การเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสระหว่างสารสกัดที่แตกต่างกัน 3 ชนิดจากลำต้นชะเอมเหนือ

นางสาวศิริพร ริยะจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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COMPARISON OF ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY ACTIVITIES AMONG THREE DIFFERENT *DERRIS RETICULATA* CRAIB. STEM EXTRACTS

Siriporn Riyajan

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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COMPARISON OF ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY ACTIVITIES AMONG THREE DIFFERENT *DERRIS RETICULATA* CRAIB. STEM EXTRACTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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DERRIS RETICULATA CRAIB./PHYTOCHEMICAL SCREENING/ ANTIOXIDANT/CYTOPROTECTIVE/α-GLUCOSIDASE

The aqueous extract of *Derris reticulata Craib.* (Leguminosae) has been shown to exhibit an anti-diabetic activity both in *in vitro* study and in alloxan-induced diabetic rats. It was proposed that the anti-diabetic activity was due to its cytoprotective effect; probably by its antioxidant activity. Moreover, the activity of extract has been shown to inhibit the enzyme α-glucosidase. However, the aqueous extract used in the previous study contained many compounds; it was difficult to identify the active ingredients that are responsible for antioxidant and anti-diabetic actions. Therefore, the main objective of this study was to compare antioxidant and anti-diabetic activities of *D. reticulata* stem extract obtained by sequential extraction method with three different solvents; hexane, chloroform and water. The results from TLC showed that the compositions of the hexane extract were similar to those of the chloroform extract, but quite different from those of the aqueous extract. Phytochemical analysis revealed that all extracts consisted of triterpenoids, tannins and polyphenol whereas cardiac glycoside and anthraquinone were absent. Flavonoids and alkaloids were detected only in the hexane and chloroform extracts while saponin

was present only in the aqueous extract. The antioxidant activities were studied by DPPH, ABTS and FRAP methods. It was found that all three extracts of *D. reticulata* had *in vitro* antioxidant effects with different degrees of potency depending on the method used. The result from cytotoxicity demonstrated that the aqueous extract produced the least toxic effect to HepG2 cells since the highest dose up to 5,000 µg/ml did not cause significant cell damage compared to control. In contrast, at concentrations higher than 60 µg/ml, the hexane and chloroform extracts significantly reduced cell viability ($P<0.05$). It was found that only the aqueous extract with high doses (500 and 5,000 µg/ml) showed significant cytoprotective effects from 50.00 ± 3.48% (alloxan-treated group) to 62.41 ± 3.48% and 69.46 ± 3.77%, respectively. For the effect on α-glucosidase enzyme, the result showed that the chloroform extract produced highest degree of enzyme inhibition followed by the hexane extract. In the case of the aqueous extract, the inhibitory activity was 600 times less than the chloroform extract. Together with the results from TLC, it was suggested that the major constituent detected at the R_f = 0.854 may be responsible for α-glucosidase inhibitory activity of the hexane and chloroform extracts. However, further experiments, such as the isolation and identification of this compound as well as the examination of its antioxidant, α-glucosidase inhibitory and anti-diabetic activities, are needed for verification.

School of Pharmacology Student's Signature _____

Academic Year 2014 Advisor' Signature _____

ศิริพร ริยะจันทร์ : การเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งเอนไซม์แอลฟา กลูโคซิเดสระหว่างสารสกัดที่แตกต่างกัน 3 ชนิดจากลำต้นชะเอมเหนือ (COMPARISON OF ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY ACTIVITIES AMONG THREE DIFFERENT *DERRIS RETICULATA* CRAIB. STEM EXTRACTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 94 หน้า.

มีรายงานว่าสารสกัดด้วยน้ำของต้นชะเอมเหนือ (*Derris reticulata*, วงศ์ Leguminosae) มีฤทธิ์ต้านเบาหวานจากการศึกษาทั้งในหลอดทดลอง และหนูขาวที่ถูกชักนำให้เกิดเบาหวานด้วยสาร alloxan ผลการทดลองชี้แนะว่าฤทธิ์ในการต้านเบาหวานดังกล่าวเป็นผลจากฤทธิ์ปกป้องเซลล์ ซึ่งน่าจะเนื่องมาจากฤทธิ์ต้านอนุมูลอิสระของสารสกัด นอกจากนี้ยังพบอีกว่าสารสกัดมีฤทธิ์ในการต้านเบาหวานผ่านทางกลไกการยับยั้งเอนไซม์กลูโคซิเดส อย่างไรก็ตามสารสกัดด้วยน้ำที่ใช้ในการศึกษาก่อนหน้านี้ประกอบด้วยสารมากมายซึ่งยากที่จะหาสารประกอบที่เป็นตัวออกฤทธิ์ในการต้านอนุมูลอิสระและต้านเบาหวาน ดังนั้น การศึกษานี้จึงมีวัตถุประสงค์หลักเพื่อเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านเบาหวานของสารสกัดจากต้นชะเอมเหนือที่ได้จากการสกัดเป็นลำดับขั้นด้วยตัวทำละลายที่แตกต่างกัน 3 ชนิด คือ เฮกเซน คลอโรฟอร์มและน้ำ ผลจากการทำ TLC เผยให้เห็นว่า สารประกอบในสารสกัดเฮกเซนและคลอโรฟอร์มมีความคล้ายคลึงกันแต่ค่อนข้างแตกต่างจากสารสกัดด้วยน้ำ ผลการทดสอบพฤษเคมีเบื้องต้นได้แสดงให้เห็นว่าทุกสารสกัดมีส่วนประกอบที่เป็น ไตรเทอร์ฟีนอยด์ แทนนิน และโฟลีฟีนอล แต่ไม่พบสารกลุ่มคาร์ดิแอค-ไกลโคไซด์และแอนทราควิโนน สำหรับฟลาโวนอยด์และแอลคาลอยด์ถูกพบเฉพาะในสารสกัดเฮกเซนและคลอโรฟอร์ม ฤทธิ์ต้านอนุมูลอิสระของสารสกัดถูกตรวจสอบโดยวิธี ABTS, DPPH และ FRAP ผลการทดสอบชี้ว่า สารสกัดทั้งสามของลำต้นชะเอมเหนือมีฤทธิ์ต้านอนุมูลอิสระที่แตกต่างกันออกไปขึ้นอยู่กับวิธีที่ใช้ในการทดสอบ ผลจากการทดสอบความเป็นพิษแสดงให้เห็นว่าสารสกัดด้วยน้ำมีความเป็นพิษต่อเซลล์ตับน้อยที่สุด เนื่องจากความเข้มข้นที่สูงถึง 5,000 μg/ml ไม่ทำให้เกิดความเสียหายต่อเซลล์อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม ในทางตรงกันข้ามพบว่าสารสกัดเฮกเซนและคลอโรฟอร์มที่ความเข้มข้นมากกว่า 60 μg/ml ลดการรอดตายของเซลล์อย่างมีนัยสำคัญ และพบต่อไปอีกว่ามีเพียงสารสกัดด้วยน้ำที่ความเข้มข้นสูงที่ 500 และ 5,000 μg/ml เท่านั้นที่มีฤทธิ์ปกป้องเซลล์ได้อย่างมีนัยสำคัญ คือเพิ่มการรอดตายของเซลล์จาก 50.00 ± 3.48% ไปเป็น 62.41 ± 3.48% และ 69.46 ± 3.77% ตามลำดับ สำหรับการศึกษาฤทธิ์ต่อเอนไซม์แอลฟา-กลูโคซิเดสพบว่าสารสกัดคลอโรฟอร์มมีฤทธิ์ในการยับยั้งเอนไซม์สูงที่สุดตามด้วยสารสกัด

เฮกเซน ในส่วนของสารสกัดด้วยน้ำพบว่ามีฤทธิ์ในการยับยั้งเอนไซม์ต่ำกว่าสารสกัดคลอโรฟอร์ม ถึง 600 เท่า เมื่อนำผลการทดลองจาก TLC มาพิจารณาร่วมกัน พบว่าเป็นไปได้ที่สารประกอบหลักที่ R_f = 0.854 บนแผ่น TLC อาจจะมีความเกี่ยวข้องกับฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดสของสารสกัดเฮกเซนและคลอโรฟอร์ม อย่างไรก็ตามสำหรับข้อเสนอแนะนี้ยังต้องมีการทดลองเพิ่มเติม เช่น การแยกสารสกัดให้บริสุทธิ์และระบุชนิดของสารนี้ รวมไปถึงการทดสอบฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดสและฤทธิ์ต้านเบาหวาน เพื่อยืนยันความถูกต้องต่อไป

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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Siriporn Riyajan

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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µg	=	Microgram
µl	=	Microliter
ABTS	=	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
BHT	=	Butylated hydroxytoluene
^{o}C	=	Degree Celsius
CAT	=	Catalase
cm	=	Centimeter
DI	=	Distilled water
DPPH	=	2,2-diphenyl-l-picrylhydrazyl
DMEM	=	Dulbecco’s modified eagle’s medium
DMSO	=	Dimethyl sulphoxide
ECD	=	Endothelium cell dysfunction
ESRD	=	End-stage renal diseases
$FeCl_3$	=	Ferric chloride
$FeSO_4$	=	Ferrous sulfate
FRAP	=	Ferric reducing antioxidant power
g/ml	=	Gram per milliliter
GAE	=	Gallic acid equivalents
GPx	=	Glutathione peroxidase

LIST OF ABBREVIATIONS (Continued)

h	=	Hour
HCl	=	Hydrochloric acid
HepG2	=	Human hepatocarcinoma cell
$HgCl_2$	=	Murcuric chloride
H_2O_2	=	Hydrogen peroxide
H_2SO_4	=	Sulphuric acid
$^{\bullet}OH$	=	Hydroxyl radical
IC_{50}	=	Inhibitory concentration at 50%
KI	=	Potassium iodide
$K_2S_2O_8$	=	Potassium persulphate
M	=	Molar
mg/dL	=	Milligram per deciliter
mg/kg	=	Milligram per kilogram
mg/ml	=	Milligram per milliliter
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	=	Normal
Na_2CO_3	=	Sodium carbonate
NaOH	=	Sodium hydroxide

LIST OF ABBREVIATIONS (Continued)

Nm	=	Nanometer
$^1O_2$	=	Singlet oxygen
OD	=	Optical density
PBS	=	Phosphate buffer saline
PNP-G	=	p-nitrophenyl-α-D-glucopyranoside
PDGF	=	Platelet derived growth factor
R_f	=	Retention factor
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SOD	=	Superoxide dismutase
TGF-β	=	Transforming growth factor-β
TLC	=	Thin layer chromatography
TPTZ	=	2,4,6-tripyridyl-*S* -triazine
U	=	Unit activity
UV	=	Ultraviolet
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1. Rationale

Nowadays, advanced technology brings about the knowledge and understanding in human diseases, especially diabetes mellitus. Diabetes mellitus is not only a major public health problem worldwide but also causes 5% of all deaths globally each year (Joshi and Shrestha, 2010). Moreover, the prevalence of this disease for all age groups has been predicted to be double in 2020 (Shaw, Sicree, and Zimmet, 2010). Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from reduction of insulin secretion and/or insulin resistance. Eventually, the main cause of death for diabetic patients does not come from the disease itself. The patients with diabetes mostly die from various complications for example cardiovascular diseases, hypertension and nephropathy (American Diabetes Association, 2012). It has been suggested that oxidative stress is partly involved in these complications (Dominique, 2002; Mshelia, 2004).

Oxidative stress is the condition that the body has less amount of antioxidant compared to the amount of free radicals. Free radicals are unstable molecules with unpaired electrons in their outer orbits. They are reactive molecules generated by cellular processes and environmental stress such as immune system, electron transfer, cellular respiration, chemical substances and UV radiation. The excess of free radicals damages DNAs, proteins and lipids leading to cellular dysfunction, cellular injury

and cell death (Kannan and Jain, 2000). Normally, the free radicals mentioned above can be removed from the body by antioxidant pathways, either enzymatic or non-enzymatic systems, such as superoxide dismutase, catalase, glutathione peroxidase, and thiol antioxidant. Moreover, the excess free radicals can also be destroyed by external antioxidants such as supplements containing vitamin C, flavonoids and carotenoids, which can be found in plants, vegetables and fruits (Valko et al., 2007). Therefore, natural products from plants have been used as alternative medicines for the treatment of many diseases. Moreover, it has been accepted that they usually cause fewer side effects than synthetic chemicals (Blasa, Gennari, Angelino, and Ninfali, 2010).

Derris reticulata Craib. is a medicinal plant in the Leguminosae family. This climbing plant is known in Thai as Cha-em-nuea. It has long been traditionally used as expectorant to relieve cough. Moreover, anti-inflammatory activity in arthritis and anti-herpes simplex virus type I (Laupattarakasem, Houghton, Hoult, and Itharat, 2003; Wisetsutthichai, Techamahamaneerat, Junyaprasert, and Soonthornchareonnon, 2005) have been reported. Recently, the aqueous extract of *D. reticulata* has been shown to exhibit an anti-diabetic activity by both *in vitro* and *in vivo* studies (Kumkrai, Kamonwannasit, and Chudapongse, 2014). It was proposed that the anti-diabetic activity of *D. reticulata* was partly due to its cytoprotective effect, probably by an antioxidant activity (Kumkrai, Weeranantanapan, and Chudapongse, 2015). However, the aqueous extract used in the previous study contained many compounds; it was difficult to identify the active ingredients that are responsible for antioxidant and anti-diabetic activities. Therefore, in the present study *D. reticulata* stem was successively extracted with different solvents in the increasing polarity order (hexane,

chloroform and water). Phytochemical screening test and the examination of their compositions and antioxidant activities were performed. Moreover, the cytoprotective effects of the extracts on human hepatoma HepG2 cells were evaluated. Finally, the inhibitory activities against α-glucosidase enzyme, which is one of the known target molecules of anti-diabetic agents, were conducted and compared. The results from this study will provide useful information for the future investigation to identify active ingredients that are responsible for anti-diabetic activity of *D. reticulata.*

2. Research objectives

2.1 To compare the phytochemical compositions and phenolic contents among hexane, chloroform and aqueous extracts of *D. reticulata* stem.

2.2 To compare antioxidant activities and cytoprotective effects of the extracts.

2.3 To compare α-glucosidase inhibitory activities of the extracts.

3. Research hypothesis

The three extracts possess different degrees of antioxidant activity, cytoprotective potential and α-glucosidase inhibitory effect. The hexane extract shows the highest degree of all activities.

4. Scope and limitation of study

All of the pharmacological activities in the present study were evaluated only by *in vitro* experiments.

CHAPTER II

LITERATURE REVIEW

2.1 *Derris reticulata* Craib.

Several plants in *Derris* genus, which belong to the Leguminosae family and are distributed in the tropical regions of Asia and East Africa, are known to be used as traditional medicines (Tewtrakul, Cheenpracha, and Karalai, 2009). Some of *Derris* plants have been used as fish poison and insecticide (Luitgards-Moura, Castellón Bermudez, Rocha, Tsouris, and Rosa-Freitas, 2002; Hu et al., 2005; Kongjinda, 2004). However, many pharmacological activities of the plants in *Derris* genus have also been documented. For example, they have been used for the treatment of rheumatism and nociceptive (Laupattarakasem et al., 2003; Srimongkol et al., 2007). Some species in this genus plants show expectorant activity, therefore they are used to relieve cough (Siri Ruckhachati Nature Park, 2010). Moreover, antimicrobial activities of *Derris* plants have been cumulatively reported as well. Microbes in 25 species have been reported to susceptible to the extracts from *D. elliptica*, *D. indica* and *D. trifoliate* (Khan, Omoloso, and Barewai, 2006). The ethyl acetate extract of *D. indica* shows an inhibitory effect on growth of *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* (Sittiwet and Puangpronpitag, 2009). In addition, anti-inflammatory activity of *D. scanaens* (Laupattarakasem et al., 2003), and intestinal α-glycosidase inhibitory activity of *D. indica* (Rao et al., 2009) have been established. Not long ago, the antidiarrheal activity of *D. trifoliate* Lour has been shown and its active ingredients

have been identified (Mamoon and Azam, 2012). The biological actions of *Derris* plants are listed in Table 2.1.

Table 2.1 Biological activities of *Derris* plants.

Sources	**Part of use**	**Ethnomedical activity**	**References**
D. amazonica	Root	Fish poison Insecticide	Luitgards-Moura et al., 2002
D. elliptica	Root	Insecticide Antimicrobial	Hu et al., 2005 Khan et al., 2006
D. scandens	Stem Root	Rheumatism Anti-inflammatory activity Analgesic	Laupattarakasem et al., 2003 Srimongkol et al., 2007
D. spruceana	Leaf	Fish poison	Kongjinda, 2004
D. reticulata	Stem bark Stem Leaf	Anti-coughing Expectorant Anti-diabetic Intestinal α-glucosidase inhibition Antioxidant Cytotoxicity Anti-inflammation Anti-herpes simplex virus type 1	Siri Ruckhachati Nature Park, 2010 Laupattarakasem et al., 2003 Wisetsutthichai et al., 2005 Kumkrai et al., 2014
D. indica	Stem Root Leaf	Antimicrobial Intestinal α-glucosidase inhibitory	Khan et al., 2006
D. trifoliate	Stem Root Leaf	Antimicrobial Antidiarrheal	Khan et al., 2006

D. reticulata (Figure 2.1), which is known in Thai as Cha-aim-nuea or Oi-sam-suan, has been traditionally used as an anti-coughing and anti-inflammatory agents (Siri Ruckhachati Nature Park, 2010; Laupattarakasem et al., 2003). It has also been demonstrated that the extract from *D. reticulata* possesses an antiviral against herpes simplex virus type 1 (Laupattarakasem et al., 2003; Wisetsutthichai et al., 2005).

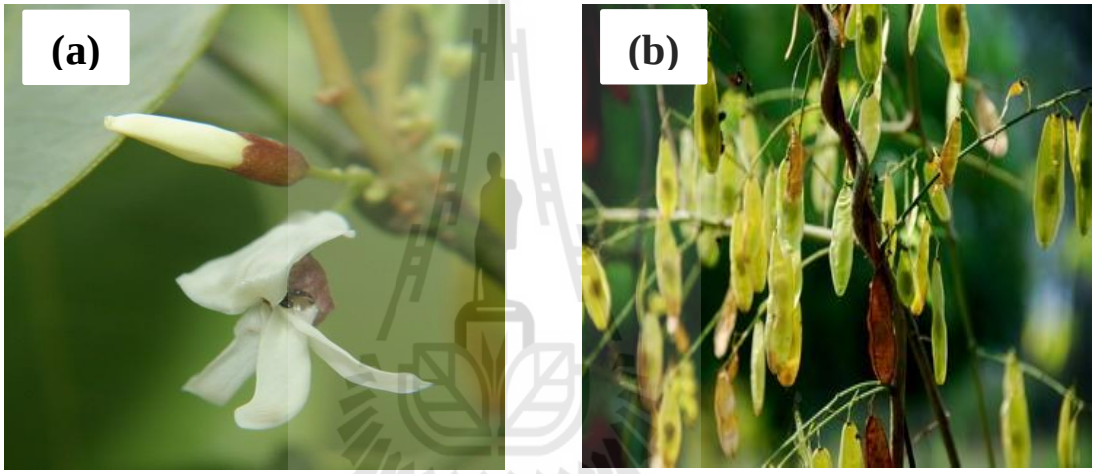


Figure 2.1 Flowers (a) and seed pods (b) of *Derris reticulata* Craib. (Siri Ruckhachati Nature Park, 2010).

Medicinal plants contain some organic compounds which provide definite physiological or pharmacological actions on the human body such as antioxidant activity, antimicrobial effect, stimulation of the immune system and anticancer property (Saxena, Saxena, Nema, Singh, and Gupta, 2013) as listed in Table 2.2. These bioactive substances include alkaloids, flavonoids, saponins, tannins, triterpenoids, cardiac glycosides, anthraquinones, and polyphenols (Dillard and

German, 2000). From phytochemical screening study, the stem of *D. reticulata* consists of terpenoids, flavonoids, saponin and tannins (Kumkrai et al., 2014).

Table 2.2 Examples of the bioactive phytochemicals in plants (Saxena et al., 2013).

Classification	**Main groups of compounds**	**Biological functions**
NSA (non-starchpoly saccharides)	Cellulose Hemicellulose Gums Mucilages Pectins Lignins	Water holding capacity Delay in nutrient absorption Binding toxins and bile acids
Antibacterial and antifungal	Terpenoids Alkaloids Phenolics	Inhibotors of micro-oraganisms Reduced the risk of fungal Infection
Antioxidants	Polyphenolic compounds Flavonoids Carotenoids Tocopherols Ascorbic acid Polyphenol	Oxygen free radical quenching Inhibition of lipid peroxidation
Anticancer	Carotenoids Polyphenols Flavonoids	Inhibitors of tumor Inhibited development of lung cancer Anti-metastatic activity
Detoxifying agents	Reductive acids Tocopherols Phenols Indoles Aromatic isothiocyanates Coumsrins Flavones Carotenoids Retinoids	Inhibitors of procarcinogen activation Inducers of drug binging of carcinogens Inhibitors of tumourogenesis

Flavonoids have been demonstrated to possess a variety of biological activities including antibacterial, antiviral, antioxidant, antiulcerogenic, antineoplastic, antihepatotoxic, anti-inflammatory activities and cytotoxicity (Cushnie and Lamb, 2005; Pengelly, 2004). Lupinifolin is a prenylated flavanone which was found to be a major compound in stem and stem bark of *D. reticulata* Benth (Mahidol et al., 1997). However, it can be isolated from other medicinal plants, such as *Myriopteron extensum* (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004), *Eriosema chinense* (Prasad et al., 2013) and *Albizia myriophylla* (Joycharat et al., 2013). Lupinifolin is a compound of whose characteristics is less soluble in water, but easily soluble in methanol, ethanol, trichoromethane, and other organic solvents. It has been shown to exhibit antimicrobial activities against several pathogens such as herpes simplex virus type 1 (Soonthornchareonnon et al., 2004), *Mycobacterium tuberculosis* (Sutthivaiyakit et al., 2009), *Bacillus cereus*, *Corynebacterium diphtheria* and *S. epidermidis* (Soonthornchareonnon et al., 2004; Sutthivaiyakit et al., 2009).

Recently, the aqueous extract of *D. reticulata* has been reported to possess anti-diabetic activity. This therapeutic potential has been studied both *in vitro* and *in vivo* (Kumkrai et al., 2014). Moreover, Kumkrai and co-workers have demonstrated that the mechanisms underlying the anti-diabetic activity of *D. reticulata* aqueous extract are the combination of cytoprotective effect, suppression of intestinal glucose absorption and inhibition of α-glucosidase activity. However, the active ingredients have not yet been identified. Further studies should include the isolation and identification of the active chemical(s) responsible for the protective effect and α-glucosidase activity of *D. reticulata* extract.

2.2 Free radicals and antioxidant

Free radicals are unstable molecules with unpaired electrons in their outer orbits. These free radicals can withdraw electron from other molecules. They are reactive molecules generated by cell processes and environmental stress such as immune system, electron transfer, cellular respiration, chemical substances and UV radiation. The free radicals can damage DNAs, proteins and lipids leading to cellular dysfunction, cell injury and cell death. Excessive free radicals cause inflammation, cancer, premature aging disorder, atherosclerosis and diabetes (Kannan and Jain, 2000).

Among various free radicals, reactive oxygen species (ROS) is the most important class generated in living systems. ROS can be classified into two categories as follows: free oxygen radicals and non-radical ROS for example hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical ($OH^{\cdot}$).

Superoxide (O_2^-) is the primary ROS occurred from electron transport chain in mitochondria of the cell. Moreover, O_2^- can further interact with other molecules to generate secondary ROS. For example, the O_2^- is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidase and xanthine oxidase. Then, the O_2^- is dismutated by superoxide dismutase to H_2O_2. Finally, the H_2O_2 can split to $OH^{\cdot}$ by Fenton reaction (Figure 2.2).

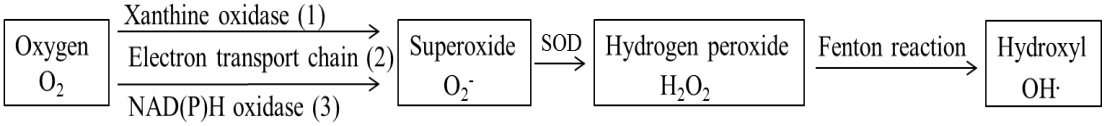


Figure 2.2 The pathways of ROS formation.

The free radicals mentioned above can be removed from the body by antioxidant pathways including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as shown in Figure 2.3. In addition, the excess free radicals can be destroyed by external antioxidants such as supplements containing vitamin C, flavonoids and carotenoids, which can be found in plants, vegetables and fruits (Valko et al., 2007).

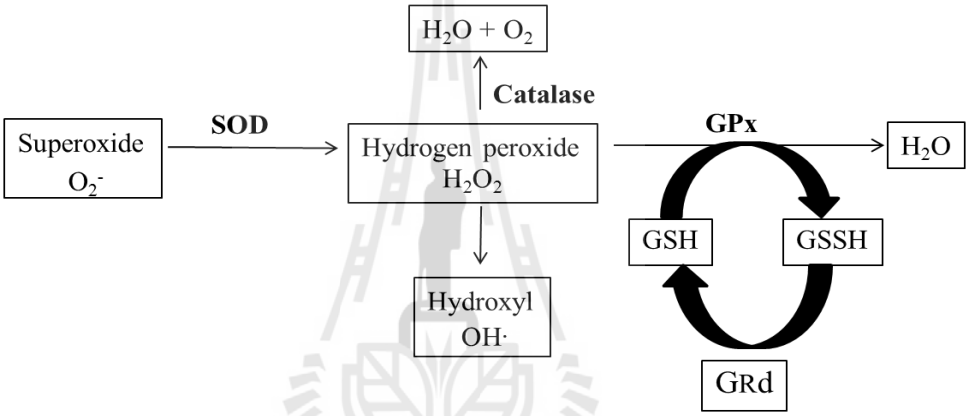


Figure 2.3 The pathways of antioxidant in scavenging free radicals.

2.3 Diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from reduction of insulin secretion and/or insulin resistance. Insulin is an important hormone involved in the regulation of the blood glucose level by stimulating removal of glucose from the blood as shown in Figure 2.4 (Yeo and Sawdon, 2013). If the insulin cannot function efficiently, the glucose cannot be used as a primary source of energy. Therefore, the body shifts to use fat and protein as an energy source (American Diabetes Association, 2012).

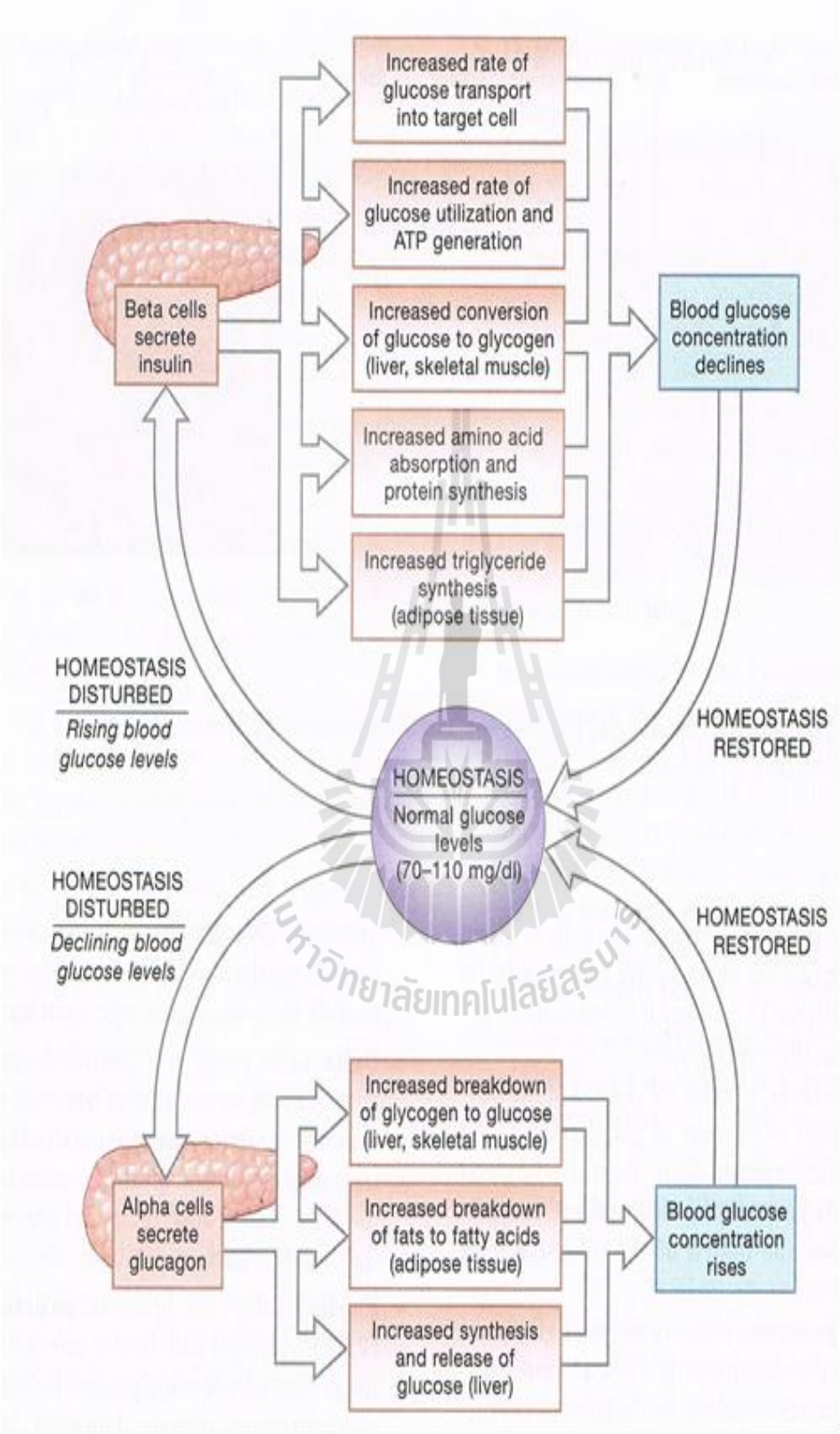


Figure 2.4 The role of insulin in control of blood glucose (Martini, Ober, Garrison, Welch, and Hutchings, 2006).

2.3.1 Symptoms of diabetes mellitus

Diabetes results in metabolic imbalance of carbohydrate, lipid and protein. The fasting plasma sugar more than 126 mg/dL or more than 200 mg/dL for glucose tolerance test are considered as diabetes (Olokoba, Obateru, and Olokoba, 2012). Moreover, most of diabetes symptoms of patient are polyuria, polydipsia and polyphagia including glucosuria (American Diabetes Association, 2012). The symptoms of diabetes can be explained as follows:

1) Due to the level of blood sugar being higher than the capability of the kidneys to reabsorb; therefore the excess blood sugar is excreted to the urine. The presence of blood sugar in urine increases the urine quantity because of the ability of glucose molecules to absorb the water. This condition is called glucosuria.

2) Polyuria or increased urination frequently in a diabetic patient leads to body dehydration and eventually to polydipsia or increased thirst.

3) The polyphagia or excessive appetite of the diabetes patient results from the failure of glucose utilization, leading to more consumption in order to take more energy from the other sources.

2.3.2 Types of diabetes mellitus

Diabetes mellitus can be classified into 2 types (Table 2.3) as follows: type 1 diabetes (insulin dependent diabetes mellitus) and type 2 diabetes (non-insulin dependent diabetes mellitus). Diabetes type 1 is caused from beta-cell damage. The damage of beta cells makes insulin production decrease. Therefore, the diabetes patients need insulin injections to acquire the normal blood sugar level. Diabetes type 2 is caused from insulin resistance, which occurs when cells fail to respond to the

normal actions of the hormone insulin. This may be caused by genetics, aging or obesity.

Table 2.3 The two major types of diabetes mellitus.

	Type 1 diabetes (insulin dependent diabetes mellitus)	**Type 2 diabetes (non-insulin dependent diabetes mellitus)**
Cause of disease	Destruction of β-cells	Reduction of insulin secretion and/or insulin resistance
Prevalence of disease	About 5-10%	About 90-95%
Age	< 20 year old	>20 year old
Symptom	Hyperglycemia	Hyperglycemia
Treatment	Insulin	Insulin secretagogues Metformin α-Glucosidase inhibitors, etc.

2.3.3 Diabetic complications

An abnormality of blood glucose concentration can lead to many health problems and subsequently to death (Yeo and Sawdon, 2013). The main cause of death for diabetes patients does not come from the disease itself. Patients with diabetes mostly die from various complications for example cardiovascular diseases, hypertension and nephropathy (American Diabetes Association, 2012). However, oxidative stress is partly involved in these complications as shown in Figure 2.5 (Dominique, 2002; Mshelia, 2004).

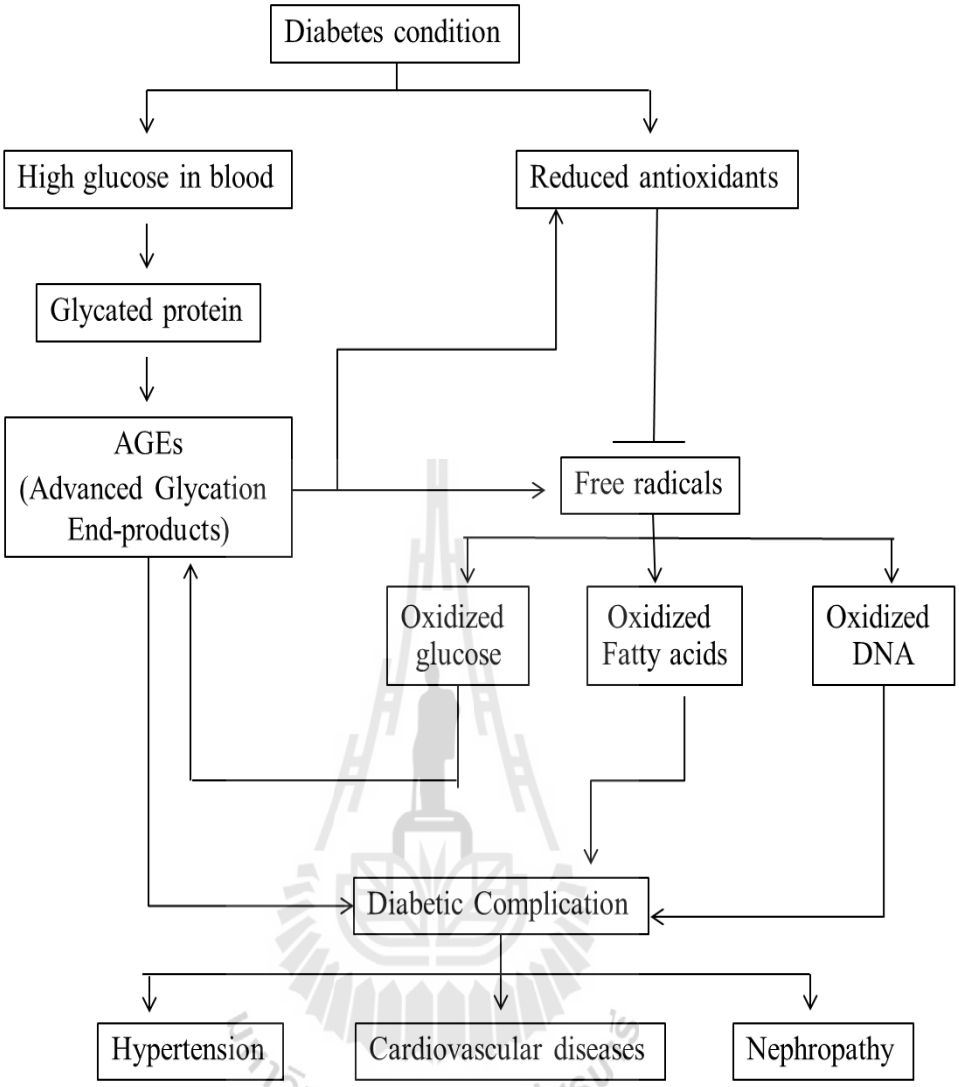


Figure 2.5 The relationship of free radicals and diabetic complications.

2.3.4 Glucose and formation of free radical

Previous studies showed the relationship between the development of diabetes and the level of oxidative stress in diabetic patients (Mshelia, 2004). The increasing sugar level causes an elevation of oxidant level and/or decreases the function of antioxidants (Dominique, 2002), resulting in an oxidative stress. The formation of free radicals in the body has also been found in different mechanisms, such as glucose oxidation, non-enzymatic glycation of proteins, polyol pathway,

hexosamine pathway and electron transport chain (Bierhaus, Hofmann, Ziegler, and Nawroth, 1998; Dominique, 2002), as shown in Figure 2.6-2.8.

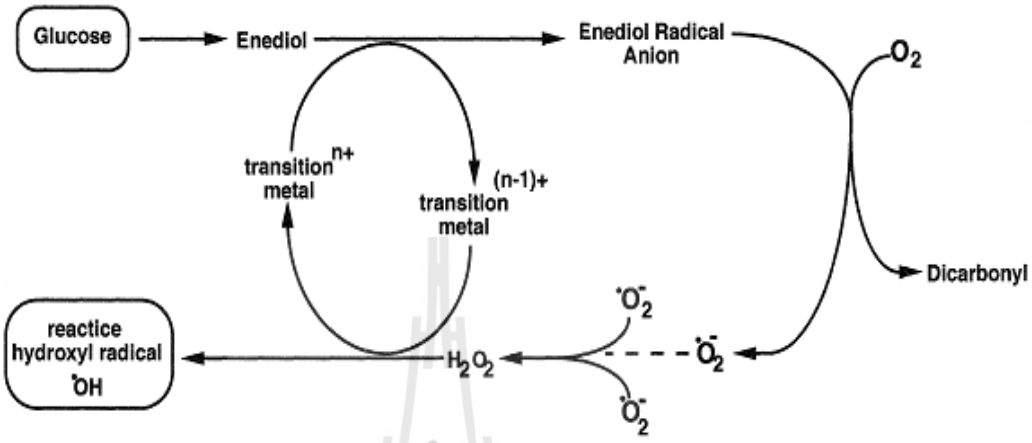


Figure 2.6 The formation of ROS under metal catalyzed autoxidation of glucose (Bierhaus et al., 1998).

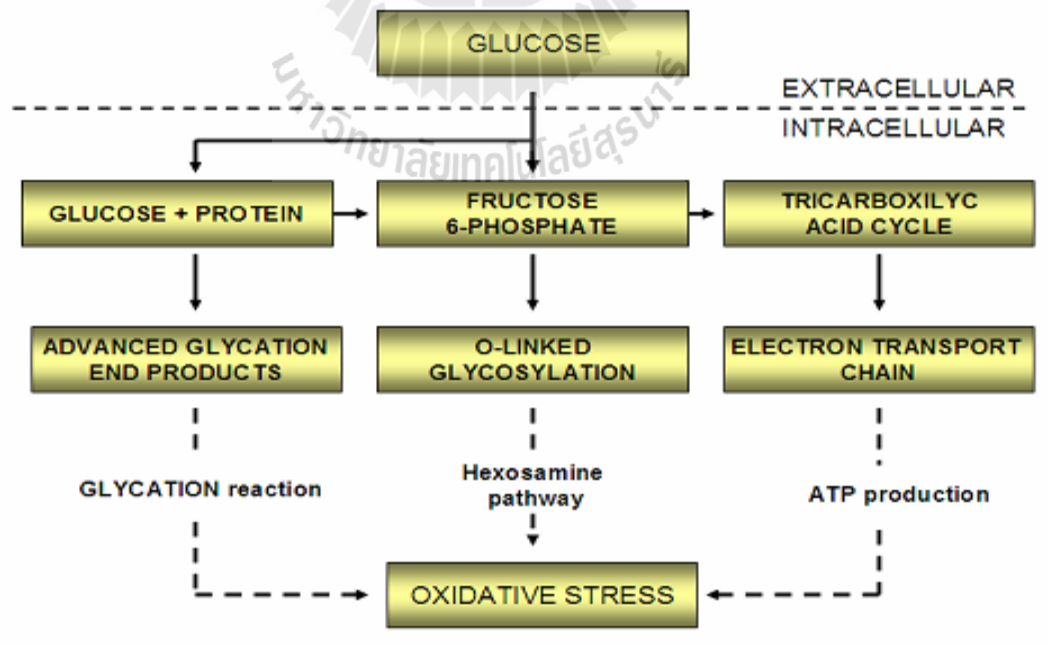


Figure 2.7 Sources of ROS under the influence of hyperglycemia (Radoi, Lixandru, Mohora, and Virgolici, 2012).

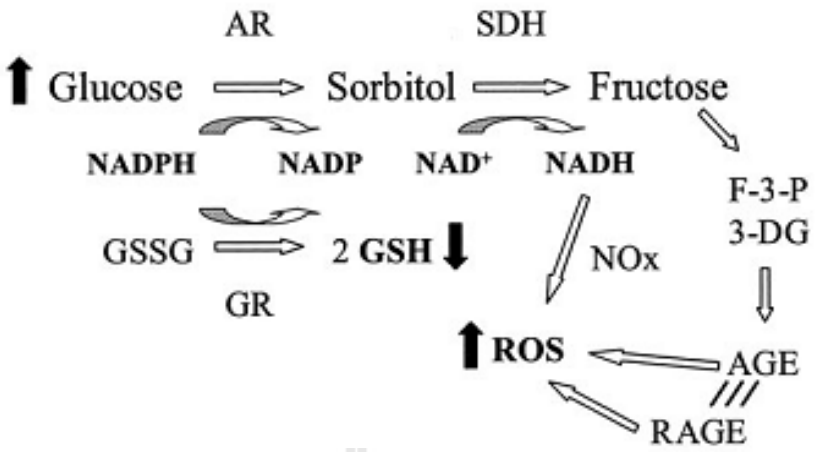


Figure 2.8 Polyol pathway-induced oxidative stress. The polyol pathway decreases function of antioxidant via NADPH and produces free radical (Chung, Ho, Lam, and Chung, 2003).

2.4 Free radical on nephropathy

Diabetic nephropathy is one of the complications which are causes of death for diabetic patients. The pathophysiology of diabetic nephropathy starts with the endothelial cell dysfunction (ECD) and ends with renal failure (Mshelia, 2004). High glucose can directly cause ECD or indirectly increase oxidative stress in glomerular mesangial cells which is a target cell in diabetic nephropathy (Figure 2.9). As shown in Figure 2.10, oxidative stress induces mRNA expression of NFkB gene which in turn promotes production of proinflammatory such as TGF, IL-6 and PDGF. These cytokine are part of the abnormal blood pressure deregulation and renal fibrosis.

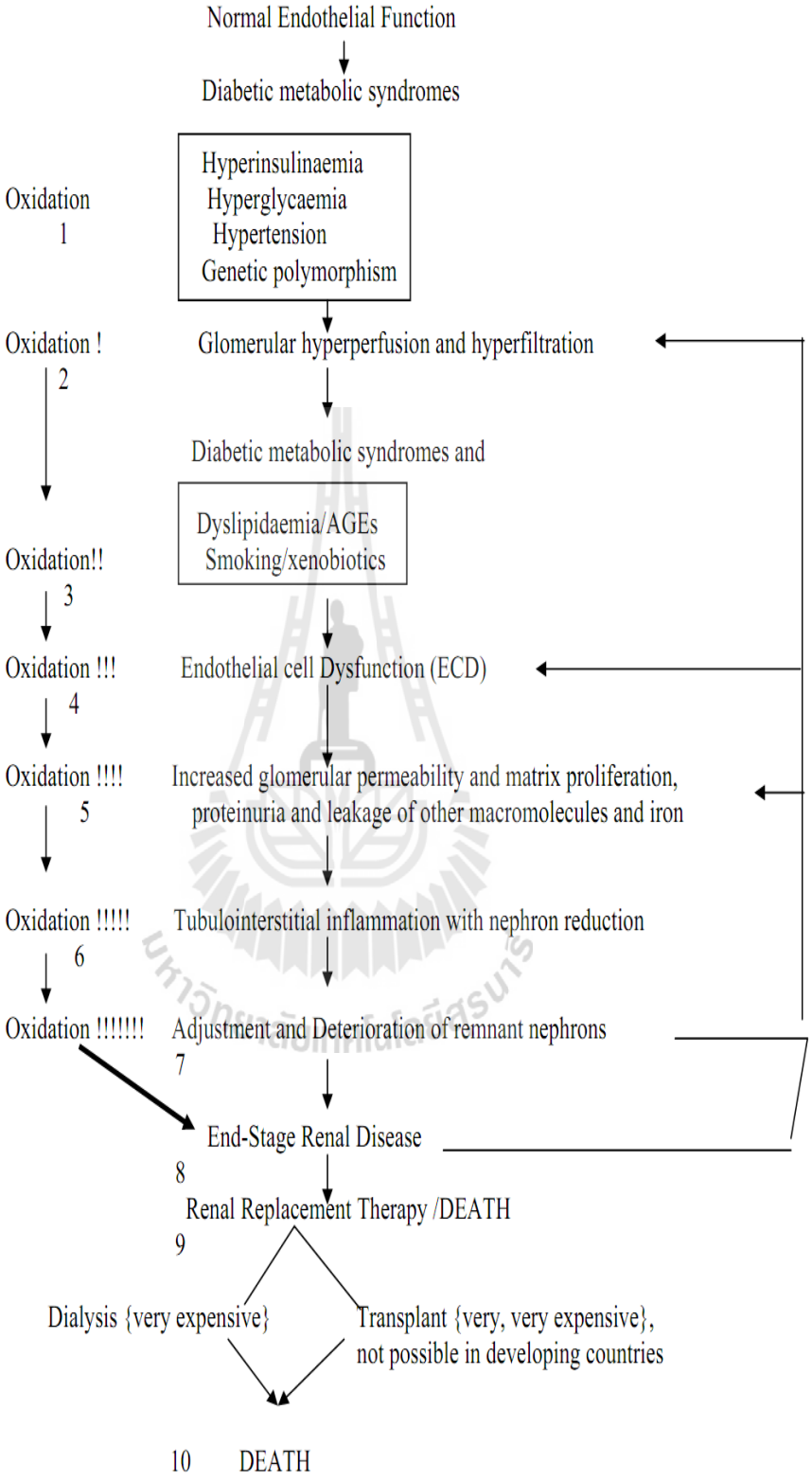


Figure 2.9 The sequence of events in the pathogenesis of diabetic nephropathy (Mshelia, 2004).

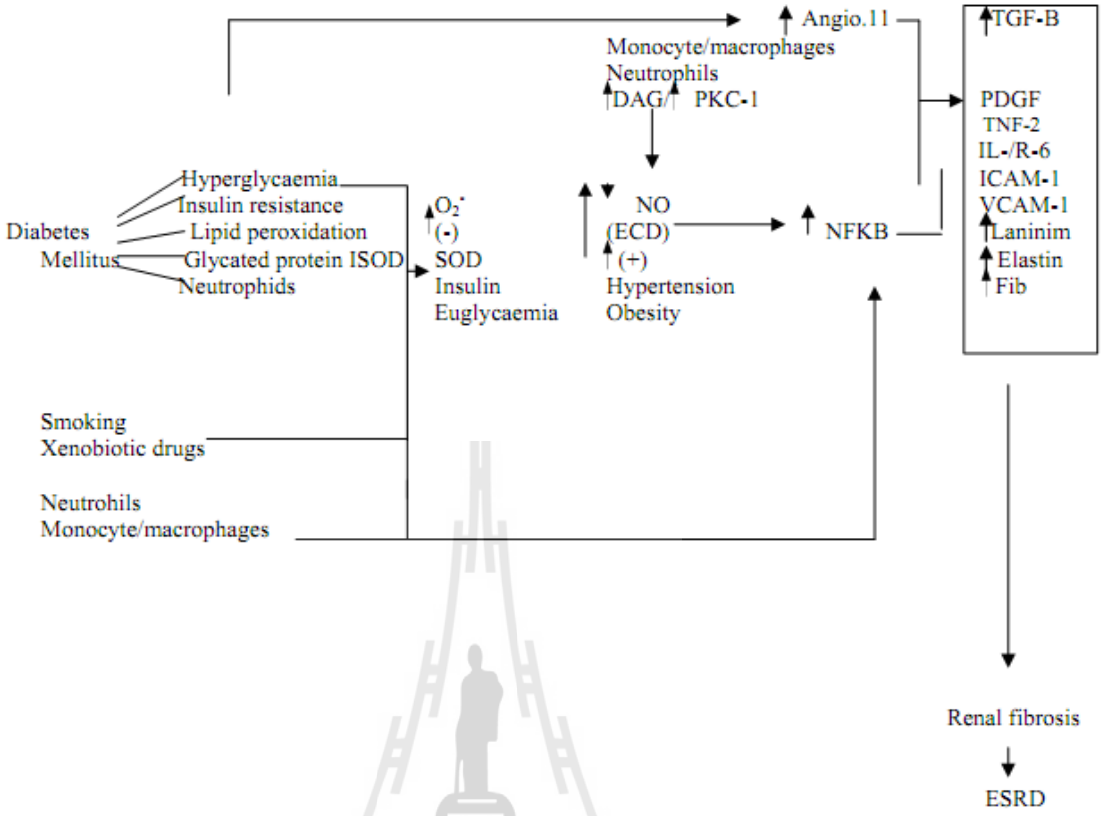


Figure 2.10 The cytokines in the pathogenesis of diabetic nephropathy (Mshelia, 2004).

2.5 Thin layer chromatography

Thin layer chromatography (TLC) is a method for analyzing the number of components in a mixture by separating the compounds in the mixture. Moreover, TLC can used for identification and purification of a compound (Bele and Khale, 2011; Sherma and Fried, 2003). TLC is generally regarded as a simple, rapid, and inexpensive method for the separation, tentative identification, and visual semiquantitative assessment of a wide variety of substances (Harborne, 1998; Wagner and Bladt, 1996). The steps of TLC are shown in Figure 2.11. If components of the reaction are colored, no visualization method is required because spots can be seen

directly on the silica layer. However, many substances cannot be seen easily, therefore one of the non-destructive methods such as viewing ultraviolet-active compounds under an ultraviolet lamp or one of destructive methods such as staining with some reagents should be used to reveal the spots. Moreover, there are many available TLC visualization reagents, for example aluminium chloride for detection flavonoids, formaldehyde/sulfuric acid for detection of alkaloids, and formaldehyde/phosphoric acid for detection of steroid alkaloids, steroid sapogenins and phenothiazine derivative.

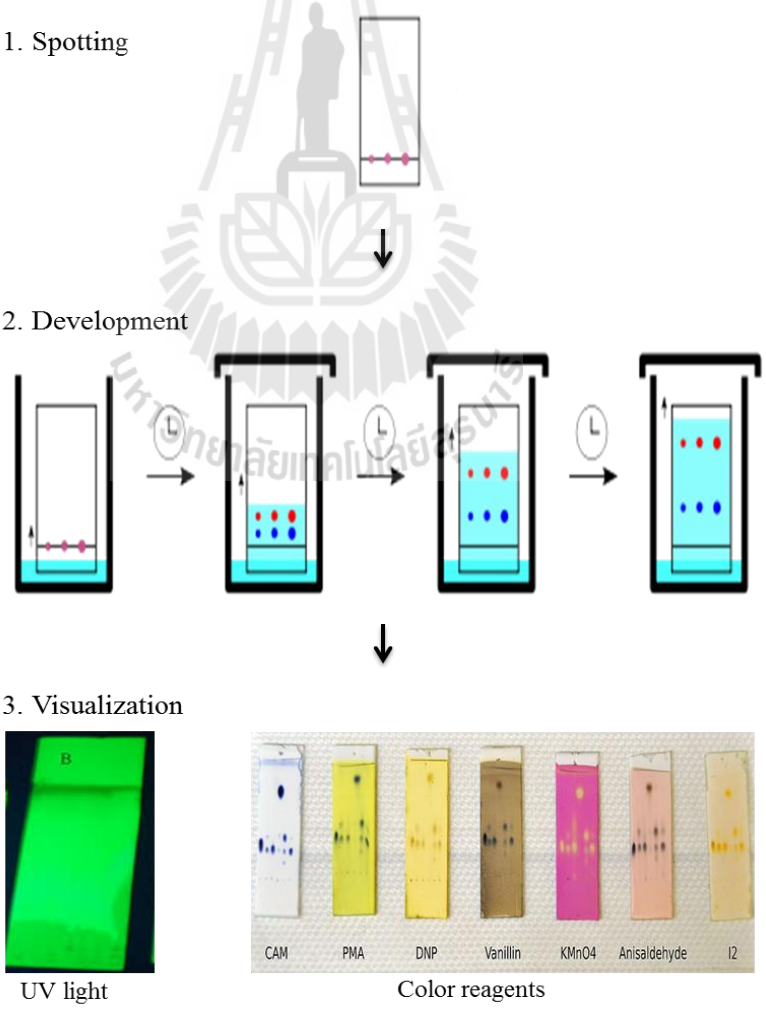


Figure 2.11 The three steps of TLC (Bele and Khale, 2011).

2.6 Methods for antioxidant measurement

The methods for antioxidant measurement can be classified into two types based on the reaction involved, including hydrogen atom transfer (HAT) and electron transfer (ET) reactions (Prior, Wu, and Schaich, 2005; Karadag, Ozcelik, and Saner, 2009). The ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change correlates with the antioxidant activity while HAT-based assays apply a competitive reaction. Oxygen radical absorbance capacity (ORAC) is a sample of HAT reaction. The ORAC assay is considered to be more relevant because it utilizes a biologically relevant radical source (Cao, Alessio, and Cutler, 1993). Although ORAC assay is sensitive, this assay requires detection by fluorometer, which may not be routinely available in analytical laboratories. Conversely, ABTS, DPPH and FRAP assays are examples of ET reaction. These assays are routinely available in laboratories and usually used for initial screening for antioxidative compounds because they are simple, inexpensive, and rapid (Antolovich, Prenzler, Patsalides, Mcdonald, and Robards, 2002; Prior et al., 2005).

2.6.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assay is a method for determination of antioxidant activity by using 2,2'-azino-bis(3-ethylbenzoline-6-sulfonic acid) diammonium salt (ABTS) as stable radical (Miller, Rice-Evans, Davies, Gopinathan, and Milner, 1993). The ABTS changes to $ABTS^{\cdot+}$ radical cation by potassium permanganate or potassium persulfate; this solution shows green color. The ability in scavenging free radicals of ABTS assay will be detected by spectrometer at 734 nm for measuring the reduction in color. The color of $ABTS^{\cdot+}$ radical cation decreases

when reacting with an antioxidant compound (Figure 2.12). The degree of color change correlates with the antioxidant activity (Antolovich et al., 2002). $ABTS^{\cdot+}$ can be solubilized in both aqueous and organic media. Thus, this method can determine both the hydrophilic and lipophilic nature of the compounds (Karadag et al., 2009).

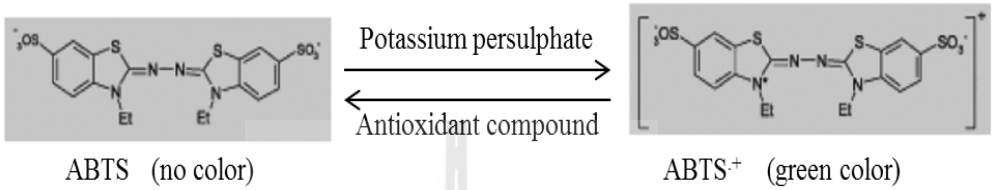


Figure 2.12 The reaction of ABTS assay.

2.6.2 DPPH radical scavenging activity assay

DPPH radical scavenging activity assay is a method to determine antioxidant capacity (Blois, 1958). This method uses DPPH reagent as stable radical. The DPPH reagent shows purple color in methanol solution. The ability in scavenging free radicals in DPPH assay is detected by spectrometer at 517 nm. The purple color of the DPPH radical will change to yellow color when the DPPH reacts with the antioxidant compound (Figure 2.13). Yellow color which occurs after reaction correlates with the antioxidant activity. However, the DPPH method limits is of limited use with hydrophilic oxidants because the DPPH can only be dissolved in alcoholic media (Karadag et al., 2009).

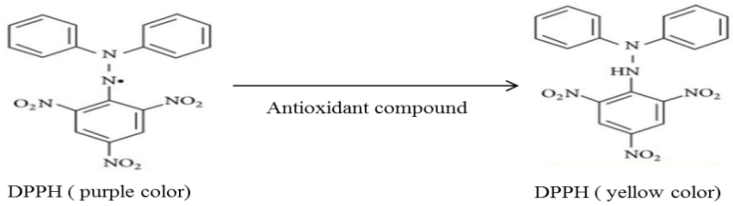


Figure 2.13 The reaction of DPPH assay.

2.6.3 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is a method for determination of antioxidant capacity (Benzie and Strain, 1996). This method is based on the reduction of the Fe^{3+} complex (Fe (TPTZ) $^{3+}$) to Fe^{2+} complex (Fe (TPTZ) $^{2+}$) by antioxidants (Figure 2.14). The Fe (TPTZ) $^{2+}$ complex shows blue color which is correlated with the reducing power. The blue color occurring after the reaction can be measured by spectrometer at 593 nm. The reduction of (Fe (TPTZ) $^{3+}$) to (Fe (TPTZ) $^{2+}$) is defined as antioxidant power, reflecting to its action of reducing reactive species (Karadag et al., 2009).

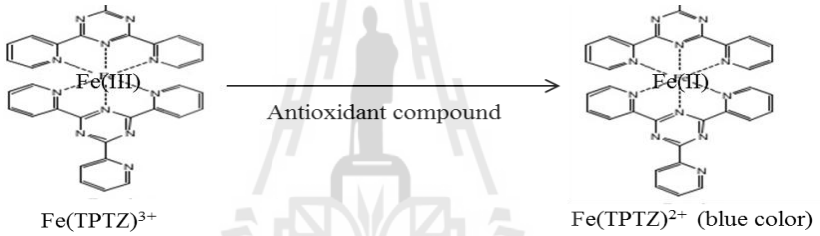


Figure 2.14 The reaction of FRAP assay.

2.7 Alloxan

Alloxan is the most prominent chemical compound used to induce diabetes in animals such as rabbits (Alam, Khan, Sirhindi, and Khan, 2005), mice (Heikkila, 1977), and rats (Raut and Gaikwad, 2006). The structure of alloxan resembles that of glucose (Figure 2.15). Its molecule is hydrophilic and does not penetrate the lipid bilayer of the plasma membrane. Thus, its process to penetrate to the cell depends on the GLUT2 glucose transporter (Elsner, Tiedge, Guldbakke, Munday, and Lenzen, 2002). Alloxan induces diabetes by generating reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals. These radicals are ultimately

responsible for the death of the beta cells (Lenzen, 2008). Moreover, GLUT2 glucose transporter can be found in liver and kidney cells. In this study, alloxan was used as oxidative stress agent for studying the protective effects of the extracts of *D. reticulata.*

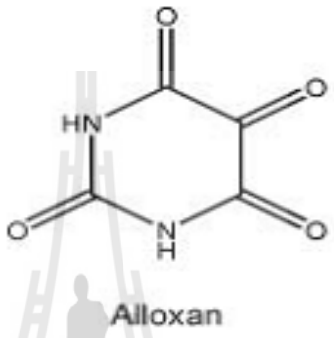


Figure 2.15 Chemical structure of alloxan (Lenzen, 2008).

2.8 HepG2 cell

HepG2 cell or hepatocellular carcinoma is a perpetual cell line which was derived from the liver tissue of a 15-year old Caucasian American male. The morphological features of HepG2 cells are illustrated in Figure 2.16. HepG2 cells are suitable for *in vitro* model system for the study of polarized human hepatocytes, for example liver metabolism (Hirayama et al., 1993), cytoprotective effect (Thabrew, Hughes, and Mcfarlane, 1997), and hepatotoxicity study (Neuman, Koren, and Tiribelli, 1993). The liver plays many essential roles in maintaining normal physiology. It is also a vulnerable target of many drugs or other chemicals because the liver is involved in complex metabolism. Therefore, the liver is one of the primary organ tested in drug and chemical safety evaluations. In this study, HepG2 cell was

used as a representative cell type to study the protective effects of *D. reticulata* extracts.

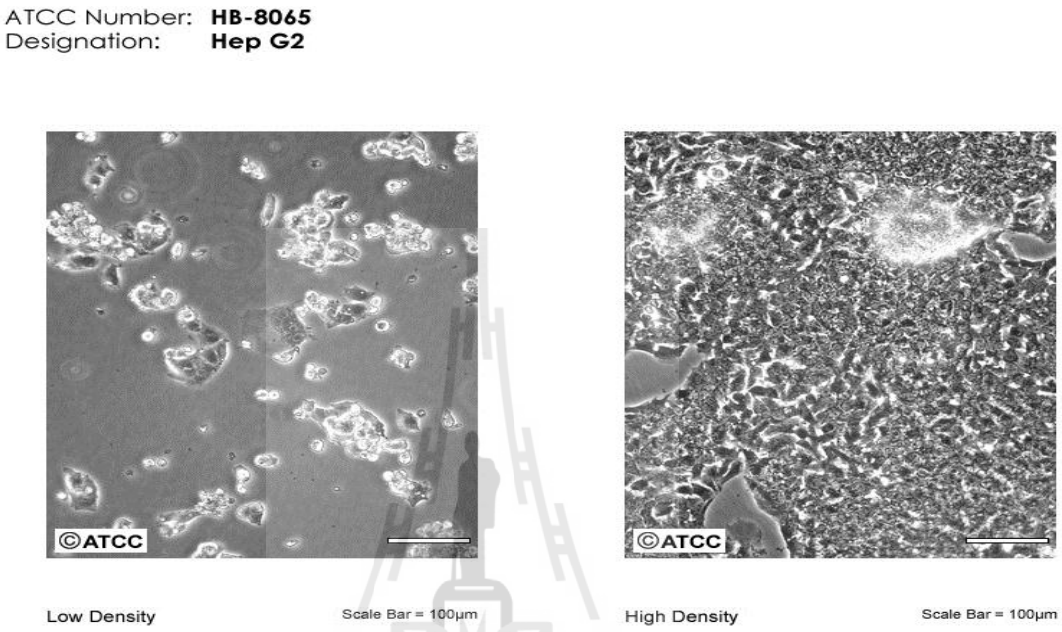


Figure 2.16 Photographs of HepG2 cells (American Type Culture Collection, 2014).

2.9 Methods for cell viability measurement

Quantification of cell viability is an indispensable tool for various biological studies, including cancer biology, microbiological resistance, and compound toxicity (Marks, Belov, Davey, Davey, and Kidman, 1992; Montoro et al., 2005; Wu, Liao, and Chang, 1993).

2.9.1 Trypan blue exclusion method

The trypan blue exclusion method is the stain most commonly used to distinguish viable from nonviable cells. The principle of this method is based on cell membrane integrity (Strober, 2001). Dead cells absorb TB into the cytoplasm and stain

intracellular components making dead cells stained blue because of loss of membrane selectivity, whereas live cells remain unstained (Figure 2.17). Thus, the relative number of dead and live cells is obtained by optical microscopy by counting the number of stained (dead) and unstained (live) cells by using haemocytometer (counting chamber, Figure 2.18). In this study, the trypan blue exclusion method was used for counting cells in cell preparation.

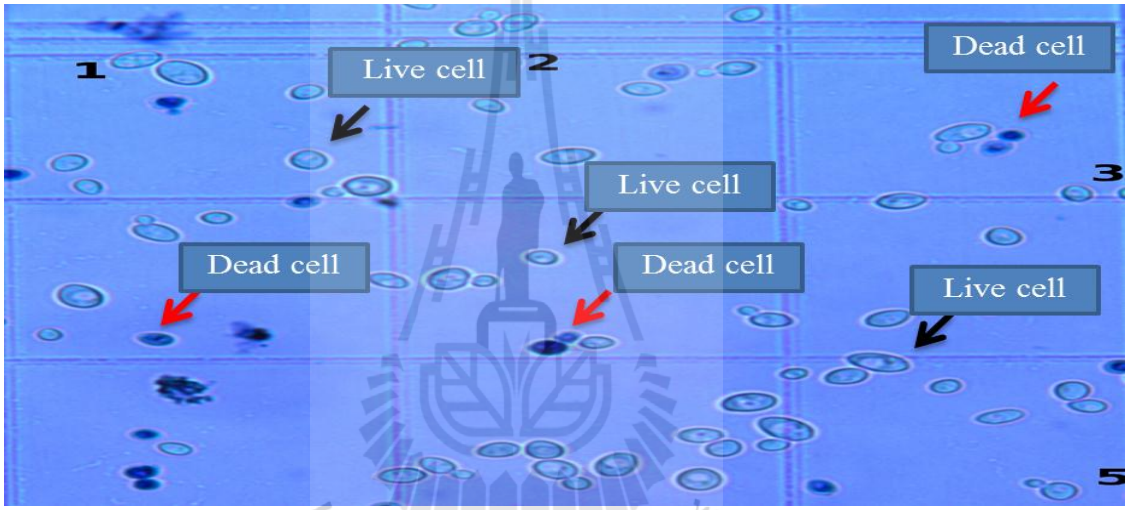


Figure 2.17 Dead and live cells on trypan blue exclusion method.

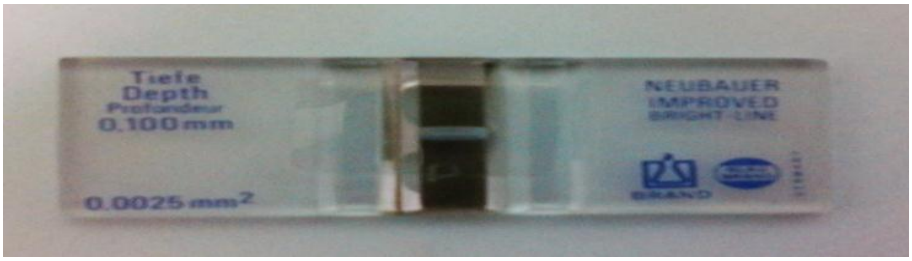


Figure 2.18 Haemocytometer.

2.9.2 MTT method

The MTT method is a quantitative colorimetric method to determine cell proliferation and cell viability. The assay is based on the cleavage of the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to formazan by cellular mitochondrial dehydrogenases (Van Meerloo, Kaspers,and Cloos, 2011), which has a purple color (Figure 2.19). The mitochondrial enzyme is only active in viable cells. The more viable cells are present, the more formazan purple product is produced. The formazan can be measured by spectrophotometer at 570 nm.

Figure 2.19 The formazan purple product of tetrazolium salt of MTT assay.

2.10 α-Glucosidase

The α-glucosidase enzyme, known by other names such as maltase, and glucosidosucrase, is one of several enzymes that help digest carbohydrates (International Union of Biochemistry and Molecular Biology, 2010). It is located in the brush border of the small intestine and takes part in the breakdown of

carbohydrates into monosaccharide before absorption. The enzymes responsible for carbohydrate digestion in the gastrointestinal system are summarized in Table 2.4. The inhibition on intestinal α-glucosidase is one of the therapeutic approaches to decrease blood glucose level in the treatment of diabetics patients (Burkhard and Christine, 1998), because the inhibition on intestinal α-glucosidase would delay the digestion of carbohydrates and reduce the rate of glucose absorption. α-Glucosidase inhibitors, such as acarbose, delay carbohydrate digestion by competitively inhibiting α-glucosidase in the small intestine (Clissold and Edwards, 1988). They are clinically useful for the control of postprandial hyperglycemia.

Table 2.4 Summary of carbohydrate digestion.

Site of synthesis	**Site of action**	**Enzyme**	**Substrate**	**Product**
Salivary gland	Mouth	α-amylase	Starches	Polysaccharide, Disaccharides
	Stomach	No digestion of carbohydrate		
Pancreas	Small intestine	β-amylase	Starches	Maltose
Small intestine	Small intestine	Sucrose	Sucrose	Glucose Fructose
		Maltase (α-glucosidase)	Maltose	2 glucoses
		Lactase	Lactose	Glucose Galactose

CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials

3.1.1 Collection of plants

D. reticulata was collected from Prachinburi province, Thailand by a former Ph. D. student (Dr. Pakarang Kumkrai). Its authenticity was identified and confirmed using taxonomy based on the definition previously described (Bangkok Herbarium, 2009; Siri Ruckhachati Nature Park, 2010; Songsak, 1995). The voucher specimen (Pharm-Chu-006) was kept at School of Pharmacology, Suranaree University of Technology. The collected stems were cut into small pieces and dried at 50ºC in a hot air oven, then kept at -20ºC until used.

3.1.2 Preparation of the extract

The extracts were obtained by sequential extraction method. First, 60 g of dried plants extract with 400 ml of hexane, and then with the same amount of chloroform, at 80ºC for 8 h for each solvents by using Soxhlet extractor. After extraction with hexane and chloroform the residues were subjected to water decoction at 100ºC for 10 min. This process was repeated twice. After that the aqueous extract was filtered and centrifuged at 2500 × g for 10 min. All of extracts were collected and concentrated by using a rotary evaporator. After that, the concentration was lyophilized into powder by a lyophilizer at -40ºC for four days. Finally, the dried

extract was stored at -20ºC until used. The percent yield of extract was calculated as:

$$\% \text{ Yield } = \frac{W_F\,(g)}{W_I\,(g)} \times 100 \quad (1)$$

where W_F is the weight of extract powder at terminal of extraction

W_I is the weight of dried plant at initial of extraction

3.2 Thin layer chromatography (TLC)

TLC was carried out for screening the number of components in the extracts as follows. The aqueous extract was dissolved in distilled water whereas the hexane and chloroform extracts were dissolved in ethanol. Then, 10 µl (5 µg) of the extracts solution were applied on 4×8 cm of TLC plates. After drying, TLC plates were transferred to TLC developing tank containing mobile phase [Dichloromethane: Methanol (95:5) and Chloroform: Methanol: Water (10:30:1)]. Compositions of each extract were first observed under UV lamp. After that, TLC plates were stained with 10% sulfuric acid for image analysis and R_f value was calculated for the main component of the extract.

$$R_f \text{ value} = \frac{Y}{X} \quad (2)$$

where Y is the distance in movement of component

X is the distance in movement of mobile phase

3.3 Phytochemical screening test

The phytochemical screening test was conducted for identification of components of the extracts. The test involved screening for alkaloids, flavonoids,

saponins, tannins, triterpenoids, cardiac glycosides, anthraquinones, and polyphenol by using standard procedures (Harborne, 1998; Saxena and Saxena, 2012).

3.3.1 Test for alkaloids

Mayer's test

Mayer's reagent was prepared by dissolving 1.358 g of mercuric chloride ($HgCl_2$) in 60 ml distilled water, and 5 g of potassium iodide (KI) in 10 ml distilled water; then mixed together. Distilled water was added to the mixture to bring the volume to 100 ml. Mayer's reagent (2-3 drops) was added to 1 ml of test sample (0.2 g/ml) and shaken. The appearance of cream or pale-yellow precipitate indicates the presence of alkaloids.

Wagner's test

Wagner's reagent was prepared by dissolving 2 g of iodine and 6 g of KI in 100 ml of distilled water. 2-3 drops of Wagner's reagent were added to 1 ml of the sample solution (0.2 g/ml) and shaken. The appearance of brown or reddish brown precipitate indicates the presence of alkaloids.

3.3.2 Test for flavonoids

Shinoda test

A few fragments of magnesium ribbon were added to 1 ml of the extract solution (0.2 g/ml) followed by 2-3 drops of concentrated HCl. The appearance of pink, crimson red, or green to blue color indicates the presence of flavonoids.

Ferric chloride test

A few drops of 1% ferric chloride ($FeCl_3$) were added to 1 ml of the extract solution (0.2 g/ml) and shaken. The appearance of intense green color indicates the presence of flavonoids.

3.3.3 Test for saponins

Froth test

Distilled water (2-3 ml) was added to 0.2 g of the dried powdered extract and then, boiled for 10 min. The extract solution was shaken. The appearance of honey comb froth that is stable for 15-30 min indicates the presence of saponins.

3.3.4 Test for tannins

Ferric chloride test

A few drops of 1% ferric chloride ($FeCl_3$) were added to 1 ml of the extract solution (0.2 g/ml) and shaken. The appearance of intense green, purple, blue or black color indicates the presence of tannins.

Lead acetate test

A few drops of 10% lead acetate solution were added to 1 ml of the extract solution (0.2 g/ml) and shaken. The appearance of precipitate indicates the presence of tannins.

3.3.5 Test for triterpenoids

Salkowski's test

Chloroform was added to 1 ml of the extract solution (0.2 g/ml), followed by a few drops of concentrated H_2SO_4. The sample was shaken and left standing at room temperature for 5 min. The appearance of a yellow lower layer indicates the presence of triterpenoids.

3.3.6 Test for cardiac glycosides

Keller Killiani test

Glacial acetic acid containing 2-3 drops of 2% $FeCl_3$ solution was added to 1 ml of the extract solution (0.2 g/ml). Then, the mixed solution was transferred to a new

tube containing 0.5 ml of concentrated H_2SO_4. The appearance of blue color in the acetic acid layer indicates the presence of cardiac glycosides.

3.3.7 Test for anthraquinones

Borntrager's test

0.2 g of dried extract was shaken with 1 ml of benzene until the extract solution showed organic layer. Then, 1 ml of 10% ammonia solution was added to the solution. The appearance of pink, red or violet coloration in the ammonical phase indicates the presence of anthraquinones.

3.3.8 Test for polyphenol

Ferric-chloride-ferricyanide test

Ferric chloride (1%) and potassium ferricyanide (1%) solutions were added to 1 ml of the extract solution (0.2 g/ml). The appearance of radish blue color indicates the presence of polyphenol.

3.4 Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method and performed according to the previous report (Prior, Wu, and Schaich, 2005) with some modifications. In brief, 100 µl of 2.5 mg/ml extract was mixed with 2 ml of 2% Na_2CO_3 and incubated for 2 min at room temperature. After incubation, 100 µl of Folin-Ciocalteu reagent (diluted in methanol 1:1 v/v) was added to the reaction mixture and incubated in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 750 nm by using spectrophotometer. The total phenolic content of extract was determined from a standard curve of Gallic acid and results were expressed as mg gallic acid equivalents (GAE) per gram extract.

3.5 Antioxidant activity determination

3.5.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to the method previously described (Re et al., 1999) with some modifications. The $ABTS^{\cdot+}$ radical cation stock solution was prepared by mixing 5 ml of 14 mM ABTS with 5 ml of 4.9 mM potassium persulphate ($K_2S_2O_8$) and incubated in the dark at room temperature for 16 h. Before use, the $ABTS^{\cdot+}$ radical cation stock solution was diluted with ethanol to attain absorbance of 0.7 ± 0.02 at 734 nm. After that, 50 µl of various concentrations of the extract (0-6,000 µg/ml) was mixed with 1.5 ml of diluted $ABTS^{\cdot+}$ stock solution. Then, the mixture was incubated in the dark at room temperature for 6 min. After incubation, the absorbance was measured at 734 nm by using spectrophotometer. Butylated hydroxytoluene (BHT) was used as standard. Results were expressed as IC_{50} (the concentration required for 50% scavenge for free radical). The control is the reaction without the extract (0 µg/ml).

$$ABTS^{\cdot+} \text{ radical scavenging activity } (\%) = \frac{[OD_{control} - OD_{sample}]}{OD_{control}} \times 100 \quad (3)$$

where $OD_{control}$ is the absorbance of the control reaction

OD_{sample} is the absorbance of sample reaction

3.5.2 DPPH radical scavenging activity assay

DPPH radical scavenging activity assay was performed according to the previous report (Bor, Chen, and Yen, 2006) with some modifications. DPPH stock solution was prepared by dissolving 0.2 M DPPH in methanol and then stored at -20ºC until needed. 1 ml of various extract concentrations (0-6,000 µg/ml) was added to 4 ml of 0.2 M DPPH solution. Then, the reaction mixture was incubated in the dark at room

temperature for 30 min and measured the absorbance at 517 nm using spectrophotometer. Ascorbic acid (vitamin C) was used as standard. Results were expressed as IC_{50} (the concentration required for 50% scavenge for free radical). The control is the reaction without the extract (0 µg/ml).

$$\text{DPPH radical scavenging activity (\%)} = \frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}]}{\text{OD}_{\text{control}}} \times 100$$

where $\text{OD}_{\text{control}}$ and $\text{OD}_{\text{sample}}$ have the same meanings as in the Equation (3).

3.5.3 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the method described by (Benzie and Strain, 1996) with some modifications. The FRAP reagent was prepared by mixing 10 mM TPTZ solution (2,4,6-tripyridyl-s-triazine), 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6), in ratio 1:1:10 (v/v/v). Then, FRAP reagent was incubated in water bath at 37ºC until used. A 50 µl of 5 mg/ml extract was mixed with 1.5 ml of the FRAP reagent. The reaction mixture was incubated at room temperature for 4 min and measured the absorbance at 593 nm. The reducing potential of extract was determined from a standard curve of $FeSO_4$ and the FRAP value were expressed as µmol Fe^{2+}/mg dried extract.

3.6 Determination of cytoprotective effect

3.6.1 Cells and culture conditions

HepG2, *Homo sapiens* (Human) cell line was obtained from the American Type Culture Collection (ATCC number: HB-8065). HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic solution, 1% HEPES, 1% sodium pyruvate and incubated at 37ºC in a

humidified atmosphere containing 5% CO_2. Cells were grown as adherent cells and spread in culture flask. The cells were sub-cultured at 70-90% confluence. The cells were trypsinized with 0.25% trypsin-EDTA.

3.6.2 Determination of alloxan-induced cell death

In order to find the suitable concentration of alloxan for induction of 50% cell damage, HepG2 cells with density 4.5×10^4 cells per well were seeded in 96 well plates and incubated in a humidified incubator containing 5% CO_2 at 37ºC for 16-18 h. Then, the cells were treated with alloxan at range of concentration 0-15 mM (dissolved in 50 mM citrated buffer pH 3.0). After 2 h of incubation, the medium was removed and cells were further incubated for 23 h. Then, the cells were centrifuged 2500 rpm at 4ºC for 5 min and removed medium. 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added for determination of cell viability. After that, the samples were incubated in the dark at 37ºC for 4 h. After incubation, the cells were centrifuged 2500 rpm at 4ºC for 5 min. The culture medium was discarded and the purple formazan crystals were dissolved with DMSO (50 µl per well). MTT solution was measured at 570 nm using a microplate reader. Results were expressed as percentage of cell viability.

$$\%\text{Cell viability} = [\frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}] \times 100 \qquad (4)$$

Where $\text{OD}_{\text{sample}}$ is the absorbance of the sample after test with MTT

$\text{OD}_{\text{control}}$ is the absorbance of control after test with MTT

3.6.3 Determination of the effect of the extracts on cell viability

The effects of *D. reticulata* extracts on the viability of HepG2 cell was first tested to find the range of non-toxic concentrations for further experiments. HepG2 cells were seeded in 96 well plates at the concentration of 4.5×10^4 cells per well and incubated in a humidified incubator containing 5% CO_2 at 37ºC for 16-18 h. After that, cells were treated with various concentrations of *D. reticulata* extracts at the doses of 0-5000 µg/ml for 24 h. After treatment, the cells were centrifuged 2500 rpm at 4ºC for 5 min and removed medium. Then, 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added for determination of cell viability. After that, the samples were incubated in the dark at 37ºC for 4 h. After incubation, the cells were centrifuged 2500 rpm at 4ºC for 5 min. The culture medium was discarded and the purple formazan crystals were dissolved with DMSO (50 µl per well). MTT solution was measured at 570 nm using a microplate reader. Results were expressed as percentage of cell viability.

$$\%\text{Cell viability} = [\frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}] \times 100$$

where $\text{OD}_{\text{control}}$ and $\text{OD}_{\text{sample}}$ have the same meanings as in the Equation (4).

3.6.4 Determination of the effect of *D. reticulata* extracts on alloxan-induced HepG2 cell damage

HepG2 cells were plated in a 96 well plate (4.5×10^4 cell per well) and incubated in a humidified incubator containing 5% CO_2 at 37ºC for 16-18 h. Then, the cells were treated with 14 mM alloxan (which caused about 50% of cell death) for 2 h. After incubation, the medium was removed. The cells were treated with 60 µg/ml of hexane and chloroform extracts while aqueous extract the cells were treated with 60, 500 and 5000 µg/ml (500 and 5000 µg/ml of aqueous extract shown not toxic to cells)

for 22 h. After that, the cells were centrifuged 2500 rpm at 4ºC for 5 min and removed from the medium. At the end of experiment, the viable of cells were determined by MTT method.

3.7 Determination of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined according to the previous reports (Choi et al., 2010; Dong, Li, Zhu, Liu, and Huang, 2012) with some modifications. The reaction mixture consisted of 10 µl of 0.1 U/ml α-glucosidase enzyme, 50 µl of 0.1 M potassium phosphate buffer pH 6.8 and 20 µl of the test sample (the extracts or acarbose as a positive control). Then, the reaction mixture was mixed and incubated at room temperature for 10 min. After that, 10 µl of 1 mM p-nitrophenyl-α-D-glucopyranoside (PNP-G) was added into the reaction mixture and incubated at room temperature for 30 min. After incubation, the mixture was added with 50 µl of 0.1M Na_2CO_3 to stop the reaction and the absorbance was measured at 405 nm. Results were expressed as IC_{50} (the concentration of inhibitors required for inhibiting 50% for α-glucosidase activity). The control is 100 percent of enzyme activity in the reaction (added enzyme and PNP-G but not added extract or acarbose).

$$\%\ \text{Inhibition of}\ \alpha-\text{glucosidase activity}\ = \frac{[\mathrm{OD}_{\mathrm{control}} - \mathrm{OD}_{\mathrm{sample}}]}{\mathrm{OD}_{\mathrm{control}}} \times 100$$

where $OD_{control}$ and OD_{sample} have the same meanings as in the Equation (3).

3.8 Data analysis

Data are expressed as mean ± SD and the comparisons among different groups were performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P*-values less than 0.05 were considered as significant difference.

CHAPTER IV

RESULTS

4.1 Yield of *Derris reticulata* extracts

The results in Table 4.1 show that the aqueous extract of *D. reticulata* gave the highest yield (10.56%) compared to the chloroform and hexane extracts with 2.68% and 2.02% yields, respectively.

Table 4.1 Yields of the chloroform, hexane, and aqueous extracts from *D. reticulata* stem.

Extract	**Total dry plant (g) (6 times, 60 g each)**	**Powder (g)**	**% Yield**
Hexane	360	7.279	2.02
Chloroform	360	9.65	2.68
Aqueous	360	38.02	10.56

4.2 Compositions of the extracts detected by TLC

The TLC finger prints shown in Figure 4.1 revealed that the chloroform and hexane extracts contained some similar constituents, and the major compound in both extracts was the compound with R_f = 0.854. In addition, more compounds were observed in the chloroform extract than in the hexane extract. In the case of aqueous extract, the compounds were not separated well in both types of mobile phase used. However, it is clear that a much smaller amount of the major compound mentioned earlier (R_f = 0.854) was found in the aqueous extract.

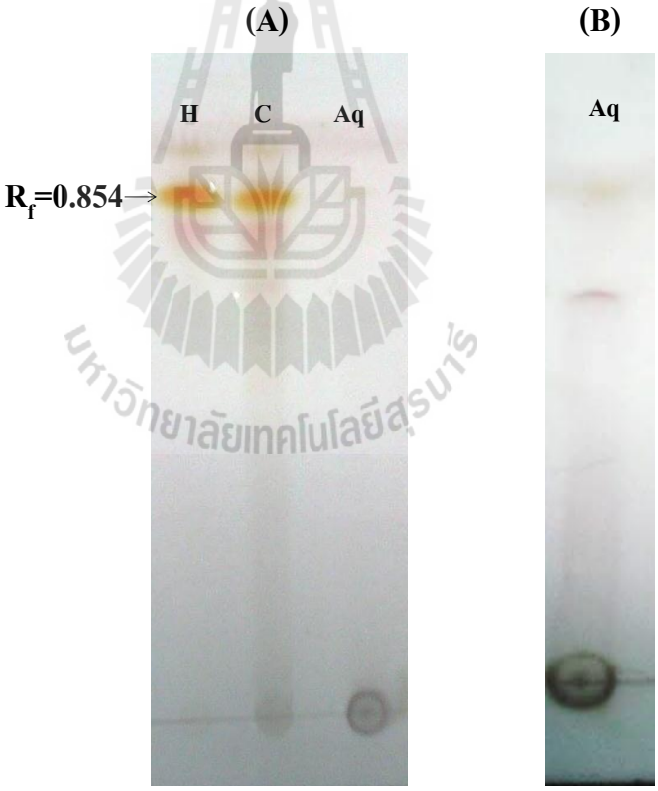


Figure 4.1 TLC finger prints of three *D. reticulata* extracts. (A) TLC plate in Dichloromethane: Methanol (95:5) and (B) TLC plate in Chloroform: Methanol: Water (10:30:1). H, C and Aq denote the type of extracts (hexane, chloroform and aqueous extracts, respectively).

4.3 Phytochemical compositions

Phytochemical screening analysis (Table 4.2) demonstrated the presence of alkaloids (Mayer's test and Wagner's test), triterpenoids (Salkowski's test), flavonoids (Shinoda test and $FeCl_3$ test), tannins ($FeCl_3$ test and Lead acetate test) and polyphenols (Ferric-Chloride-Ferricyanide test) for the hexane and chloroform extracts, whereas saponin (Froth test), triterpenoids, tannins and polyphenols were present in the aqueous extract. However, cardiac glycosides (Keller Killiani test) and anthraquinone (Borntrager's test) were absent from all extracts.

4.4 Total phenolic content

The Folin-Ciocalteau method was employed to determine the phenolic content, which was expressed in term of mg GAE/g extract. The mg GAE/g extract was calculated by using the gallic acid standard curve (Appendix A). As shown in Table 4.3, total phenolic contents of the hexane and chloroform extracts were not different (52.29 ± 1.33 and 53.89 ± 0.68 mg GAE/g extract, respectively), but significantly higher than that of the aqueous extract (33.21 ± 0.31 mg GAE/g extract, $p<0.05$).

4.5 Antioxidant activity

4.5.1 ABTS assay

The results from ABTS radical scavenging assay of three extracts of *D. reticulata* and BHT (standard) are shown in Appendix B (B.1-B.4). The extracts and BHT expressed scavenging activity in a concentration-dependent manner. The IC_{50} values for radical scavenging activity of extracts are summarized in Table 4.3. The

three extracts showed significant differences (p<0.05) in radical scavenging activity.

Table 4.2 Phytochemical compositions of the chloroform, hexane, and aqueous extracts from *D. reticulata* stem.

Phytochemical Screening Tests	**Extract**		
	Hexane	**Chloroform**	**Aqueous**
Alkaloids			
Mayer's test	+	+	-
Wagner's test	+	+	-
Saponins			
Froth test	-	-	+
Triterpenoids			
Salkowski's test	+	+	+
Flavanoids			
Shinoda test	+	+	-
$FeCl_3$ test	+	+	-
Tannins			
$FeCl_3$ test	+	+	+
Lead acetate test	+	+	+
Cardiac glycosides			
Keller Killiani test	-	-	-
Anthraquinone			
Borntrager's test	-	-	-
Polyphenol			
Ferric-chloride-Ferricyanide test	+	+	+

(+) Presence, (-) Absence

It was found that chloroform extract had the lowest IC_{50} value (441.55 ± 13.56 µg/ml), followed by hexane extract (632.69 ± 23.02 µg/ml) and aqueous extract (1252.68 ± 110.00 µg/ml). However, the antioxidant capacities of all extracts were lower than BHT of which IC_{50} in ABTS assay was 108.12 ± 8.41 µg/ml.

4.5.2 DPPH assay

The dose-response curves from DPPH radical scavenging assay of three extracts and ascorbic acid (as a standard) are shown in Appendix B (B.5-B.8) while their IC50 are summarized in Table 4.3. The results reveal that the aqueous extract had the lowest IC_{50} value (236.99 ± 10.98 µg/ml), followed by chloroform extract (570.56 ± 22.02 µg/ml) and hexane extract (717.37 ± 36.31 µg/ml). Ascorbic acid showed much more powerful in scavenging DPPH than the extracts with the IC_{50} of 1.86 ± 0.12 µg/ml.

4.5.3 FRAP assay

The reducing potential of the extracts were calculated by using the $FeSO_4$ standard curve (Appendix A). The result was expressed as µmol Fe^{2+}/mg dried extract. The FRAP value of extracts were not statistically different among all three extracts of *D. reticulata* as shown in Table 4.3.

4.6 Effect of alloxan on the viability of HepG2 cells

Figure 4.2 shows the results from the cytotoxicity study of alloxan on HepG2 cell. The half maximal inhibitory concentration (IC_{50}) of alloxane on cell viability was found at 13.72 ± 0.27 mM, which was used to study the cytoprotective effect of extracts on alloxan-induced cells damage in the next experiment.

Table 4.3 Total phenolic content and antioxidant activities of *D. reticulata* extracts.

Extract	Total phenolic compound (mg GAE/g extract)	ABTS assay (IC_{50} µg/ml)	DPPH assay (IC_{50} µg/ml)	FRAP assay (µmol Fe^{2+}/mg dried extract)
Hexane	52.29 ± 1.33[a]	441.55 ± 13.56[a, b, c]	714.37 ± 36.31 [a, b, c]	0.047 ± 0.036
Chloroform	53.89 ± 0.68[a]	632.69 ± 23.02[a, c]	570.56 ± 22.02 [a, c]	0.121 ± 0.028
Aqueous	33.21 ± 0.31	1252.68 ± 110.00[c]	236.99 ± 10.98 [c]	0.091 ± 0.026
BHT	-	108.12 ± 8.41	-	-
Ascorbic acid	-	-	1.86 ± 0.12	-

Values are expressed as mean ± SD (n = 3).
[a] $p<0.05$ statistically significant difference from aqueous extract.
[b] $p<0.05$ statistically significant difference from chloroform extract.
[c] $p<0.05$ statistically significant difference from standard.

4.7 Effect of the extracts on the viability of HepG2 cells

As shown in Figures 4.3A and 4.3B, after treatment for 24 h, hexane and chloroform extracts at the concentrations higher than 60 µg/ml significantly decreased the viability of HepG2 cells compared to control (p<0.05); While, the aqueous extract did not significantly decrease the cell viability at all concentrations (Figure 4.3C). Therefore, the hexane and chloroform extract at the concentration at 60 µg/ml were chosen for studying their cytoprotective effects on alloxan-induced cell damage. In case of the aqueous extract, the concentrations at 60, 500 and 5000 µg/ml were used since all of them did not show significant toxicity to the cell when compared to control. It should be noted that the vehicle (0.1% NaOH) did not cause any damage to the cells.

4.8 Cytoprotective effect of the extracts on alloxan-induced HepG2 cell damage

The cytoprotective effect of extracts was evaluated on cell damage induced by alloxan at 14 mM for 2 h, which decreased cell viability to about 50% of control (Figure 4.4). After posttreatment of all extracts at the doses of 60µg/ml for 22 h, HepG2 cell viability was not significant altered compared to alloxan-treated cells. However, the aqueous extract at the concentrations at 500 and 5000 µg/ml showed significant protective potential by increasing HepG2 cell viability up to 62.41 ± 3.48% and 69.46 ± 3.77%, respectively.

4.9 Inhibitory effect of the extracts on α-glucosidase activity

The α-glucosidase inhibition curves of three extracts of *D. reticulate* are shown in Appendix C. The effectiveness of enzymatic inhibition of extracts and acarbose were compared by their calculated IC_{50} (Table 4.4). The IC_{50} value acarbose, a positive control, was 157.95 ± 5.91 µg/ml. The three extracts showed significant differences (p<0.05) in α-glucosidase inhibition activity. It was found that chloroform extract showed the highest potential with the IC_{50} of 0.68 ± 0.10 µg/ml, followed by the hexane (IC_{50}; 4.19 ± 0.28 µg/ml) and aqueous extracts (IC_{50}; 411.62 ± 15.01 µg/ml).

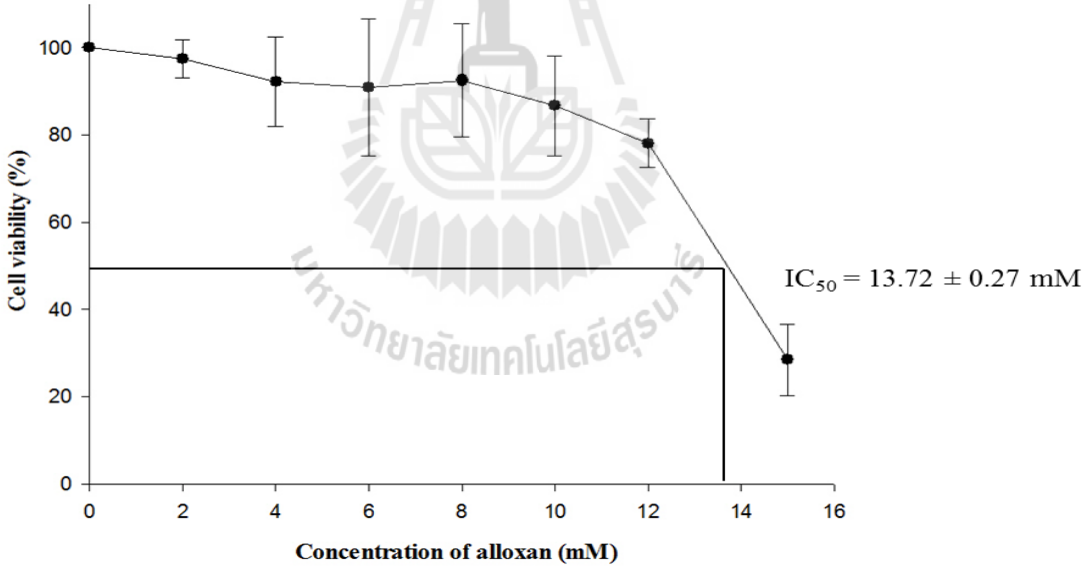


Figure 4.2 Cytotoxicity effect of alloxan on HepG2 cell viability. Values are expressed as mean ± SD (n = 3).

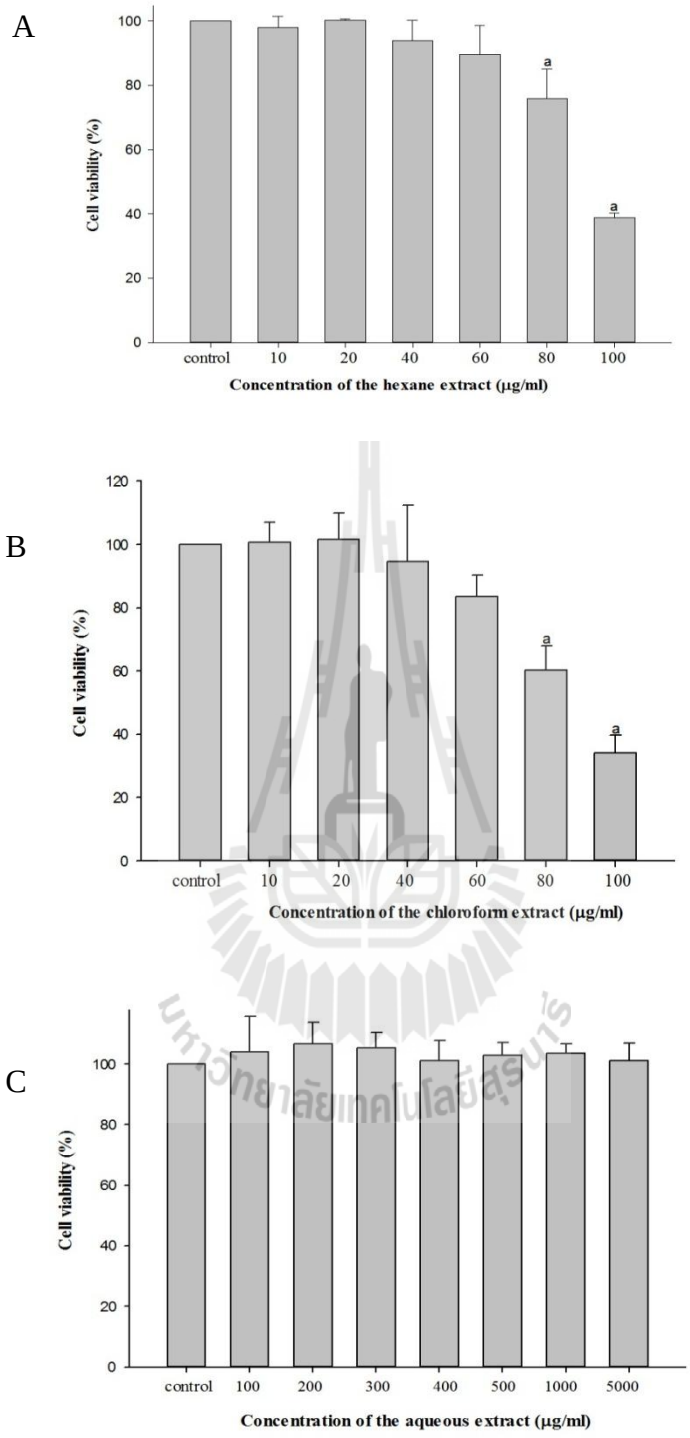


Figure 4.3 Cytotoxicity effects of extracts. Panels A, B and C are cell viability of HepG2 cells after treated at various concentrations of hexane, chloroform and aqueous extracts, respectively. Values are expressed as mean ± SD (n = 3).

[a] $p<0.05$ statistically significant difference from control.

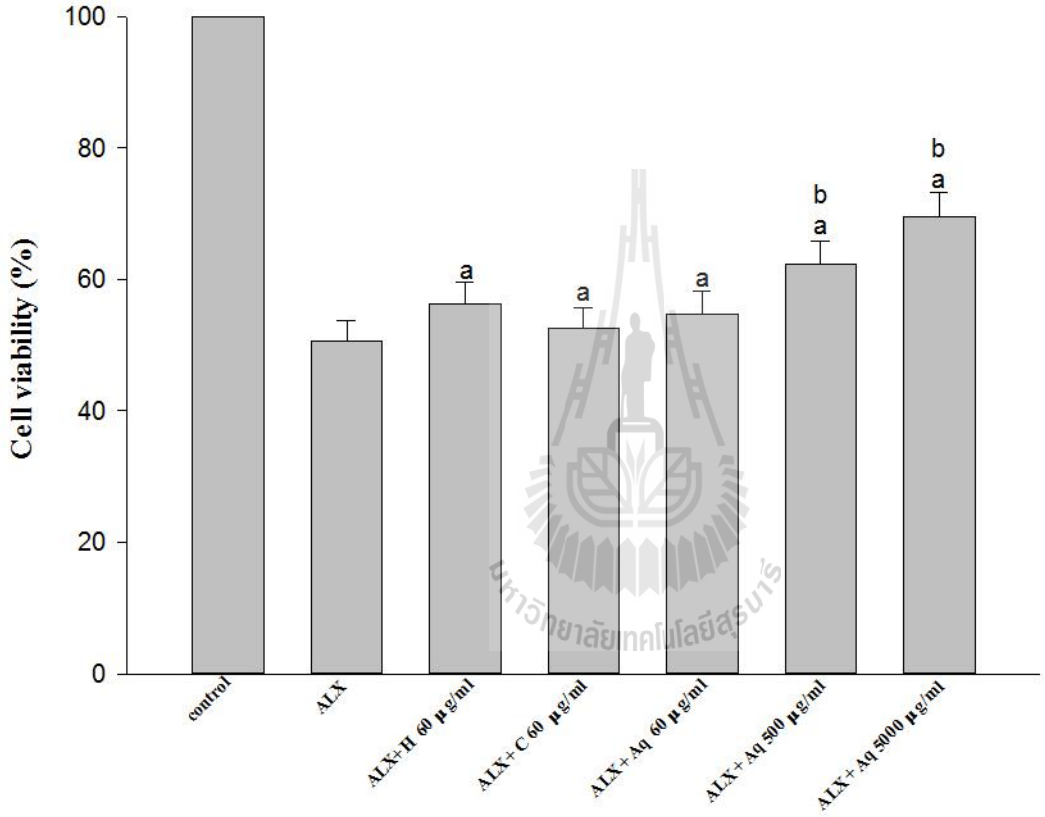


Figure 4.4 The effect of *D. reticulata* extracts on alloxan-induced HepG2 cell damage. ALX, H, C and Aq denote the type of chemical or extracts (alloxan, hexane extract, chloroform extract and aqueous extract, respectively).Values are expressed as mean ± SD (n = 3).

[a] $p<0.05$ statistically significant difference form control. [b] $p< 0.05$statistically significant difference from alloxan.

Table 4.4 α-Glucosidase inhibition activity of the chloroform, hexane, and aqueous extracts from *D. reticulata* stem.

	α – glucosidase inhibitory assay (IC_{50} µg/ml)
Hexane extract	4.19 ± 0.28 [a, b, c]
Chloroform extract	0.68 ± 0.10 [a, c]
Aqueous extract	411.62 ± 15.01[c]
Acarbose	157.95 ± 5.91

Values are expressed as mean ± SD (n = 3).
[a] $p<0.05$ statistically significant difference from aqueous extract.
[b] $p<0.05$ statistically significant difference from chloroform extract.
[c] $p<0.05$ statistically significant difference from acarbose.

CHAPTER V

DISCUSSION AND CONCLUSION

D. reticulata known in Thai as Cha-aim-nuea or Oi-sam-suan, has been traditionally used as an anti-coughing agent (Siri Ruckhachati Nature Park, 2010). The pharmacological activities of this plant discovered by scientific methods have also been documented, for example anti-rheumatism, anti-inflammatory and anti-nociceptive (Laupattarakasem et al., 2003; Srimongkol et al., 2007) and antimicrobial (Khan et al., 2006). Recently, the aqueous extract of *D. reticulata* has been reported to possess anti-diabetic activity (Kumkrai et al., 2014). Moreover, mechanisms underlying the hypoglycemic activity of *D. reticulata* aqueous extract have been proposed. It has been suggested that the combination of cytoprotective effect, suppression of intestinal glucose absorption and inhibition of α-glucosidase activity are responsible for the anti-diabetic activity (Kumkrai et al., 2014; Kumkrai et al., 2015). However, the active ingredients have not yet been identified.

In general, it is difficult to isolate and identify active compounds that are responsible for a specific activity when a crude extract is used in an experiment because it contains too many compounds. Since the crude extract by hot water was used to study anti-diabetic activity of *D. reticulata* in the previous study, it is least likely to specify the active ingredient. In the present study, a sequential extraction method was used in order to separate several compounds in the stem of *D. reticulata* into three fractions in three different solvents, including hexane, chloroform and water.

It was expected that all of the compounds could be extracted by these solvents which are different in polarity (from less to more polar: hexane, chloroform and water).

From the previous report (Kumkrai et al., 2014), the aqueous extract of *D. reticulata* stem contained 16.7 yield % (w/w) of dried plant. In the present study, the extracts obtained were different from the previous report in terms of yield. Due to the polarity of the solvent, the hexane extract contained compounds which are more hydrophobic than the chloroform and aqueous extracts. *D. reticulata* stem appeared to contain more hydrophilic than hydrophobic compounds because the percentage of yield from the aqueous extract was about 5 times to the others (about 10% compare to about 2%).

Due to hydrophobic property of the compounds in hexane and chloroform extracts, there were difficulties in finding a suitable solvent to dissolve the extract for future experiments when water media was needed. It was found that solubility of the extracts was improved after adjusting to higher pH. It is worth noting that NaOH at all concentrations used to improve the solubility was tested and it appeared to have no effect in all experiments.

The constituents of all extracts were first screened by TLC. As seen in Figure 4.1, fewer components were found in the hexane extract compared to the chloroform extract. However, at least one major compound (R_f = 0.854) was found in both extracts. Compared to the previous report from a group of researchers (Laupattarakasem et al., 2003), it was suggested that this compound would be lupinifolin. However, this prediction needs to be clarified with further experiments.

In the case of aqueous extract, the compounds were not separated well in both types of mobile phase used. Nevertheless, it can be seen that the constituents of

aqueous extract are different from those of the other extracts. Moreover, it is quite clear that the amount of the major compound mentioned earlier (R_f= 0.854) was much less or not found in the aqueous extract.

The results from phytochemical analysis revealed that none of the extracts from *D. reticulata* stem contained cardiac glycosides and anthraquinone, whereas triterpenoids, tannins and polyphenol were detected in all three extracts. Saponin appeared to be found only in the aqueous extract. Flavanoids and alkaloids seemed to be completely extracted by hexane and chloroform because these types of compound could not be detected in the aqueous extract by the phytochemical screening methods used. In the case of flavonoids, this is in accordance with the major compound lupinifolin that was mentioned earlier. Lupinifolin is a flavanoid that has been isolated from several plants in *Derris* genus, including *D. reticulata*. It is water insoluble, but very soluble in organic solvents, especially in hexane, chloroform and ethanol. This could be an explanation that flavonoids were not found in the aqueous extract and the band with R_f at 0.854 in the aqueous extract was not seen on TLC.

D. reticulata extract has been shown to possess antioxidant and anti-diabetic activities. It has been proposed that the anti-diabetic activity of aqueous extract is partly due to cytoprotective effect by its antioxidant compounds (Kumkrai et al., 2014). It is suggested that an increase in sugar level induces an elevation of oxidant level and/or a decreased function of antioxidants (Mshelia, 2004; Dominique, 2002). Thus, these two factors are causes of oxidative stress and oxidative stress partly involved in diabetes complications such as cardiovascular diseases, hypertension and nephropathy (American Diabetes Association, 2012; Dominique, 2002; Mshelia, 2004). Normally, the excess free radicals can be removed from the body by

antioxidant pathways such as superoxide dismutase, catalase, glutathione peroxidase, and thiol antioxidant (Bierhaus et al., 1998; Dominique, 2002). In addition, the excess free radicals can also be destroyed by external antioxidants such as supplements containing vitamin C, flavonoids and carotenoids, which can be found in plants, vegetables and fruits (Valko et al., 2007).

It has been reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds (Xin-Hua, Chang-Qing, Xing-Bo, and Lin-Chun, 2001). In this study, it was found that the aqueous extract contained fewer total phenolic compounds than the other two extracts. Antioxidant activities of the extracts were further examined and it was found that the hexane and chloroform extracts exhibited significantly more antioxidant potential than the aqueous extract by ABTS assay. Conversely, with DPPH scavenging method, the aqueous extract showed strongest antioxidant activity with the lowest IC_{50} among three extracts. The results found in the present study are in accordance with the study reported by a group of researchers (Antolovich et al., 2002) that the DPPH scavenging activity is correlated with phenolic compounds, but not flavonoids. Since flavonoids were detected in both hexane and chloroform, but not in the aqueous extract, the results implicate that major compounds in both hexane and chloroform extract were flavonoids of which antioxidant activity would not be measured by DPPH scavenging method. Therefore, the IC_{50} of the hexane and chloroform extracts obtained from DPPH scavenging assay were found higher than the aqueous extract, in spite of the fact that they contained more total phenolic contents.

Together with preliminary phytochemical analysis, it was suggested that the major compounds that were responsible for antioxidant activities of hexane and

chloroform extracts could be flavonoids or tannins which have been reported to possess antioxidant activity (Saxena et al., 2013). In contrast, as flavonoids were not detected in the aqueous extract, other classes of phytochemicals, such as tannin and polyphenol, may be responsible for its antioxidant effects (Saxena et al., 2013).

Ferric reducing antioxidant power (FRAP) assay were also performed to determine the antioxidant activity of *D. reticulata* extracts. As shown in Table 4.3, the three extracts possessed no significant differences in reducing power activity.

The methods for determination of antioxidant activities discussed earlier are useful only for estimating antioxidant capacities of chemical *in vitro* because living cells are not involved in the experiments. To be more relevant, the antioxidant activity of *D. reticulata* extracts were tested in the human hepatoma HepG2 cells to show a biologically relevance using alloxan as a free radical source.

The liver is an organ which plays an essential role in maintaining normal physiology and it is also a vulnerable target of many drugs or other chemicals. The safety and protective evaluation in this study was performed using alloxan as an oxidative stress agent to induce cell damage. The IC_{50} of alloxan (14 mM) found in the present study was similar to that from the previous study (Kumkrai et al., 2014), using RINm5F cells (9 mM).

Cytotoxicity studies of all extracts were conducted and the results showed that the aqueous extract had less toxicity to HepG2 cells than the other extracts. After treated with the aqueous extract at the concentrations up to 5000 µg/ml overnight, cell viability was not significantly decreased compared to control. In contrast, after exposure of hexane and chloroform extracts at the concentrations higher than 60 µg/ml, cell death increased significantly. Therefore, in further experiments, the

concentrations of hexane and chloroform extracts were limited at 60 µg/ml, for otherwise the extracts themselves might have synergized the toxicity of alloxan, instead of protection.

In the cytoprotective study, the result revealed that all extracts at the concentration of 60 µg/ml were not be able to protect HepG2 cells from the damage induced by alloxan. However, the aqueous extract at the doses of 500 and 5000 µg/ml possessed cytoprotective effect by increasing cell viability from about 50% up to 70%. The results obtained from this experiment support the proposed mechanism for anti-diabetic action of *D. reticulata* aqueous extract by its cytoprotective effect. However, the mechanisms underlying this protective effect and whether or not it is involved in antioxidant activity still remain elusive.

Except for the antioxidant activity to protect oxidative stress from oxidant agents, another mechanism which was proposed to responsible for anti-diabetic activity of *D. reticulata* extract was the inhibition of α-glucosidase enzyme (Kumkrai et al., 2014). This enzyme has the property to breakdown carbohydrates to monosaccharide before absorption. The inhibition of intestinal α-glucosidase delays the digestion of carbohydrates and reduces the rate of glucose absorption (International Union of Biochemistry and Molecular Biology, 2010; Burkhard and Christine, 1998). Thus, in this study α-glucosidase inhibitory effects of three *D. reticulata* extracts were compared. As shown in Table 4.4, the result indicates that all of *D. reticulata* extracts have α-glucosidase inhibitory activity. However, the chloroform extract showed the highest potency (IC_{50}; 0.68 ± 0.10 µg/ml). The hexane extract also exhibited high potential in inhibiting the enzyme activity with the IC_{50} of 4.19 ± 0.28 µg/ml which is lower than acarbose, a standard drug. The active ingredient

may be the chemical on TLC at R_f = 0.854. The IC_{50} of the aqueous extract was found at 411.62 µg/ml which implied that it had much lower inhibitory effect than the other extracts. The activity of the aqueous extract in this study was comparable to the activity of the aqueous extract of *D. reticulata* reported previously (917.29 µg/ml). As mentioned earlier, the major compound present in the hexane and chloroform extracts was not detected in the aqueous extract. Taken together, it is possible that the compound on TLC at R_f = 0.854 may be the active ingredient for inhibition of α-glucosidase enzyme. The flavonoid lupinifolin has been reported to be a major compound in *D. reticulata* (Chivapat, Chavalittumrong, Attiwist, and Soonthornchareonnon, 2009). Hence, the compound which was detected at R_f = 0.854 in this study might be lupinifolin and may be responsible for α-glucosidase inhibitory activity of both hexane and chloroform extracts. However, further experiments are necessary for verification. Thus, the isolation and identification of this compound including the examination of its antioxidant, α-glucosidase inhibitory and anti-diabetic activities are interesting for the future study.

In conclusion, the present study has demonstrated that the compositions found in hexane and chloroform extracts by phytochemical screening test were similar, but different from those found in the aqueous extract. The major compound of the hexane and chloroform extracts appeared to be the same which was detected at R_f = 0.854 on TLC. Instead, this compound was likely not present in the aqueous extract. All three extracts of *D. reticulata* possessed *in vitro* antioxidant activities with different degrees of power depend on the method used. From the cytotoxic test, hexane and chloroform extracts produced more toxic effects than the aqueous extract on HepG2 cell viability. All extracts at the dose of 60 µg/ml failed to protect cells

from the damage induced by alloxan, however, higher doses of the aqueous extracts produced a cytoprotective effect to HepG2 cells. Finally, the chloroform extract exhibited the strongest inhibitory effect on α-glucosidase activity followed by hexane extract, whereas the aqueous extract showed the weakest effect. Its IC_{50} was 600 times higher than the IC_{50} of chloroform extract.

REFERENCES

REFERENCES

Alam, S. A., Khan, A. H., Sirhindi, G. A., and Khan, S. (2005). Alloxan induced diabetes in rabbits. **Pakistan Journal of Pharmacology.** 22(2): 41-45.

American Diabetes Association. (2012). Diagnosis and classification of diabetes mellitus. **Diabetes Care.** 35: 64-71.

American Type Culture Collection. (2014). **HepG2 cells** [On-line]. Available: http://www.atcc.org/products/all/HB-8065.aspx#characteristics.

Antolovich, M., Prenzler, P. D., Patsalides, E., Mcdonald, S., and Robards, K. (2002). Methods for testing antioxidant activity. **The Analyst.** 127: 183-198.

Bangkok Herbarium. (2009). **Type specimens in Bangkok herbarium volume III : Guttiferae to Myrcinaceae.** Bangkok: Agricultural cooperatives association of Thailand.

Bele, A. A., and Khale, A. (2011). An overview on thin layer chromatography. **International Journal of Pharmacy and Pharmaceutical Sciences.** 2: 256-267.

Benzie, I. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. **Analytical Biochemistry.** 239: 70-76.

Bierhaus, A., Hofmann, M. A., Ziegler, R., and Nawroth, P. P. (1998). AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. the AGE concept. **Cardiovascular Research.** 37(3): 586-600.

Blasa, M., Gennari, L., Angelino, D., and Ninfali, P. (2010). **Bioactive foods in promoting health** (ed.). San Diego: Academic Press.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. **Nature.** 181: 1199-1200.

Bor, J. Y., Chen, H. Y., and Yen, G. C. (2006). Evaluation of antioxidant activity and inhibitory effect on nitric oxide production of some common vegetables. **Journal of Agricultural and Food Chemistry.** 54: 1680-1686.

Burkhard, G., and Christine, H. R. (1998). The evolving role of α-glucosidase inhibitors. **Diabetes Metabolism Reviews.** 14: 31-38.

Cao, G., Alessio, H. M., and Cutler, R. G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. **Free Radical Biology and Medicine.** 14(3): 303-311.

Chivapat, S., Chavalittumrong, P., Attiwist, A., and Soonthornchareonnon, N., (2009). Toxicity study of lupinifolin from stem of *Derris reticulata* Craib. **Journal of Thai Traditional and Alternative Medicine.** 7:146-155.

Choi, C.W., Choi, Y. H., Cha, M. R., Yoo, D. S., Kim, Y. S., Yon, G. H., Hong, K. S., Kim, Y. H., and Ryu, S. Y. (2010). Yeast alpha-glucosidase inhibition by isoflavones from plants of Leguminosae as an in vitro alternative to acarbose. **Journal of Agricultural and Food Chemistry.** 58: 9988-9993.

Chung, S. S., Ho, E. C., Lam, K. S., and Chung, S. K. (2003). Contribution of polyol pathway to diabetes-induced oxidative stress. **Journal of the American Society of Nephrology.** 14: 233-236.

Clissold, S., and Edwards, C. (1988). Acarbose. **Drugs.** 35: 214-243.

Cushnie, T. P., and Lamb, A. J. (2005). Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agents**. 26: 343-356.

Dillard, C. J., and German, J. B. (2000). Phytochemicals: nutraceuticals and human health. **Journal of the Science of Food and Agriculture.** 80: 1744-1756.

Dominique, B. R. (2002). Glucose and reactive oxygen species. **Current Opinion in Clinical Nutrition Metabolic Care.** 5: 561-568.

Dong, H. Q., Li, M., Zhu, F., Liu, F. L., and Huang, J. B. (2012). Inhibitory potential of trilobatin from *Lithocarpus polystachyus* Rehd against α-glucosidase and α-amylase linked to type 2 diabetes. **Food Chemistry.** 130: 261-266.

Elsner, M., Tiedge, M., Guldbakke, B., Munday, R., and Lenzen, S. (2002). Importance of the GLUT2 glucose transporter for pancreatic beta cell toxicity of alloxan. **Diabetologia.** 45(11): 1542-1549.

Harborne, J. B. (1998). **Phytochemical methods a guide to modern techniques of plant analysis** (3rd ed.). London: Chapman and Hall Press.

Heikkila, R. E. (1977). The prevention of alloxan-induced diabetes in mice by dimethyl sulfoxide. **European Journal of Pharmacology.** 44(2): 191-193.

Hirayama, M., Kohgo, Y., Kondo, H., Shintani, N., Fujikawa, K., Sasaki, K., and Nhtsu, Y. (1993). Regulation of iron metabolism in HepG2 cells: a possible role for cytokines in the hepatic deposition of iron. **Hepatology.** 18(4): 874-880.

Hu, M., Zhong, G., Sun, Z. T., Sh, G., Liu, H., and Liu, X. (2005). Insecticidal activities of secondary metabolites of endophytic *Pencillium* sp. in *Derris elliptica* Benth. **Journal of Applied Entomology.** 129(8): 413-417.

International Union of Biochemistry and Molecular Biology. (2010). **The enzyme list class-3-hydrolases**-[on-line].-Available:http://www.enzymedatabase.org/query.php?ec=3.2.1.

Joshi, S. K., and Shrestha, S. (2010). Diabetes mellitus: a review of its associations with different environmental factors. **Kathmandu University Medical Journal.** 8: 109-115.

Joycharat, N., Thammavong, S., Limsuwan, S., Homlaead, S., Voravuthikunchai, S. P., Yingyongnarongkul, B. E., Dej-Adisai, S., and Subhadhirasakul, S. (2013). Antibacterial substances from *Albizia myriophylla* wood against cariogenic *Streptococcus mutans*. **Archives of Pharmacal Research.** 36:723-730.

Kannan, K., and Jain, S. K. (2000). Oxidative stress and apoptosis. **Pathophysiology.** 7: 153-163.

Karadag, A., Ozcelik, B., and Saner, S. (2009). Review of methods to determine antioxidant capacities. **Food Analytical Methods.** 2(1): 41-60.

Khan, M. R., Omoloso, A. D., and Barewai, Y. (2006). Antimicrobial activity of the *Derris elliptica*, *Derris indica* and *Derris trifoliata* extractives. **Fitoterapia.** 77: 327-330.

Kongjinda, V. (2004). **Chemical constituents and its biological activities from *Derris malaccensis*.** M.Sc. thesis, Kasetsart University.

Kumkrai, P., Kamonwannasit, S., and Chudapongse, N. (2014). Cytoprotective and anti-diabetic effects of *Derris reticulata* aqueous extract. **Journal of Physiology and Biochemistry.** 70: 675-684.

Kumkrai, P., Weeranantanapan, O., and Chudapongse, N. (2015). Antioxidant, α-glucosidase inhibitory activity and sub-chronic toxicity of *Derris reticulata* extract: its antidiabetic potential. **BMC Complementary and Alternative Medicine.** 15(1): 35.

Laupattarakasem, P., Houghton, P. J., Hoult, J. R. S., and Itharat, A. (2003). An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. **Journal of Ethnopharmacology.** 85: 207-215.

Lenzen, S. (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. **Diabetologia.** 51(2): 216-226.

Luitgards-Moura, J. F., Castellón Bermudez, E. G., Rocha, A. F. I. D., Tsouris, P., and Rosa-Freitas, M. G. (2002). Preliminary assays indicate that *Antonia ovata* (Loganiaceae) and *Derris amazonica* (Papilionaceae), ichthyotoxic plants used for fishing in Roraima, Brazil, have an insecticide effect on *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae). **Memórias do Instituto Oswaldo Cruz.** 97(5): 737-742.

Mahidol, C., Prawat, H., Ruchirawat, S., Lihkitwitayawuid, K., Lin, L.-Z., and Cordell, G. A. (1997). Prenylated flavanones from *Derris reticulata*. **Phytochemistry.** 45:825-829.

Mamoon, A. S., and Azam, M. G. (2012). Preliminary phytochemical screening and antidiarrhoeal activity of *Derris trifoliata* Lour. **International Journal of Pharmaceutical Sciences.** 3: 97-100.

Marks, D. C., Belov, L., Davey, M. W., Davey, R. A., and Kidman, A. D. (1992). The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. **Leukemia Research.** 16(12): 1165-173.

Martini, F. H., Ober, W. C., Garrison, C. W., Welch, K., and Hutchings, R. T. (2006). **Fundamenttals of Anatomy and Physiology** (7th ed.). San Francisco: Daryl Fox.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., and Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. **Clinical Science.** 84: 407-407.

Montoro, E., Lemus, D., Echemendia, M., Martin, A., Portaels, F., and Palomino, J. C. (2005). Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. **Journal of Antimicrobial Chemotherapy.** 55(4): 500-505.

Mshelia, D. S. (2004). Role of free radicals in pathogenesis of diabetes nephropathy. **Annals of African Medicine.** 3: 55-62.

Neuman, M. G., Koren, G., and Tiribelli, C. (1993). *In vitro* assessment of the ethanol-induced hepatotoxicity on HepG2 cell line. **Biochemical and Biophysical Research Communications.** 197(2): 932-941.

Olokoba, A. B., Obateru, O. A., and Olokoba, L. B. (2012). Type 2 diabetes mellitus: a review of current trends. **Oman Medical Journal.** 27(4): 269-273.

Pengelly, A. (2004). **The constituents of medicinal plants.** United States of America: CABI international.

Prasad, S. K., Laloo, D., Kumar, M., and Hemalatha, S. (2013). Antidiarrhoeal evaluation of root extract, its bioactive fraction, and lupinifolin isolated from *Eriosema chinense*. **Planta Medica.** 79:1620-1627.

Prior, R. L., Wu, X., and Schaich, K. (2005). Standardized method for the determination of antioxidant capacity and phenolics in food and dietary supplements. **Journal of Agricultural and Food Chemistry.** 53: 4290-4302.

Radoi, V., Lixandru, D., Mohora, M., and Virgolici, B. (2012). Advanced glycation end products in diabetes mellitus: mechanism of action and focused treatment. **Publishing House of the Romanian Academy.** 1: 9-19.

Rao, R. R., Tiwari, A. K., Reddy, P. P., Babu, K. S., Ali, A. Z., Madhusudana, K., and Rao, J. M. (2009). New furanoflavanoids, intestinal α-glucosidase inhibitory and free-radical (DPPH) scavenging, activity from antihyperglycemic root extract of *Derris indica* (Lam.). **Bioorganic & Medicinal Chemistry.** 17: 5170-5175.

Raut, N. A., and Gaikwad, N. J. (2006). Antidiabetic activity of hydro-ethanolic extract of *Cyperus rotundus* in alloxan induced diabetes in rats. **Fitoterapia.** 77(7): 585-588.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine.** 26: 1231-1237.

Saxena, M., Saxena, J., Nema, R., Singh, D., and Gupta, A. (2013). Phytochemistry of medicinal plants. **Journal of Pharmacognosy and Phytochemistry.** 1(6): 168-182.

Shaw, J. E., Sicree, R. A., and Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. **Diabetes Research and Clinical Practice.** 87: 4-14.

Saxena, M., and Saxena, J. (2012). Phytochemical screening of *Acorus calmus* and *Lantana camara*. **International Research Journal of Pharmacy.** 3: 324-326.

Sherma, J., and Fried, B. (2003). **Handbook of thin-layer chromatography** (3rd ed.). New York: CRC Press.

Siri Ruckhachati Nature Park. (2010). ***Derris reticulata*** **Craib** [on-line]. Available : http://www.pharmacy.mahidol.ac.th/siri/.

Sittiwet, C., and Puangpronpitag, D. (2009). Antimicrobial properties of *Derris scandens* aqueous extract. **Journal of Biological Sciences**. 9:607-611.

Songsak, T. (1995). **Phytochemistry of stem bark and pharmacognostic specification of *Derris reticulata* Craib.** M.Sc. thesis, Chulalongkorn University.

Soonthornchareonnon, N., Ubonopas, L., Kaewsuwan, S., and Wuttiudomlert, M. (2004). Lupinifolin, a bioactive flavanone from *Myriopteron extensum* (Wight) K. Schum. stem. **Thai Journal of Phytopharmacy.** 11: 19-27.

Srimongkol, Y., Warachit, P., Chavalittumrong, P., Sriwanthana, B., Pairour, R., Inthep, C., and Wongsinkongman, P. (2007). A study of the efficacy of *Derris scandens* (Roxb.) Benth. extract compared with diclofenac for the alleviation of low back pain. **Journal of Thai Traditional & Alternative Medicine.** 5: 17-23.

Strober, W. (2001). **Current protocols in immunology.** Hoboken: John Wiley and Sons.

Sutthivaiyakit, S., Thongnak, O., Lhinhatrakool, T., Yodchun, O., Srimark, R., Dowtaisong, P., and Chuankamnerdkarn, M. (2009). Cytotoxic and antimycobacterial prenylated flavonoids from the roots of *Eriosema chinense*. **Journal of Natural Products.** 72: 1092-1096.

Tewtrakul, S., Cheenpracha, S., and Karalai, C. (2009). Nitric oxide inhibitory principles from *Derris trifoliata* stems. **Phytomedicine.** 16(6-7): 568-572.

Thabrew, M., Hughes, R. D., and Mcfarlane, I. G. (1997). Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. **Journal of Pharmacy and Pharmacology.** 49(11): 1132-1135.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry and Cell Biology.** 39: 44-84.

Van Meerloo, J., Kaspers, G. J., and Cloos, J. (2011). Cell sensitivity assays: the MTT assay. **Cancer Cell Culture.** 731: 237-245.

Wagner, H., and Bladt, S. (1996). **Plant drug analysis: A thin layer chromatography** Atlas (2nd ed.). New York: Springer Science and Business Media.

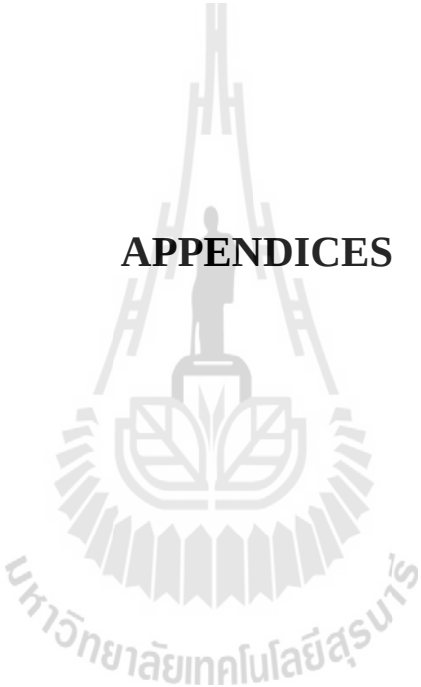
Wisetsutthichai, S., Techamahamaneerat, S., Junyaprasert, V., and Soonthornchareon-non, N. (2005). Gel preparation development from *Derris reticulata* Craib. extract as an anti-herpes simplex drug. **Thai Journal of Phytopharmacy.** 12: 11-22.

Wu, F. Y. H., Liao, W. C., and Chang, H. M. (1993). Comparison of antitumor activity of vitamins K 1, K 2 and K 3 on human tumor cells by two (MTT and SRB) cell viability assays. **Life Sciences.** 52(22): 1797-1804.

Xin-Hua, W., Chang-Qing, L., Xing-Bo, G., and Lin-Chun, F. (2001). A comparative study of *Phyllanthus amarus* compound and interferon in the treatment of chronic viral hepatitis B. **Southeast Asian Journal of Tropical Medicine and Public Health.** 32(1): 140-142.

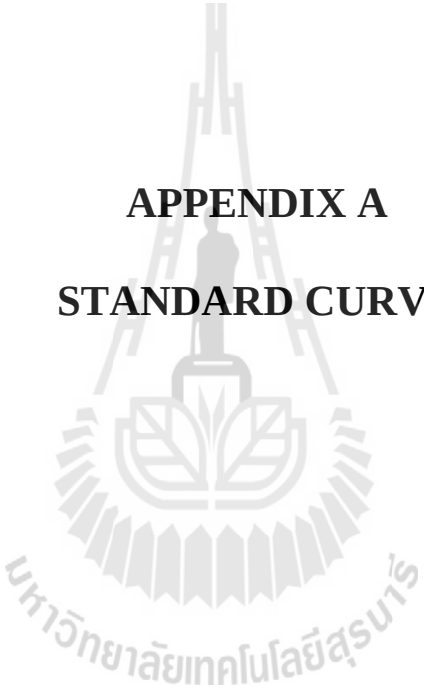
Yeo, R., and Sawdon, M. (2013). Hormonal control of metabolism: Regulation of plasma glucose. **Anaesthesia and Intensive Care Medicine.** 14: 296-300.

APPENDICES

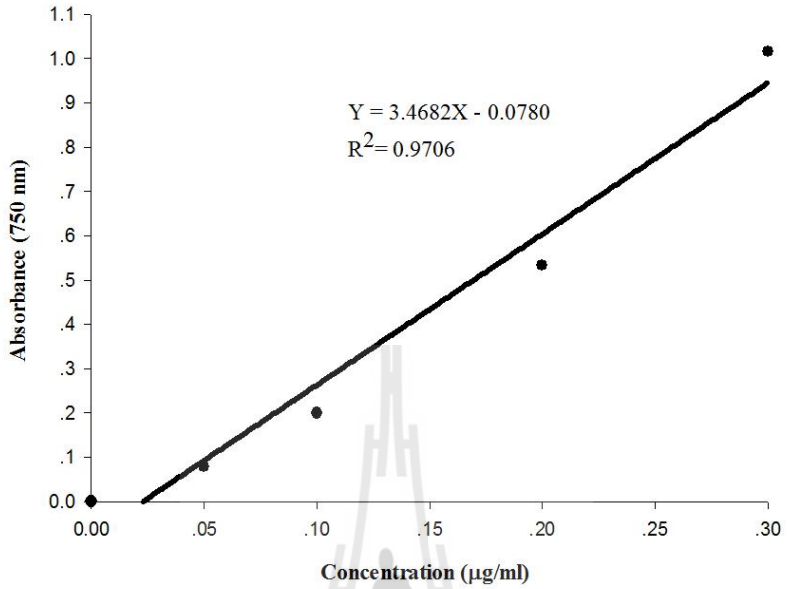


APPENDIX A

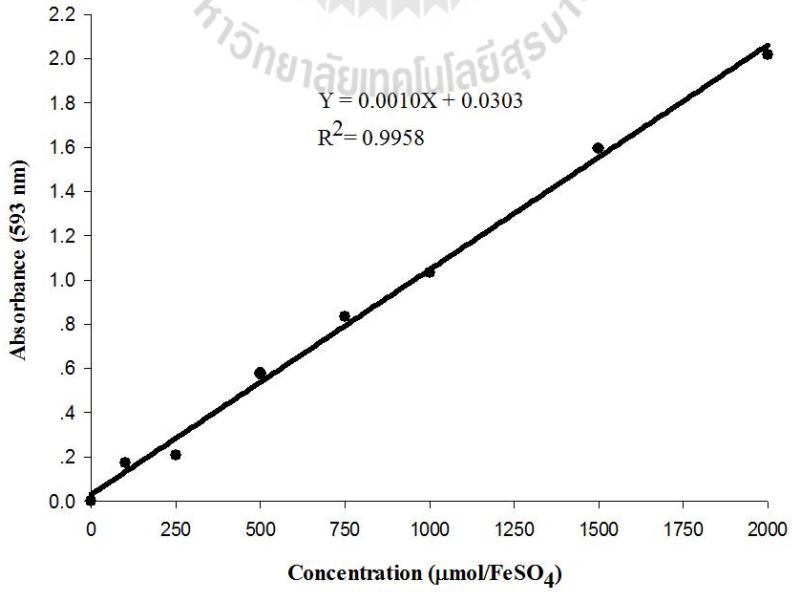
STANDARD CURVE



A.1 Standard gallic acid



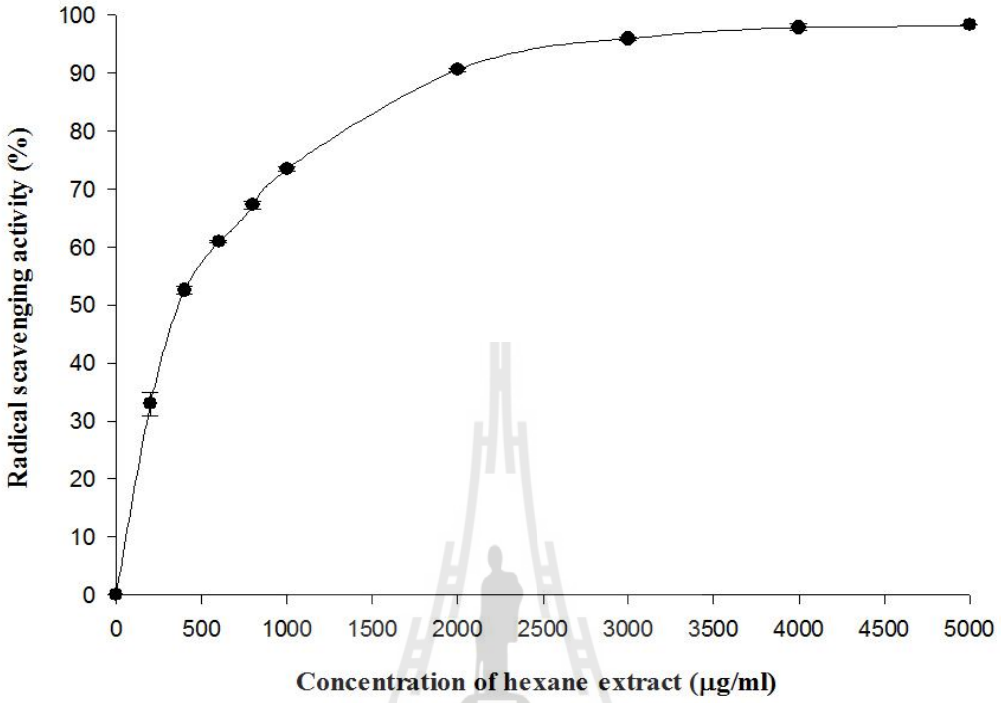
A.2 Standard $FeSO_4$



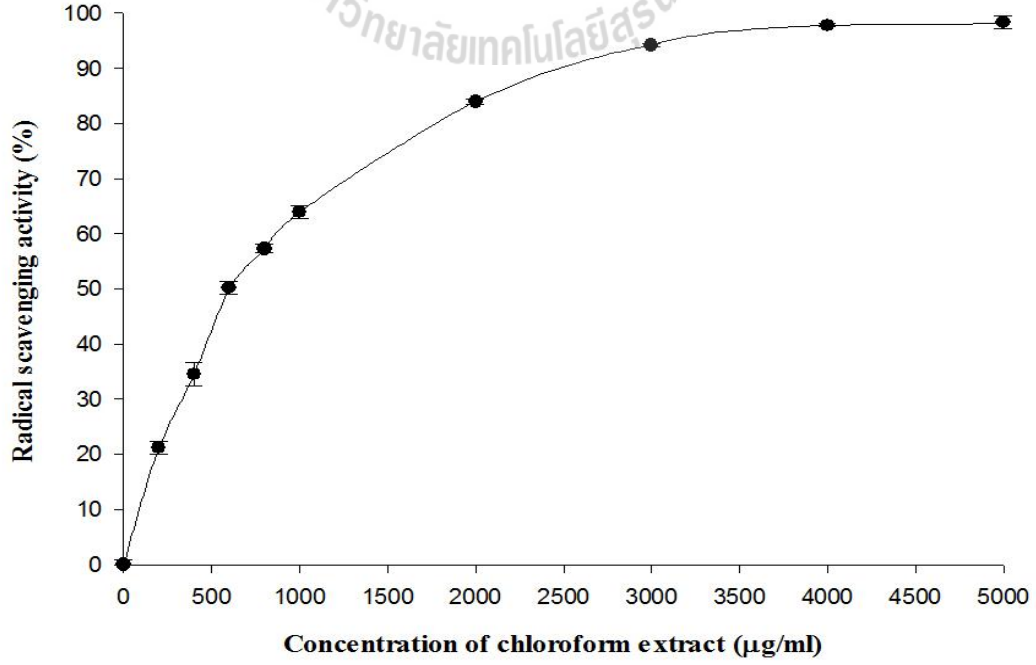
APPENDIX B

ANTIOXIDANT ACTIVITY

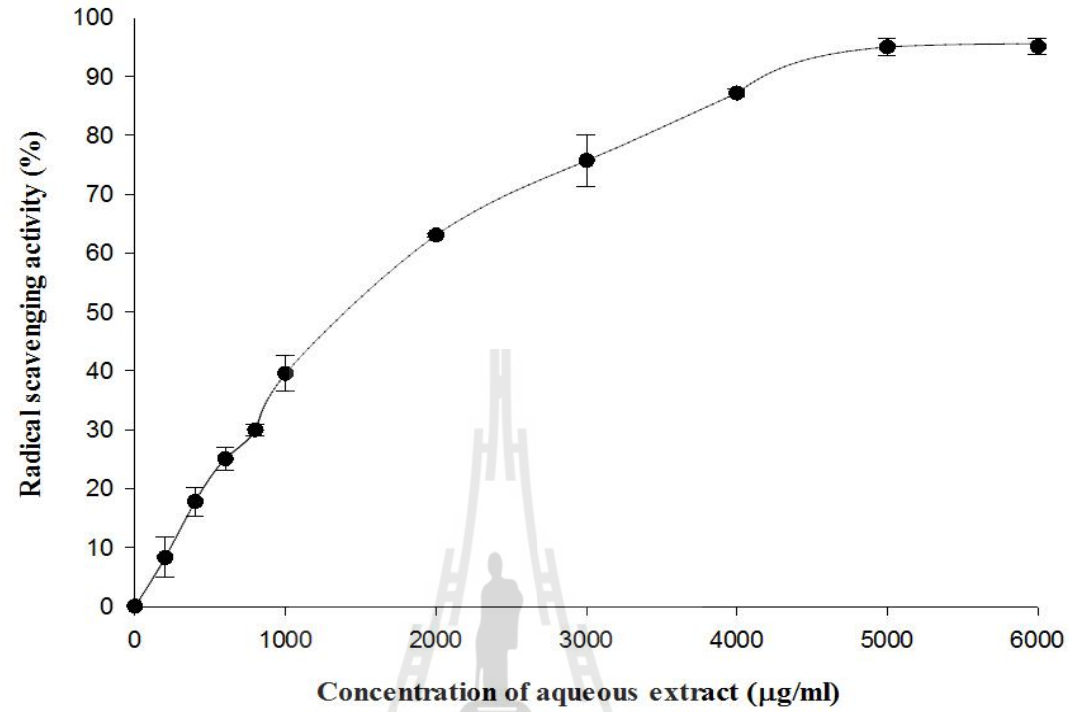
B.1 ABTS assay



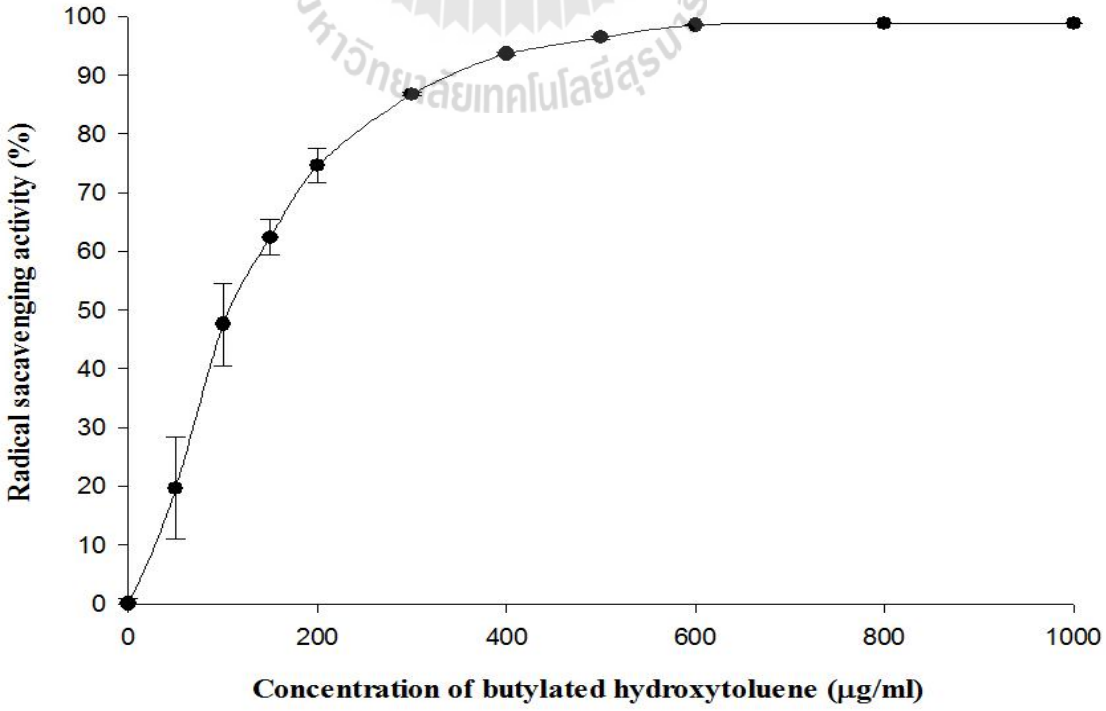
B.2 ABTS assay



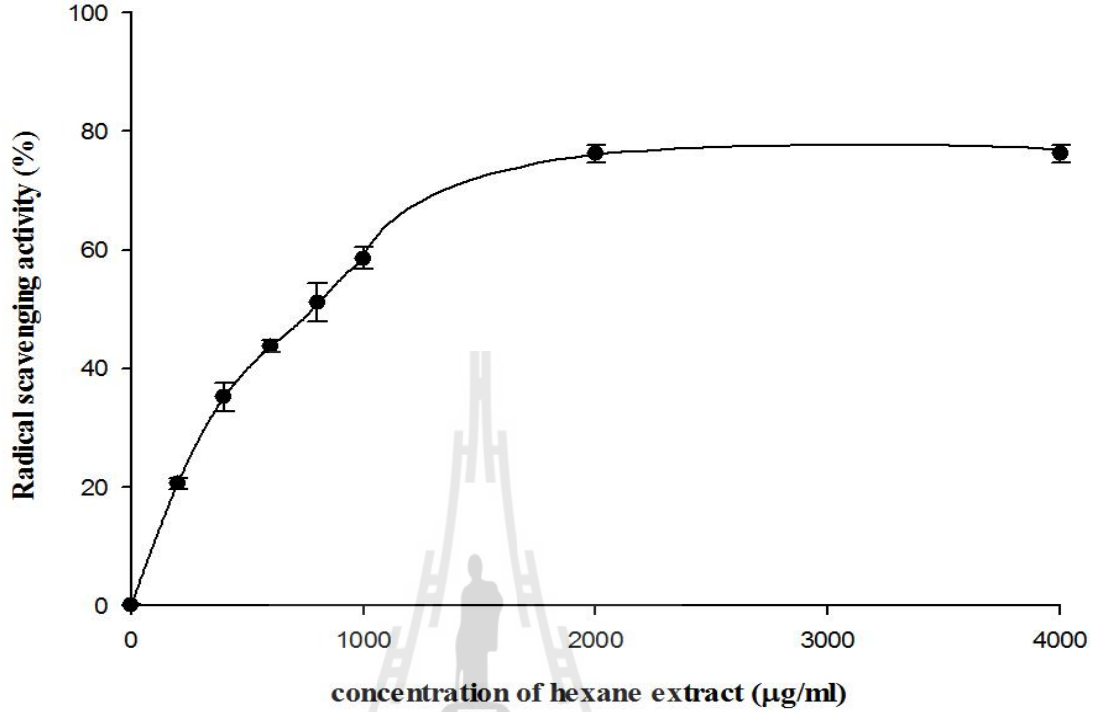
B.3 ABTS assay



B.4 ABTS assay



B.5 DPPH assay



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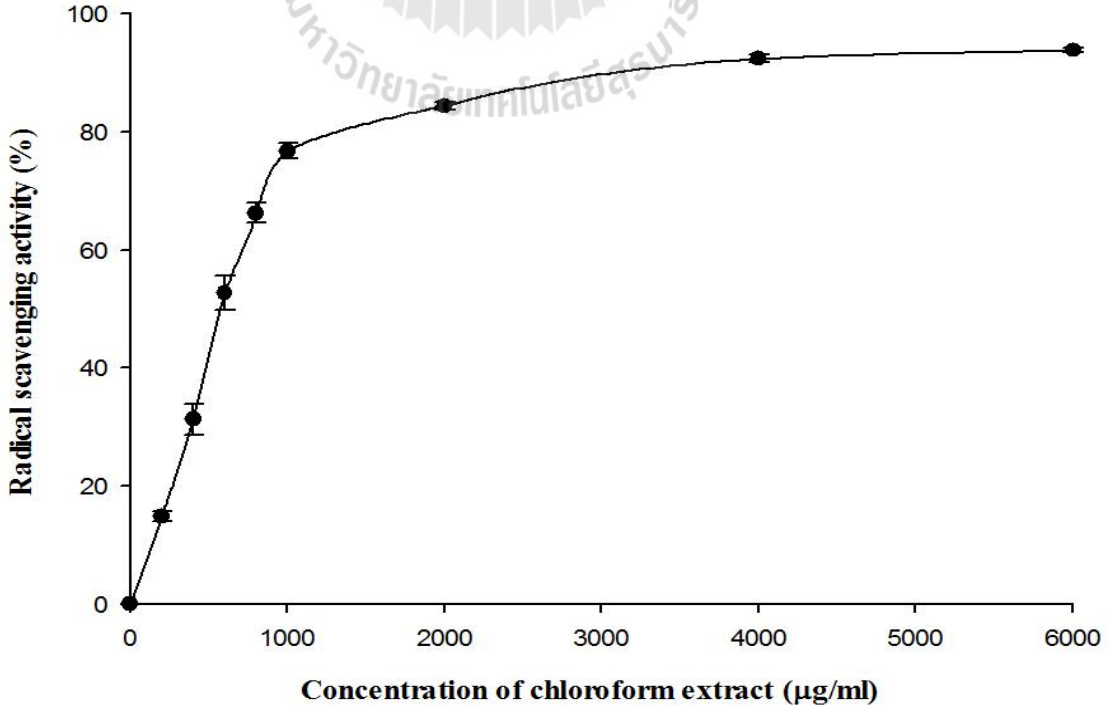

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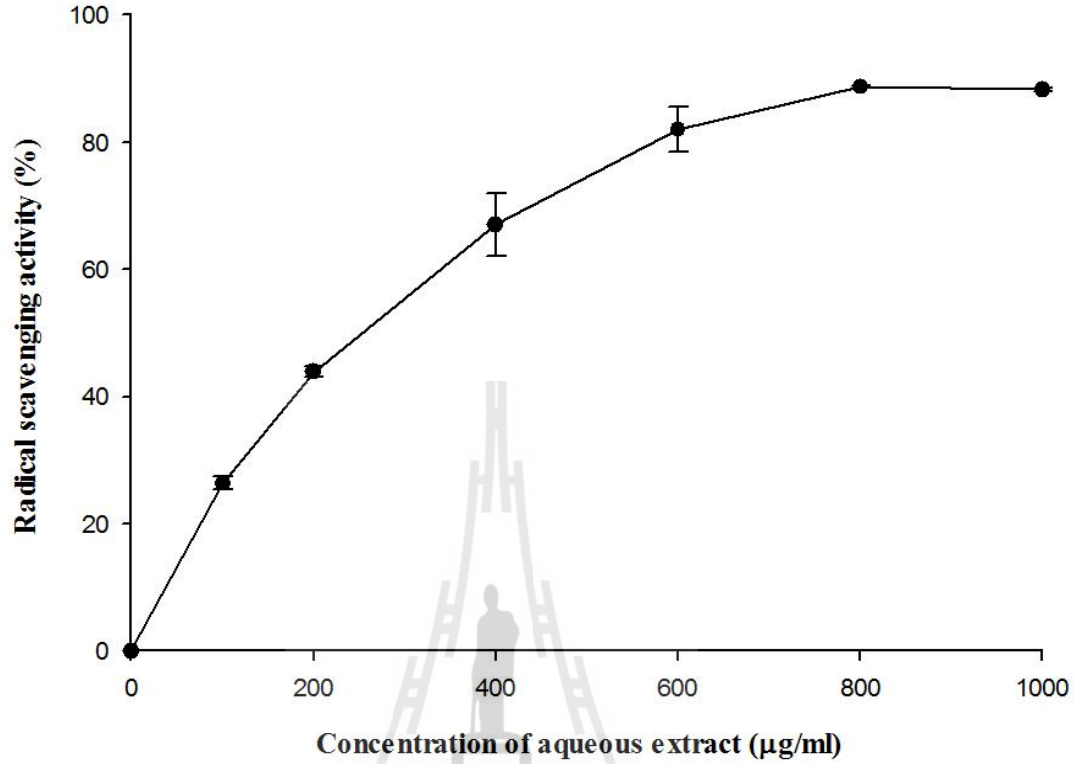

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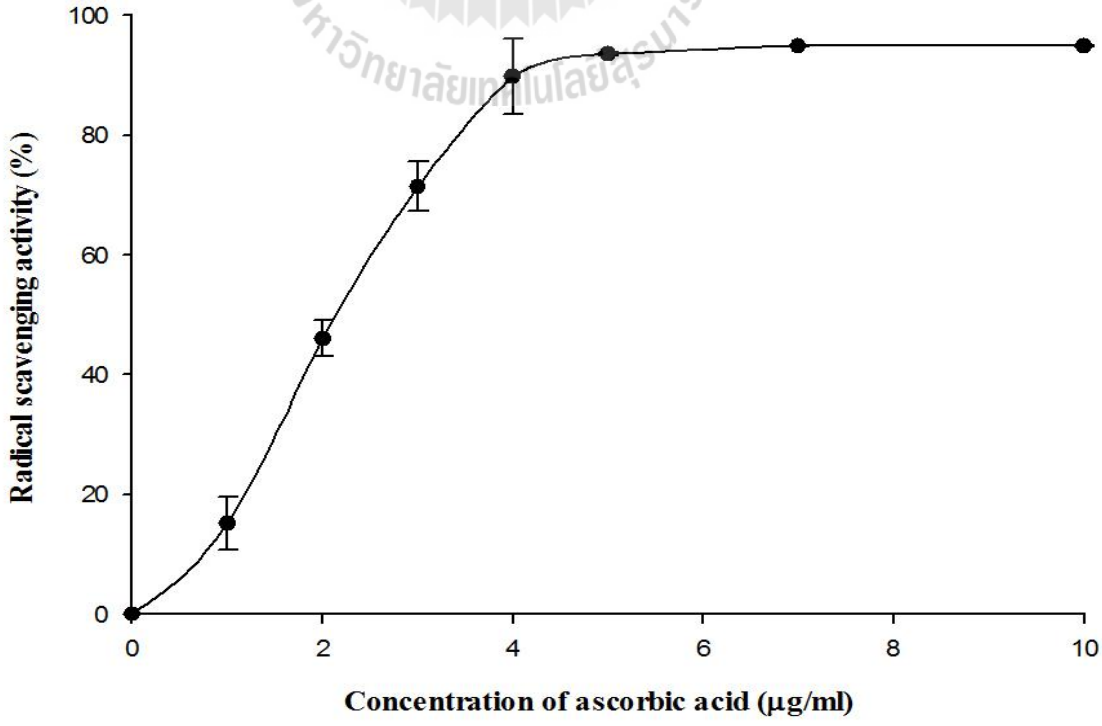
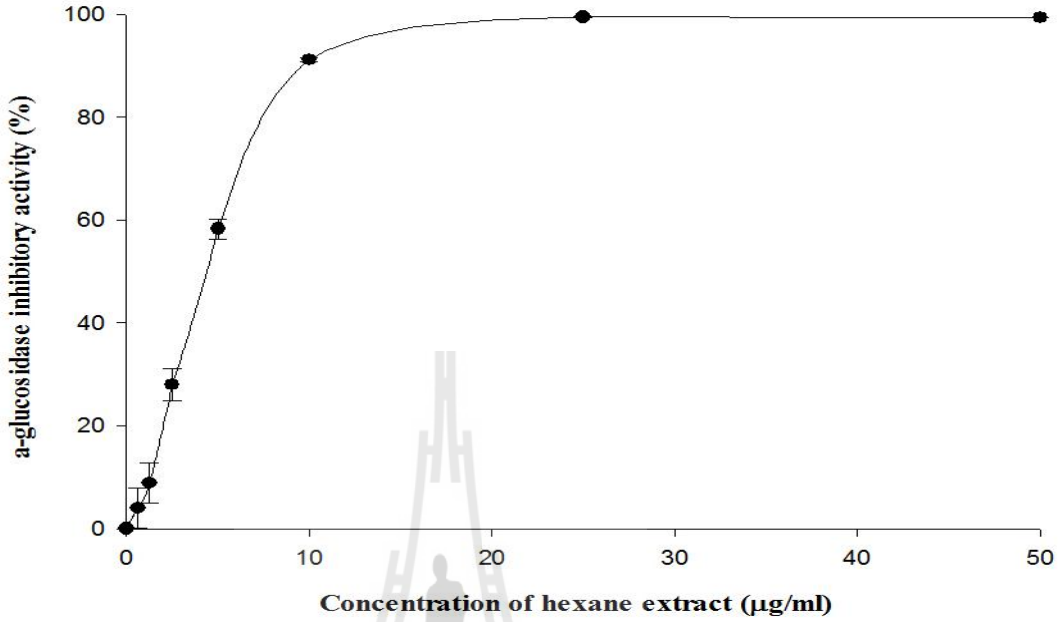


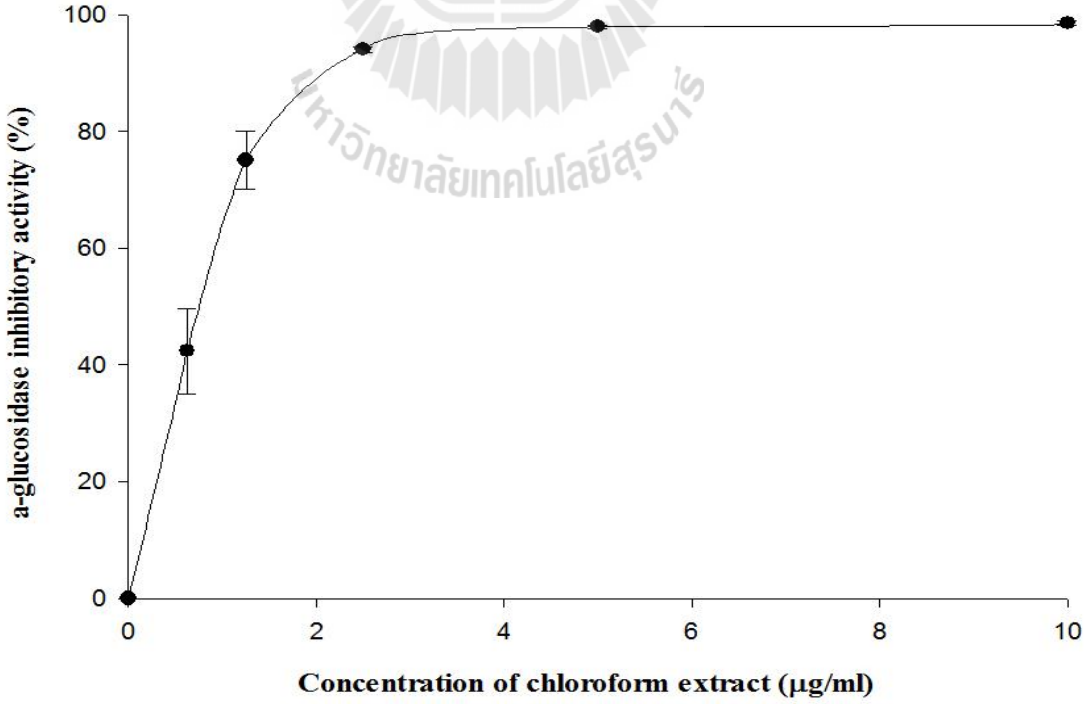
APPENDIX C

α-GLUCOSIDASE INHIBITORY ACTIVITY

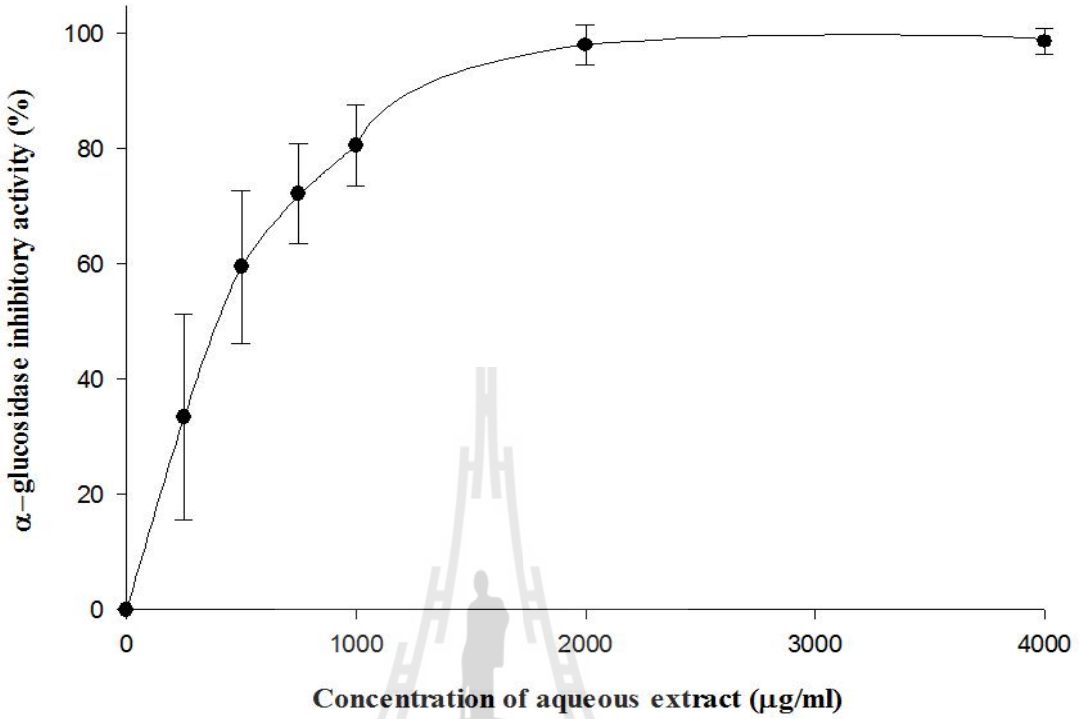
C.1 α-glucocidase inhibitory activity assay



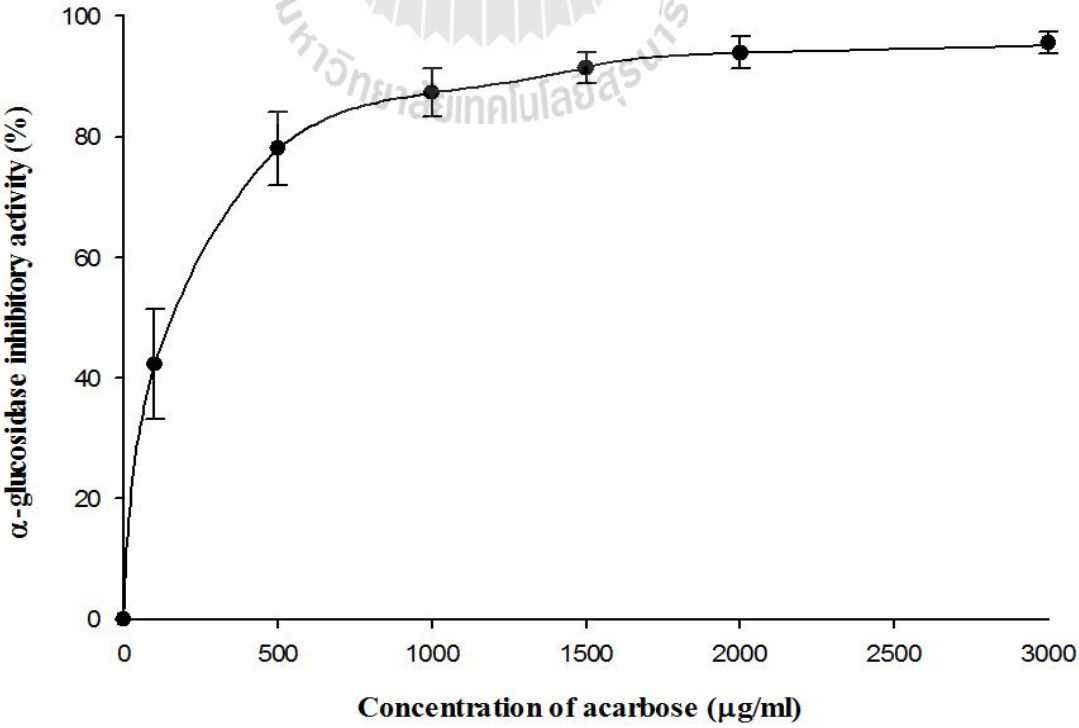
C.2 α-glucocidase inhibitory activity assay



C.3 α-glucocidase inhibitory activity assay



C.4 α-glucocidase inhibitory activity assay



APPENDIX D

CHEMICALS AND INSTRUMENTS

Chemicals and instruments

All chemicals and instruments used in this study are listed in Table 1 and 2.

Table 1 List of chemicals used in the study.

Name	**Source**
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS)	Sigma
2,2-diphenyl-1-picrylhydrazyl (DPPH) radical	Aldrich
2,4,6-tri(2-pyridyl)-s-triazine (TPTZ)	Flukar
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Invitrogen
Acetic acid glacial	Carlo erba
Ammonia	Panreac
Antibiotic-antimitotic solution	Gibco
Acarbose	Fluka
Ascorbic acid	Carlo erba
Acetone	Loba chemic
Benzene	Loba chemic
Butylated hydroxytoluene (BHT)	Flukar
Chloroform	Analar nornapur

Table 1 List of chemicals used in the study (Continued).

Name	**Source**
Dichloromethane	Carlo erba
Dimethylsulphoxide (DMSO)	Carlo erba
Dulbecco's modified eagle medium (DMEM)	Gibco
Ethanol	Aci labscan
Ethyl alcohol	Univar
Ferric chloride ($FeCl_3$)	Fisher chemical
Ferrous sulfate ($FeSo_4$)	Carlo erba
Fetal bovine serum	Gibco
Folin-Ciocalteau reagent	Carlo
Gallic acid	Fluka
HEPES	Gibco
Hexane	Aci labscan
Hydrochloric acid (HCl)	Carlo erba
Iodine	Univar
Lead acetate	Loba chemie
Magnesium ribbon	Aldrich
Mercury chloride ($HgCl_2$)	Univar

Table 1 List of chemicals used in the study (Continued).

Name	**Source**
Methanol	Lobal chemie
Sodiun hydroxide (NaOH)	Univar
Potassium iodide (KI)	Univar
Potassium ferricyanide	Univar
Sodium acetate trihydrate	Gibco
Sodium carbonate	Carlo
Sodium pyruvate	Gibco
Sulphuric acid	Univar
TLC silica gel 60 F_{254}	Merck kgaa
Trypan blue	Gibco
0.25% trypsin-EDTA	Gibco
Tween 80	Bio-rad

Table 2 List of instruments used in the study.

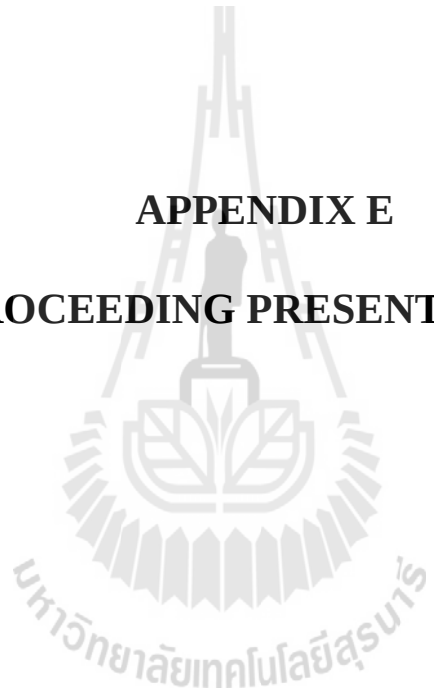
Name	**Source**
96-well plate	Nunc
Analytical balance	Ohaus

Table 2 List of instruments used in the study (Continued).

Name	**Source**
Autoclave	Hirayama
Centrifuge machine	Hettich
ELISA plate reader	Bio-rad
Haemocytometer	Fisher Scientific
Heating mantle with digital	Tops
Hot air over	Memmert
Lyophilizer	Labconco
Micropipette	Eppendorf
Microscope (model CKX4)	Olympus
Microscope (model CX21)	Olympus
Rotary evaporator	Buchi
Soxhlet extractor	Sigma Adldrich
Spectrophotometer	Cecil
Tissue culture flask	Nunc
TLC developing tank	Sigma Adrich

APPENDIX E

PROCEEDING PRESENTATION



34th The National Graduate Research Conference

MMP56

Phytochemical Screening and Antioxidant Activities of Hexane, Chloroform, and Aqueous Extracts of *Derris reticulata* stem

พฤกษเคมีเบื้องต้นและฤทธิ์ต้านอนุมูลอิสระของต้นชะเอมเหนือที่สกัดด้วยเฮกเซน คลอโรฟอร์มและน้ำ

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ABSTRACT

Recently, the aqueous extract of *Derris reticulata Craib.* (Leguminosae) has been shown to exhibit an anti-diabetic activity in alloxan-induced diabetic rats. It was proposed that the anti-diabetic activity was due to its cytoprotective effect, probably by its antioxidant activity. The main objective of this study was aim to compare antioxidant activity of *D. reticulata* stem extract obtained by sequential extraction method with three different solvents, hexane, chloroform and water. The results from phytochemical analysis showed that the compositions of hexane extract were similar to those of the chloroform extract, but quite different from those of the aqueous extract. It was found that the aqueous extract contained the least total phenolic compounds. In accordance with phenolic contents, the hexane and chloroform extracts exhibited more antioxidant potential than the aqueous extract. The results from TLC revealed that the major constituent of hexane and chloroform extracts was the compound observed on TLC with R_f 0.854. Taken together, the results suggested that this major compound may be responsible for antioxidant activities of hexane and chloroform extracts. Now, isolation and identification of this compound as well as the examination of its antioxidant and anti-diabetic activities are in progress.

บทคัดย่อ

เมื่อไม่นานมานี้มีรายงานพบว่าสารสกัดด้วยน้ำของต้นชะเอมเหนือ (*Derris reticulate*, วงศ์ Leguminosae) มีฤทธิ์ต้านเบาหวานจากการศึกษาในหนูขาวที่ถูกชักนำให้เกิดเบาหวานด้วย alloxan โดยฤทธิ์ดังกล่าวได้รับการเสนอว่าเป็นผลจากฤทธิ์ปกป้องเซลล์ซึ่งอาจจะเนื่องมาจากฤทธิ์ต้านอนุมูลอิสระของสารสกัด การศึกษานี้มีวัตถุประสงค์หลักเพื่อเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากต้นชะเอมเหนือที่ได้การสกัดเป็นลำดับขั้นด้วยตัวทำละลายที่แตกต่างกัน 3 ชนิด คือ เฮกเซน คลอโรฟอร์มและน้ำ ผลจากการตรวจสอบพฤกษเคมีเบื้องต้นแสดงให้เห็นว่าส่วนประกอบของสารสกัดด้วยเฮกเซนและคลอโรฟอร์มมีความคล้ายคลึงกันแต่ค่อนข้างแตกต่างจากสารสกัดด้วยน้ำ และพบว่าสารสกัดด้วยน้ำมีปริมาณสารฟีนอลิกน้อยที่สุด นอกจากนั้นยังพบว่าสารสกัดด้วยเฮกเซนและคลอโรฟอร์มมีฤทธิ์ต้านอนุมูลอิสระมากกว่าสารสกัดด้วยน้ำ ซึ่งผลดังกล่าวนี้สอดคล้องกับปริมาณสารฟีนอลิกที่พบในสารสกัดทั้งสามชนิด ผลจากการทำ TLC เผยให้เห็นว่าสารประกอบหลักในสารสกัดด้วยเฮกเซนและคลอโรฟอร์มน่าจะเป็นสารชนิดเดียวกันซึ่งปรากฏเป็นค่า R_f เท่ากับ 0.854 บนแผ่น TLC ผลการทดลองทั้งหมดที่ได้ชี้แนะว่า สารประกอบหลักที่พบชนิดนี้อาจจะเป็นสารออกฤทธิ์ในการต้านอนุมูลอิสระของสารสกัดเฮกเซนและคลอโรฟอร์ม ซึ่งขณะนี้กำลังอยู่ระหว่างการสกัดสารนี้ให้บริสุทธิ์เพื่อพิสูจน์เอกลักษณ์ รวมทั้งทดสอบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านเบาหวาน

Key Words: *Derris reticulata*, Antioxidant, Phytochemical screening

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Introduction

Nowadays, antioxidants are topics of interest in many research areas including nutrition, health and cosmetics due to their ability to interrupt free radical process. Free radicals are unstable molecules with unpaired electrons in their outer orbits. Free radicals are reactive molecules generated by cellular processes and environmental stress such as immune system, electron transfer, cellular respiration, chemical substances and UV radiation. Free radical can damage DNAs, proteins and lipids leading to cellular dysfunction, injury and death. Excess free radicals cause inflammation, cancer, premature aging disorder, atherosclerosis and diabetes (Kannan and Jain, 2000). Free radicals mostly found in cellular systems are reactive oxygen species and reactive nitrogen species for example hydrogen peroxide, superoxide radical, hydroxyl radical and nitric oxide. Normally, free radical mentioned above can be removed from the body by antioxidant pathways, either enzymatic or non-enzymatic systems, such as superoxide dismutase, catalase, glutathione peroxidase, vitamin C and thiol antioxidant. However, the excess free radicals can also be destroyed by external antioxidants such as supplements containing vitamin C, flavonoid and carotenoid, which can be found in plants, vegetables and fruits (Valko et al., 2007).

It has long been widely accepted that plants are one of the major sources of antioxidants. These external antioxidants have been proven for their efficiency in interrupting free radical processes with fewer side effects than synthetic chemicals. Therefore, the natural products from medicinal plants are more interesting for reducing the risk of several ailments such as diabetes, cancer, ischemia injury and aging (Blasa et al., 2010). One of our research projects has been focused on antioxidant effects of *Derris reticulata* Craib. (Leguminosae). This climbing plant is known in Thai as Cha-em-nuea. It has long been traditionally used as expectorant to relieve cough. Moreover, anti-inflammatory activity in arthritis and anti-herpes simplex virus type I have been reported (Laupattarakasem et al., 2003; Wisetsutthichai et al., 2005). Recently, the aqueous extract of *D. reticulata* has been shown in alloxan-induced diabetic rats. by our group (Kumkrai et al., 2014). It was proposed that the anti-diabetic activity of *D. reticulata* was partly due to its cytoprotective effect, probably by an antioxidant activity. However, the aqueous extract used in our previous study contained many compounds; it was difficult to identify the active ingredients that are responsible for antioxidant and anti-diabetic activities. In the present study, we sequentially extracted *D. reticulata* stem with three solvents differ in polarity (hexane, chloroform and water), screened for phytochemical compounds and finally compared their antioxidant potentials. The results from this study would provide useful information for the future investigation to identify the active ingredients responsible for anti-diabetic activity of *D. reticulata*.

Objective of the study

The aim of this study was to compare antioxidant activities among the hexane, chloroform, and aqueous extracts from *D. reticulata* stem. Phytochemicals and phenolic contents of the extracts were investigated. Thin layer chromatography (TLC) was also conducted to examine major compounds of the extracts.

Methodology

Plant collection and extraction

The stems of *D. reticulata* were obtained from Prachinburi province, Thailand (January - April, 2011). Plant verification has been confirmed by a plant taxonomist, Dr. Paul J. Grote. Voucher specimens (Pharm-Chu-006) were preserved at School of Pharmacology, Suranaree University of Technology (SUT). The stems were cut into small pieces and dried at 50 °C in hot air oven. The extracts were obtained by sequential extraction method with hexane and chloroform using Soxhlet extractor at 80°C. Then the residues were subjected to water decoction at 100 °C.

Phytochemical screening

The phytochemical screening was carried out for identification of components of the extracts. They were screened for alkaloids, flavonoids, saponins, tannins, triterpenoids, cardiac glycosides, and polyphenol by using standard procedures (Harborne, 1998; Saxena and Saxena, 2012).

Determination of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu method and performed according to (Prior et al., 2005) with some modifications. In brief, 100 µl of 2.5 µg/ml extract was mixed with 2 ml of 2% Na_2CO_3 and incubated for 2 min at room temperature. After incubation, 100 µl of Folin-Ciocalteu reagent (diluted in methanol 1:1 v/v) was added in the reaction mixture and incubated in the dark at room temperature for 30 min. The absorbance at 750 nm was measured using spectrophotometer. The total phenolic content of extract was determined from a standard curve of Gallic acid and results are expressed as mg gallic acid equivalents (GAE) per gram extract.

Antioxidant activity determinations

ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to the method previously described (Re et al., 1999) with some modifications. The $ABTS^{\cdot+}$ radical cation stock solution was prepared by mixing 5 ml of 14 mM ABTS with 5 ml of 4.9 mM potassium persulphate ($K_2S_2O_8$) and incubated in the dark at room temperature for 16 h. Before use, the $ABTS^{\cdot+}$ radical cation stock solution was diluted with ethanol to attain absorbance of 0.7 ± 0.02 at 734 nm. After that, 50 µl of various concentrations of the extract (0-5,000 µg/ml) was mixed with 1.5 ml of diluted $ABTS^{\cdot+}$ stock solution. Then, the mixture was incubated in the dark at room temperature for 6 min. After incubation, the absorbance was measured at 734 nm using spectrophotometer. Butylated hydroxytoluene (BHT) was used as standard. Results were expressed as IC_{50} (the concentration required for 50% scavenge for free radical).

Ferric reducing antioxidant power assay

FRAP assay was performed according to the method described by (Benzie and Strain, 1996) with some modifications. The FRAP reagent was prepared by mixing 10 mM TPTZ solution (2,4,6-tripyridyl-s-triazine), 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6), in ratio 1:1:10 (v/v/v). Then, FRAP reagent was incubated at 37 °C until used. A 50 µl of 5 µg/ml extract was mixed with 1.5 ml of the FRAP reagent. The reaction mixture was incubated at room temperature for 4 min and measured the absorbance at 593 nm. The reducing potential of extract was determined from a standard curve of $FeSO_4$ and the FRAP value was expressed at µmol Fe^{2+}/mg dried extract.

Thin layer chromatography (TLC)

TLC was carried out for screening the number of components in extracts solution as follow. The aqueous extract was dissolved in distilled water whereas the hexane and chloroform extracts were dissolved in ethanol. Then, 10 µl (5 µg) of the extract solution was applied on 4×8 cm of TLC plates. After drying, TLC plates was transferred to TLC developing tank containing mobile phase [Dichloromethane: Methanol (95:5) and Chloroform: Methanol: Water (10:30:1)]. Compositions of each extract were first observed under UV lamp and then stained with 10% sulfuric acid for image analysis.

Statistical analysis

Data are expressed as mean ± SD. Comparisons among different groups were performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P*-values less than 0.05 were considered as significantly different.

Results

% Yield and phytochemical compositions

The results in Table 1 showed that the aqueous extract of *D. reticulata* gave the highest yield (10.56%) compared to the chloroform and hexane extracts with 2.68% and 2.02% yields, respectively.

As shown in Table 1, phytochemical screening analysis demonstrated the presence of alkaloids, triterpenoids, flavonoids, tannins and polyphenols for the hexane and chloroform extracts, whereas saponin, triterpenoids, tannins and polyphenols were presented in the aqueous extract.

Total phenolic compounds and antioxidant activity

Total phenolic contents and antioxidant activities of the extracts are shown in Table 2. Total phenolic contents of the hexane and chloroform extracts were similar (52.29±1.33 and 53.89±0.68 mg GAE/g extract, respectively), but significantly higher than that of the aqueous extract (33.21±0.31 mg GAE/g extract). For antioxidant activity by ABTS method, the hexane extract exhibited the highest potential. The IC_{50} of hexane and chloroform extracts were 441.55±13.56 and 632.69±23.02 µg/ml, respectively, whereas that of the aqueous extract was 1252.68±110.00 µg/ml. However, in this study, the

Table 1 Yields and phytochemical compositions of the chloroform, hexane, and aqueous extracts from *D. reticulata* stem

Phytochemical Screening Tests	Extract		
	Hexane	Chloroform	Aqueous
Alkaloids			
Mayer's test	+	+	-
Wagner's test	+	+	-
Saponins			
Froth test	-	-	+
Triterpenoids			
Salkowski's test	+	+	+
Flavanoids			
Shinoda test	+	+	-
$FeCl_3$ test	+	+	-
Tannins			
$FeCl_3$ test	+	+	+
Lead acetate test	+	+	+
Cardiac glycosides			
Keller Killiani	-	-	-
Polyphenol			
Ferric-chloride-Ferricyanide test	+	+	+
% Yield (dry weight)	**2.02**	**2.68**	**10.56**

reducing powers measured by FRAP assay were not statistically different among all three extracts (p-value = 0.064).

Compositions of the extracts detected by TLC

TLC finger prints shown in Figure 1 revealed that the chloroform and hexane extracts contained some similar constituents, and the major compound in both extracts was the compound with R_f= 0.854. In addition, more compounds were observed in the chloroform extract than in the hexane extract. In the case of aqueous extract, the compounds were not separated well in both types of mobile phase used. However, it is clear that the amount of the major compound mention earlier (R_f= 0.854) was much less found in the aqueous extract.

Table 2 Total phenolic content and antioxidant activities of *D. reticulata* extracts

Extract	Total phenolic compound (mg GAE/g extract)	ABTS assay (IC_{50} µg/ml)	FRAP assay (µmol Fe^{2+}/mg dried extract)
Hexane	52.29±1.33[a]	441.55±13.56[a, b]	0.047±0.036
Chloroform	53.89±0.68[a]	632.69±23.02[a]	0.121±0.028
Aqueous	33.21±0.31	1252.68±110.00	0.091±0.026
BHT	-	108.12±8.41	-

Values are expressed as mean ± SD (n = 3).
[a] $p< 0.05$ statistically significant difference compared to aqueous extract.
[b] $p< 0.05$ statistically significant difference compared to chloroform extract.

Discussion and conclusion

From our previous report, the aqueous extract of *D. reticulata* stem contained 16.7 yield % (w/w) of dried plant. In the present study, the extracts were obtained from sequential extraction method which was different from the previous report. Due to the polarity of solvent, the hexane extract contained compounds which are more hydrophobic than the chloroform and aqueous extracts, respectively. The results from phytochemical analysis revealed that all extracts from *D. reticulata* stem did not contain cardiac glycosides, whereas triterpenoids, tannins and polyphenol were detected in all three extracts. Saponin appeared only in the aqueous extract. Flavanoids and alkaloids seemed to be completely extracted by hexane and chloroform

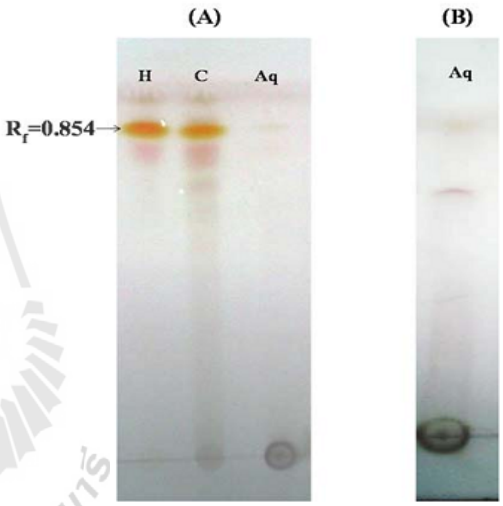

Figure 1 TLC finger print of three *D. reticulate* extracts. (A) TLC plate in Dichloromethane: Methanol (95:5) and (B) TLC plate in Chloroform: Methanol: Water (10:30:1). H, C and Aq denote the type of extracts (hexane, chloroform and aqueous extracts, respectively).

because these types of compound could not be detected in the aqueous extract by the phytochemical screening method used.

D. reticulata extract has been shown to possess antioxidant and anti-diabetic activities. It has been reported that the antioxidant activity of plant

materials was well correlated with the content of their phenolic compounds (Xin-Hua et al., 2001). In this study, it was found that the aqueous extract contained less total phenolic compounds than the other two extracts. We further examined antioxidant activity of the extracts and found that in accordance with the phenolic content, the hexane and chloroform extracts exhibited significantly more antioxidant potential than the aqueous extract.

As shown in Figure 1, the major compound of hexane and chloroform extracts obtained from the present study was the compound shown in TLC with R_f 0.854. The flavonoid lupinifolin has been reported to be a major compound in *D. reticulate* (Chivapat et al., 2009). Taken together, the compound detected at R_f 0.854 in this study could be lupinifolin and may be responsible for antioxidant activities of both hexane and chloroform extracts. Now the isolation and identification of this compound as well as the examination of its anti-diabetic and antioxidant activities are in progress.

References

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996; 239: 70-6.

Blasa M, Gennari L, Angelino D, Ninfali P. Fruit and vegetable antioxidants in health. In: Preedy RRWR, editor. Bioactive foods in promoting health. San Diego : Academic Press; 2010. p. 37-58.

Chivapat S, Chavalittumrong P, Attawish A, Soonthornchareonnon N. Toxicity study of lupinifolin from stem of *Derris reticulata* Craib. J Thai Tradit Altern Med 2009; 7: 146-55.

Harborne JB. Phytochemical methods a guide to modern techniques of plant analysis. 3rd ed. London: Chapman & Hall; 1998.

Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology 2000; 7: 153-63.

Kumkrai P, Kamonwannasit S, Chudapongse N. Cytoprotective and anti-diabetic effects of *Derris reticulata* aqueous extract. J Physiol Biochem 2014; 70: 675-84.

Laupattarakasem P, Houghton PJ, Hoult JR, Itharat S. An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. J Ethnopharmacol 2003; 85: 207-15.

Prior RL, Wu X, Schaich K. Standardized method for the determination of antioxidant capacity and phenolics in food and dietary supplements. J Agric Food Chem 2005; 53: 4290-302.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999; 26: 1231-7.

Saxena M, Saxena J. Phytochemical screening of *Acorus calmus* and *Lantana camara*. IRJP 2012; 3: 324-6.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39: 44-84.

Wisetsutthichai S, Techamahamaneerat S, Junyaprasert V, Soonthornchareonnon N. Gel preparation development from *Derris reticulata* Craib. extractas an anti-herpes simplex drug. Thai Journal of Phytopharmacy 2005; 12: 11-22.

Xin-Hua W, Chang-Qing L, Xing-Bo G, Lin-Chun F. A comparative study of *Phyllanthus amarus* compound and interferon in the treatment of chronic viral hepatitis B. Southeast Asian J Trop Med Public Health 2001; 32: 140-2.

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Riyajan, S., Yusook, K., Kumkrai, P., and Chudaponges, N. Phytochemical screening and antioxidant activity of hexane, chloroform and aqueous extracts of *Derris reticulata* stem. (2015)**. Proceeding of the 34th The National Graduate Research Conference. Khon Kaen University, Thailand.**