO_3 and the absorbance was measured at the wavelength of 405 nm. Analyzed each sample in triplicate. The percentage of alpha-glucosidase inhibition activity was calculated as equation below and EC_{50} was calculated.

$$\%\ Alpha\ glucosidase\ inhibition\ = \left[1 - \left(\frac{A1 - A2}{B}\right)\right] \times 100$$

Where, A1 = absorbance intensity of extract

A2 = absorbance intensity of extract without enzyme

B = absorbance intensity of negative control

Hypnotic Activity

Sodium Pentobarbital Induced Sleeping Time Test

Sodium pentobarbital induced sleeping time test was done according to Hossein *et al.*, 2009 and Carvalho *et al.*, 2002[57, 58]. Adult male Albeno ICR mice (35-45 g) were randomized and used as experimental animals, housed in Faculty of Pharmaceutical Sciences, Chulalongkorn University's Animal House, under standard environmental conditions of temperature, relative humidity and light (24 °C, 60-70% humidity, 12 h light: 12 h dark cycle).Food and water were starved for two hours before the experimental procedures. The different concentrations of extract suspended in Tween 80(2% v/v in saline) were administered (10 ml/kg) orally by gavage (P.O.). The control group was received only vehicle (Tween 80) at the same volume as the treated groups. Sodium pentobarbitone (20 mg/kg) was injected to induce animal hypnosis by intra peritoneally (I.P.) after 30 minutes of extract administration. The sleeping time was recorded by observing time of righting reflex. The sodium pentobarbital induced sleeping time of treated groups was compared to control group by Dunnett t-tests following ANOVA. This research protocal has been reviewed and approved by ethics committee of Research Institute, Rangsit University number RSEC 01/2556.

CHAPTER IV

RESULTS

Determination of Secondary Metabolites

The results indicated secondary metabolites of *Leptocarpus disjunctus* were shown in detail (Table 3). *L. disjunctus* whole plant were tested with various reagents and revealed the chemical composition of coumarin glycoside, tannin, steroid and sugar.

Table 3. Determination of secondary metabolites of *Leptocarpus disjunctus* whole plants

Secondary metabolite	*Leptocarpus disjunctus*
1. Anthraquinone glycoside	
- Brontrager test	-
2. Coumarin glycoside	+
3. Cardiac glycoside	
- Legal test	-
- Libermann-Burchard test	+
- Keller-Kiliani test	+
4. Flavonoid	
- Cyanidin test	-
5. Saponin	-
6. Tannin	
- Ferric chloride test	+
7. Alkaloid	
- Wagner's test	-
- Mayer's test	-
- Marme's test	-
- Dragendorff's test	-
- Hager's test	-

Positive and negative results were expressed as + and – respectively.

Ethanolic Extract of *L. disjunctus* Fresh Whole Plants

Crude extract of *L. disjunctus* fresh whole plants were performed by maceration in 95% ethanol until exhaustion and evaporated to dryness *in vacuo*. The yield of fresh ethanolic extract was 2.66 % w/w.

Antimicrobial Test

The antibacterial and antifungal activities of *L. disjunctus* ethanolic extract were determined *in vitro*. Thirteen microorganisms including five gram positive, six gram negative bacteria and two fungal strains were tested.

The ethanolic extract was firstly tested by agar well diffusion method against 13 species of microorganisms as mentioned. It was found that *L. disjunctus* ethanolic extract presented no inhibitory activity against any tested microorganisms. The results were demonstrated in Table 4.

Table 4. The antimicrobial activities of *L. disjunctus* ethanolic extract

Microorganism	Inhibition zone (mm)			
	Extract	DMSO	Ampicillin	Amikacin
Bacillus subtilis ATCC 6633	NA	NA	17.67±0.58	30.67±0.58
Staphylococcus aureus ATCC 6538P	NA	NA	45.00±0.00	23.33±0.58
Micrococcus luteus ATCC 9341	NA	NA	48.33±0.58	27.67±0.58
Staphylococcus epidermidis (isolates)	NA	NA	30.33±0.58	29.00±0.00
Salmonella typhi (isolates)	NA	NA	33.00±1.00	26.00±1.00
Escherichia coli ATCC 25922	NA	NA	29.00±0.00	28.33±0.58
Pseudomonas aeruginosa ATCC 9027	NA	NA	25.23±0.00	23.00±0.00
Bacillus cereus ATCC 11778	NA	NA	35.67±0.58	28.00±0.00
Shigella sp. (isolates)	NA	NA	32.00±0.00	26.00±1.00
Salmonella typhimurium (isolates)	NA	NA	32.00±0.00	30.33±0.58
Enterobacter aerogenes ATCC 13048	NA	NA	20.19±0.00	19.5±0.00
Candida albicans ATCC 10230	NA	NA	NA	NA
Saccharomyces cerevisiae ATCC 9763	NA	NA	NA	NA

Mean±SD, NA = no activity, each experiment was done in triplicate

Antioxidant activity

DPPH Radical Scavenging Assay

The effect of radical scavenging is based on the hydrogen donating ability [12]. Results demonstrated that the ethanolic extract of the *L. disjunctus* showed the radical scavenging activity with EC_{50} of 0.78 mg/ml (Figure 15) and positive control (BHT) showed EC_{50} of 5.96 mg/ml (Figure 16).

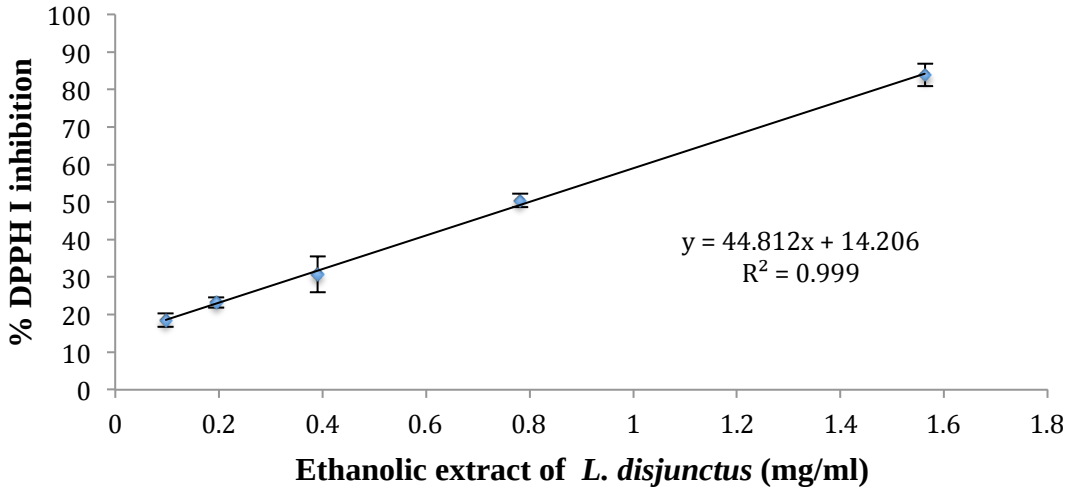


Figure 15. Percent DPPH inhibition of *L. disjunctus* whole plant

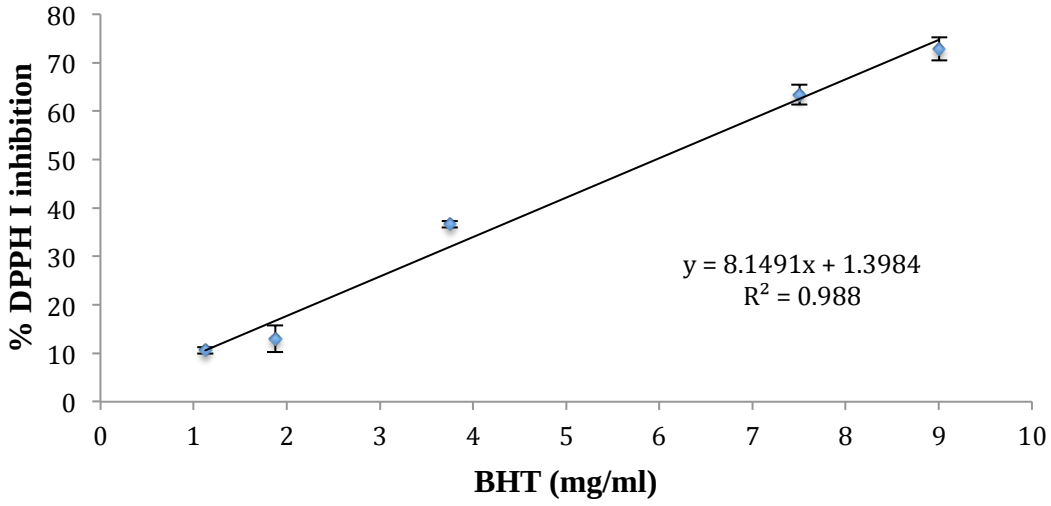


Figure 16. Percent DPPH inhibition of positive control (BHT)

Ferric Reducing Antioxidant Power (FRAP)

The reducing power potential of *L. disjunctus* ethanolic extract and positive control (ascorbic acid) were shown in term of ferrous sulphate ion concentration. The ferrous sulphate ion concentration was calculated according to the equation of standard curve of ferrous sulphate as shown in Figure 17. *L. disjunctus* ethanolic extract exhibited ferrous sulphate ion concentration of 6, 23 and 50 mM at the concentration of 25, 50 and 100 mg/ml respectively. While ascorbic acid exhibited ferrous sulphate ion concentration of 3, 17, 40 and 85 mM at the concentration of 0.03125, 0.0625, 0.125 and 0.25 mg/ml respectively.

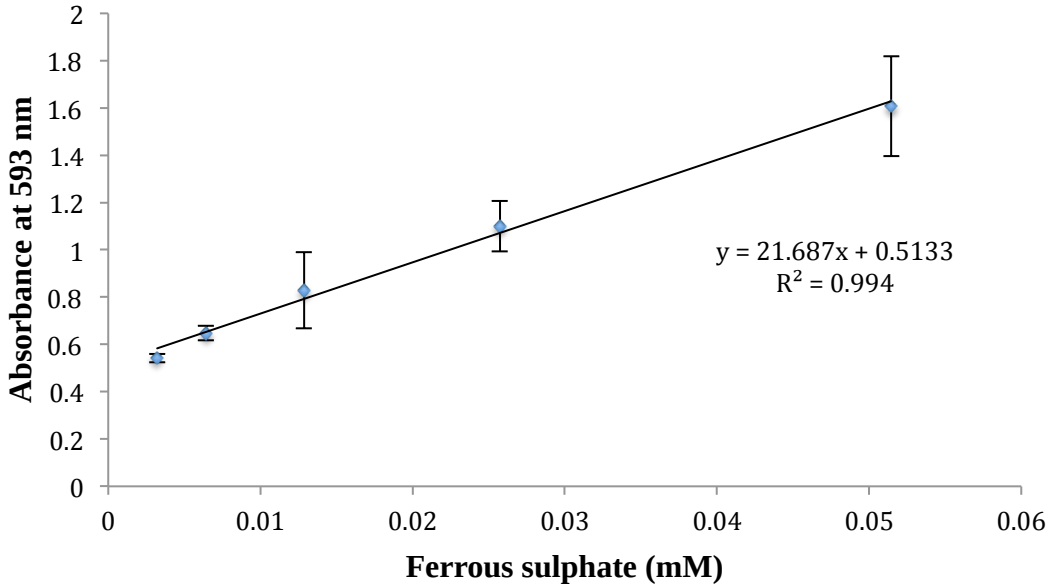


Figure 17. Standard curve for determination of antioxidant capacity by FRAP reducing antioxidant power

Nitric Oxide Radical Inhibitory Activity

Results demonstrated that the ethanolic extract of the *L. disjunctus* showed the nitric oxide radical scavenging activity with EC_{50} of 2.32 mg/ml (Figure 18) and positive control (quercetin) showed EC_{50} of 0.29 mg/ml (Figure 19).

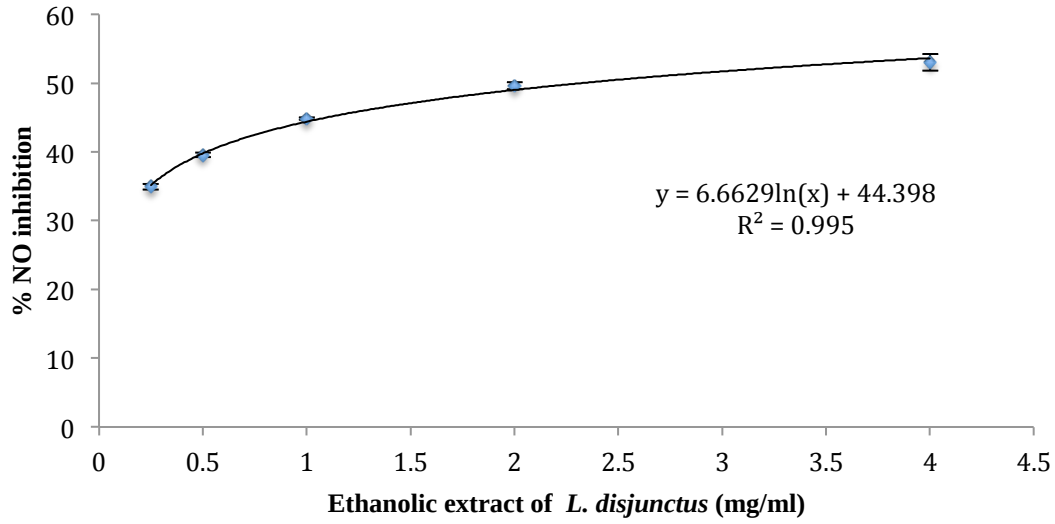


Figure 18. Percent NO inhibition of *L. disjunctus* whole plant extracts

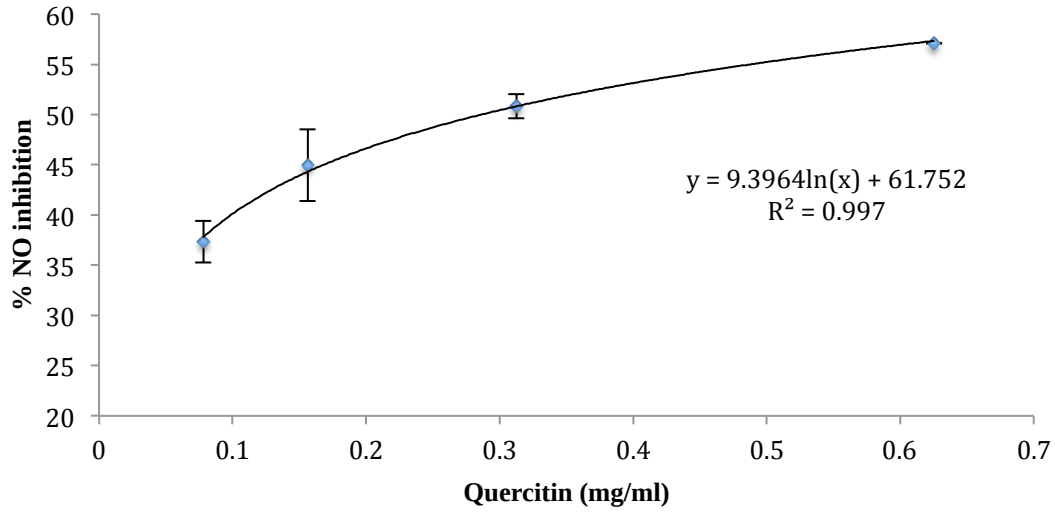


Figure 19. Percent NO inhibition of positive control (quercetin)

β-carotene Bleaching Assay

Anti β-carotene bleaching effects of *L. disjunctus* were shown in Figure 20. The activity was concentration relationship. However, *L. disjunctus* showed borderline antioxidant activity compared to positive control (BHT). The antioxidant activity of *L. disjunctus* and BHT were 3.88 and 73.98 % respectively (Figure 21).

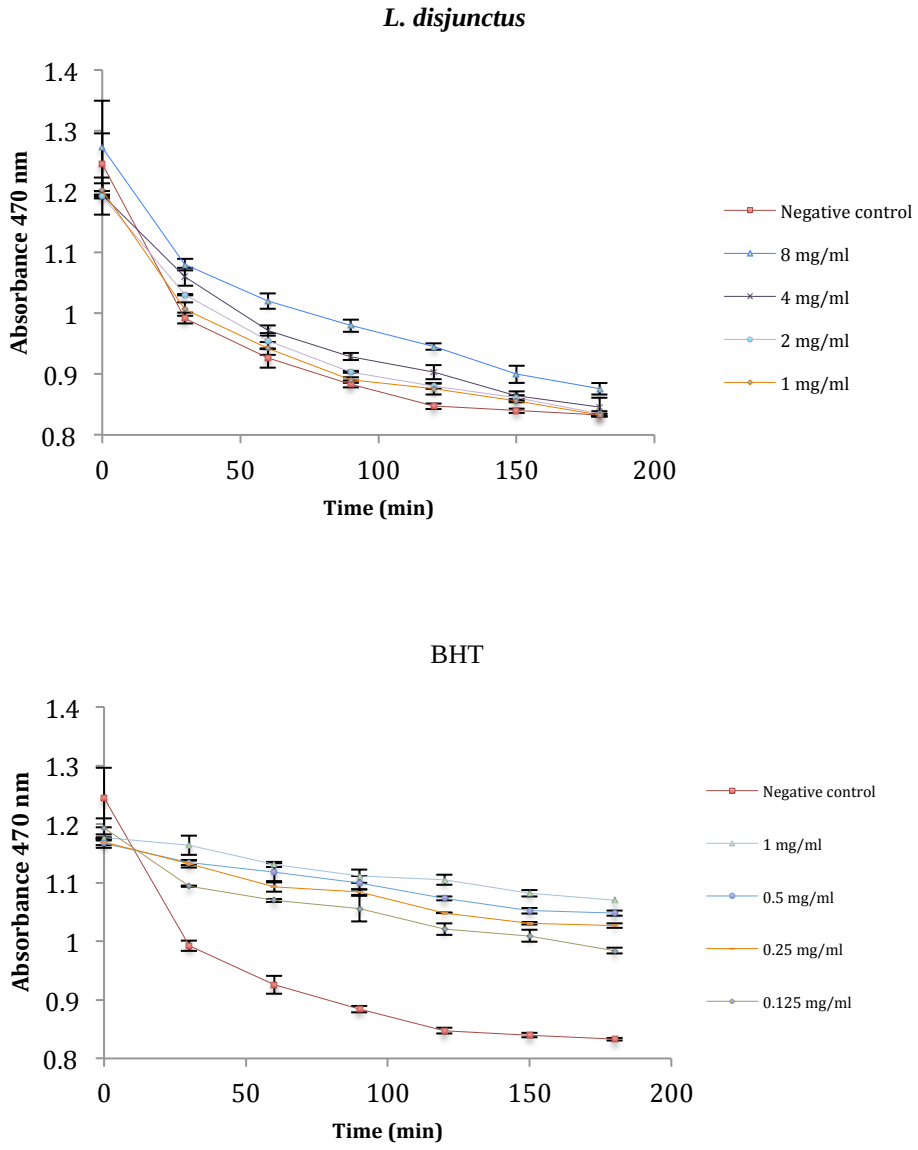


Figure 20. Anti β-carotene bleaching effect of various concentration of *L. disjunctus* and positive control (BHT).

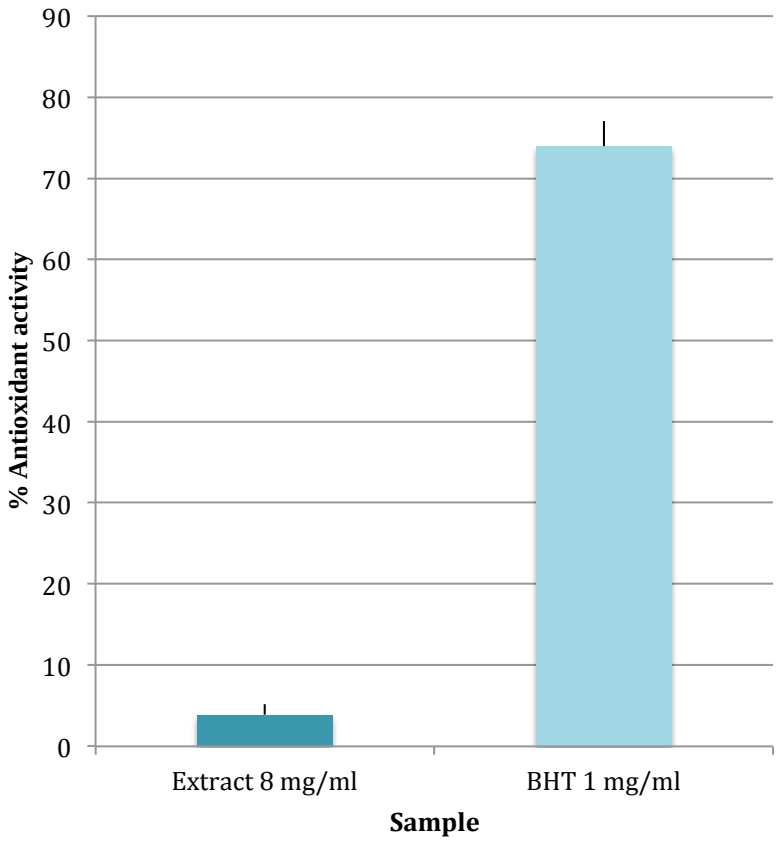


Figure 21. The antioxidant activity of *L. disjunctus* compared to positive control (BHT)

Toxicity Test

Brine Shrimp Lethality Assay

Result of brine shrimp lethality assay demonstrated that all of *A. salina* nauplii survived during 48 hours incubation with *L. disjunctus* fresh extracts at the concentration >500 mg/ml as shown in Table 5.

Table 5. Number of survivor nauplii at each time among various concentrations of fresh extracts

Concentration (mg/ml)	**Time (hr.)**				
	6	**12**	**18**	**24**	**48**
31.25	10	10	10	10	10
62.5	10	10	10	10	10
125	10	10	10	10	10
250	10	10	10	10	10
500	10	10	10	10	10

Phototoxic Activity

The *in vitro* phototoxic activity of *L. disjunctus* and antibiotic drug was determined against thirteen microorganisms, bacteria and yeast strains with unexposed and exposed to UV at wavelength 360 nm overnight. *L. disjunctus* and antibiotic drugs showed no phototoxic potential by microorganisms test as shown in Table 6.

Table 6. Phototoxic activity among *L. disjunctus* fresh extract, negative control and positive control

Microorganism	**Inhibition zone (mm)**			
	LD extract	**DMSO**	**Ampicillin**	**Amikacin**
Bacillus subtilis ATCC 6633	NA	NA	17.67±0.58	30.67±0.58
Staphylococcus aureus ATCC 6538P	NA	NA	45.00±0.00	23.33±0.58
Micrococcus luteus ATCC 9341	NA	NA	48.33±0.58	27.67±0.58
Staphylococcus epidermidis (isolates)	NA	NA	30.33±0.58	29.00±0.00
Salmonella typhi (isolates)	NA	NA	33.00±1.00	26.00±1.00
Escherichia coli ATCC 25922	NA	NA	29.00±0.00	28.33±0.58
Pseudomonas aeruginosa ATCC 9027	NA	NA	25.23±0.00	23.00±0.00
Bacillus cereus ATCC 11778	NA	NA	35.67±0.58	28.00±0.00
Shigella sp. (isolates)	NA	NA	32.00±0.00	26.00±1.00
Salmonella typhimurium (isolates)	NA	NA	32.00±0.00	30.33±0.58
Enterobacter aerogenes ATCC 13048	NA	NA	20.19±0.00	19.5±0.00
Candida albicans ATCC 10230	NA	NA	NA	NA
Saccharomyces cerevisiae ATCC 9763	NA	NA	NA	NA

Mean±SD, NA = no activity, each experiment was done in triplicate

Enzyme Inhibitory Test

Antityrosinase Activity

The ethanolic extract of the *L. disjunctus* showed the tyrosinase inhibition activity with EC_{50} of 15.15 mg/ml (Figure 22) and positive control (glutathione) showed EC_{50} of 0.02 mg/ml (Figure 23).

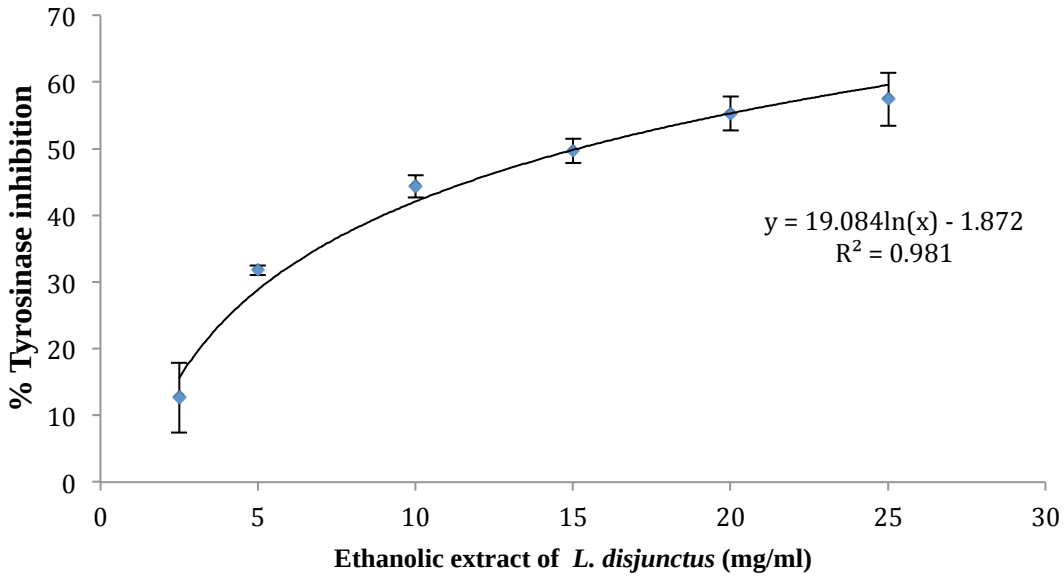


Figure 22. Percent tyrosinase inhibition of *L. disjunctus* whole plant extract

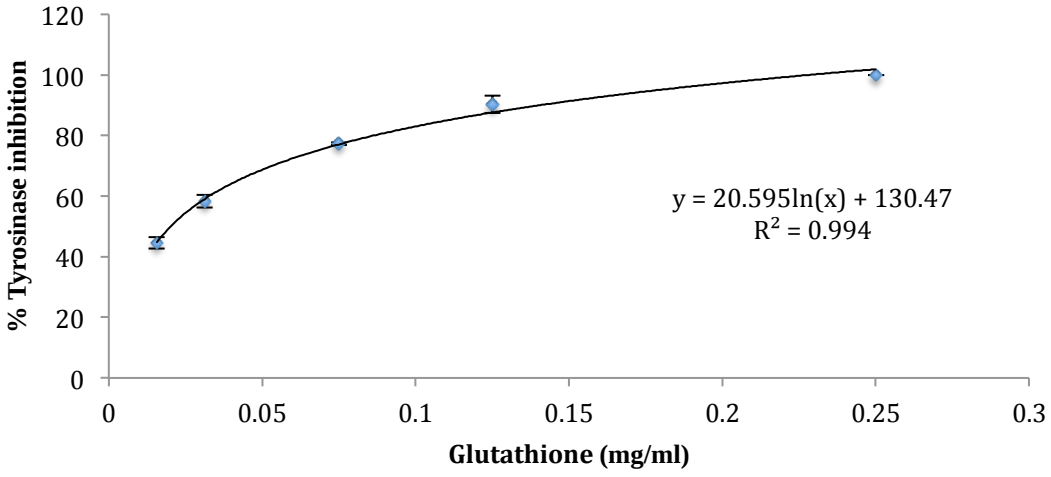


Figure 23. Percent tyrosinase inhibition of glutathione

Alpha-glucosidase Inhibition Testing

The ethanolic extract of the *L. disjunctus* showed the alpha-glucosidase inhibition with EC_{50} of 6.13 mg/ml (Figure 24) and positive control (1-deoxynojirimycin) showed EC_{50} of 0.0124 mg/ml of stock solution (Figure 25).

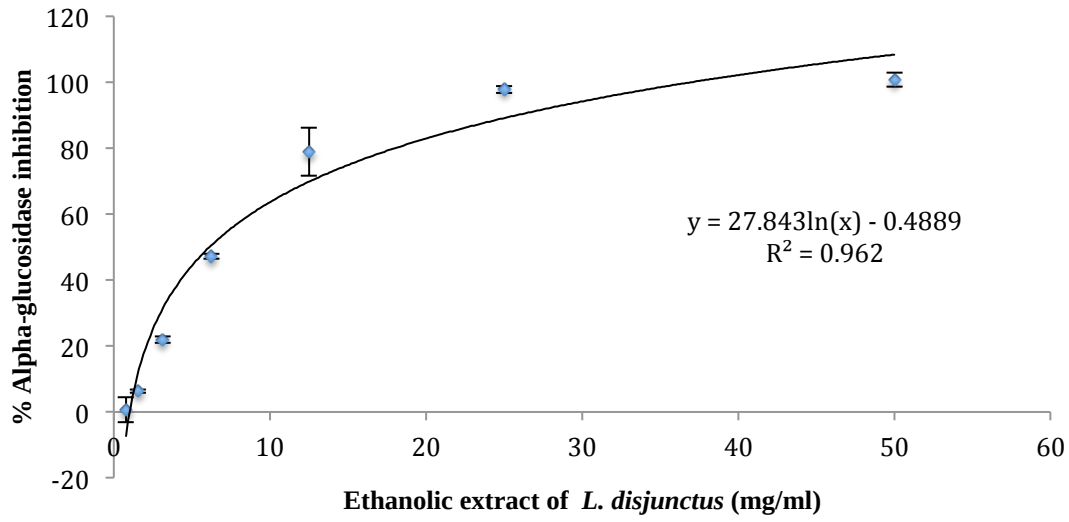


Figure 24. Percent alpha-glucosidase inhibition of *L. disjunctus* whole plant extract

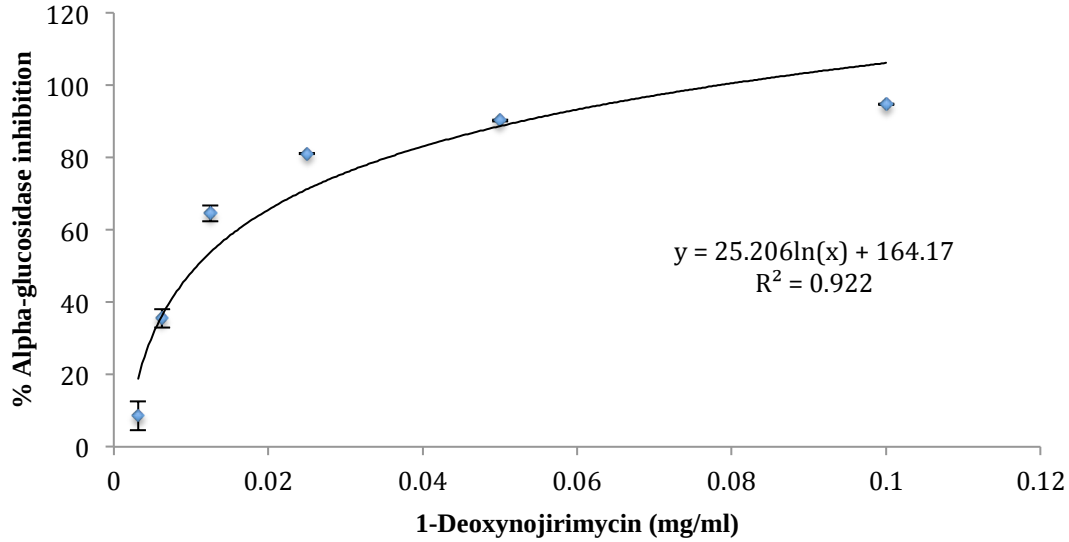


Figure 25. Percent alpha-glucosidase inhibition of 1-Deoxynojirimycin

Hypnotic activity

Sodium Pentobarbitone Induced Sleeping Time Test

Sodium pentobarbitone induced sleeping time's data were obtained and submitted to statistical analysis. *L. disjunctus* ethanolic extract significantly prolonged sleeping time induced by sodium pentobarbitone. The hypnotic activity of *L. disjunctus* was shown to be concentration response relationship (Figure 26).

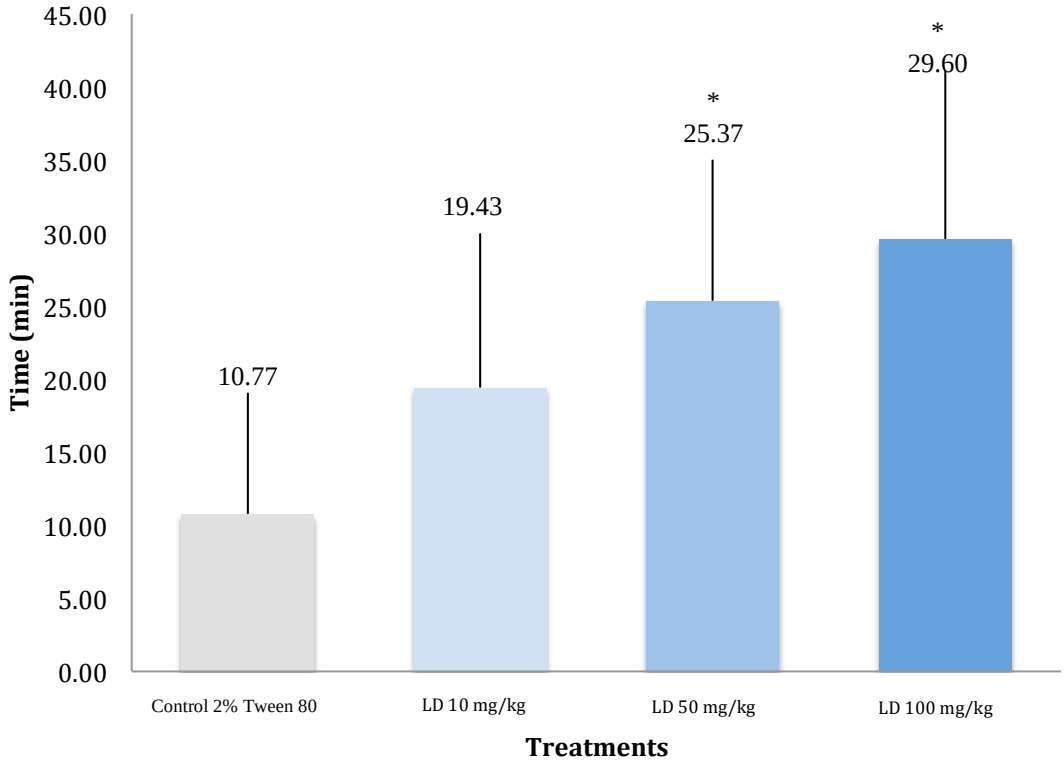


Figure 26. Effect of *L. disjunctus* extract on sleeping time induced by sodium pentobarbital (*p<0.05)

CHAPTER V

DISCUSSION AND CONCLUSION

Traditionally, *Leptocarpus disjunctus* is commonly eaten in Southern Thailand as a side dish plant and there is the indigenous warning about its side effects for dizziness and intoxicated symptoms. There are a lot of plant secondary metabolites being employed in anxiolytic effect especially for anxiety in traditional medicine practice, most of which directly or indirectly affect the central nervous system, $GABA_A$ and other neurotransmitters activities [68].

The purposes of this study were to evaluate selected *in vitro* and *in vivo* biological activities *L. disjunctus* ethanolic whole plants extract. Hypnotic evaluation was done by pentobarbital induced sleeping time assay in mice. The secondary metabolites of ethanolic extract were determined by color tests. This study provides scientific information and scientific evidence to validate the plant to be phytopharmaceutical.

The ethanolic extract of *L. disjunctus* showed negative results in all alkaloid tests, which related to study of William J.J., 1995 [63] that no alkaloid was discovered in Restionaceae family. *L. disjunctus* showed negative result by using Legal test, on the other hand it showed positive results with Liebermann-Burchard test and Keller-Killiani test. This means that *L. disjunctus* has steroid and sugar [10]. *L. disjunctus* showed green fluorescence under UV 365 nm on filter paper exposed to NaOH and vapor of boiling plant. The reaction represented trans-cinnamic acid formation from cis-cinnamic acid, which exhibited the presentation of coumarin. *L. disjunctus* showed positive ferric chloride test of tannins, which related to the previous study that some tannins could be found in Restionaceae family [63]. Furthermore the determination of anthraquinone glycoside, saponin and flavonoid gave negative results. The previous study of *Leptocarpus brownil* and *Leptocarpus tenax* also reported negative flavonoid results [16, 17].

Antimicrobial test by agar diffusion method is based on the principle of diffusion. Tested substance is able to diffuse into the agar that was cultivated with interested microorganisms. In this study, *L. disjunctus* ethanolic extract showed no inhibition zone against each microorganism that was shown in Table 4 meanwhile amikacin and ampicillin showed inhibition zone against most microorganisms, which ranged from 19.5±0 to 30.67±0.58 and from 17.67±0.58 to 48.33±0.58 respectively. From the previous study, amikacin and ampicillin have been used as positive control, which gave the sterility effect at the nearly range of this study [42].

Antioxidant compounds are important in medicinal role. It is a health-protecting factor along with the scientific evidences that antioxidants can be the diseases reducing factor especially cancer [64]. Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods. Many types of reactive oxygen species have been used to evaluate the capacity of foods to scavenge or reduce these radicals *in vitro*. The antioxidant activities of *L. disjunctus* ethanolic extract were assessed based on distinctive radical reduction including DPPH, metal ion, nitric oxide and peroxide radical.

DPPH, the stable free radical, is always used for estimation of radical scavenging potential of the natural substance. Reduction of DPPH is used as an indicator of radical scavenger property. DPPH radical has a deep violet color in methanolic solution with the absorbance range between 515-520 nm and it becomes colorless or pale yellow when neutralized by hydrogen atom donated from the antioxidant [65]. The discoloration degree indicates the scavenging potential of plant extract. The color changing is monitored by using spectrophotometry. In this study, the ethanolic extract of *L. disjunctus* was able to decolorized DPPH free radical in methanolic solution with EC_{50} of 0.8 mg/ml. The scavenging activity of *L. disjunctus* was more potent than positive control (BHT) with EC_{50} of 5.96 mg/ml. Basically, a higher DPPH radical scavenging activity is associated with a lower EC_{50} value. The other studies were carried out to evaluate the antioxidant activity of BHT that used as positive control and showed nearly EC_{50} of BHT against DPPH [42, 69].

Metal ion transition such as Fe^{3+}/Fe^{2+} and Cu^{3+}/Cu^{2+} plays a role in oxidative stress pathway of living organism [66]. Metal reducing power is one of parameters representing antioxidant capacity. Ferric reducing antioxidant power assay is an antioxidant capacity assay that measures the power of ferric reduction. This method uses TPTZ as the coupling agent to form colorless Fe^{3+}-TPTZ complex, which turns to a blue color complex of Fe^{2+}-TPTZ by reducing power of antioxidant [40]. In this study, the ethanolic extract of *L. disjunctus* was able to reduce ferric ion then colorize FRAP working solution. The result indicated that the reducing power of *L. disjunctus* ethanolic extract at the concentration of 25 mg/ml had a value equivalent to ferrous sulphate 6 mM and the reducing power of ascorbic acid at the concentration of 0.031 mg/ml had a value equivalent to ferrous sulphate 3 mM. From the previous studies, ascorbic acid was used as positive control and shown high reducing power of 0.34 mM at the concentration of 3 µg/ml [71, 72].

Nitric oxide is an important for the body and affects physiological processes. It is free radical that causing the oxidative stress, the tissue damage and destroys antioxidant system in body. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide that produce nitrite ions when react with oxygen. The nitric oxide can be estimated by using Griess reagent, which give blue color [67]. In this study, *L. disjunctus* was able to reduce production of nitrite ions by observing reagent colorize with EC_{50} of 2.32 mg/ml. However, the activity of quercetin was more pronounced with EC_{50} of 0.29 mg/ml. Nitric oxide scavenging activity of quercetin in this study was in accordance with the previous study that showed EC_{50} of 0.2 mg/ml [42].

The β-carotene bleaching assay is commonly used in research to evaluate *in vitro* antiperoxidant capacity. Polyunsaturated fatty acid such as linoleic acid can form lipid peroxides under oxygen atmosphere, which further oxidise β-carotene resulting β-carotene bleaching. The determination of the antioxidant activity against lipid peroxidation is based on the ability to delay the bleaching of yellow color of β-carotene. The rate of β-carotene bleaching can be delayed, which indicated the presence of antioxidants. In this study, the ethanolic extract of *L.* presented antioxidant activity only 4% compared to the positive control (BHT), which showed

antioxidant capacity of 74%.

Brine shrimp lethality and phototoxic activity tests are the assay for determining the toxicity potential of medicinal plant. In this study, *L. disjunctus* was shown non-toxicity according to Mayer *et al.*, 1982 [57] category using brine shrimp lethality test. Phototoxic activity test showed no inhibition zone among all microorganisms after ultraviolet exposure. These preliminary studies can be concluded that the ethanolic extract of *L. disjunctus* has no cytotoxicity and genotoxicity against brine shrimp and microorganisms respectively.

Tyrosinese is the enzyme in melanosomes of melanocyte, which involves in melanin biosynthesis. Antityrosinase activities were examined by using dopachrome method [59, 60]. L-dopa was used as substrate of tyrosinese *in vitro.* Dopaquinone product chemically changed to color substance, dopachrome. As the results shown in Figure 22 and Figure 23, the ethanolic extract of *L. disjunctus* was able to inhibit the enzyme with EC_{50} of 15.15 mg/ml that less effective than positive control (glutathione) with EC_{50} of 0.02 mg/ml. Meanwhile the other herbs, for example *Psidium guajava* Linn. showed EC_{50} of 9.9 mg/ml and *Morus nigra* Linn. showed EC_{50} of 12.1 mg/ml [73, 74].

Alpha-glucosidase is an enzyme act for digesting carbohydrates to monosaccharaides. Alpha-glucosidase inhibitors are saccharides that competitively inhibit enzymes activity on carbohydrates digestion. Inhibition of these enzyme systems reduces the rate of digestion and less glucose is absorbed. In this study, alpha-glucosidase inhibition was assayed using p-nitrophenyl α-D-glucopyranoside as substrate, which was hydrolyzed into p-nitrophenol and glucose by alpha-glucosidase. The product was monitored using UV-Visible spectrophotometry [61, 62]. As the results shown in Figure 24 and Figure 25, the ethanolic extract of *L. disjunctus* was able to inhibit enzyme with the EC_{50} of 6.13 mg/ml that less effective than positive control (1- deoxynojirimycin) with EC_{50} of 0.0124 mg/ml. There were previous researches reported about vegetables and weeds that could inhibit alpha-glucosidase such as *Curcuma longa* and *Sida acuta* with EC_{50} of 5.24 and 5.5 mg/ml respectively [76, 77]. This study demonstrated hypoglycemic potential of *L. disjunctus*, which

belonged to Restionaceae family.

The possible anxiolytic effect of *L. disjunctus* ethanolic extract was investigated by using pentobarbitone induced sleeping time test, the popular hypnotic test. The principal is based on observing time between the loss and recovery of righting reflex after receive testing substance followed by sodium pentobarbital, which is short acting barbiturate. The results of the present study showed that ethanolic extracts of *L. disjunctus* whole fresh plant exhibited dose relationship of hypnotic effect among control and treatment groups. According to the previous study, the plant in Restionaceae family also reported the anxiolytic effect [70]. Thus *L. disjunctus* should be further studied on the general locomotors activity to confirm its anxiolytic and hypnotic activity.

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APPENDICES

APPENDIX A

Chemical Preparation

Phytochemical Screening

Wagner's Reagent

Dissolved 2 g of Iodine and 6 g of KI in 100 mldistilled water.

Mayer's Reagent

Dissolvedsolution A, 1.358 g of $HgCl_2$ in 60 ml distilled water and mix with solution B, 5 g of KI in 10 ml distilled water, added sufficient water to make the volume up to 100 ml.

Marme's Reagent

Added 2 g of CdI_2 to a boiling solution of 4 g of KI in 12 ml of distilled water and mixed with 12 ml of saturated KI solution.

Dragendorff's Reagent

Solution A,dissolved 1.7 g bismuth (III) nitrate and 20 g glacial acetic acid in 80 ml distilled water.

Solution B, dissolved 16 g potassium iodide in 40 ml distilled water

Stock Solution, mixed equal parts of solution A and solution B, working solution, dissolved 10 g glacial acetic acid in 50 ml distilled water and added 10 ml to the stock solution.

Hager's Reagent

Dissolved 1 g of picric acid in 100 ml of distilled water.

Antimicrobial Test

Mueller Hinton Agar (MHA)

Suspended 38.0 grams in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring.

Mueller Hinton Broth (MHB)

Suspended 21.0 grams in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring.

Sabouraud Dextose Agar (SDA)

Suspended 65.0 grams in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring.

Sabouraud Dextose Broth (SDB)

Suspended 30.0 grams in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring.

Antioxidant Activity

DPPH Radical Ethanolic Solution

120 µM DPPH, dissolved 0.00474 g in 100 ml distilled water.

FRAP Reagent

Solution A300 mM Acetate buffer (pH 3.6), dissolved 4.68 ml glacial acetic acid and 0.2699 g sodium acetate trihydrate to 100 ml distilled water.

Solution B TPTZ (2, 4, 6-tripyridyl-*s*-triazine) 10 mM in 40mM HCl, chemical A 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine), dissolved 3.1233 g TPTZ in 1000 ml distilled water.Chemical B 40mM HCl, dissolved 4.05 ml hydrochloric acid in 1000 ml distilled water.

Solution C $FeCl_3.6H_2O$ 20 mM, dissolved 5.406 g iron(III) chloride hexa hydrate in 1000 ml distilled water.Combined all FRAP reagent to FRAP working solution in 10:1:1 ratio respectivelyby freshly prepare.

5 mM Sodium Nitroprusside

Dissolved 0.13096 g sodium nitroprusside in 100 ml phosphate buffer pH 7.5

Phosphate Buffer (pH 7.5)

Phosphate buffer pH 7.5 with a buffer strength of 0.1 M is obtained by adding 25.9631 g monosodium phosphate monohydrate and 217.5789 g disodium phosphate, heptahydrate to 100 ml distilled water.

Griess Reagent

Solution A 0.2% naphthylethylenediaminedihydrochloride, dissolved 0.2 g naphthylethylenediaminedihydrochloride in 100 distilled water.

Solution B 2%(w/v)sulphanilamide in 5%(v/v) phosphoric acid, dissolved 2 g sulphanilamide in 5% phosphoric acid

Enzyme Inhibition Test

0.5 U/ml α-glucosidase from *Saccharomyces cerevisiae*

α-glucosidase from *Saccharomyces cerevisiae*3.85 mg solid, 26 units/mg solid, 37 units/mg protein by weighed 0.0193 mg in 1 ml phosphate buffer pH 6.8

31 U/ml Tyrosinasesolution

Tyrosinase from mushroom 26.58 mg solid, 1881 unit/mg solid by weighed 0.0165 mg in 1 ml phosphate buffer pH 6.8

Phosphate Buffer (pH 6.8)

Phosphate buffer pH 6.8 with a buffer strength of 0.1 M is obtained by adding 123.9896 g monosodium phosphate monohydrate and 74.1546 g disodium phosphate, heptahydrate to 100 ml distilled water.

APPENDIX B

Data of bioactivities of *L. disjunctus* ethanolic extract

Table 7. DPPH scavenging activity of ethanolic extracts of *L. disjunctus*

Ethanolic extracts of *L. disjunctus* (mg/ml)	**OD_{517} (reaction mixture)**			**DPPH scavenging (%)**				
	exp 1	**exp 2**	**exp 3**	**exp 1**	**exp 2**	**exp 3**	**Mean**	**SD**
0.0	0.083	0.086	0.085					
0.098	0.102	0.099	0.1	20.47	16.93	18.11	18.50	1.80
0.195	0.105	0.103	0.105	24.02	21.65	24.02	23.23	1.36
0.391	0.115	0.107	0.11	35.83	26.38	29.92	30.71	4.77
0.781	0.129	0.126	0.127	52.36	48.82	50.00	50.39	1.80
1.563	0.153	0.156	0.158	80.71	84.25	86.61	83.86	2.97

Table 8. DPPH scavenging activity of positive control (BHT)

BHT(mg/ml)	**OD_{517} (reaction mixture)**			**DPPH scavenging (%)**				
	exp 1	**exp 2**	**exp 3**	**exp 1**	**exp 2**	**exp 3**	**Mean**	**SD**
0.0	0.083	0.086	0.085					
1.125	0.076	0.076	0.075	10.63	10.24	10.24	11.42	0.68
1.875	0.071	0.075	0.075	12.99	16.14	11.42	11.42	2.73
3.75	0.053	0.054	0.054	36.61	37.40	36.22	36.22	0.68
7.5	0.03	0.033	0.03	63.39	64.57	61.02	64.57	2.05
9	0.021	0.025	0.023	72.83	75.20	70.47	72.83	2.36

Table 9. The calculated FRAP value of *L. disjunctus* ethanolic extract, which calculated by using the equation from standard curve of furrous sulphate tripyridyl triazine

Ethanolic extracts of *L. disjunctus* (mg/ml)	**Absorbance at 593 nm**				**Ferrous sulphate equivalent (mM)**
	First	**Second**	**Third**	**$\bar{X}$± SD**	
25	0.580	0.656	0.717	0.651±0.0686	0.006
50	1.142	0.903	0.992	1.012±0.1208	0.023
100	1.560	1.559	1.656	1.592±0.0557	0.050

Table 10. The calculated FRAP value of ascorbic acid, which calculated by using the equation from standard curve of furrous sulphate tripyridyl triazine

Ascorbic acid concentration (mg/ml)	**Absorbance at 593 nm**				**Ferrous sulphate equivalent (mM)**
	First	**Second**	**Third**	**$\bar{X}$± SD**	
0.03125	0.559	0.56	0.585	0.568±0.01	0.003
0.0625	0.858	0.864	0.902	0.874±0.02	0.017
0.125	1.170	1.429	1.526	1.375±0.184	0.040
0.25	2.441	2.430	2.180	2.350±0.147	0.085

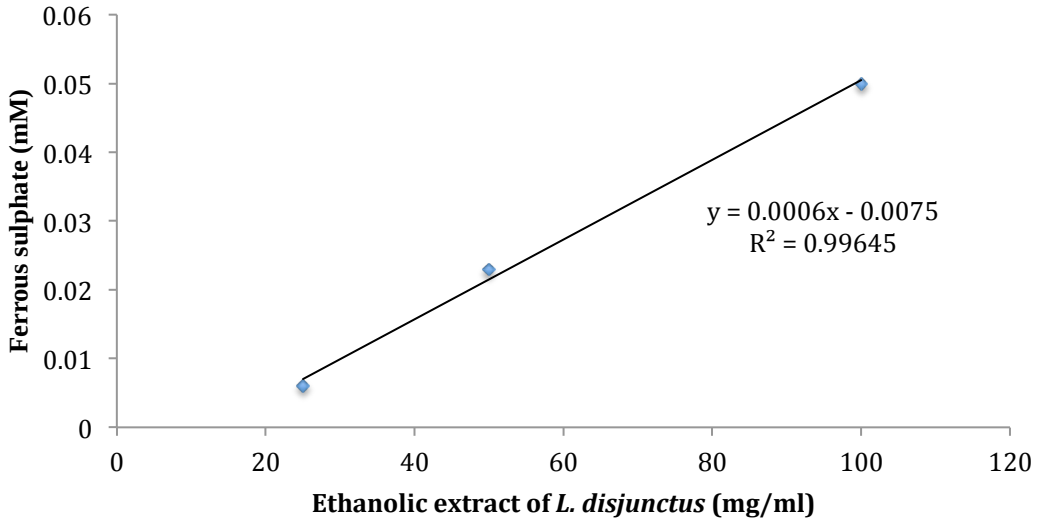


Figure 27. Ferrous sulphate equivalent of *L. disjunctus* whole plant

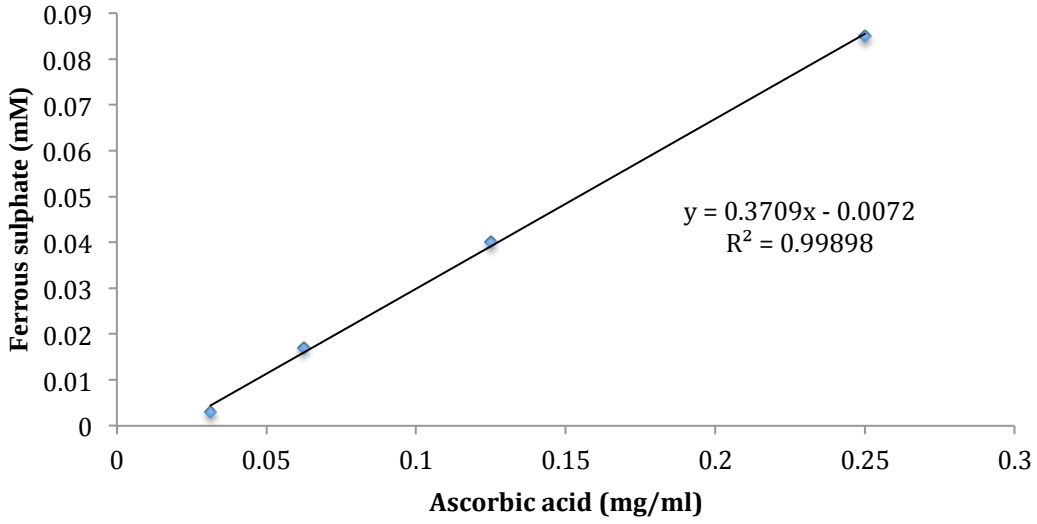


Figure 28. Ferrous sulphate equivalent of positive control (ascorbic acid)

Table 11. Nitric oxide radical inhibition activity of ethanolic extracts of *L. disjunctus*

Ethanolic extracts of *L. disjunctus* (mg/ml)	**OD_{450} (reaction mixture)**			**NO scavenging (%)**				
	exp 1	**exp 2**	**exp 3**	**exp 1**	**exp 2**	**exp 3**	**Mean**	**SD**
0.0	0.1384	0.1369	0.1335					
0.25	0.0893	0.0884	0.0882	34.47	35.13	35.27	34.47	0.43
0.5	0.0829	0.0821	0.0821	39.16	39.75	39.75	39.16	0.34
1	0.0754	0.075	0.0751	44.67	44.96	44.89	44.67	0.15
2	0.068	0.0685	0.0694	50.10	49.73	49.07	50.10	0.52
4	0.0622	0.0654	0.0645	54.35	52.01	52.67	54.35	1.21

Table 12. Nitric oxide radical inhibition activity of positive control (quercitin)

Quercitin(mg/ml)	**OD_{450} (reaction mixture)**			**NO scavenging (%)**				
	exp 1	**exp 2**	**exp 3**	**exp 1**	**exp 2**	**exp 3**	**Mean**	**SD**
0.0	0.3384	0.2999	0.2904					
0.078125	0.1915	0.1792	0.1841	38.14	42.11	40.53	40.26	2.00
0.15625	0.1738	0.1605	0.1529	43.86	48.15	50.61	47.54	3.42
0.3125	0.1489	0.1445	0.1418	51.90	53.32	54.19	53.14	1.16
0.625	0.1267	0.1265	0.1267	59.07	59.14	59.07	59.09	0.04

Table 13. β-carotene bleaching assay's absorbance of *L. disjunctus* ethanolic extract

***L. disjunctus* (mg/ml)**	**Time**	**OD_{470} (reaction mixture)**				
		exp 1	**exp 2**	**exp 3**	**Average**	**SD**
1	0	1.213	1.203	1.191	1.202	0.011
	30	1.019	0.999	1.002	1.007	0.011
	60	0.947	0.949	0.930	0.942	0.010
	90	0.893	0.885	0.893	0.890	0.005
	120	0.877	0.866	0.885	0.876	0.010
	150	0.853	0.857	0.858	0.856	0.003
	180	0.835	0.831	0.832	0.833	0.002
2	0	1.228	1.174	1.176	1.193	0.031
	30	1.031	1.031	1.029	1.030	0.001
	60	0.958	0.966	0.941	0.955	0.013
	90	0.902	0.905	0.903	0.903	0.002
	120	0.881	0.884	0.875	0.880	0.005
	150	0.856	0.861	0.865	0.861	0.005
	180	0.834	0.831	0.839	0.835	0.004
4	0	1.199	1.188	1.197	1.195	0.006
	30	1.076	1.047	1.056	1.060	0.015
	60	0.968	0.965	0.981	0.971	0.009
	90	0.929	0.935	0.923	0.929	0.006
	120	0.916	0.895	0.898	0.903	0.011
	150	0.865	0.858	0.871	0.865	0.007
	180	0.863	0.837	0.837	0.846	0.015
8	0	1.229	1.362	1.226	1.272	0.078
	30	1.078	1.071	1.090	1.080	0.010
	60	1.006	1.024	1.031	1.020	0.013
	90	0.977	0.991	0.971	0.980	0.010
	120	0.949	0.947	0.939	0.945	0.005
	150	0.887	0.915	0.897	0.900	0.014
	180	0.867	0.886	0.875	0.876	0.010
Negative control	0	1.228	1.302	1.205	1.245	0.051
	30	1.002	0.987	0.987	0.992	0.009
	60	0.911	0.925	0.942	0.926	0.016
	90	0.881	0.880	0.890	0.884	0.006
	120	0.842	0.851	0.849	0.847	0.005
	150	0.836	0.840	0.843	0.840	0.004
	180	0.830	0.834	0.834	0.833	0.002

Table 14. β-carotene bleaching assay's absorbance of BHT

BHT (mg/ml)	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
0.125	0	1.175	1.203	1.203	1.1937	0.0162
	30	1.093	1.095	1.094	1.0940	0.0010
	60	1.067	1.072	1.070	1.0697	0.0025
	90	1.073	1.031	1.064	1.0560	0.0221
	120	1.026	1.009	1.026	1.0203	0.0098
	150	1.019	0.999	1.010	1.0093	0.0100
	180	0.987	0.978	0.986	0.9837	0.0049
0.25	0	1.167	1.176	1.166	1.1697	0.0055
	30	1.128	1.139	1.129	1.1320	0.0061
	60	1.101	1.085	1.092	1.0927	0.0080
	90	1.084	1.079	1.088	1.0837	0.0045
	120	1.048	1.049	1.048	1.0483	0.0006
	150	1.029	1.033	1.030	1.0307	0.0021
	180	1.031	1.023	1.026	1.0267	0.0040
0.5	0	1.174	1.168	1.159	1.1670	0.0075
	30	1.133	1.139	1.131	1.1343	0.0042
	60	1.115	1.135	1.104	1.1180	0.0157
	90	1.094	1.113	1.093	1.1000	0.0113
	120	1.071	1.077	1.071	1.0730	0.0035
	150	1.056	1.053	1.047	1.0520	0.0046
	180	1.044	1.053	1.047	1.0480	0.0046
1	0	1.183	1.170	1.179	1.1773	0.0067
	30	1.167	1.158	1.167	1.1640	0.0052
	60	1.119	1.125	1.150	1.1313	0.0164
	90	1.115	1.106	1.113	1.1113	0.0047
	120	1.116	1.093	1.106	1.1050	0.0115
	150	1.073	1.082	1.090	1.0817	0.0085
	180	1.066	1.068	1.076	1.0700	0.0053
Negative control	0	1.228	1.302	1.205	1.2450	0.0507
	30	1.002	0.987	0.987	0.9920	0.0087
	60	0.911	0.925	0.942	0.9260	0.0155
	90	0.881	0.880	0.890	0.8837	0.0055
	120	0.842	0.851	0.849	0.8473	0.0047
	150	0.836	0.840	0.843	0.8397	0.0035
	180	0.830	0.834	0.834	0.8327	0.0023

Table 15. Alpha-glucosidase inhibition testing of 1-Deoxynojirimycin

1-Deoxynojirimycin (mg/ml)	OD_{504} (reaction mixture)			Alpha-glucosidaseinhibition(%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	1.055	1.041	1.015					
0.003125	0.907	0.947	0.99	4.53	8.68	12.54	8.58	4.01
0.00625	0.656	0.651	0.699	32.59	37.22	36.74	35.52	2.55
0.0125	0.349	0.36	0.393	62.1	65.28	66.35	64.58	2.21
0.025	0.197	0.195	0.197	81	81.2	81	81.07	0.12
0.05	0.099	0.101	0.104	89.97	90.26	90.45	90.23	0.24
0.1	0.055	0.056	0.052	94.99	94.6	94.7	94.76	0.20

Table 16. Alpha-glucosidase inhibition testing of ethanolic extracts of *L. disjunctus*

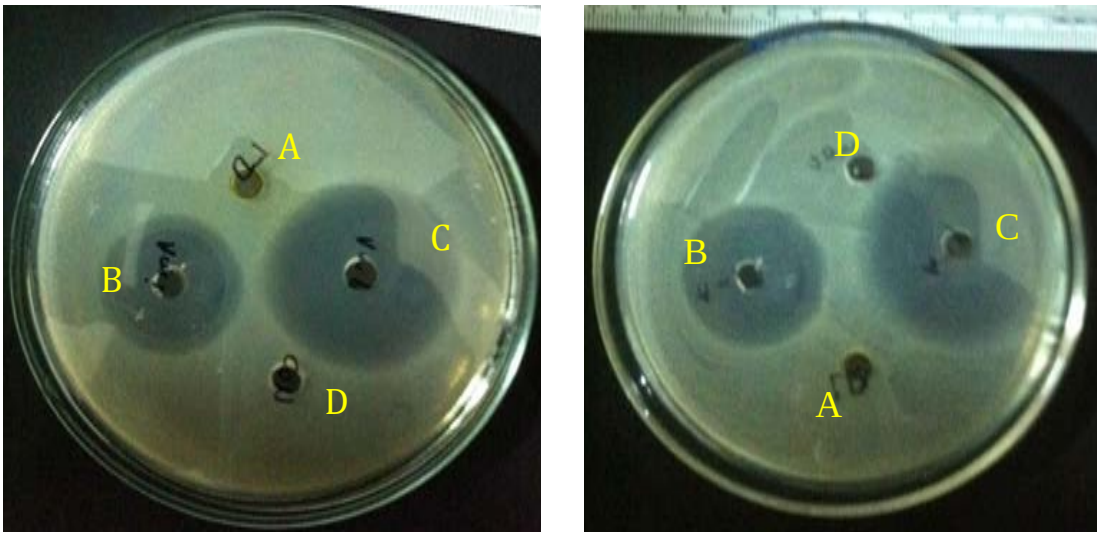
Ethanolic extracts of *L. disjunctus* (mg/ml)	OD_{504} (reaction mixture)			Alpha-glucosidase inhibition(%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	1.055	1.041	1.015					
0.78125	1.034	0.956	0.989	4.63	7.81	0.29	4.24	3.77
1.5625	0.928	0.936	0.939	9.45	9.74	10.51	9.90	0.55
3.125	0.763	0.771	0.784	24.4	25.65	26.42	25.49	1.02
6.25	0.504	0.506	0.519	51.4	51.21	49.95	50.85	0.79
12.5	0.256	0.185	0.105	89.87	82.16	75.31	82.45	7.28
25	-0.006	-0.011	-0.027	100.58	101.06	102.6	101.41	1.06
50	-0.02	-0.058	-0.058	101.93	105.59	105.59	104.37	2.11

Table 17. Tyrosinase inhibition testing of glutathione

Glutathione (mg/ml)	OD_{475} (reaction mixture)			Tyrosinaseinhibition(%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	0.137	0.138	0.138					
0.015625	0.074	0.079	0.076	46.30	42.67	44.85	44.61	1.83
0.03125	0.059	0.059	0.054	57.18	57.18	60.81	58.39	2.09
0.075	0.032	0.031	0.031	76.78	77.50	77.50	77.26	0.42
0.125	0.018	0.011	0.011	86.94	92.02	92.02	90.32	2.93
0.25	0	0	0	100.00	100.00	100.00	100.00	0.00

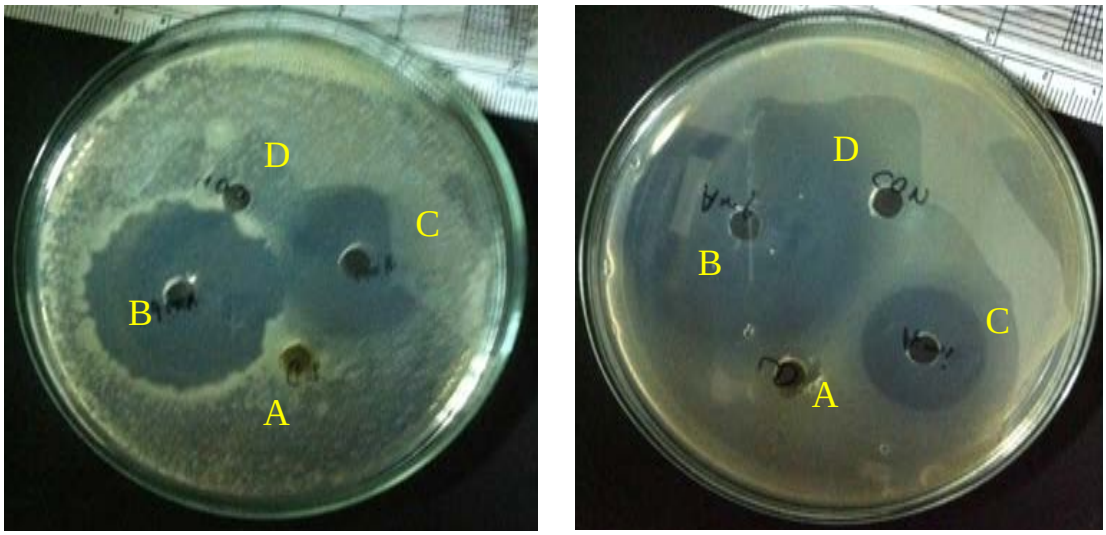
Table 18. Tyrosinase inhibition testing of ethanolic extracts of *L. disjunctus*

Ethanolic extracts of *L. disjunctus* (mg/ml)	OD_{475} (reaction mixture)			Tyrosinase inhibition(%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	0.137	0.138	0.138					
2.5	0.065	0.058	0.062	10.01	18.72	9.29	12.68	5.25
5	0.067	0.072	0.069	31.06	32.51	31.79	31.79	0.73
10	0.078	0.078	0.074	43.40	43.40	46.30	44.36	1.68
15	0.095	0.093	0.094	51.38	47.75	49.93	49.69	1.83
20	0.124	0.112	0.125	52.83	57.91	55.01	55.25	2.55
25	0.154	0.147	0.146	57.18	61.54	53.56	57.43	4.00



Shigella sp.
(Isolates)

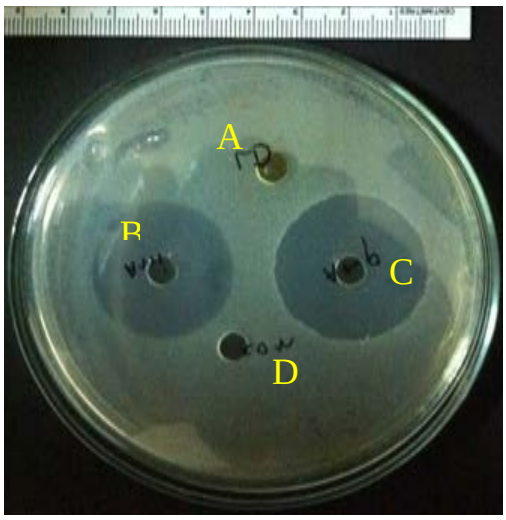
Salmonella typhimurium
(Isolates)



Bacillus cereus
ATCC 11778

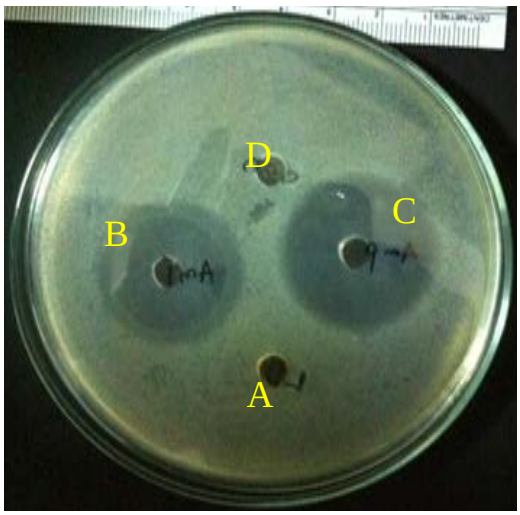
Staphylococcus aureus
ATCC 6538P

Figure 29. Inhibition zone of agar diffusion test among *L. disjunctus* ethanolic extract (A), amikacin (B), ampicillin (C) and negative control (D)



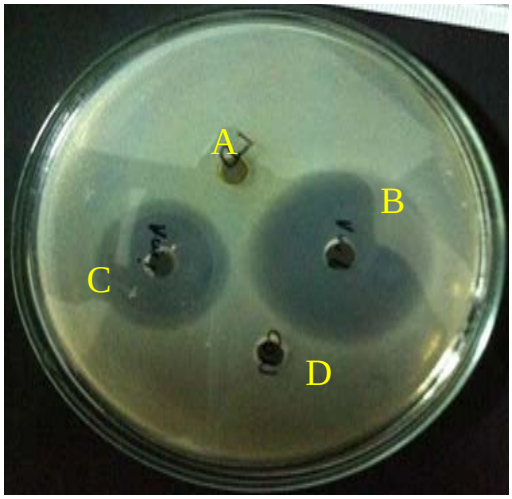
Straphylococcus epidermidis

(Isolates)



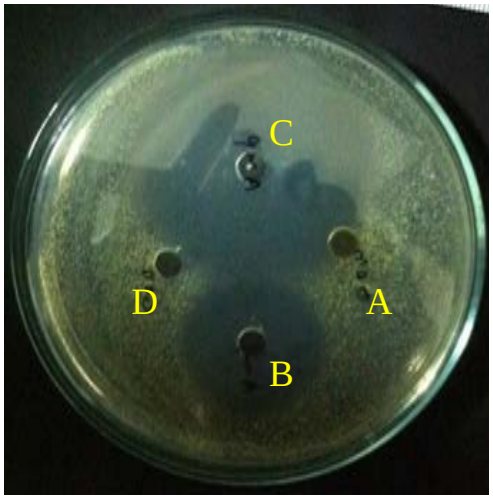
Escherichia coli

ATCC 25922



Salmonella typhi

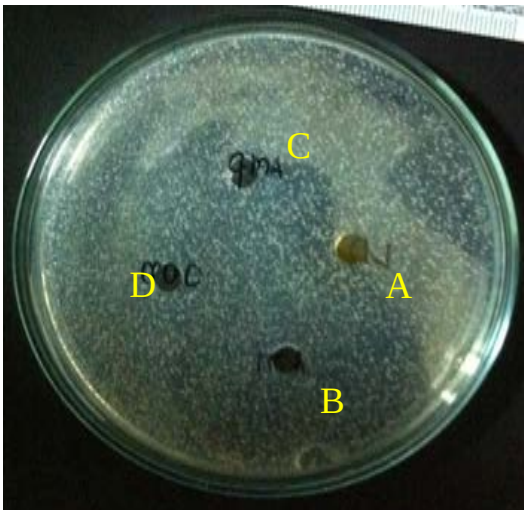
(Isolates)



Micrococcus luteus

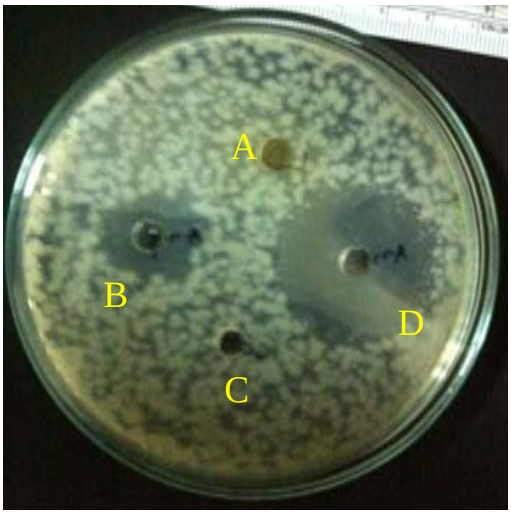
ATCC 9341

Figure 29. Inhibition zone of agar diffusion test among *L. disjunctus* ethanolic extract (A), amikacin (B), ampicillin (C) and negative control (D)



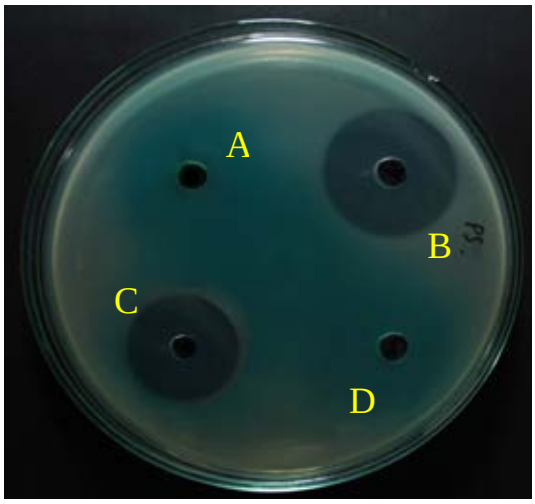
Candida albicans

ATCC 10230



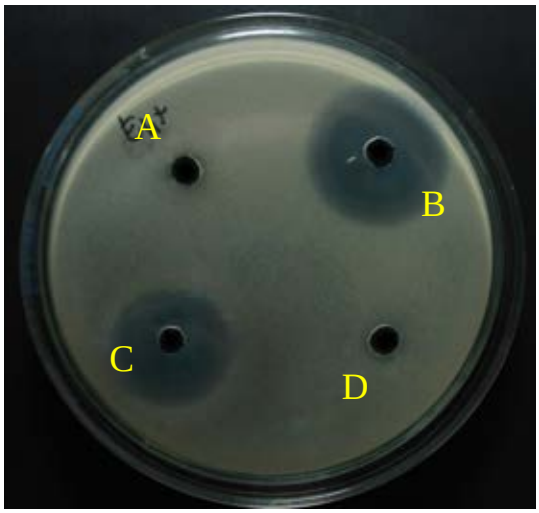
Bacillus subtilis

ATCC 6633



Pseudomonas aeruginosa

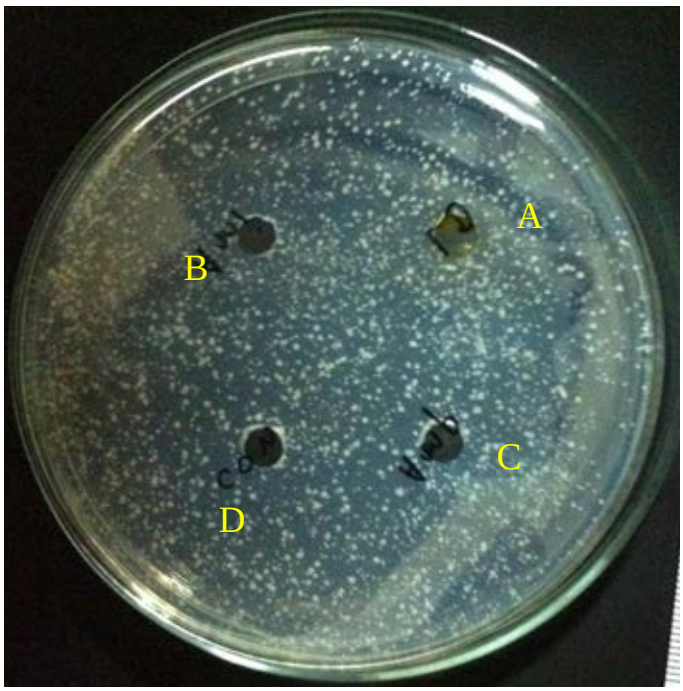
ATCC 9027



Enterobacter aerogenes

ATCC 13048

Figure 29. Inhibition zone of agar diffusion test among *L. disjunctus* ethanolic extract (A), amikacin (B), ampicillin (C) and negative control (D)



Saccharomyces cerevisiae

ATCC 9763

Figure 29. Inhibition zone of agar diffusion test among *L. disjunctus* ethanolic extract (A), amikacin (B), ampicillin (C) and negative control (D)

Table 19. Sleeping time interval of pentobarbitone induced sleeping time test

Dose	Time			Dose	Time			Dose	Time			Dose	Time		
2% Tween 80	Initial	End	Duration[f]	10 mg/kg	Initial	End	Duration[f]	50 mg/kg	Initial	End	Duration[f]	100 mg/kg	Initial	End	Duration[f]
1	10.40	14.17	3.77	**1**	12.04	31.39	19.35	**1**	0	0	0	**1**	7.3	56.19	48.89
2	10.14	35.17	25.03	**2**	8.47	15.21	6.74	**2**	8.33	45.38	37.05	**2**	7.35	45.19	37.84
3	10.09	27.12	17.03	**3**	10.46	20.05	9.59	**3**	8.45	42.12	33.67	**3**	8.35	26.45	18.1
4	10.20	36.43	26.23	**4**	11.43	34.13	22.7	**4**	8.35	38.36	30.01	**4**	0	0	0
5	16.45	21.32	4.87	**5**	10.45	20.25	9.8	**5**	7.06	26.25	19.19	**5**	10.08	40.58	30.5
6	11.12	15.34	4.22	**6**	7.17	42.11	34.94	**6**	7.23	29.01	21.78	**6**	8.55	46.18	37.63
7	8.27	14.45	6.18	**7**	8.21	39.21	31	**7**	11.48	21.31	9.83	**7**	9.5	27.45	17.95
8	7.37	17.08	9.71	**8**	7.29	39.2	31.91	**8**	7.04	45.57	38.53	**8**	6.4	47.03	40.63
9	18.26	23.18	4.92	**9**	8.36	17.19	8.83	**9**	12.43	37.35	24.92	**9**	8.08	28.14	20.06
10	9.25	15.02	5.77	**10**	7.34	53.18	45.84	**10**	9.02	22.37	13.35	**10**	7.54	22.3	14.76

*f is time between initial time and ending period(period of losing righting reflex)

VITAE

Mr. Watchara Damjuti was born on October31, 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. He attended to study Master of Science Program in Public Health Sciences in 2012 at College of Public Health Sciences, Chulalongkorn University, Thailand.

Publication

Damjuti, W., Towiwat, P., Palanuvej, C. and Ruangrungsi, N.*In vitro* and *In vivo* bioactivities of *Leptocarpus disjunctus* ethanolic extract.Proceeding of The 1st International Conference on Herbal Medicines Herbal Remedies: The Art of Science, pp. 55-64. Pathumthani, 2012