การเปรียบเทียบฤทธิ์ทางเภสัชวิทยาของสารสกัดหยาบจากลูกใต้ใบ 3 ชนิด

นางสาวกาญจนา พุ่มภาชี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2552

COMPARISON OF THE PHARMACOLOGICAL ACTIVITIES AMONG CRUDE EXTRACTS FROM THREE *PHYLLANTHUS* SPECIES

Kanchana Poompachee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Biology

Suranaree University of Technology

Academic Year 2009

COMPARISON OF THE PHARMACOLOGICAL ACTIVITIES AMONG CRUDE EXTRACTS FROM THREE *PHYLLANHUS* SPECIES

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

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กาญจนา พุ่มภาชี : การเปรียบเทียบฤทธิ์ทางเภสัชวิทยาของสารสกัดหยาบจากลูกใต้ใบ 3 ชนิด (COMPARISON OF THE PHARMACOLOGICAL ACTIVITIES AMONG CRUDE EXTRACTS FROM THREE PHYLLANTHUS SPECIES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ เภสัชกรหญิง ดร.นวลน้อย จูฑะพงษ์, 83 หน้า.

ในการศึกษานี้มีวัตถุประสงค์เพื่อทำการเปรียบเทียบฤทธิ์ทางเภสัชวิทยาของสารสกัดหยาบจากลูกใต้ใบ 3 ชนิด ได้แก่ *Phyllanthus virgatus*, *P. amarus* และ *P. urinaria* โดยทำการศึกษาฤทธิ์การต้านอนุมูลอิสระ ฤทธิ์ต้านมะเร็ง และผลต่อกระบวนการหายใจของไมโทคอนเดรียที่แยกจากตับของหนูขาว จากการตรวจหาปริมาณของสารประกอบฟีนอลิกที่พบในสารสกัดด้วย 50% เมธานอลของ *P. virgatus* มีปริมาณของสารประกอบฟีนอลิกสูงกว่าสารสกัดของ *P. urinaria* และสารสกัดของ *P. amarus* และจากการทดสอบโดยใช้วิธี 2, 2–dipenyl-1-picrylhydrazyl radical (DPPH) พบว่า สารสกัดของ *P. virgatus* มีฤทธิ์ต้านอนุมูลอิสระได้ดีที่สุด นอกจากนั้นยังสามารถยับยั้งการเกิด lipid peroxidation ของ linoleic acid system ได้ดีที่สุดด้วย และเมื่อทำการทดสอบความเป็นพิษของสารสกัดลูกใต้ใบต่อเซลล์มะเร็ง HepG2 โดยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) และ trypan blue พบว่าสารสกัดของ *P. virgatus* มีความเป็นพิษต่อเซลล์ HepG2 มากกว่าสารสกัดของ *P. amarus* และ *P. urinaria* ซึ่งสามารถยืนยันได้จากภาพถ่ายที่แสดงการเปลี่ยนแปลงโครงสร้างทางสัณฐานวิทยาของเซลล์ HepG2 และเมื่อใช้สารสกัดที่ความเข้มข้นเดียวกันคือ 500 µg/mL พบว่า *P. virgatus* กระตุ้นให้เซลล์มะเร็ง HepG2 มีการใช้ออกซิเจนมากที่สุด เนื่องจากไมโทคอนเดรียเป็นออร์กาเนลล์ที่สำคัญที่สุดในการใช้ออกซิเจนของเซลล์ ดังนั้นจึงได้ทำการศึกษาผลของสารสกัดลูกใต้ใบทั้ง 3 ชนิดต่อกระบวนการหายใจของไมโทคอนเดรียที่แยกจากตับของหนูขาวซึ่งพบว่าสารสกัดลูกใต้ใบที่ความเข้มข้นสูงตั้งแต่ 250 µg/mL กระตุ้นอัตราการใช้ออกซิเจนใน state 4 แต่ลดอัตราการใช้ออกซิเจนใน state 3 ซึ่งมีผลทำให้ค่าดัชนีควบคุมการหายใจลดลง เมื่อใช้กลูตาเมทและมาเลท หรือซักซิเนตเป็นสับสเตรท ผลการศึกษานี้แสดงให้เห็นว่าสารสกัดลูกใต้ใบมีฤทธิ์ 2 ประการต่อไมโทคอนเดรีย กล่าวคือสารสกัดลูกใต้ใบมีฤทธิ์เป็นสารอันคัปเปอร์และยับยั้งกระบวนการออกซิเดทีฟ ฟอสฟอริเลชัน ผลการศึกษานี้ชี้แนะว่าฤทธิ์ต่ อไมโทคอนเดรียดังกล่าวอาจมีบทบาทสำคัญในการเกิดพิษของสารสกัดลูกใต้ใบต่อเซลล์ HepG2

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

KANCHANA POOMPACHEE : COMPARISON OF THE PHARMACO LOGICAL ACTIVITIES AMONG CRUDE EXTRACTS FROM THREE *PHYLLANTHUS* SPECIES. THESIS ADVISOR : ASST. PROF. NUANNOI CHUDAPONGSE, Ph.D. 83 PP.

PHYLLANTHUS SPECIES/ANTIOXIDANT/ANTICANCER/MITOCHONDRIA

The purpose of this study was to compare the pharmacological activities among crude extracts from three *Phyllanthus* species, namely *P. virgatus*, *P. amarus* and *P. urinaria.* The pharmacological activities of the *Phyllantus* extracts were compared, including antioxidant and anticancer activities as well as the effects on mitochondrial respiration. The total phenolic content of *P. virgatus* extract was higher than those of *P. urinaria* extract and *P. amarus* extract. *P. virgatus* showed the highest antioxidant property by using 2, 2–dipenyl-1-picrylhydrazyl radical (DPPH) assay and highest inhibition of peroxidation in linoleic acid system. Additionally, the *in vitro* cytotoxicity of the *Phyllanthus* extracts on human hepatoma HepG2 cells was also investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) and trypan blue assay. *P. virgatus* was more toxic than *P. amarus* and *P. urinaria*. Furthermore, all of the *Phyllanthus* extracts caused morphology changes. *P. virgatus* produced the highest stimulation on oxygen consumption of HepG2 cells which may involve in the cytotoxicity action of the *Phyllanthus* extracts on HepG2 cells. Hence, the effects of three *Phyllanthus* extracts on respiratory function of isolated rat liver mitochondria have been investigated. *Phyllanthus* extracts at high concentration (≥ 250 µg/mL) stimulated state 4 respiration, decreased state 3

respiration and respiratory control ratio when used glutamate plus malate or succinate as substrates. These results suggested that *Phyllanthus* extracts had two acitions on the mitochondria, namely uncoupler and inhibitor of oxidative phosphorylation. These results suggested that mitochondrial effects may play an important role in the cytotoxicity of *Phyllantus* extracts on HepG2 cells.

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Academic Year 2009

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ACKNOWLEDGEMENTS

The thesis has been completed with the support and assistance from many people. I would like to express my sincere thanks to all the people who directly or indirectly encourage and help me.

I am deeply grateful to my thesis advisor Asst. Prof. Dr. Nuannoi Chudapongse who gave good advice and guidance to this thesis since the beginning until completion. My co-advisor, Asst. Prof. Dr. Benjamart Chitsomboon, I thank her for helpful comments on the experiments. I also thank Assoc. Prof. Dr. Prakorn Chudapongse for kindness and helpful suggestion for the experiment on the measurement of mitochondrial respiration. The valuable comment of Dr. Nawarat Nantapong and Dr. Pongrit Krubphachaya, members of my thesis committee, is much appreciated. Also, I am very much indebt to Miss Matcha Kamkhunthod for her technical assistance throughout this work.

I am grateful to the Graduate School, Suranaree University of Technology for the major financial support in my graduate school training.

Finally, my graduation would not be achieved without best wish from my parents who help me in everything and financial support until the completion of this study.

Kanchana Poompachee

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LIST OF ABBREVIATIONS

ADP	=	Adenosine 5´- diphosphate
ATP	=	Adenosine 5´- triphosphate
ATPase	=	Adenosinetriphosphatase
CAT	=	Catalase
CCCP	=	Carbonyl cyanide *m*-chlorophynylhydrazone
CoQ	=	Coenzyme Q, ubiquinone
COX-2	=	Cyclooxygenases-2
°C	=	Degree celcius
CTX	=	Cyclophosphamide
DCCD	=	Dicyclohexylcarbodiimide
DLA	=	Dalton's Lymphoma Ascites
DMSO	=	Dimethylsulphoxide
DNA	=	Deoxyribonucleic acid
DNP	=	2, 4 – Dinitrophenol
DOX	=	Doxorubicin
$DPPH^{\bullet}$	=	2, 2–Dipenyl-1-picrylhydrazyl radical
EC_{50}	=	50% effective concentration
EAC	=	Ehrlich Ascite Carcinoma
FAD	=	Flavin Adenine Dinucleotide
$FADH_2$	=	Reduced Flavin Adenine Dinucleotide

LIST OF ABBREVIATIONS (Continued)

FBS	=	Fetal Bovine Serum
FCCP	=	*p*-trifluoromethoxy carbonyl cyanide phenylhydrazone
FMN	=	Flavin Mononucleotide
Fe-S	=	Iron-sulfur center
g	=	Gram
g	=	Centrifugal force unit (gravity)
GAE	=	Gallic acid
H^+	=	Proton
HBV	=	Hepatitis B virus
HEPES	=	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IC_{50}	=	50% inhibitory concentration
kg	=	Kilogram
M	=	Molar
µg	=	Microgram
µL	=	Microliter
µM	=	Micromolar
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
NAD^+	=	Nicotinamide adenine dinucleotide

LIST OF ABBREVIATIONS (Continued)

NADH	=	Reduced nicotinamide adenine dinucleotide
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
O_2	=	Oxygen
Pi	=	Inorganic phosphate
RCR	=	Respiratory Control Ratio
S.E.	=	Standard error of mean
TB	=	Trypan Blue
TPC	=	Total phenolic compound
v/v	=	Volume by volume
w/v	=	Weight by volume
/	=	Per
%	=	Percent

CHAPTER I

INTRODUCTION

According to the World Health Organization, almost 80% of the world's inhabitants in developing countries still use traditional medicine in their primary health care, due to poverty and lack of access to modern medicine (WHO-TRM, 1998, quoted in Calixto, 2000; Mahady, 2001; Howe et al., 2004). Of all diseases, thirty percent are serious and require modern medicine and health care specialists while seventy percent are less serious (Howe et al., 2004). The latter are often preventable diseases that can be treated using traditional or folk medicines. Therefore, safety and efficacy data are crucial for these drugs. The field of herbal medicine and medicinal plants requires additional research and more financial support in the future.

In Thailand, herbal medicine has long been practiced by Thai people in all regions, especially in rural areas, because the western medical treatments are too expensive or not available. Herbal treatment is easy to obtain with cheap cost. In addition, Thai herbs are necessary to everyday Thai life because they have been used as both food and medicine. Medicinal herbs have been successfully employed to cure the Thais of a wide range of sicknesses. Knowledge of using Thai medicinal plants has been passed from father to son. Over the years, it has grown and sought for the effects and mechanisms. Up to date, medicinal plants have been used extensively due to a global renaissance of both herbal medicine and traditional health practices. Hence, Thailand is also alerted to this global trend.

Among medicinal plants especially *Phyllanthus* species have all long been prescribed in traditional medicine for treating various diseases such as fever, jaundice, ascites, hemorrhoid, diabetes and particularly for liver disorders. Because it is widely distributed in all tropical regions of the world and each species is closely related in appearance, phytochemical structure and history of use, it is difficult and confusing among scientists for plant information and evaluation of published research.

Oftentimes, one name is indicated to be synonymous with another and sometimes both names are used interchangeably as if referring to one plant. Besides, the same species of herb may be known by different names in different areas. Nowadays the *Phyllanthus* genus is reorganized by scientists.

Herbal product of *Phyllanthus* sp. has been sold in Thailand and in foreign countries. However, because each species are closely related in appearance and phytochemical structure, products of *Phyllantus* sp. are often harvested from different species which may have different pharmacological activities. It has been shown that three *Phyllanthus* species collected from India and China give a different therapeutic effect to patients with chronic hepatitis B (Wang et al., 1995).

In Thailand, the pharmacological activity of each *Phyllanthus* sp. has never been compared. Moreover, the product of *Phyllanthus* sp. which has been sold in market, known as Luk-tai-bai, is mixed with several species and has not been identified.

In this study, pharmacological activities of the extract of three *Phyllanthus* species found in Nakhon Ratchasima province, Thailand were investigated. They are *P. amarus*, *P. urinaria* and *P. virgatus*. The pharmacological activities in the present study include antioxidant and anticancer activities as well as the effects on

mitochondrial respiration. This study will provide additional evidence that supports the best choice in the use of *Phyllanthus* sp. in Thailand for an effective medical care.

CHAPTER II

LITERATURE REVIEWS

2.1 *Phyllantus* species

The *Phyllanthus* genus (family Euphorbiaceae) contains more than 600 species, found throughout the tropics and subtropics, including central and southern India, the Philippines, Nigeria, Brazil and the United States in Florida and Texas. In Thailand, *Phyllanthus* contains 36 species but three *Phyllanthus* species are known as Luk-tai-bai, including *P. amarus*, *P. urinaria* and *P. virgatus* (Department of Forest Herbarium, National Park, Wildlife and Plant Conservation, Online, 2006).

***Phyllanthus amarus* Schumach. & Thonn.**

P. amarus is widely distributed throughout the country. It is a common pantropical weed that grows well in moist, shady and sunny places. Some common names of *P. amarus* in northern, central and southern Thailand are Ma-kham-pom-din, Luk-tai-bai and Ya-tai-bai-khao, respectively. *P. amarus* is an erect annual herb, up to 70 cm high, branchlets terete, glabrous. It is monoecious or homogamous. The leaves are simple, alternate, on petioles 0.2-0.8 mm long, blade oblong, and elliptic-oblong or obovate, base slightly unequal, obtuse or rounded and apex obtuse or rounded. The flowers are alone or usually one male and female are in each leaf axil together. Its staminate flowers have 4 or 5 sepals, on pedicel 0.5-1.0 mm long, ovate or elliptic and light yellowish. Its pistillate flowers have 5 sepals, obovate-oblong or

lanceolate, glabrous, apex acute and 5-lobed or laciniate. The fruit is a three-lobed capsule about 0.8-2.0 mm diameter, light green or light yellow green. When the fruits burst open the seeds are hurled away. Seeds are triangular, light brown, 0.5-1.2 mm long, with 5 or 6 ribs on the back (Department of Forest Herbarium, National Park, Wildlife and Plant Conservation, Online, 2006).

Figure 2.1 *Phyllanthus amarus* Schumach. & Thonn.

The secondary metabolites present in *P. amarus* are alkaloids, flavanoids, hydrolysable tannins, major lignans and polyphenols. *P. amarus* has undergone much research in recent years. It has been used in Thai folk medicine for the treatment of fever, jaundice, ascites, hemorrhoid and diabetes (Wongnawa et al., 2006). Apart from these medicinal uses, several reports showed antiviral activity, including against hepatitis B virus (HBV; Liu et al., 2001). *P. amarus* down-regulates HBV mRNA transcription by a specific mechanism involving interaction between HBV enhancer I

and C/EBP transcription factors (Thyagarajan et al., 1997) and has remarkable effect for chronic viral hepatitis B in recovery of liver function and inhibition of the replication of HBV (Xin-hua et al., 2001). *P. amarus* inhibited hepatitis B virus polymerase activity and decreased episomal hepatitis B virus DNA content in HepG2 2.2.15 cells, which is a stable cell line containing the hepatitis B virus ayw strain genome. Moreover, it suppressed virus release into culture medium (Lee et al., 1996). It has been reported that *P. amarus* blocks the retroviral reverse transcriptase and inhibits HIV replication. The aqueous solution of *P. amarus* extracts has been shown to inhibit HIV-1 replication in HeLa $CD4^+$ cells with 50% effective concentration (EC_{50}) values ranging from 0.9 to 0.76 µg/mL (Notka et al., 2003). The anti-inflammatory activity of *P. amarus* has also been reported. It inhibits iNOS, COX-2 and cytokines via the NF-κB pathway (Kiemer et al., 2003). The niranthin isolated from *P. amarus*, exhibits anti-inflammatory and anti-allodynic actions which are probably mediated through its direct antagonistic action on the platelet activating factor receptor binding sites (Kassuya et al., 2006). Moreover, the aqueous and methanol extracts of *P. amarus* produced an inhibition of rat paw edema induced by carrageenan up to 42% (Raphael et al., 2003). *P. amarus* has antispasmodic properties including uterine relaxant effect and it is finally concluded that smooth muscle relaxation within the urinary or biliary tract probably facilitates the expulsion of kidney or bladder calculi (Santos et al., 2000). The pain relieving effects of *P. amarus* were performed in experimental animal models. *P. amarus* showed inhibitory effect on different phlogistic agents-induced paw edema, carrageenan-induced leukocyte infiltration and exudates formation (Mahat et al., 2009). In addition, *P. amarus* produced marked inhibition of paw oedema formation and inhibited the myelo-

peroxidase activity, both in the paw and in the injured sciatic nerve were induced by complete Freund's adjuvant (Kassuya et al., 2003). *P. amarus* has also effective for its antiulcerous properties and to protect the gastric tract (Shokunbi et al., 2008). The aqueous extract of *P. amarus* exhibited potent anticarcinogenic activity against 20-methylcholanthrene induced sarcoma development and increased the survival of tumor harboring mice. The extract administration was also found to prolong the life span of Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascite Carcinoma (EAC) bearing mice (Rajeshkumar et al., 2002) and reduced the volume of transplanted solid tumors by inhibiting aniline hydroxylase, an enzyme P-450 (Bhaskarannair et al., 2006). Simultaneous administration of *P. amarus* extract along with the carcinogen has been reported to inhibit the hepatocellular carcinoma development induced by *N*-nitrosodiethylamine. The extract also caused a decrease in serum γ-glutamyl tran-peptidase activity indicating decreased proliferation of tumor cells (Rajeshkumar and Kuttan, 2000). The aqueous extract of *P. amarus* produced protective effects against the mutagenic effect of 2-aminofluorene, 2-aminoanthracene, 4-nitroquinolone-1-oxide, N-ethyl-N-nitrosoguanidine, 2-nitrofluorene and sodium azide in the test bacteria and the extract antagonizes DNA damage caused by dimethylnitrosamine in hamster liver cells (Sripanidkulchai et al., 2002; Rajeshkumar et al., 2002). The lignans (phyllanthin and hypophyllanthin) of *P. amarus* has remarkable effect in decreasing the growth of solid tumor mass induced by EAC cells (Islam et al., 2008). Furthermore, *P. amarus* can reduce the toxic side effects of cyclophosphamide (CTX) without interfering with the antitumor efficiency of CTX (Kumar and Kuttan, 2005). The extract of *P. amarus* has been administered orally (750 mg/kg and 250 mg/kg body weight) in the radiation (6 Gy) induced BALB/c mice for its protective activity

against carcinogenesis. The WBC count, bone marrow cellularity and α-esterase activity increased as compared to radiation only. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPX) and glutathione reductase, both in blood and tissue were increased after received *P. amarus* and radiation as compared to radiation alone (Kumar and Kuttan, 2004). It shows a potential to inhibit lipid peroxides and protect mouse chromosomes and intestine from radiation induced damages (Bhaskaran et al., 2007). The antidiabetic activity of the aqueous extracts of leaf and seed of *P. amarus* was studies at oral dose of 150, 300 and 600 mg/kg body weight. The experiment showed dose-dependent decrement of the fasting plasma glucose level and cholesterol content and reduction of body weights in treated mice (Adeneye et al., 2006). The hexane extract of *P. amarus* caused inhibition of α-amylase and decreased glucose, which oleanolic and ursolic were found to be relevant to this effect (Ali et al., 2006). The extract of *P. amarus* has blood pressure lowering effect which may be mediated by the combined effects of myocardial depression, muscarinic receptor mediated vascular smooth muscle relaxation and by the calcium channel ion blockade in vascular smooth muscle (Amaechina and Omogbai, 2007). The antiamnesic effects of *P. amarus* was observed which may be due to its inhibition of brain acetyl-cholinesterase enzyme, thereby elevating acetylcholine concentration in brain homogenate and improved memory of mice. Hence, a combination of neuropro-tective, anticholinesterase and nootropic effects exhibited by *P. amarus* may be responsible for the memory improving effect (Joshi et al., 2007). An antihepa-totoxicity effect of *P. amarus* has been proved in animals with induced liver damage. *P. amarus* can antagonize ethanol effects both in primary cultures of rat hepatocyte

and rats. Efeect of *P. amarus* is similar to silymarin, which is hepatoprotective agent. *P. amarus* decreased the release of transaminase (Pramyothin et al., 2007). Moreover, it has potent hepatoprotection against paracetamol, alflatoxin B_1 and carbon tetrachloride. *P. amarus* decreased several enzyme levels, such as SGOT, SGPT, ALK and serum bilirubin, which have been raised in toxin control (Naaz et al., 2007; Wongnawa et al., 2006; Yadav et al., 2008; Islam et al., 2008). However, *P. amarus* extracts are found to be nontoxic in mice but it is slightly cytotoxic to the human adenocarcinoma cell line Caco-2 (Lawson-Evi et al., 2008).

***Phyllanthus urinaria* Linnea.**

P. urinaria is widely distributed in Southern America and in many countries in Asia, such as Thailand, India, China, Indonesia, Philippines and Japan. All parts of the plants have been used medicinally. *P. urinaria* is very similar to *P. amarus*. Its young leaves are for treatment of cough in children and substitute for *P. amarus* in traditional Thai recipes. Moreover, this plant is used for several conditions such as blennorrhagia (gonorrhea), diabetes, dysentery, flu, tumors, jaundice, headache, fever, conjunctivitis, menstrual disorders and dyspepsia.

Some common names of *P. urinaria* in Thailand are Fai-duean-ha (Chon Buri), Ma-kham-pom-din (Northern), Ya-tai-bai (Ang Thong, Surat Thani) and Mak-khai-lang (Loei). *P. urinaria* is an erect annual herb up to 60 cm high. Stem and branchlets are flat and green and often with dark red. The leaves are alternate and simple, on petioles 0.3 to 0.9 mm long with 10 to 42 leaves. Shape of the leaves is oblong or oblong-ovate, obtuse to rounded and sometimes slightly unequal at base. Otherwise, the leaves are dark green above and pale light green underneath. The flowers are unisexual glomerules. Its pistillate flowers have about 5-20 in proximal

axils, sepals 6, linear-oblong or oblanceolate, apex obtuse and pedicel 0.1-0.4 mm long. Its staminate flowers have 5-7 together in distal axils, sepals 6, elliptic or oblong-ovate and pedicel 0.1-0.4 mm long. The fruit is a globular capsule about 2-4 mm in diameter. Seeds are 3-angled with sharp transverse ridges on the back and pits on the sides, light brown (Department of Forest Herbarium, National Park, Wildlife and Plant Conservation, Online, 2006).

Figure 2.2 *Phyllanthus urinaria* Linnea.

The secondary metabolites present in *P. urinaria* are lignans, alkaloid, tannin, flavanoids, carboxylic acid, methyl and ethyl gallates and other unknown structures (Chang et al., 2003; Wei et al., 2005; Xiangrong et al., 2008). Recent research has been placed on antiviral, anti-inflammatory, muscle relaxation effect, anticancer and antioxidant activities of *P. urinaria*. It has been shown *in vivo* that the aqueous extract inhibits tumor growth of Lewis lung carcinoma cells transplanted on C57BL/6J mice

which associated with the down-regulation of Bcl-2 gene expression and induced apoptosis (Huang et al., 2003; 2006). In HL-60 cells, the extract induced Fas receptor/ligand expression through ceramide-mediated apoptosis (Huang et al., 2004). Recently, 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan from the ethyl acetate extract of *P. urinaria* has been shown to exhibit anticancer activity by inducing apoptosis through the inhibition of telomerase activity and Bcl-2 expression, and activation of c-myc and caspases leading to apoptosis (Giridharan et al., 2002). Moreover, *P. urinaria* has also been demonstrated to inhibit an intracellular hepatitis B s-antigen formation in hepatic cells (Shin et al., 2005). Clinically, *P. urinaria* has been shown to seroconvert hepatitis B e-antigen status in the patients from positive to negative as well as the seroconvert of hepatitis B e-antibody from negative to positive (Wang et al., 1995). Further, geraniin and 1,3,4,6-tetra-*O*-galloyl-β-D-glucose isolated from *P. urinaria* can against herpes simplex virus type 1 and type 2 injection (Yang et al., 2005, 2006). The methanolic extracts obtained from callus cultures of *P. urinaria* significantly inhibited both neurogenic and inflammatory phase of the formalin test (Catapan et al., 2000). The alcoholic extracts of *P. urinaria* induced guinea pig urinary bladder contraction which may involve direct action on smooth muscle (Paulino et al., 1999) and relies on the mobilization of extracellular calcium influx unrelated to activation of L- and N-type calcium channels or activation of protein kinase C mechanisms (Dias et al., 1995). In addition, *P. urinaria* protection against doxorubicin (DOX) cardiotoxicity has been suggested to mediate through multiple pathways and this plant may serve as an alternative source of antioxidants for prevention of DOX cardiotoxicity (Chularojmontri et al., 2005).

***Phyllanthus virgatus* G. Forst.**

Phyllanthus virgatus G. Forst. (Synonyms: *Phyllanthus simplex* Retz.) is known as Khang-amphai (Phrae), Phaeng-kham-hoi (Si Sa Ket) and Luk-tai-bai (central) in Thailand. *P. virgatus* is widely distributed in India, Burma, Indochina, China, Malaysia and Australia. *P. virgatus* is an annual or perennial herb, up to 70 cm high, erect or prostrate, branchlets compressed, glabrous. The leaves are blade narrowly to broadly oblong-lanceolate on petioles 0.5 to 0.9 mm long. The flowers are unisexual fascicles with 2-4 staminate flowers and 1 pistillate flower per axil. The fruit is a capsule about 1.5-2.3 mm in diameter (Department of Forest Herbarium, National Park, Wildlife and Plant Conservation, Online, 2006).

Figure 2.3 *Phyllanthus virgatus* G. Forst.

The knowledge of activity of *P. virgatus* is relatively limited compared to *P. amarus and P. urinaria.* Different parts of the plant are used to cure gonorrhea,

jaundice, liver disease and mammary abscess (Chopra et al., 1980, quoted in Shabeer et al., 2009). Fresh leaves are used to cure itching and pruritus. It had been reported to possess astringent, diuretic and cathartic properties. In a previous study, *P. virgatus* has been tested for anti-human hepatitis B virus *in vitro*. It suppressed effectively both HBsAg and HBeAg expression (Huang et al., 2003), probably through the actions of lignan components, a hydrolyzable tannin and flavonol sulfonates (Huang et al., 1998). It has been demonstrated that the active constituents of *P. virgatus* contains (-)-7,8-cis-8,8́-trans-7-(3,4-(methylenedioxy)phenyl)-7́-(3́,4́-dimethoxyphenyl)-8,8́ bis (methoxymethyl) tetrahydrofuran (called name virgatusin), hinokinin, hypophyllanthin, isolintetralin, niranthin, nirtetralin, indole-3-carboxylic acid, (+)-8-(3,4 (methylenedioxy)benzyl)-8́-(3́,4́ dimethoxybenzyl) butyrolactone, phyltetralin, norlignan, a hydrolyzable tannin (virganin), and flavonol sulfonates (Huang et al., 1996; 1998). In 1996, the constituents of *P. virgatus* were examined by chromatographic separation. A new tetrahydrofuran lignan, virgatusin has been found and purified from *P. virgatus* (Huang et al., 1996). Later, it has been shown that virgatusin has antibacterial and antifungal activities (Maruyama et al., 2007; Akiyama et al., 2007). Virgatusin, isolated from *P. virgatus*, has been used as a herbal drug for liver treatment (Huang et al., 1996, quoted in Akiyama et al., 2007) Furthermore, the ethanolic extract of *P. simplex* (a synonym of *P. virgatus*) significantly reduced the malondialdehyde content which is a measure of lipid peroxidation and showed antioxidant activity in both *in vitro* and *in vivo* experiments (Kumar et al., 2007). Recently, the methanol and aqueous extracts of *P. simple* showed antihyperglycemic effect. It increased antioxidant enzymes level in liver and kidney and the body weight of diabetic rats which was induced by alloxan monohydrate. Hence, the antioxidant status of *P.*

simplex might be responsible for antidiabetic action of this plant (Shabeer et al., 2009).

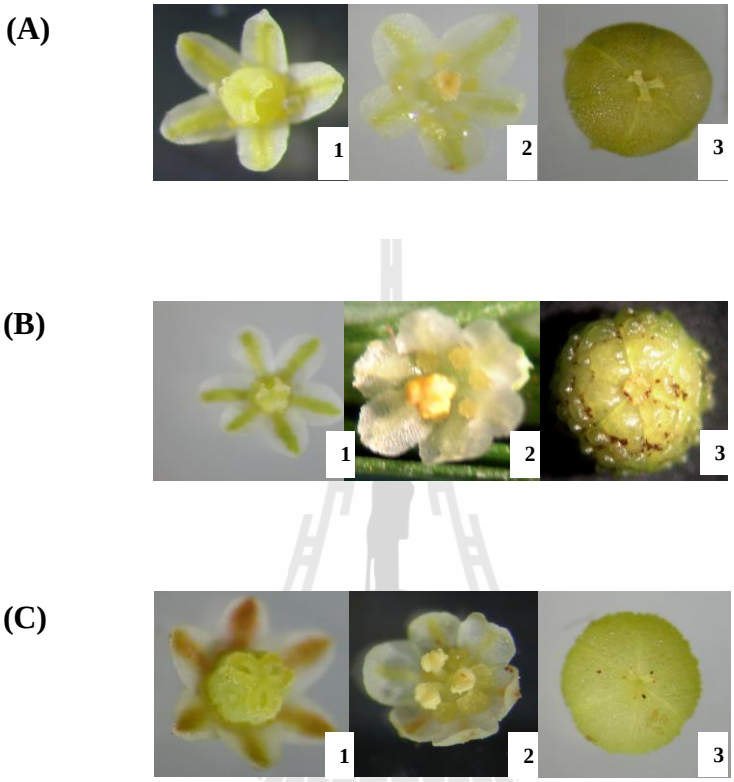


Figure 2.4 Morphology of three *Phyllanthus* species. (A) *Phyllanthus amarus* (B) *Phyllanthus urinaria* and (C) *Phyllanthus virgatus*. (1) Pistillate flower (2) Staminate flower (3) Fruit (4) X-section of fruit.

2.2 Free radicals, reactive oxygen species (ROS) and antioxidant systems

Free radicals are atoms, molecules or ions with unpaired electrons. Free radicals are very reactive and highly unstable molecules that attempt to achieve a more stable state by reacting with other atoms or molecules in the cell. Examples of

reactive free radicals are superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), sulphur ($RS^{\cdot}$) and transition metals (e.g., Fe, Cu, Zn, etc; Wu and Cederbaum, 2003).

ROS refer to oxygen containing free radicals or non free radical active molecules. ROS that are not free radicals include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3). ROS are small molecules and highly reactive, thus they are prominent toxicologic intermediates and involved in oxidative stress. This term is an imbalance between the production of reactive oxygen species and the scavenging capacity of antioxidative defense mechanism of the organism (Aruoma, 1998).

Free radicals derived from oxygen represent the most important class of such species generated in living systems. Further, free radicals play an important role in a number of biological processes and are necessary for life such as the intracellular killing of bacteria by neutrophil granulocytes. Free radicals have been implicated in certain cell signaling processes. When produced in excess, ROS can cause damage to cell structure. ROS can be produced from both endogenous and exogenous substances. Endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisome and inflammatory cell activation. The major source of ROS production in the cell is the mitochondrial respiratory chain. It has been estimated about 2 to 3 percents of O_2 consumed by the respiratory chain is converted to ROS. However, they are unstable and rapidly react with additional electrons and protons. Most of these ROS are converted to water before they can damage cells. Besides the ROS generation occuring naturally in the body, humans are exposed to ROS from exogenous sources such as exposure to certain chemicals, radiation, ultraviolet light,

alcohol, cigarette smoke and air pollutants (Valko et al., 2006). Environmental stress can dramatically increase ROS levels. If the body can not eliminate ROS, they caused damages to cell structures. Generally, harmful effects of reactive oxygen species on cells are most often due to damage of DNA, oxidation of polydesaturated fatty acids in lipids, oxidation of amino acids in proteins, oxidatively inactivate specific enzymes by oxidation of co-factors. In human, oxidative stress is involved in many diseases such as atherosclerosis, Parkinson's diseases, cancer and hepatic injury (Figure 2.5).

Since ROS are produced by a variety of pathways and accumulate in the body, humans and other animals have evolved defense mechanisms against these free radicals. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Because ROS production is a naturally occurring process, a variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS. Enzymes involved in the elimination of ROS, as show in table 2.1, include superoxide dismutase (SODs), catalase (CAT) and glutathione peroxidase (GPx). SODs catalyze the decomposition of superoxide anion into oxygen and hydrogen peroxide. In mammals, there are three forms of SODs. SOD 1 is a Cu/Zn containing metalloenzyme in the cytoplasm. SOD 2 is a Mn-containing metalloenzyme in the mitochondria. SOD 3 is a Fe-containing metalloenzyme in the extracellular. Catalase is an iron-containing enzyme found in the small membrane-enclosed cell components called peroxisomes. Catalase and the glutathione peroxidase system both help to remove hydrogen peroxide. Catalase eliminates hydrogen peroxide by catalyzing a reaction between two hydrogen peroxide molecules, resulting in the formation of water and oxygen.

Glutathione peroxidase, a selenium-containing antioxidant, is important for the decomposition of hydrogen peroxide and lipid peroxide. In addition, nonenzymatic antioxidant systems consist of scavenging molecules that are endogenously produced (glutathione (GSH), ubichinol, uric acid) or those derived from natural foods such as vitamins C and E, β-carotene, and α-tocopherol are known to possess antioxidant potential (Valko et al., 2006). Moreover, there are several herbs which have antioxidant activity. The majority of the active antioxidant compounds in plants are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins and isocatechins (Aqil et al., 2006).

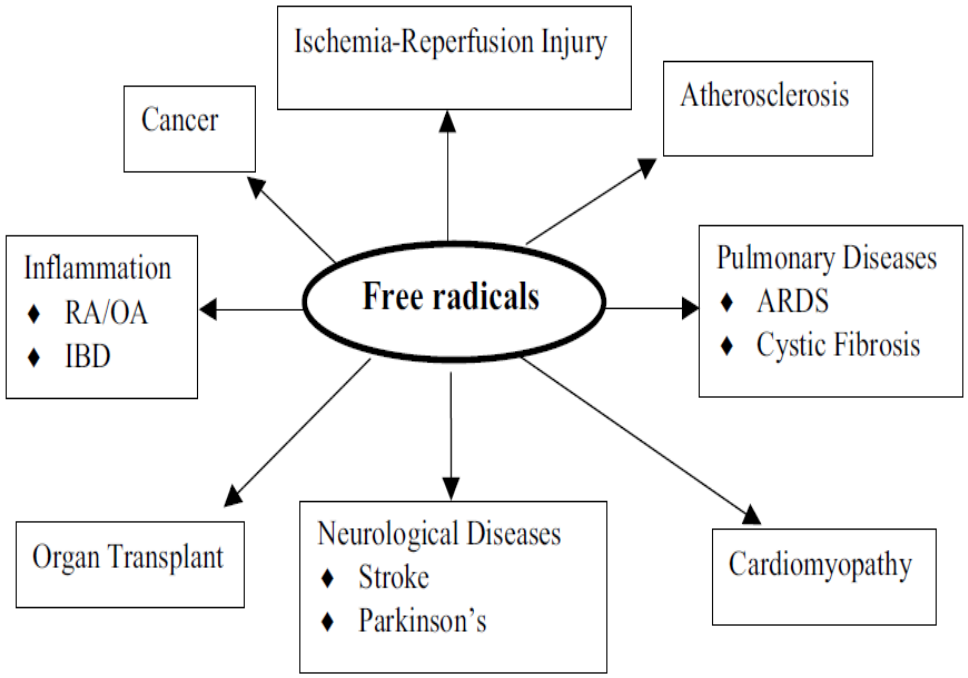


Figure 2.5 Free radicals and diseases: ARDS, acute respiratory distress syndrome; IBD, inflammatory bowel disease; OR, osteoarthritis; RA, rheumatoid arthritis. (Phromgate, 1999).

2.3 The mitochondria

The discovery of mitochondria as distinctive cytoplasmic organelles dated back to the mid nineteenth century before scientists began to recognize the relationship between the process of oxidation and phosphorylation inside the cells (Engelhardt, 1930; 1932; Kalckar, 1939). Oxygen uptake by tissue homogenates and action of simple respiratory inhibitors was first studied when it was realized that electrons flow from substrates to oxygen. In the early 1940's, it was demonstrated that cellular respiration is a process connected with the esterification of inorganic phosphate and the average value of 3 for the P/O ratio for the complete oxidation of pyruvate to CO_2 and H_2O was calculated (Ochoa, 1943). In 1948, Hogeboom et al. and Green et al. independently reported a method for isolating mitochondria from tissue homogenates.

A mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells. Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as cell signaling, cell differentiation, cell death, as well as the control of the cell cycle and cell growth (Campbell and Reece, 2004).

A mitochondrion contains outer and inner membranes composed of phospholipid bilayers and proteins. The space between the inner and outer membranes is referred to as intermembrane space. Several enzymes that utilize ATP are found in the intermembrane space. The inner membrane is extensively folded. The folds known as cristae provide the inner membrane with a large surface area in a small

volume, enhancing its ability to produce ATP. The space inside the inner mitochondrial membrane is called the matrix. It contains most of the enzymes of TCA cycle and fatty acid oxidation. In addition, mitochondria contain circular DNA molecules, along with ribosomes and the enzymes required to synthesize proteins coded within the mitochondrial genome. The most prominent roles of the mitochondrion are the production of ATP and regulation of cellular metabolism (Frey and Mannella, 2000).

Table 2.1 Antioxidant enzymes

Enzyme	Mineral	Reaction
superoxide dismutase (SODs)	Cu/Zn Mn Fe (bacterial)	$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$
catalase (CAT)	Fe	$2H_2O_2 \longrightarrow 2H_2O + O_2$
glutathione peroxidase (GPx)	Se	$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$ $ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O$

The production of ATP is accomplished by oxidizing the major products of glucose metabolism, pyruvate and NADH, which are produced in the cytosol

(Campbell and Reece, 2004). This process of cellular respiration known as aerobic respiration, is dependent on the presence of oxygen. Aerobic respiration requires oxygen in order to generate energy (ATP). It is the preferred method of pyruvate breakdown from glycolysis and requires that pyruvate enter the mitochondrion in order to be fully oxidized by the Krebs cycle.

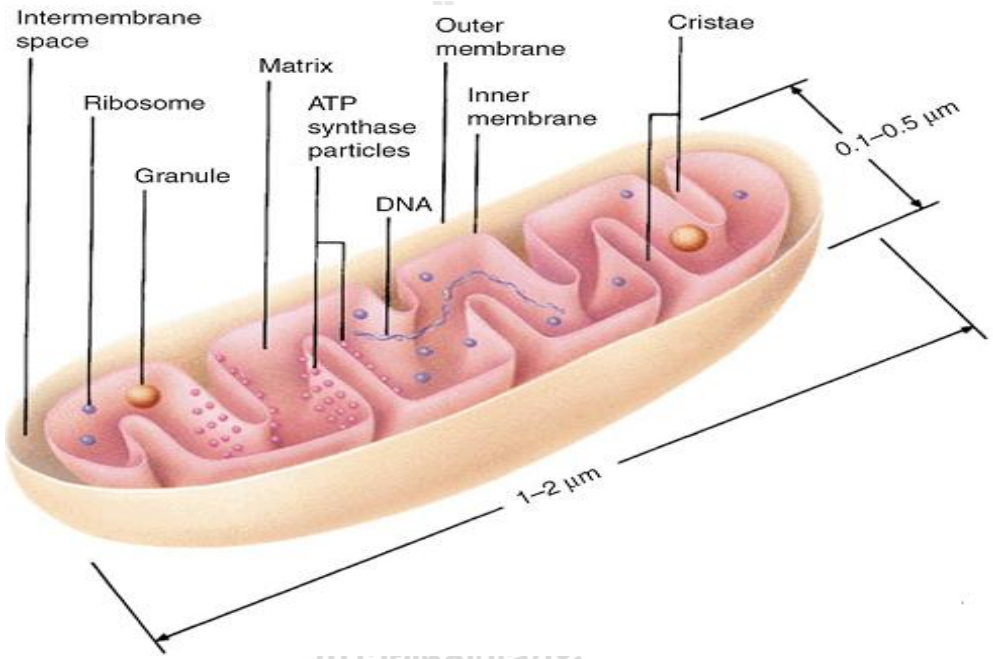


Figure 2.6 Structure of mitochondrion (Frey and Mannella, 2000).

The product of this process is adenosine triphosphate (ATP) formed by substrate-level phosphorylation, NADH and $FADH_2$. The reducing potential of NADH and $FADH_2$ is converted to more ATP by the process known as oxidative phosphorylation. In this process, the energy released when NADH and $FADH_2$ are oxidized by oxygen, the terminal electron acceptor, via electron transport chain is

used by ATP synthase to synthesize ATP from adenosine 5´- diphosphate (ADP) and inorganic phosphate (Pi). Most of the ATP produced by aerobic cellular respiration is made by oxidative phosphorylation (Figure 2.7).

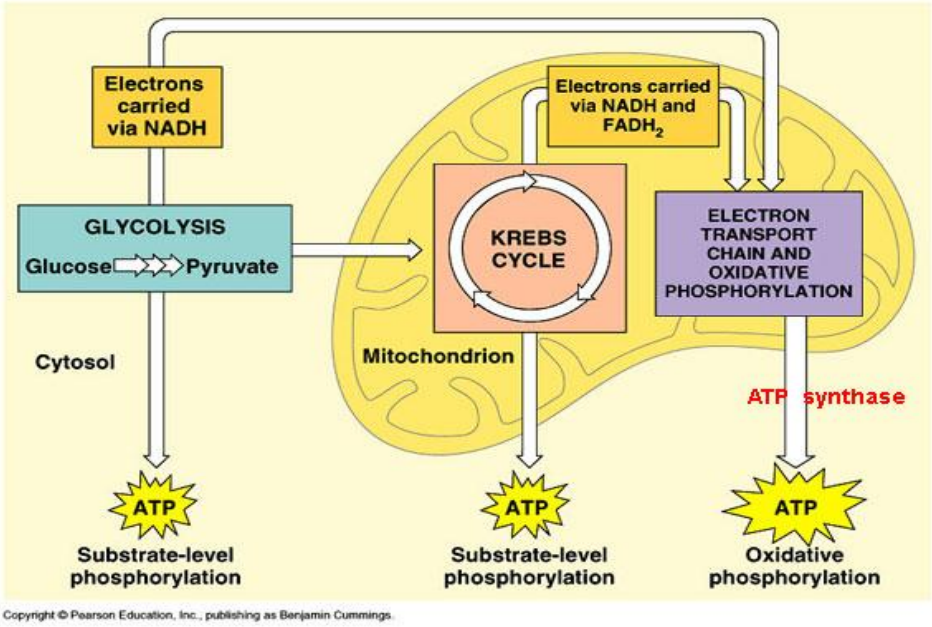


Figure 2.7 An overview of cellular respiration (Campbell and Reece, 2004).

2.3.1 The electron transport chain (respiratory chain) and oxidative phosphorylation

The components of the electron transport chain can be purified from the mitochondrial inner membrane. The respiratory chain consists of four complexes carriers, identified as complexes I, II, III and IV (Table 2.2 and Figure 2.8). Complexes I, II, III and IV catalyze the transfer of electron from NADH to ubiquinone, from succinate to ubiquinone, from ubiquinone to cytochrome *c* and from cytochrome *c* to oxygen respectively. Two components of the electron transport chain, namely ubiquinone and cytochrome *c*, are not part of the four complexes.

Ubiquinone or coenzyme Q links electrons flow between complex I and complex III, and between complex II and complex III; whereas cytochrome c links electrons flow between complexes III and IV (Devlin, 2002).

Table 2.2 Components of the mitochondrial electron transport chain (Stryer, 1995)

Enzyme complex	Mass (kd)	Subunits	Prosthetic group	Oxidant or reductant		
				Matrix side	Hydrocarbon core	Cytosolic side
NADH-Q oxidoreductase	880	≥34	FMN Fe-S	NADH	Q	
Succinate-Q reductase	140	4	FAD Fe-S	Succinate	Q	
Cytochrome oxidoreductase	250	10	Heme *b*-562 Heme *b*-566 Heme c_1 Fe-S		Q	Cyt *c*
Cytochrome oxidase	160	10	Heme *a* Heme a_3 Cu_A and Cu_B			Cyt *c*

In the process of this electron transfers, the proton concentration increases in the intermembrane space as protons are pumped out of the matrix (Figure 2.9). A strong electrochemical gradient is established across the inner membrane. Protons can return to the matrix through the ATP synthase complex and their potential energy is used to synthesize ATP from ADP and inorganic phosphate (Pi). This process is called chemiosmosis. This chemiosmotic hypothesis of oxidative phosphorylation proposed by Peter D. Mitchell, who was awarded the 1978 Nobel Prize in chemistry for this work, postulate that electron transport chain and ATP synthesis are coupled by

a proton gradient, generated by electron transport chain, across the inner mitochondrial membrane (Devlin, 2002).

In Mitchell's chemiosmotic hypothesis**,** protons are driven across the mitochondrial inner membrane from the matrix to the intermembrane space by the flow of electron in the electron transport chain. This mechanism stores the released energy of electron transport in an electrochemical potential. As protons are driven out of the matrix, the matrix pH rises and the matrix becomes negatively charged with respect to the cytosol (Figure 2.9). Therefore, proton pumping creates a pH gradient and an electrical gradient across the inner membrane, both of which tend to attract protons back into the matrix. This proton gradient is used by the ATP synthase complex to make ATP (Garrett and Grisham, 1999).

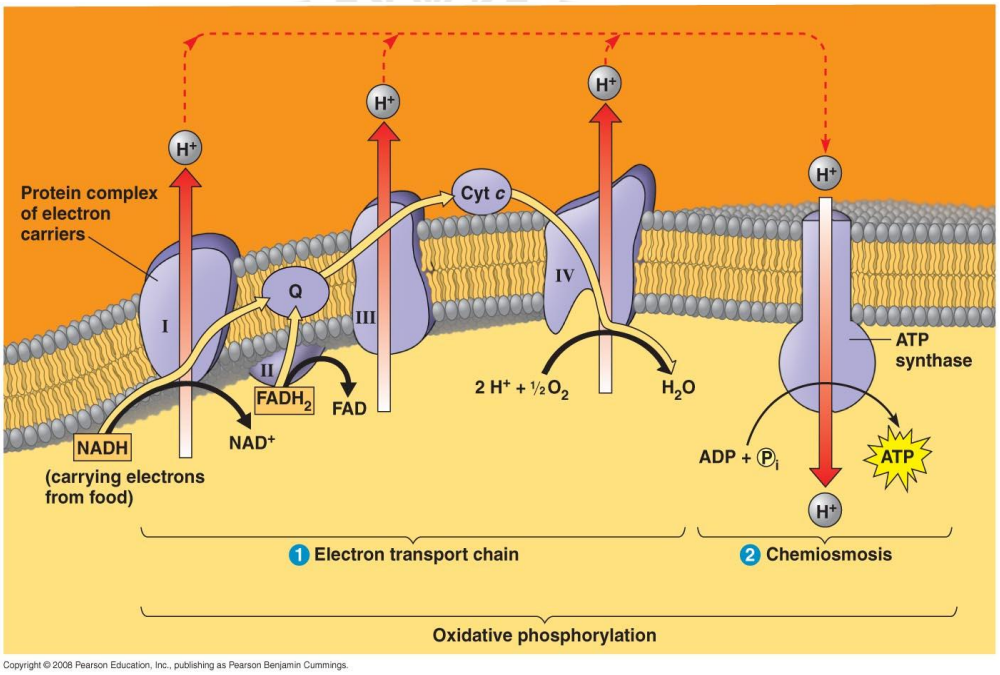


Figure 2.8 Chemiosmosis couples the electron transport chain to ATP synthesis (Campbell and Reece, 2004).

The mitochondrial complex carries out ATP synthesis is called ATP synthase or complex V. The ATP synthase is located in the inner mitochondrial membrane. It is the final enzyme in the oxidative phosphorylation pathway. ATP synthase consists of two complexes, F1 and F_0 (Figure 2.10). The F_0 component of ATP synthase acts as a highly selective proton channel allowing the return of protons back to mitochondrial matrix. The flow of proton back to the matrix through ATP synthase releases free energy; and this energy is used to drive ATP synthesis by the F_1 component of the complex (Wallace and Starkov, 2000).

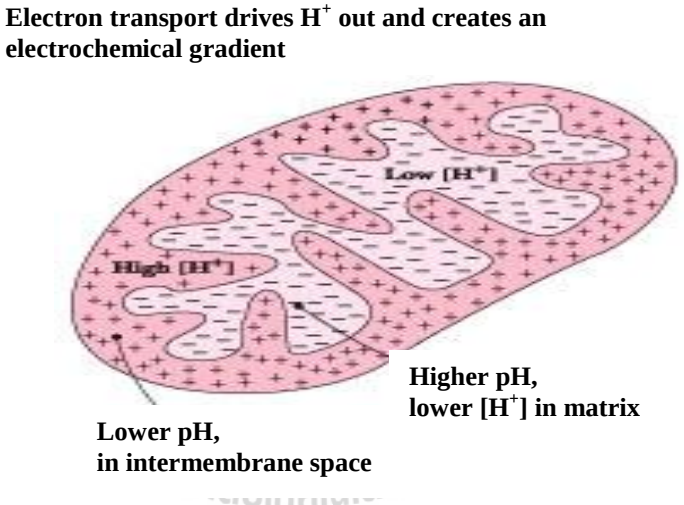


Figure 2.9 The proton electrochemical gradients existing across the inner mitochondrial membrane. The electrochemical gradient is generated by the transport of protons across the membrane (Garrett and Grisham, 1999).

2.3.2 Uncouplers and inhibitors of electron transport chain and oxidative phosphorylation

Several natural and synthetic poisons block mitochondrial respiration. Many details of electron transport and oxidative phosphorylation mechanisms have been

gained from studying the effects of particular inhibitors. The sites of inhibition by these agents are indicated in Figure 2.11

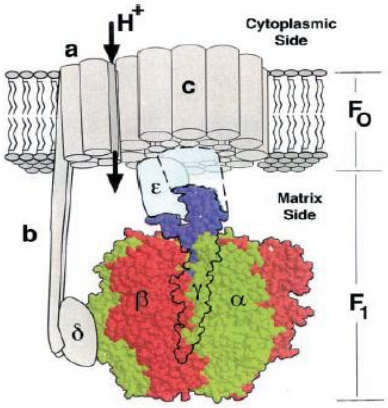


Figure 2.10 A model of the F_1 and F_0 components of the ATP synthase (Saraste et al., 1999).

Inhibitors of electron transport chain

Rotenone is a common pesticide that strongly inhibits the complex I but has no effect on the oxidation of succinate or ascorbate plus N,N,N',N'-tetramethylpara-phenylenediamine (TMPD). Ptericidin, amytal and other barbiturates, mercurial agents and the widely prescribed painkiller demerol exert inhibitory actions on this enzyme complex. All these substances appear to inhibit reduction of coenzyme Q and the oxidation of the Fe-S clusters of complex I. 2-thenoyltrifluoroacetone and carboxin and its derivatives specifically block complex II. Antimycin, an antibiotic produced by *Streptomyces griseus,* inhibits complex III by blocking electron transfer between b_H and coenzyme Q. Myxothiazol also inhibits the same complex. Complex IV, the cytochrome *c* oxidase, is specifically inhibited by cyanide (CN^-), azide (N_3^-),

and carbon monoxide (CO). Cyanide and azide bind tightly to the ferric form of cytochrome a_3, whereas carbon monoxide binds only to the ferrous form. The inhibitory actions of cyanide and azide at this site are very potent, whereas the principal toxicity of carbon monoxide arises from its affinity for the iron of hemoglobin. Cyanide can prevent both coupled and uncouple respiration with all substrates, including NADH, succinate and ascorbate plus TMPD. Inhibitors of electron transport chain block electron flow and, therefore, inhibit oxidative phosphorylation and ATP synthesis (Garrett and Grisham, 1999).

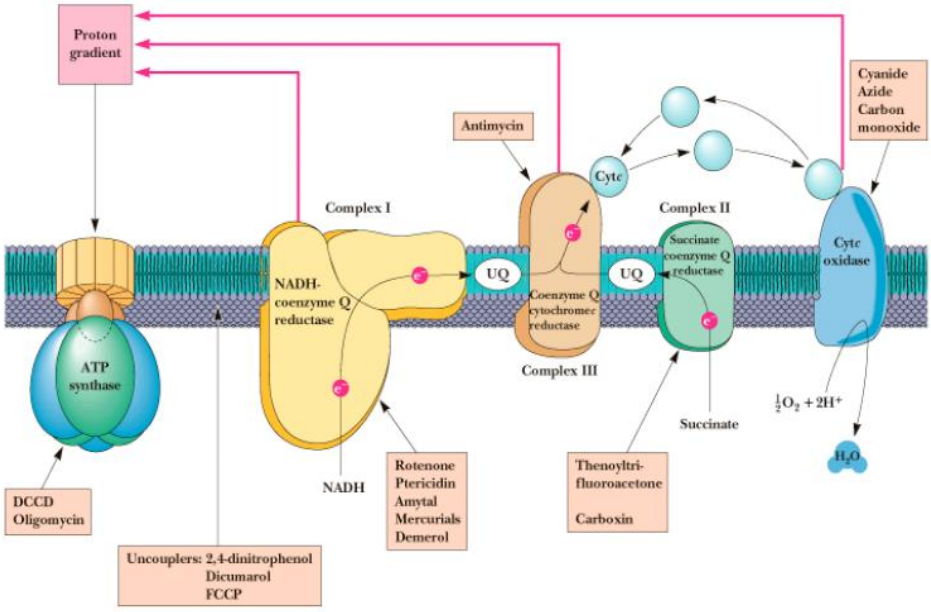


Figure 2.11 The sites of action of several inhibitors of electron transport chain and/or oxidative phosphorylation (Garrett and Grisham, 1999).

Oligomycin and DCCD are ATP synthase inhibitors

Inhibitors of ATP synthase include dicyclohexylcarbodiimide (DCCD) and oligomycin. DCCD bonds covalently to carboxyl groups in hydrophobic domains of

proteins in general and to a glutamic acid residue of the *c* subunit of F_0, the proteolipid forming the proton channel of the ATP synthase, in particular. If the *c* subunit is labeled with DCCD, proton flow through F_0 is blocked and ATP synthase activity is inhibited. Oligomycin acts directly on the ATP synthase to block the movement of protons through F_0. Because these agents block ATP synthase activity, therefore, oxidative phosphorylation and ATP synthesis are also inhibited (Garrett and Grisham, 1999).

Uncoupler

The uncouple reagents inhibit ATP synthesis but their actions do not involve direct binding to the proteins of electron transport chain or ATP synthase. These agents are known as uncouplers. They disrupt the tight coupling between electron transport and the ATP synthase by dissipating the proton gradient across the inner mitochondrial membrane created by the electron transport system. Typical examples of uncouplers include 2,4-dinitrophenol (DNP), dicumarol, carbonyl cyanide m-chloro phenylhydrazone (CCCP) and *p*-trifluoromethoxy carbonyl cyanide phenyl-hydrazone (FCCP). These compounds are lipid soluble week acid. They uncouple mitochondrial oxidative phosphorylation by carrying protons across the inner membrane into the matrix and destroying the proton gradient that couples electron transport with ATP synthase. In mitochondria treated with uncouplers, electron transport continues and protons are driven out through the inner membrane. However, no proton gradient is formed because protons leak back via the uncouplers and ATP synthesis cannot occur. Thus, the energy released in electron transport is dissipated as heat by the uncouplers (Garrett and Grisham, 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All materials, chemicals and instruments used in study were listed in Table 3.1 and 3.2 below.

Table 3.1 List of chemicals used in the studies.

Name	Source
Adenosine 5′-diphosphate monopotassium salt dihydrate	Sigma
Acetic acid glacial	Carlo
Ammonium thiocyanate	Guangdong Guanghua Chemical Factory
Antibiotic-antimycotic	Gibco
Ascorbic acid	Carlo
Alpha tocopherol	Sigma
Bio-rad protein assay (dye reagent)	Bio-rad
Bio-rad protein assay standard II	Bio-rad
Carbonyl cyanide-3-chloro-phenylhydrazone (CCCP)	Sigma
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma

Table 3.1 (Continued)

Name	Source
Dimethylsulphoxide (DMSO)	Carlo
Di-sodium hydrogen phosphate anhydrous	Carlo
DL-Malic acid	Sigma
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Sigma
Ethyl alcohol absolute	Carlo
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Fetal bovine serum (FBS)	Gibco
Folin & Ciocalteu's Phenol Reagent	Sigma
Gallic acid	Sigma
HEPES	Sigma
Iron(II) chloride 4-hydrate	BDH
Linoleic acid	Acros
L-Glutamic acid	Sigma
Magnesium sulphate	Carlo
Methyl alcohol	Carlo
Potassium chloride	Sigma
Potassium hydroxide	Carlo
Potassium phosphate monobasic	Sigma
Roswell Park Memorial Institute 1640 medium (RPMI 1640)	Gibco
Sodium bicarbonate	Carlo

Table 3.1 (Continued)

Name	Source
Sodium hydroxide anhydrous pellets	Carlo
Succinic acid	Sigma
Sucrose	Carlo
Tris (hydroxymethyl)-aminomethane	Carlo
Tris (hydroxymethyl)-aminomethane hydrochloride	Acros
Trypsin (2.5%)	Gibco
Trypan blue dye	Sigma
Tween 20	Bio-rad

Table 3.2 List of equipments and glasswares used in the studies.

Name	Source
96-well plate flat, round and U bottom	SPL
Acrodisc syringe filter 0.45µm	Pall
Bottle top filter 500 mL, 45mm. PES, 0.22 um.	Corning
Cell culture dishes 100 x 20 mm.	SPL
Centrifuge machine (model CT15RT)	Techcomp
CO_2 incubator	SHEL LAB
Cryogenic vial	Corning
ELISA plate reader	Bio-rad
Homoginizer	IKA

Table 3.2 (Continued)

Name	Source
Microliter pipette (100-1000 µL)	Denville
Multichannel pipette	Gibco
Orbital shaker	N-biotek, Inc
Oxygraph plus	Hansatech
Oven incubator	Memmert
pH meter	Consort
Rotary evaporator with vacuum (model R205)	Buchi
Spectrophotometer (model CE1011)	Cecil
TC flask 25 Sq.cm.	Corning
TC flask 75 Sq.cm.	Corning

3.2 Methods

3.2.1 Plants and extract

3.2.1.1 Collection of plants

P. amarus, *P. virgatus* and *P. urinaria* were collected from areas where they grow naturally in Nakhon Ratchasima province, Thailand. Plants were washed thoroughly to remove dirt and contaminants, and then oven dried at 40°C. The whole plants were ground to pass a 2 mm sieve and kept in well-sealed at -20°C.

3.2.1.2 Preparation of plant extract

Each dried plant (10 g) was extracted with 100 mL (1:10 w/v) of 50% methanol in distilled water by agitation with orbital shaker at a speed of 115 rpm

for 72 hours at room temperature. The supernatant was drained and the residue was extracted again with 100 mL of 50% methanol in distilled water for 24 hours. The pooled extracts were filtered through Whatman No. 4 filter paper and concentrated at 40°C using a rotary evaporator under low pressure. The residue was freeze-dried in a lyophilizer and stored at -20°C.

3.2.2 The total phenolic content (TPC)

The concentration of phenolic compounds extracted from each plant was measured according to the method as described previously (Kulkarni et al., 2007) with minor changes and calculated by using gallic acid as a standard. The reaction mixture consisted of 250 µL of the extract (1 mg/mL) or standard (0.025-0.5 mg/mL gallic acid), 2.5 mL of 2% Na_2CO_3 and 100 µL of 50% Folin-ciocalteu reagent. After 30 min of incubation, the absorbance was measured at 750 nm using a spectro-photometer. Results were expressed as milligrams per gram of gallic acid equivalents.

3.2.3 Determination of antioxidant activity

3.2.3.1 DPPH free radical scavenging activity

Radical scavenging activity of the plant extracts against stable 2,2-dipenyl-1-picrylhydrazyl (DPPH) was determined spectrophotometrically (Ahmad et al., 2005). In principle, when DPPH reacts with an antioxidant compound that can donate hydrogen, it is reduced by acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule. The changes in colour from deep violet (DPPH) to light yellow (reduced DPPH) was measured at 517 nm. Each sample (500 µL; range from 0-300 µg/mL) was added to 4.0 mL of 50 µM DPPH in methanolic solution and the final volume was adjusted to 5.0 mL with water. After vortexing, the mixture was incubated for 30 min in the dark at room

temperature. The decrease in absorbance at 517 nm was then measured using a spectrophotometer. For controls, solutions containing deionized water instead of the extract was used. The radical scavenging activity was calculated using the following equation:

Radical scavenging activity (%) = $[(A_{control} - A_{sample})/ A_{control}] \times 100$

where $A_{control}$ is the absorbance of the control reaction and A_{sample} is that of the reaction sample extract or standard compounds

3.2.3.2 Inhibition of linoleic acid oxidation

An antioxidant activity of the extract of *Phyllanthus* sp. was determined using the ferric thiocyanate method as described previously (Zhan et al., 2006). A 0.5 mL of each sample (31.25 to 2,000 µg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.2 M, pH 7.0) and 2 mL of phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 and 50 mL phosphate buffer. The reaction mixture was incubated in a test tube and placed in the dark at 60°C to accelerate the oxidation process for 8 hours. An aliquot (0.1 mL) of the mixture was diluted with 4.5 mL of 75% ethanol, followed by the addition of 0.2 mL of 30% ammonium thiocyanate and 0.2 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid. During the linoleic acid oxidation, peroxides are formed and then oxidize Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. Absorbance of the solution was measured using a spectro-photometer. High absorbance indicates high linoleic acid oxidation. α-Tocopherol and ascorbic acid were used as positive controls. Duplicate experiments were performed. The percentage of inhibition of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

Inhibition of lipid peroxidation (%) = $100 - [(A_{sample}/A_{control}) \times 100]$

where $A_{control}$ is the absorbance of the control reaction (without the extract or standard compound) and A_{sample} is that of the reaction in the presence of the sample extracts or standard compounds.

3.2.4 Cell culture

3.2.4.1 Cell lines

HepG2 liver cancer cells were cultured on cell culture dish in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium). The medium was consisted of 10% fetal bovine serum (FBS), 100 U/mL antibiotic-antimycotic. Cells were grown at 37°C with 5% carbon dioxide atmosphere in fully humidified air.

3.2.4.2 Preparation of the extract of *Phyllanthus* sp. for cell cultures

The powder of the extract of *Phyllanthus* sp. (25 mg) was dissolved in 25% dimethyl sulfoxide (DMSO) and diluted with complete medium, then stored at -20°C until used. The stock concentration of each extract was 1 mg/mL.

3.2.4.3 Cytotoxicity studies

The cells were used to assess the cytotoxicity of the extract of *Phyllanthus* sp. in order to find the range of optimal concentrations for using in experiments by the trypan blue exclusion assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay. The final concentration of DMSO in the medium was adjusted to 1% (v/v) in all cultures in order to minimize the effect of DMSO.

Trypan blue exclusion assay:

The cells in 96-well plates (5×10^5 cells/well) were treated with

various concentrations of the extract of *Phyllanthus* sp. for 24 hours. Cells were harvested after digestion with 0.25% trypsin-EDTA solution at 37°C for 5 min. The cells suspension was mixed with an equal volume of 0.4% (w/v) trypan blue. The number of viable (unstained) and dead (stained) cells were counted by using hemocytometer. The percentage of viable cells was calculated according to the following formula:

% Viable cells = (Viable cells per mL/ Total cells per mL) x 100

MTT Assay:

Cells (5 x 10^5 cells/well) were plated in triplicate in a 96-well culture plate overnight. They were treated with various concentrations of the extracts of *Phyllanthus* sp. for 24 hours. After the incubation, 20 µL of MTT (5 mg/mL) was added to each well and incubated further at 37°C for 4 hours. The growth medium was removed and then 100 µL of DMSO was added to each well to dissolve purple crystals of formazan. The absorbance was measured using spectrophotometer at a wavelength of 540 nm. Reported values were the means of three replicates and expressed as percentages of the control values.

3.2.4.4 Measurement of respiration rate of HepG2 cells

Rates of oxygen consumption of HepG2 cells were determined using a Clark oxygen electrode (Hansatech Oxygraph system). HepG2 cells (2 x 10^7 cells/mL) were added into an oxygraph chamber. Glucose was used as a substrate. After a stable rate of oxygen consumption, CCCP (as a positive control) or the extract of *Phyllanthus* sp. was added into the chamber. The respiratory rate was expressed in nmol/mL/min.

3.2.5 Determination of oxygen consumption of rat liver mitochondria

Measurement of mitochondrial oxygen consumption can be done with the instrument called oxygraph. Typical oxygen tracing illustrating the response of mitochondria to ADP and uncoupler, CCCP, is shown in Figure 3.1. Addition of ADP markedly stimulates mitochondrial oxygen consumption. After all ADP is converted to ATP, the respiration returns to the slow resting level again. This dependence of mitochondria respiration (oxygen consumption) on ADP is called respiratory control or acceptor control. Chance and Williams (1955) had defined the following metabolic states in term of substrate, ADP and oxygen levels in order to identify the steady-state condition of the mitochondria during experiment. State 1 is the condition in which both ADP and respiratory substrate are low or lacking and state 2 is a starved state in which ADP has been added in order to exhaust the endogenous substrate and all the components of the respiratory chain have become nearly completely oxidized. State 3 is the “active” state of rapid respiration and phosphorylation, with adequate supplies of substrate and ADP. State 4 is the “resting” state in which the ADP is lacking and is characterized by a low respiration rate even though substrate is present (Figure 2.16). In state 5 only oxygen is lacking and the respiratory components are all reduced. The ratio of the rate of respiration in state 3 and state 4 is called “the respiratory control ratio (RCR)” and is the measure of the degree of “tightness” of the coupling mechanism in intact mitochondria. Tightly coupled mitochondria (the term applies to the mitochondria in which the respiration is dependent on phosphate acceptor) have very high RCR values. Typical RCR values range from 3 to 10, varying with the substrate and quality of the preparation. On the other hand, loosely coupled or uncoupled mitochondria have much lower RCR values that may approach 1.

Moreover, state 3U is the state with high rate of respiration caused by addition of uncoupling agent (such as dinitrophenol or CCCP). It leads to a persistent high rate of respiration in the absence of ADP until all the oxygen has been consumed as shown in Figure 3.1.

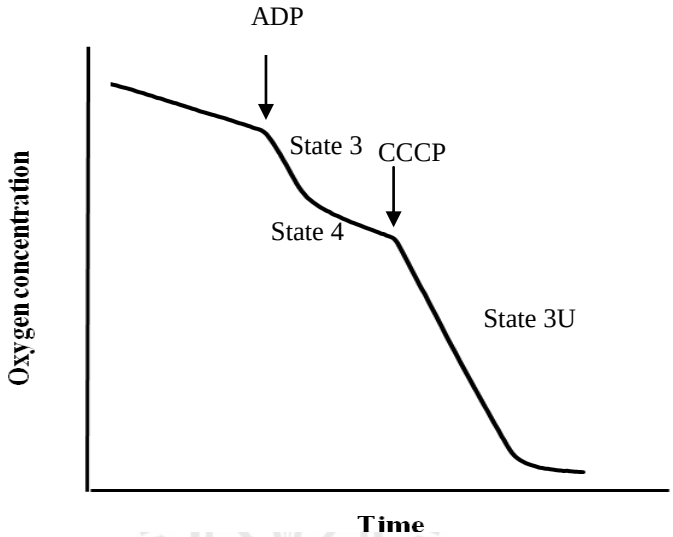


Figure 3.1 Oxygraph tracing

3.2.5.1 Experimental animals

Male albino Wistar strain rats, weighing 200-250 g, were obtained from Institutional Animal Care Building at Suranaree University of Technology, Nakhon Ratchasima. Rats were maintained in stainless steel hanging cages with hardwood bedding and provided with diet and tap water *ad libitum*. They were housed in a temperature of 25±2°C in a 12-12 hours light-dark cycle and 40-70% relative humidity.

3.2.5.2 Preparation of mitochondria from rat liver

Mitochondria were isolated by standard differential centrifuga-

tion as described by Hogeboom (1955). Rats were sacrificed by cervical dislocation. All procedures were carried out at 4°C. Livers were immediately removed, rinsed and minced in medium (containing 0.25 M sucrose, 5 mM HEPES buffer (pH 7.4) and 1 mM EGTA) and homogenized with teflon pestle tissue homogenizer. Homogenates were centrifuged at 700 x g for 7 min and the resulting supernatant further centrifuged at 4,760 x g for 10 min. Pellets were resuspended in 0.25 M sucrose and centrifuged at 16,700 x g for 10 min. The final mitochondrial pellet was washed to remove a top layer of microsome and then gently resuspended in 1 mL of 0.25 M sucrose with teflon pestle tissue homogenizer by hand. The final mitochondrial suspensions were stored in ice-bath until used, which was within 3 h after isolation. Mitochondrial protein content was determined using the Bio-Rad protein assay reagent.

3.2.5.3 Measurement of mitochondria respiration

The rate of respiration was determined with a Clark oxygen electrode (Hansatech Oxygraph system) using malate plus glutamate or succinate as substrates. Mitochondria (1 - 2 mg) were added to the chamber with medium containing 10 mM HEPES buffer (pH 7.4), 5 mM $MgCl_2$, 20 mM KCl, 225 mM sucrose and 10 mM KH_2PO_4 (Pi). Chamber contents were continually stirred by a small magnetic stirring bar. After a stable rate of oxygen consumption, the experiments were initiated by adding various concentrations of the *Phyllanthus* extracts (125-625 µg/mL) or vehicle (0.625% DMSO) followed by ADP (final concentration 0.4 mM or 0.3 mM) or CCCP (final concentration 1 µM or 0.3 µM). Respiratory control ratio (RCR) was obtained by dividing the rate of state 3 respiration with the rate of state 4 respiration.

3.2.6 Statistical analysis

Statistical analysis was performed using statistical package for social science (SPSS) version 13.0 (Trakamrungsee, 2004). For multiple comparisons, data were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($P < 0.05$).

CHAPTER IV

RESULTS

4.1 The total phenolic content (TPC) of *Phyllanthus* sp.

Phenolic compounds are commonly found in the plant kingdom and they have been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Xin-hua et al., 2001). So it is important to determine the total phenolic content of the extracts of three *Phyllanthus* species. The phenolic content of each extract was determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents (GAE). The results are represented in Table 4.1. It was found that *P. virgatus* had a significantly higher concentration of phenolic compound (435.79±4.68 mg/g GAE) than *P. urinaria* and *P. amarus* (354.63±27.3 and 342.03±7.85 mg/g GAE, respectively).

Table 4.1 Total phenolic content of the extracts of the three *Phyllanthus* species.

Extract	Phenolic content (GAE (mg g^{-1}))
P. amarus	342.03±7.85
P. virgatus	435.79±4.68*
P. urinaria	354.63±27.3

* $P < 0.05$, Values are expressed as mean ± S.E. (n=5)

4.2 Antioxidant activity of *Phyllanthus* sp.

4.2.1 DPPH free radical scavenging activity

The method with DPPH as a stable free radical to measure radical scavenging activity has been widely used. Antioxidants react with DPPH, which is a stable free radical, and convert it to 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical. The absorbance decreases when the radical is reduced by antioxidants. In this study, DPPH radical scavenging activity of the extract of three *Phyllanthus* species was observed at all concentrations tested. The scavenging effect increased with increasing concentrations from 4.7-300.0 µg/mL. The maximum inhibition of *P. urinaria*, *P. virgatus* and *P. amarus* were 92.17±0.95%, 91.81±1.00%, 90.57±0.82%, respectively, as show in Figure 4.1.

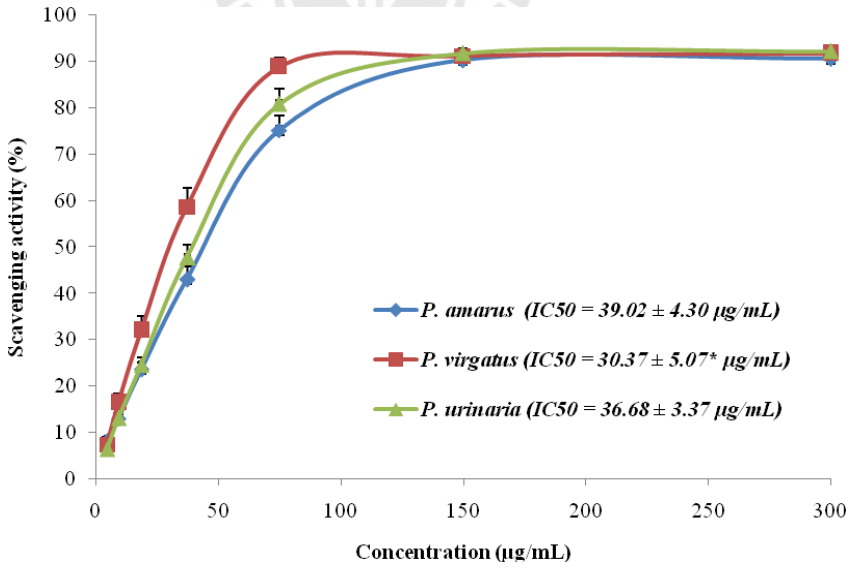


Figure 4.1 The percentage of DPPH radical scavenging of the extract of three *Phyllanthus* species. $P < 0.05$; * compared to the other species.

Generally, the concentration of antioxidant needed to decrease the initial $DPPH^{\cdot}$ concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity. The lower the IC_{50}, the higher the antioxidant activity. It was also found that the free radical scavenging activity of *P. virgatus* (IC_{50} = 30.37±5.07 µg/mL) was significantly stronger than that of *P. urinaria* (IC_{50} = 36.68±3.37 µg/mL) and *P. amarus* (IC_{50} = 39.02±4.30 µg/mL).

4.2.2 Inhibition of linoleic acid oxidation by *Phyllanthus* sp.

Ferric thiocyanate method was used to determine the amount of the peroxides of linoleic acid at an early stage of lipid peroxidation. The data obtained at different concentrations are presented in the Figure 4.2.

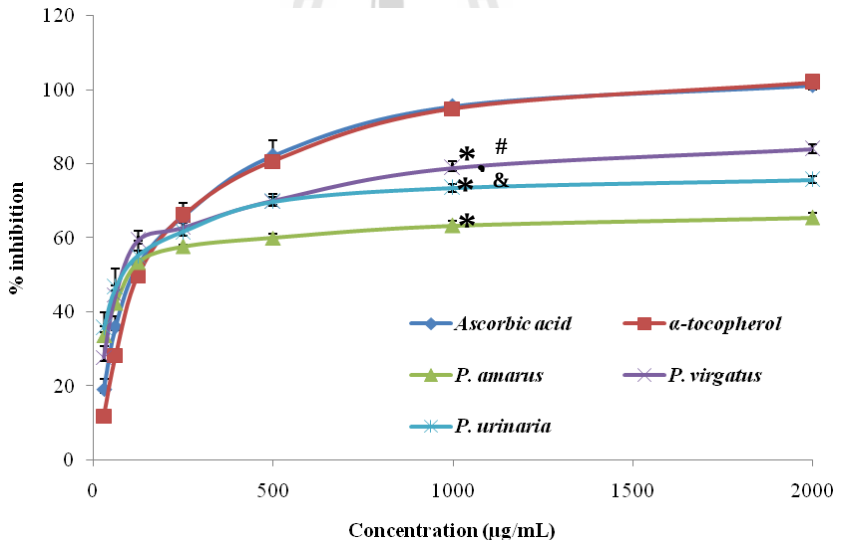


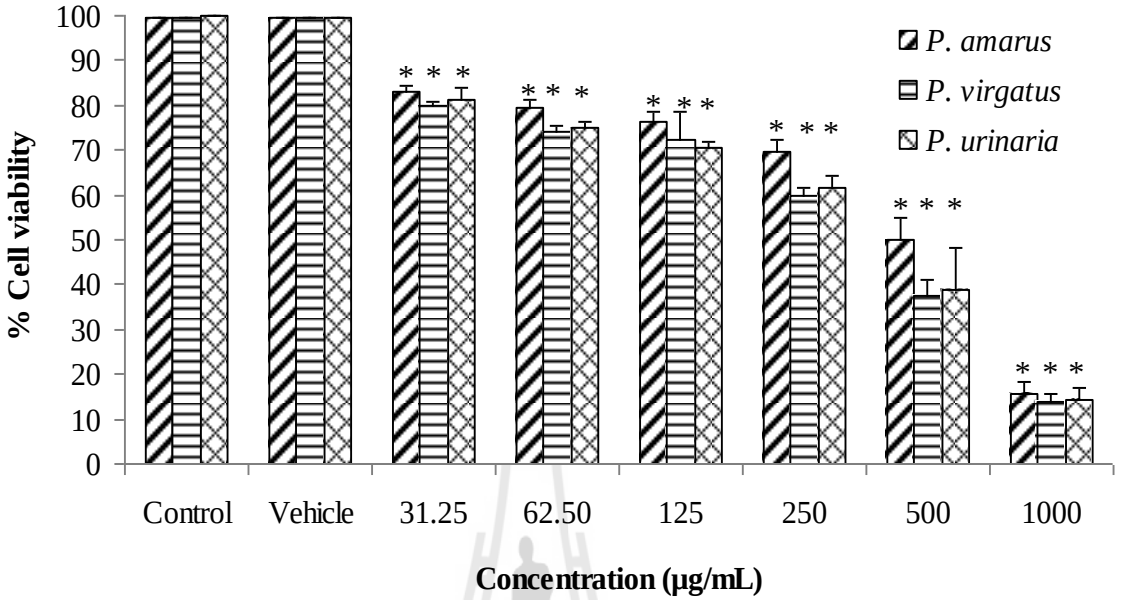
Figure 4.2 Antioxidant activities of different concentrations of the extract of three *Phyllanthus* species in comparison to positive controls as measured by ferric thiocyanate method: * results are significantly different ($P < 0.05$) from positive control and #, significantly different from *P. amarus* and *P. urinaria* and &, significantly different from *P. virgatus* and *P. urinaria*.

It was found that the inhibition of peroxidation of the extract of three *Phyllanthus* species, ascorbic acid and α-tocopherol in linoleic acid system were increased with the increasing concentration up to 2000 µg/mL. At a concentration of 2.0 mg/mL, the percentage inhibition of the extract of *P. virgatus, P. urinaria* and *P. amarus* were 83.98±1.44%, 75.77±0.97% and 65.54±1.38%, respectively, which were lower than that of ascorbic acid (100.00±0.40%) and α-tocopherol (100.00±0.89%). Statistical analysis from this study showed that there were significant ($P < 0.05\%$) differences among antioxidative activities of the extract of three *Phyllanthus* species, ascorbic acid and α-tocopherol. In addition, the extract of three *Phyllanthus* species showed significant different activity ($P < 0.05$) at a concentration of 1 mg/mL.

4.3 Cytotoxic effects of *Phyllanthus* sp.

The cytotoxicity of the extract of three *Phyllanthus* species to HepG2 cells was examined by trypan blue (TB) exclusion (Figure 4.3A) and MTT assay (Figure 4.3B). Results in Figure 4.3A and 4.3B show the effects of vehicle (final concentration of the DMSO did not exceed 1% (v/v) and DMSO did not affect the cell proliferation) and the extract of three *Phyllanthus* species on the viability of HepG2 cells as compared to control. In the present study, the plant extract induced cytotoxicity to HepG2 cells in a concentration dependent manner after 24 hour of treatment. The extract of three *Phyllanthus* species (31.25-1000 µg/mL) significantly suppress proliferation and increase cytotoxicity in HepG2 cells compared with control and vehicle ($P < 0.05$). The cytotoxic potential of the extract of three *Phyllanthus* species, expressed by their IC_{50} values from the two assays, is summarized in Table 4.2.

(A)



(B)

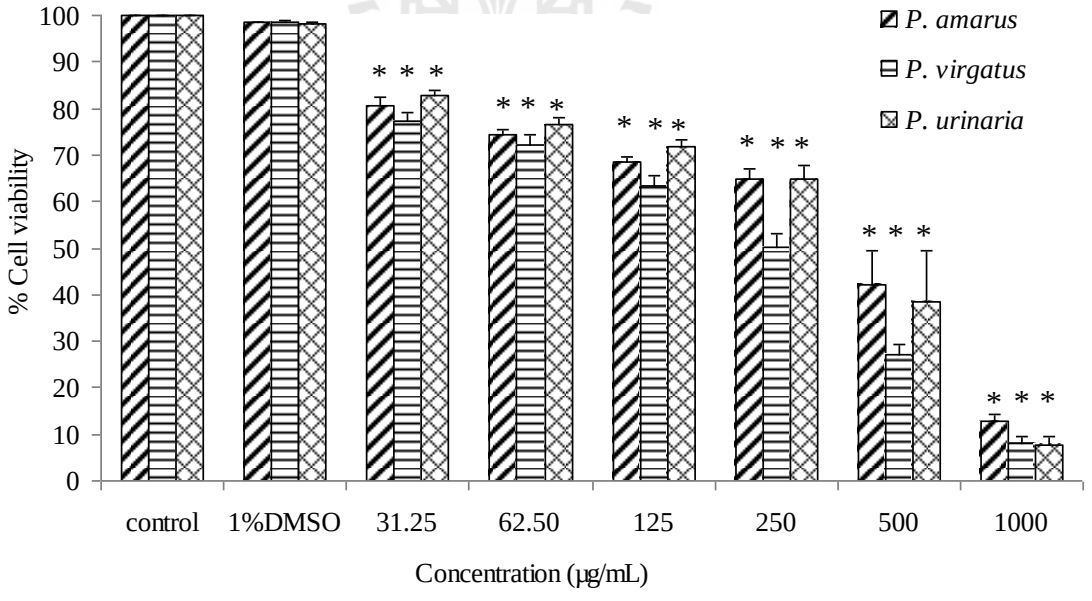


Figure 4.3 Concentration-response effects of *Phyllanthus* sp. on HepG2 cells viability measured by trypan blue exclusion (A) and MTT assay (B). *, Results are significantly different ($P < 0.05$) from control.

The IC_{50} of *P. amarus*, *P. virgatus* and *P. urinaria* measured by trypan blue exclusion assay were 514.15±44.54, 370.90±21.11 and 431.14±65.54 µg/mL, respectively. Using MTT assay, The IC_{50} of *P. amarus*, *P. virgatus* and *P. urinaria* were 463.72±68.24, 253.30±18.89 and 445.07±62.19 µg/mL, respectively. By both trypan blue exclusion and MTT assays, it was found that the cytotoxic effect of *P. virgatus* was stronger than that of *P. urinaria* and *P. amarus*.

Table 4.2 The IC_{50} of the extract of three *Phyllanthus* species on HepG2 cell viability.

Extract	Inhibiting concentration (IC_{50}) (µg/mL)	
	TB (n=5)	MTT(n=5)
P. amarus	514.15±44.54	463.72±68.24
P. virgatus	370.90±21.11	253.30±18.89*
P. urinaria	431.14±65.54	445.07±62.19

* $P < 0.05$, Values are expressed as mean ± S.E. (n=5) when compare to the other species

4.4 Effects of *Phyllanthus* sp. on morphological changes of HepG2 cells

Morphological features of cell treated with the extracts of three *Phyllanthus* species (500 µg/mL) observed under a microscope digital camera with an objective of 10X magnification were studied. The normal morphology of HepG2 maintained in RPMI 1640 was illustrated in Figure 4.4A. The epithelial-like feature forming a monolayer on the surface of the culture flask was seen. In the presence of plant extracts, the morphological changes were observed such as rounding up, loss of contact with neighboring cell and detachment from plate. This experiment, the 0.5%

DMSO did not effect to the cell proliferation and survival of HepG2 cells when compared to control.

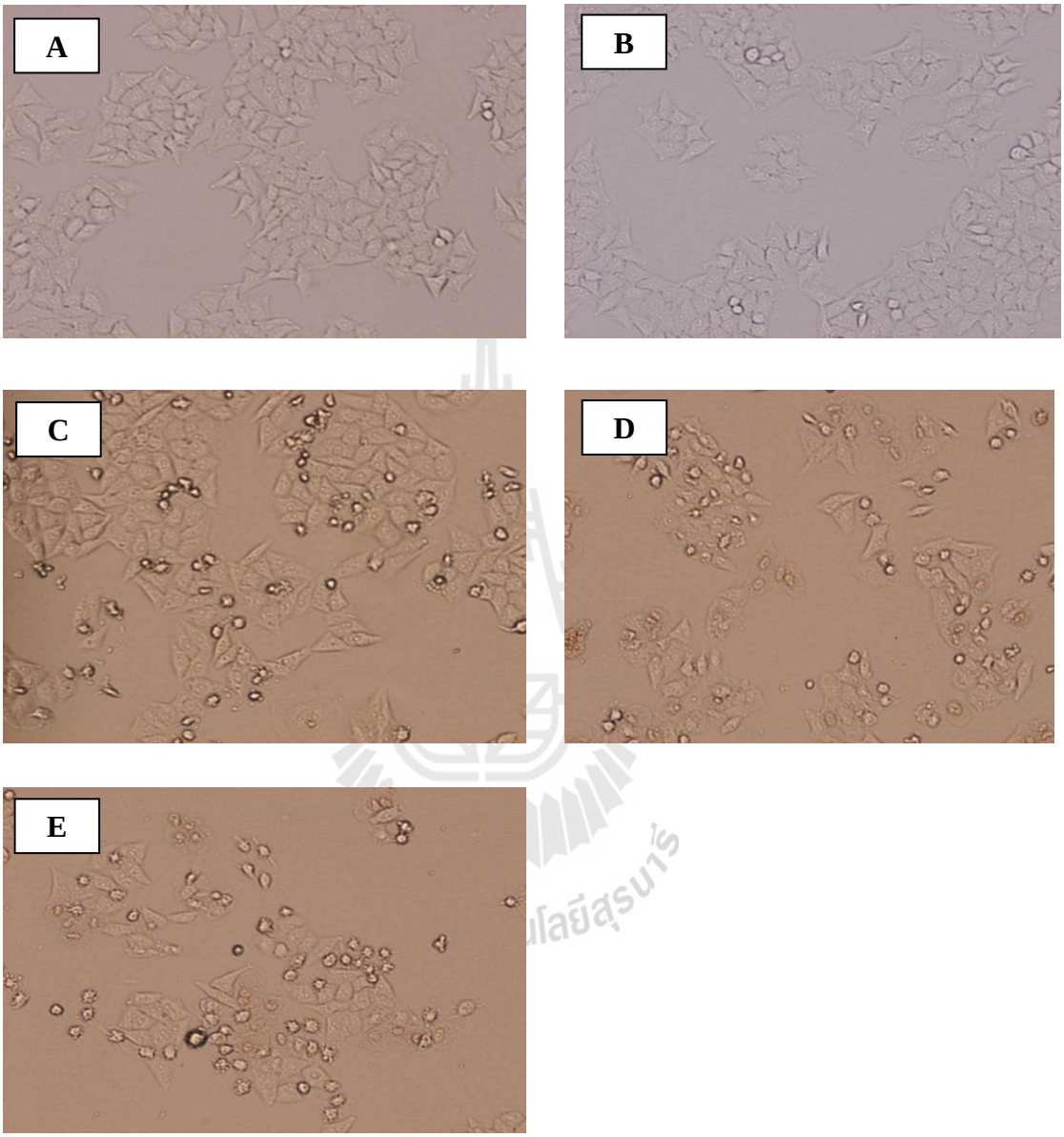


Figure 4.4 Effects of *Phyllanthus* sp. on HepG2 morphology.

(A) Control (B) Vehicle (0.5% DMSO) (C) *P. amarus* (500 µg/mL)

(D) *P. virgatus* (500 µg/mL) (E) *P. urinaria* (500 µg/mL)

4.5 The effects of *Phyllanthus* sp. on oxygen consumption of HepG2 cells

The rate of oxygen consumption by the HepG2 cells was measured in the present of CCCP, a known mitochondrial uncoupler, and the extracts of three *Phyllanthus* species (Figure 4.5 and 4.6).

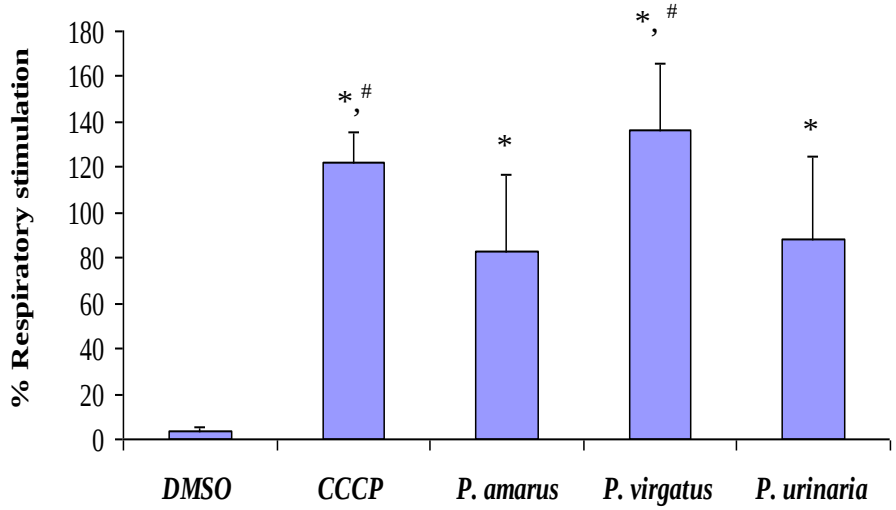


Figure 4.5 Effects of *Phyllanthus* sp. (500 µg/mL) and CCCP (1 µM) on oxygen consumption of HepG2 cells. ***P* < 0.05, significantly different from 0.5% DMSO and # *P* < 0.05, significantly different from *P. amarus* and *P. uri*naria.

It was found that at 500 µg/mL, *P. virgatus* showed the strongest respiratory stimulation among three *Phyllanthus* species, which is comparable to effect of the uncoupler CCCP (1 µM). *P. urinaria* and *P. amarus* caused slight respiratory stimulation on HepG2 respiration. The 0.5% DMSO had only slightly effect on HegG2 cells respiration.

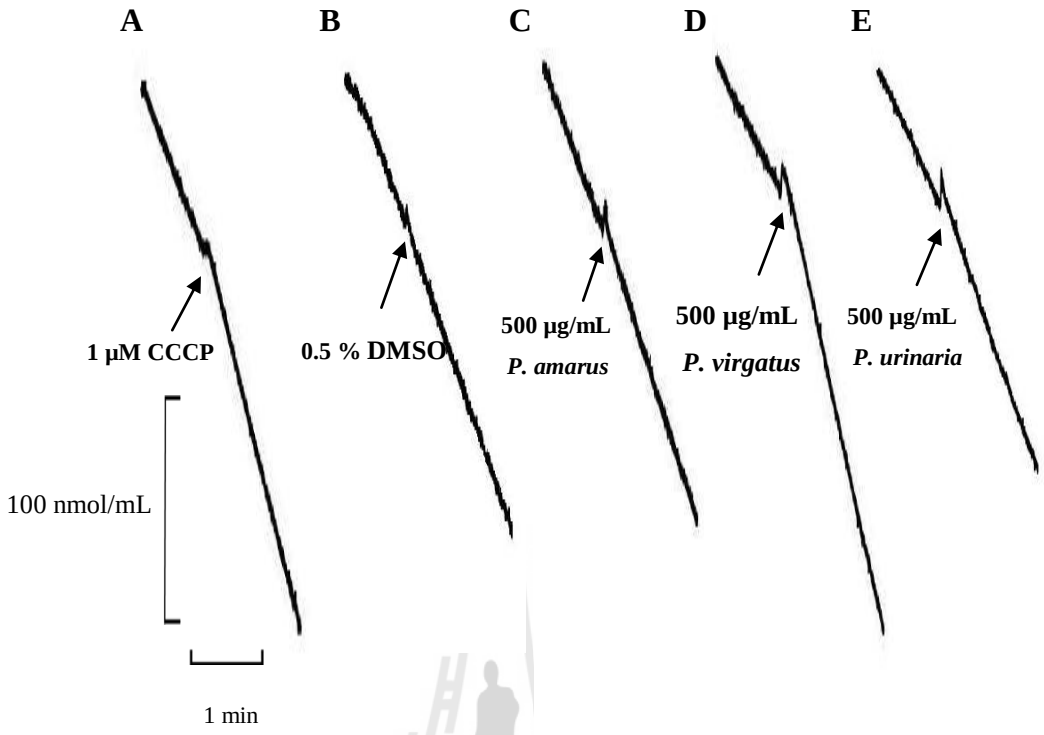


Figure 4.6 Representative recordings showing the respiratory responses of HepG2 cells to CCCP and extracts of three *Phyllanthus* species.

4.6 The effects of *Phyllanthus* sp. on state 3 and 4 respiration and respiratory control ratio (RCR) of isolated rat liver mitochondria

Figure 4.7 shows tracings of state 3 and 4 respirations of isolated rat liver mitochondria when treated with *Phyllanthus* extracts at a concentration 500 µg/mL with glutamate plus malate as substrates. Tracing 4.7A shows control respiratory response of mitochondria. Mitochondria are incubated in medium containing substrates (glutamate plus malate) and phosphate. The initial oxygen consumption rate is low. This state is called state 4 respiration. Addition of ADP caused a sudden burst of oxygen uptake as ADP was converted to ATP. This active respiring state is referred to as state 3 respiration. When all the ADP has been phosphorylated to form

ATP. The respiration returned to state 4 respiration. The ratio of the rate of respiration in state 3 and state 4 is called “the respiratory control ratio (RCR)” and is the measurement of the degree of tightness of the coupling mechanism in intact mitochondria.

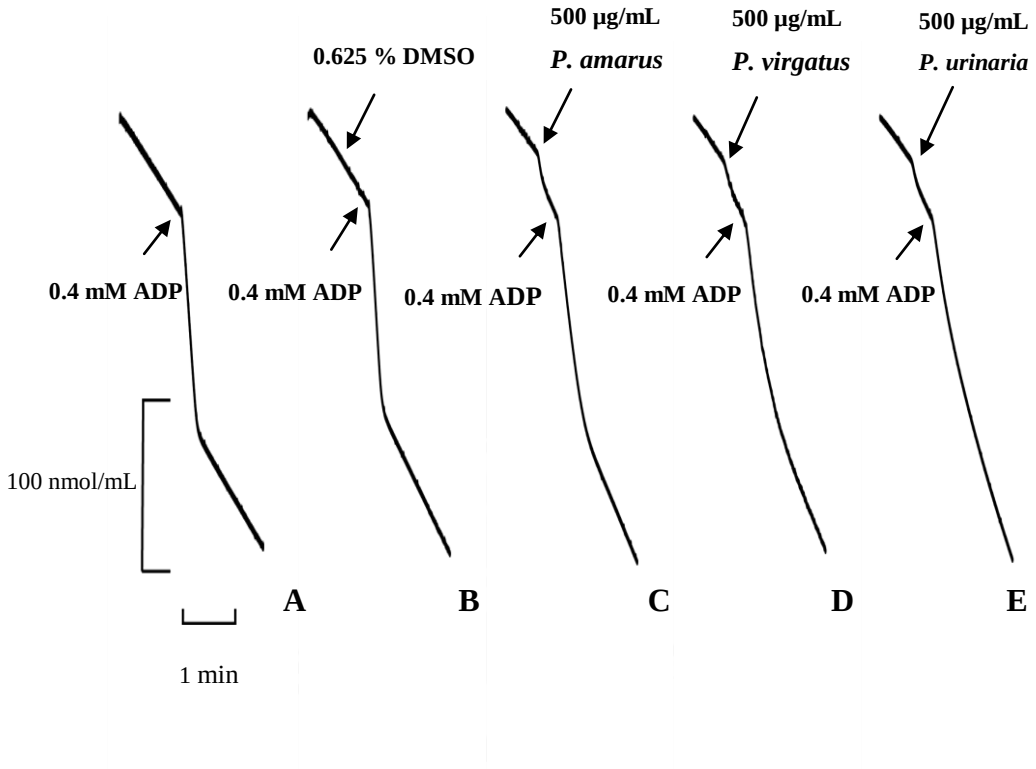


Figure 4.7 The effects of three *Phyllanthus* species (500 µg/mL) on state 3 and 4 respiration of mitochondria. Glutamate plus malate were used as substrates.

Tracing 4.7C, D and E show the effects of extracts of *P. amarus*, *P. virgatus* and *P. urinaria*, respectively, on state 3 and 4 respirations of isolated rat liver mitochondria. The extracts of three *Phyllanthus* species increased state 4 respiration but inhibited state 3 respiration. Tracing 4.7B shows that the DMSO did not affect rate of oxygen consumption.

Figure 4.8 shows tracings of state 3 and 4 respirations of isolated rat liver mitochondria when treated with *Phyllanthus* extracts at a concentration of 500 µg/mL but using succinate as a substrate. The results are similar to those described in Figure 4.7.

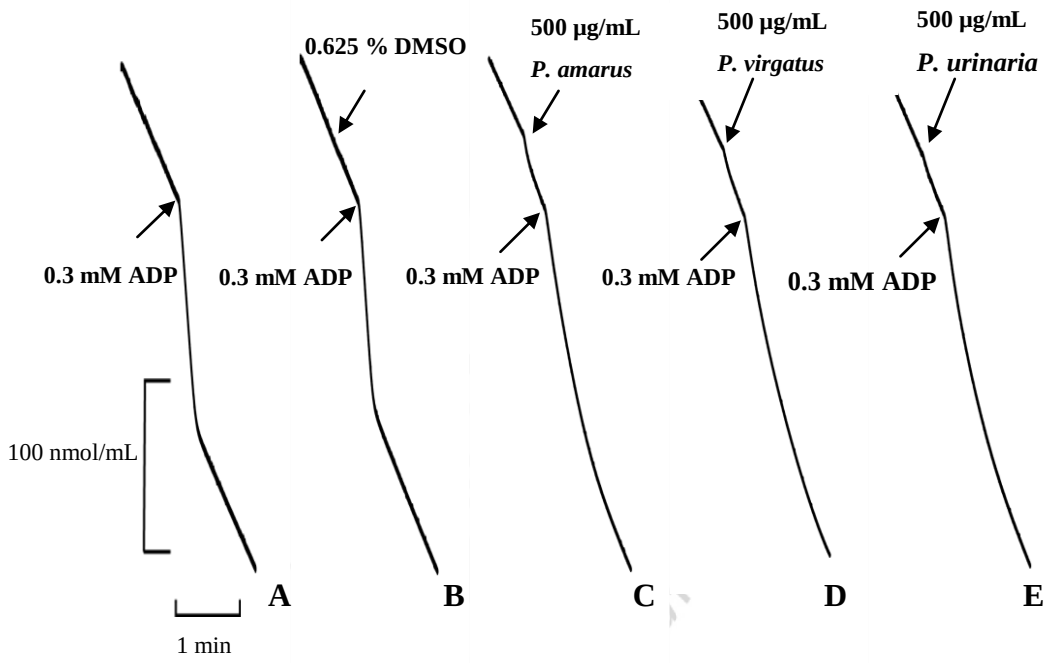


Figure 4.8 The effects of three *Phyllanthus* sp. (500 µg/mL) on state 3 and 4 respiration of mitochondria. Succinate was used as substrate.

The dose-response curves of three *Phyllanthus* sp. on state 3 and 4 respiration and respiratory control ratio of mitochondria are shown in Figure 4.9. Figure 4.9 A illustrates the effect of three *Phyllanthus* extracts on state 3 respiration with glutamate plus malate as substrates (left). State 3 respiration was progressively inhibited by the extracts of three *Phyllanthus* species. The inhibition was significantly different from

control at concentrations 250 to 625 µg/mL**.** However, no significant differences were found among *P. amarus*, *P. virgatus* and *P. urinaria* on state 3 respiration. When using succinate as substrate (right), the degree of inhibition on state 3 respiration was comparable to that observed with glutamate plus malate as substrates.

Figure 4.9 B shows the effect of the extracts of three *Phyllanthus* species on state 4 respiration with glutamate plus malate as the substrate (left). The extracts of three *Phyllanthus* species at concentration of 375 to 625 µg/mL caused significant stimulation on state 4 respiration when compared to control. Using succinate as the substrate (right), the extracts of three *Phyllanthus* species produced significant increase in state 4 respiration from concentrations 250 to 625 µg/mL.

Figure 4.9 C shows the effect of the extracts of three *Phyllanthus* species on respiratory control ratio (RCR) with glutamate plus malate (left) or succinate as the substrates (right). The extracts of three *Phyllanthus* species caused a drop of the RCR for both substrates. The RCR decreased with the increase in the extracts of *Phyllanthus* sp.

4.7 The effects of *Phyllanthus* sp. and CCCP on oxygen consumption of isolated rat liver mitochondria

This study compared the effect of *P. amarus, P. virgatus, P. urinaria* and CCCP on rate of oxygen consumption by isolated rat liver mitochondria with glutamate plus malate (Figure 4.10) or succinate (Figure 4.11) as the substrates. It is seen that all extracts, at 500 µL, stimulated oxygen consumption with both substrates while DMSO had practically no effect on respiration.

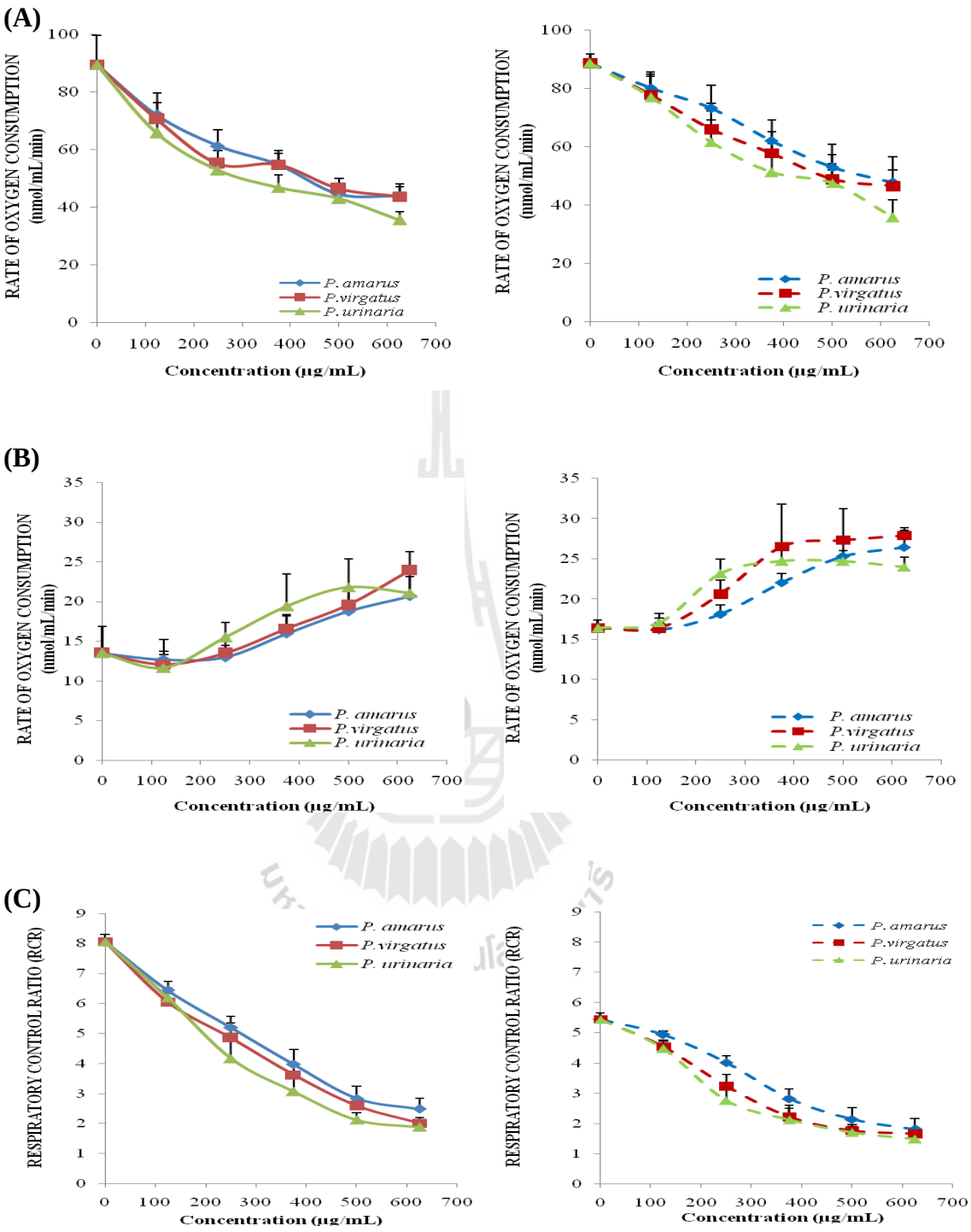


Figure 4.9 Dose-response curves of *Phyllanthus* sp. on state 3 (A) and 4 (B) respiration and respiratory control ratio (C) of mitochondria**.** Glutamate plus malate (left) or succinate (right) were used as substrate**.**

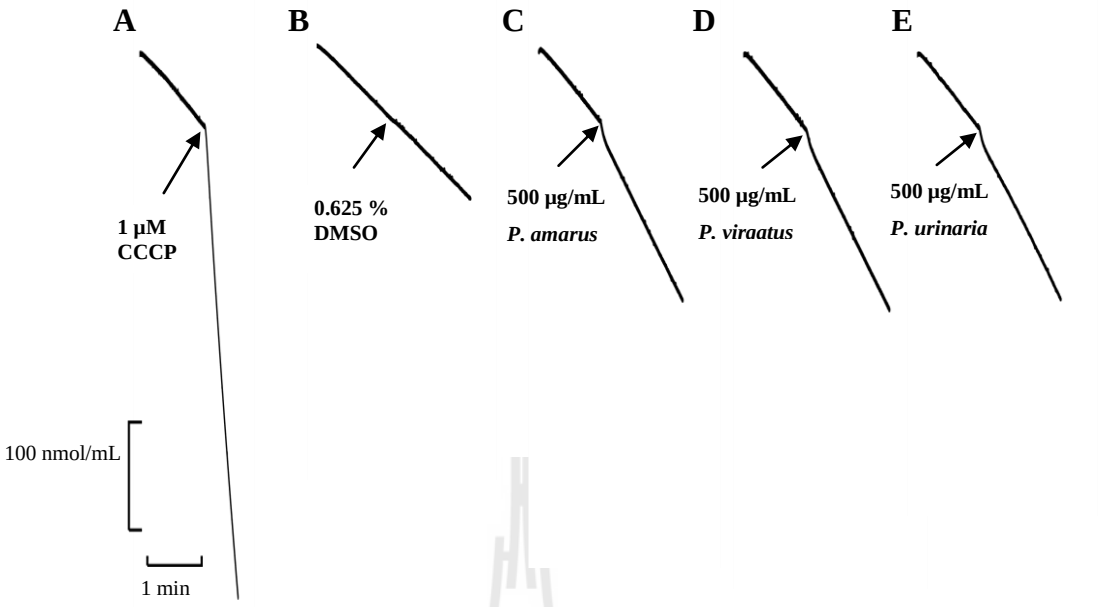


Figure 4.10 The effects of three *Phyllanthus* species (500 µg/mL) on respiration of mitochondria. Glutamate plus malate were used as substrates.

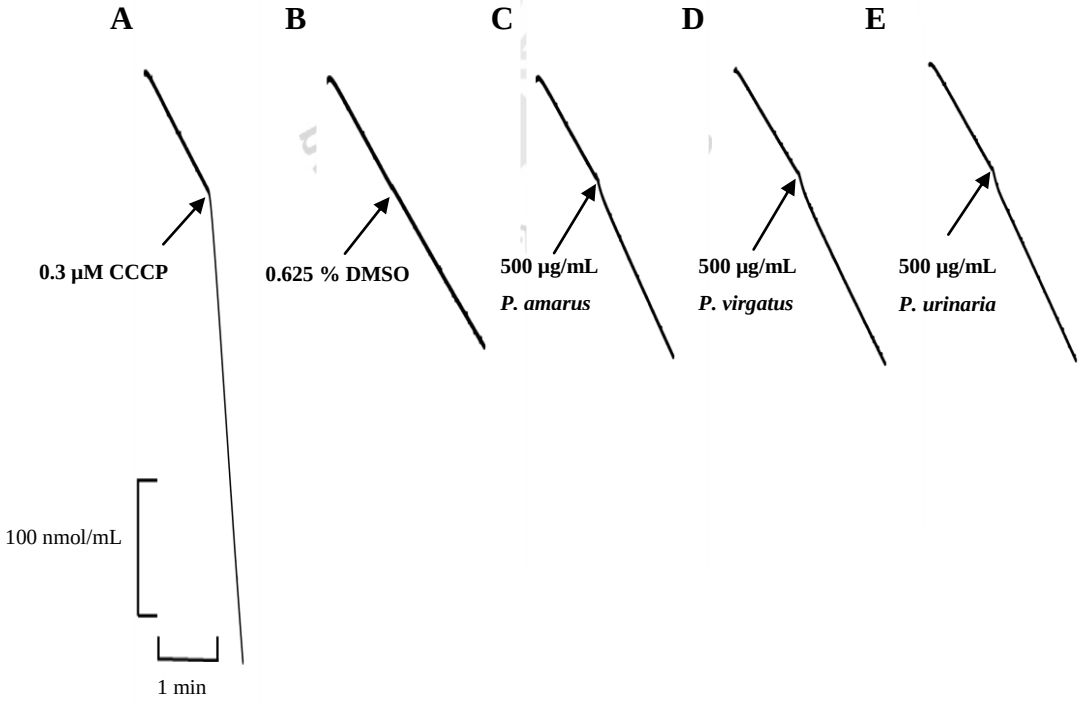


Figure 4.11 The effects of three *Phyllanthus* species (500 µg/mL) on respiration of mitochondria. Succinate was used as substrate.

However, this effect of the extracts was much less than that produced by CCCP, a potent uncoupler. The three extracts stimulated mitochondrial respiration in a dose-dependent manner (Figure 4.12) and the extract from *P. virgatus* was more active than the other two extract.

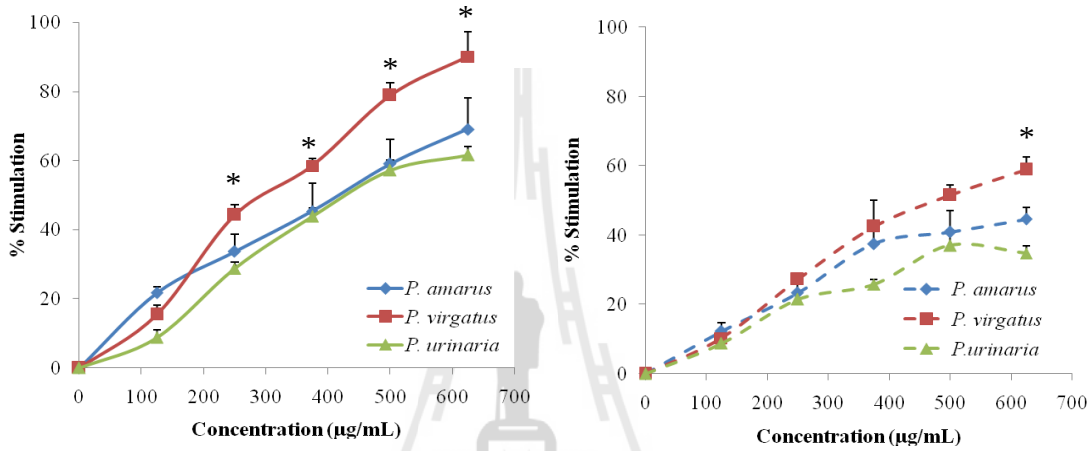


Figure 4.12 Dose-response curves of *Phyllanthus* sp. on respiration of isolated rat liver mitochondria**.** Glutamate plus malate (left) or succinate (right) was used as substrates: * $P < 0.05$, significantly difference from *P. amarus* and *P. uri*naria.

CHAPTER V

DISCUSSION

The genus *Phyllanthus* consists of several species in the family Euphorbiaceae (Jain et al., 2008). In Thailand, *P. virgatus* and other two species, *P. amarus and P. urinaria*, are closely related in appearance, phytochemical structure and have the same common local name. Therefore, it may be confusing to collect these plants for the production of herbal medicine. Frequently, the product of Luk-tai-bai in market appears as a mixture of these three *Phyllanthus* species, which may cause a much variation of activity between each batch of the production. Moreover, information on biological activities of *P. virgatus* is very limited. The purpose of this study is to investigate the differences of some pharmacological activities among these three *Phyllanthus* species.

Among the various medicinal and culinary herbs, some endemic species are of particular interest because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits. Crude extracts of fruits, herbs, vegetables, cereals, nuts and other plant materials rich in phenolics are increasingly of interest in the food industry (Kahkonen et al., 1999). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumer Phenolic compounds are commonly found in both edible and inedible plants. Phenolic

compounds comprise a large class of phytochemicals that are endowed with interesting biological properties. They possess biological properties such as antioxidant, anti-apoptosis, anti-aging, anticarcinogen, anti-inflammation, anti-arthrosclerosis, cardiovascular protection, improvement of the endothelial function, which have been attributed to their intrinsic reducing capabilities (Han et al., 2007). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g was daily ingested from a diet rich in fruits and vegetables (Tanaka et al., 1988). Moreover, previous studies have found that the three *Phyllanthus* species has antioxidant activity. Putative antioxidants from *Phyllanthus* sp. include chemicals in the group of phenolic compound such as lignans, tannins, flavonoids and other unknown structures (Rajeshkumar et al., 2002). First, the total phenolic content and antioxidative properties of each species were compared. In this study, estimation of total phenolics using the Folin-Ciocalteu reagent and gallic acid as a standard revealed that *P. virgatus* contained the highest amount of poly-phenol. Total phenolic compounds as GAE of *P. amarus* and *P. urinaria* were lower than *P. virgatus*. After that, the three *Phyllanthus* species were investigated by DPPH radical scavenging activity assay. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagenetic molecule. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The IC_{50} of scavenging effect of three *Phyllanthus* extracts with the DPPH radical is in the following order: *P. virgatus* (30.37±5.07 µg/mL) < *P. urinaria* (36.68±3.37µg/mL) < *P. amarus* (39.02±4.30 µg/mL). Previous study (Wongnawa et al., 2006) showed that *P. amarus* aqueous extract exhibited

DPPH radical scavenging activity with the EC_{50} of 45 μg/ml, which it might be related to hepatoprotective activity against paracetamol intoxication. Furthermore, Ali et al. (2008) have reviewed on 24 plants used in India for their antioxidant properties, including *P. amarus, P. virgatus* and *P. uinaria*. The most common use of these herbs was found to be in liver disease as hapatoprotective agents and in diabetics. Among different families of antioxidants, flavanoids and tannins are most recurring followed by phenolics, ascorbic acid and alkaloids. Previous experiments (Naaz et al., 2007) shown that the hepatotoxicity and damage induced by aflatoxinB1 in the liver of mice is suppressed with the administration of ethanolic extract of *P. amarus* due to the reduction in the level of reactive oxygen species (ROS) as indicated by the reduction in the level of thiobarbituric acid reactive substances (TBARS) and the induction of recovery and repair process in the liver of mice. Furthermore, several reports found that *Phyllanthus* sp. increased antioxidant enzymes level in body when they were decreased by radiation, carcinogen and other chemicals.

Several studies have reported on the relationships between phenolic content and antioxidant activity. Some authors found a correlation between the phenolic content and the antioxidant activity but some authors found no such relationship. Velioglu et al. (1998) reported a strong relationship between total phenolic content and antioxidant activity in selected fruits, vegetables and grain product. This study had no comparison of antioxidant activity and total phenolic content. However, among three *Phyllanthus* species, it was apparent that plants with high phenolic content showed greater potential to reduce free radicals *in vitro* than did plants with low phenolic content. *P. virgatus* exhibited the highest radical scavenging activity with an average IC_{50} of 30.37 μg/mL and the highest phenolic content when

compared with *P. amarus* and *P. Urinaria*. However, it should be noted that the molecular antioxidant response to free radicals varies markedly, depending on the chemical structure and the oxidation conditions. Thus, the antioxidant activity of an extract cannot be predicted on the basis of its phenolic content (Muchuweti et al., 2006).

Recently, some authors have compared the antioxidant activity and the total phenolic content of *Phyllantus* extracts (Kumaran and Karunakaran, 2007) and found that the total phenolic content and antioxidant activity of the plants species are in the order: *P. debilis* > *P. urinaria* > *P. virgatus* > *P. maderaspatensis* > *P. amarus.* The differences of the results obtained from our study compared to the previous findings may have been due to the differences in the extraction methods and environmental factor such as climatic conditions, growth, temperature and duration of storage.

The total antioxidant activities of three *Phyllanthus* species were also determined by ferric thiocyanate method in linoleic acid system. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998). Antioxidant activity in linoleic acid emulsion system, peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good oxidizing agents. They can abstract hydrogen from other molecules. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid and nucleic acids leading to structural

modification, which induce dysfunction of immune systems (Siddhuraju and Becker, 2007). The antioxidant effects of three *Phyllanthus* species were determined and compared with ascorbic acid and α-tocopherol. It was found that *P. virgatus* has protective effect against hydroperoxide generation. The inhibition of peroxidation of linoleic acid by *P. virgatus* was higher than that of *P. amarus* and *P. urinaria* but lower than that of ascorbic acid and α-tocopherol.

The cytotoxicity of the three *Phyllanthus* extracts on HepG2 hepatocellular carcinoma *in vitro* was examined by MTT and trypan blue assay. Nowadays, natural medicines have played a great role in the treatment of various disorders in human including cancer. Cancer has become an important issue in medicine as it is a major cause of death in both developed and developing countries. Human liver tumors, particularly hepatocellular carcinoma (HCC) are among the most common malignancies worldwide (Roberts et al., 2005). Furthermore, there are many reports that *Phyllanthus* sp. has anticancer activity. *P. amarus* extract administration has been shown to inhibit liver tumor development induced by *N*-nitrosodiethylamine in rats and increased the life span of hepatocellular carcinoma harboring animals (Rajeshkumar and Kuttan, 2000). Lignans such as phyllanthin, hypophyllanthin, flavanoids, quercetin, astragalin, ellagitannins and hydrolysable tannins are shown to be present in this plant. Some of these compounds have been shown to have significant activity against experimental carcinogenesis. *P. amarus* extract significantly inhibited ascites tumor induced by Dalton's lymphoma and Ehrlich ascites tumor. Inhibition of cell cycle regulation, topoisomerase II, P-450 enzymes as well as antioxidant activity may contribute to the overall activity of the extract against carcinogenesis induced in animals and may be relevant to human cancer as well

(Rajeshkumar et al., 2002). *P. amarus* is slightly cytotoxic to the human adeno-carcinoma cell line Caco-2 (Lawson-Evi et al., 2008). In addition, *P. urinaria* extract induced the apoptosis of Lewis lung carcinoma cells but it did not cause any cell loss in normal cells such as HUVECs and WRL 68 cells, suggesting that the anticancer effect of *P. urinaria* might be more specific to tumor cells (Huang et al., 2003).

The cytotoxicity of the three *Phyllanthus* extracts on HepG2 hepatocellular carcinoma obtained from this study indicated that the rate of cell survival was significantly decreased when treated with different concentrations (31.25-1,000.00 µg/mL) of three *Phyllanthus* extracts. The data showed that *P. virgatus* has stronger cytotoxic activity than *P. urinaria* and *P. amarus*, though the result obtained from trypan blue exclusion was not significantly different.

Kuo et al. (2005) suggested that several classes of natural compounds can alleviate symptoms or prevent disease, in particulary green tea, licorice, *Cirisium rhinoceros* Nakai, cloves, *Terminalia arjuna* Linn., *Euphorbia jolkini* Bioss, *Polygonum cuspidatums, Myrica rubra* Sieb et Zucc, *Centella asiatica*, *Bupleurum kaoi*, *Ochrosia elliptica* Labill, *Stephania tetrandra* and *Rhei Rhizoma*. The chemopreventive activities and active ingredients of these herbs have been studied on the various types of cancer cells. For example, flavonoid group such as acacetin in *Cirisium rhinoceros* Nakai can inhibit the growth of human prostate carcinoma LNCaP and DU145 cells (Singh et al., 2005). Group of tannin such as (-)-epigallo-catechin (EGC), one of green tea polyphenols, strongly inhibited the growth of breast cancer cell lines, MCF-7 and MDA-MB-231 (Vergote et al., 2002). From scientific evidence, many of the natural herbs have anticancer activity and the active

compounds (such as flavonoid, tannin and alkaloid) can inhibit cell growth and proliferation and induced apoptosis.

In order to search for mechanisms of the cytotoxic activities of the three *Phyllantus* extracts on the HepG2 cell, the effect of these extracts on its oxygen consumption was investigated. At concentration of 500 µg/mL *Phyllanthus* sp. stimulated oxygen uptake when compared with the basal respiration rate; and *P. virgatus* showed the strongest respiratory stimulation among three *Phyllanthus* species. Since cellular respiration is an integral indicator of metabolism. Oxygen consumption of the cells is nearly exclusively due to mitochondrial oxidative phosphorylation. Therefore, changes in respiration rate as a result of experimental treatments strongly indicate some alteration of mitochondrial activity and function. Thus, the effects of three *Phyllanthus* species on respiratory function of isolated rat liver mitochondria have been studied. Mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis. In this study, rat liver mitochondria were used because the organization and function of potential target proteins in the electron transport chain and oxidative phosphorylation is fundamentally similar among mammalian mitochondria. It was found that three *Phyllanthus* sp. stimulated state 4 respiration, decreased state 3 respiration and decreased RCR when glutamate plus malate were presented as substrates. These results suggested that *Phyllanthus* sp. interfered with the coupling between respiration and phosphorylation. Similar results were obtained with succinate as a substrate. The increase in state 4 respiration as well state 3 respiration indicates inhibition of oxidative phosphorylation and, therefore, ATP synthesis. According to the chemiosmotic view, protonophoric uncouplers such

as CCCP, which is a lipophillic weak acid, shuttle protons across the membrane in a neutral acid form, which is membrane permeable, donating protons to the matrix side. The anionic conjugate base thus produced moves back electrophoretically to the positively charge outside from negatively charged inside of the mitochondrial membrane. In the complete cycle the protonophoric uncouplers dissipate the H^+ gradient and the membrane potential across the mitochondrial membranes. Thus, the dissipation of the mitochondrial membrane potential is generally regarded as decisive for the exhibition of uncoupling activity (Michell, 1966). Thus the phenolic compounds present in *Phyllantus* extracts, because of their acidic properties, may act as protonophoric uncouplers. The action of *Phyllanthus* extracts is also similar to the actions of another medicinal plant. Elingold et al. (2008) found that prenylated flavanone 2',4'-dihydroxy-5'-(1'"-dimethylallyl)-6-prenylpinocembrin (6PP), isolated from the roots of *Dalea elegans*, has antioxidant activity and it also stimulated mitochondrial state 4, but inhibited state 3 respiration. Canton et al. (1996) explained uncoupling action of substituted phenols by a protonophoric effect. Mathlesen et al. (1996) reported that MyA, MyB and MyG effectively uncoupled oxidative phosphor-rylation in mitochondria. These flavonoids also inhibited ATP synthesis, probably as a consequence of their uncoupling activity. Since the myrigalones are lipophilic weak acids, a protonophoric action seems to be the most reasonable cause of their effects. Additionally, epigallocatechin gallate (EGCG), a major component in green tea polyphenols, was found to stimulate state 4 but decreased state 3 respiration and RCI index (Trakamrungsee, 2004).

To recapitulate, I have partially characterized the activity of three *Phyllanthus* extracts, demonstrating their toxic effects on hepatocellular carcinoma and isolated rat

liver mitochondria as well as antioxidant activity. *P. virgatus* showed the strongest in most activities studied. In view of the importance of the mitochondria in cell functions, it is proposed that the mitochondrial effects of the three *Phyllantus* extracts may intimately involve in their cytotoxic activity on HepG2 cells. However, the identification of the active ingredients of these plants needs further investigation.

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APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

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PREPARATION OF REAGENTS

1. ADP (0.1 M)

ADP	0.01	gm
Distilled water	200	µL

2. Ammonium thiocyanate (30% w/v)

Ammonium thiocyanate	3.0	gm
Distilled water	10	mL

3. CCCP (3 mM)

CCCP	6.3	mg
DMSO	10	mL

4. DPPH solution

4.1 Stock DPPH solution (2 mM)

DPPH	0.039	gm
Methanol	50	mL

4.2 Working DPPH (0.2 mM)

Stock DPPH solution (2 mM)	10	mL
Methanol	90	mL

5. EDTA (10X)

EDTA	37.22	gm
Distilled water	100	mL

(Filtrate and store at 4°C)

6. Ferrous chloride (20 mM)

$FeCl_2$	0.4	gm
3.5% hydrochloric acid	100	mL

7. Gallic acid

Gallic acid	0.50	gm
Ethanol	10	mL
Dilute to volume with distilled water		

8. Glutamate/malate 1 M (pH 7.4)

1 M glutamate acid	4.41	gm
1 M DL-malic acid	4.02	gm
Distilled water	20	mL
Adjust pH 7.4 by KOH, and then adjust volume to 30 mL		

9. Hydrochloric acid (3.5% v/v)

37% Hydrochloric acid (HCl)	9.45	mL
Distilled water	90.55	mL

10. Medium for isolate rat liver mitochondria

0.25 M sucrose	85.58	gm
5 mM HEPES	1.19	gm
1 mM EGTA	0.38	gm
Distilled water	1,000	mL

11. Medium for measurement oxygraph

225 mM sucrose	38.51	gm
20 mM KCl	0.75	gm
10 mM $KH_2 PO_4$	0.68	gm

5 mM $MgCl_2$	0.51	gm
10 mM HEPES	1.19	gm
Distilled water	450	mL
Adjust pH 7.4 by KOH, and then adjust volume to 500 mL		

12. PBS (1X)

NaCl	8.00	gm
KCl	0.20	gm
Na_2HPO_4	1.44	gm
$KH_2 PO_4$	0.24	gm
Distilled water	800	mL
Adjust pH 7.4, and then adjust volume to 1,000 mL		

13. Phosphate buffer (0.2 M, pH 7.0)

$KH_2 PO_4$	27.21	gm
Distilled water	800	mL
Adjust pH 7.0, and then adjust volume to 1,000 mL		

14. RPMI 1640 medium

RPMI 1640 (1X) with L-glutamine, with phenol red	1	pack
$NaHCO_3$	2.0	gm
HEPES	3.57	gm
MQ. Water	800	mL
Adjust pH 7.4, and then adjust volume to 1,000 mL		
Add 100 U/ml antibiotic-antimycotic	10	mL
(Filter sterile and store at 4°C)		

15. Sodium carbonate (2% w/v)

Na_2CO_3	2.0	gm
Distilled water	100	mL

16. Succinate 1 M (pH 7.4)

1 M Succinic acid	5.90	gm
Distilled water	40	mL

Adjust pH 7.4 by KOH, and then adjust volume to 50 mL

17. Sucrose (0.25 M)

Sucrose	8.58	gm
Distilled water	100	mL

18. 0.25% trypsin-EDTA solution

2.5% trypsin	5.0	mL
EDTA (10X)	5.0	mL
PBS (1X)	40	mL

APPENDIX B

DETERMINATION PROTEIN CONCENTRATION

APPENDIX B

DETERMINATION PROTEIN CONCENTRATION

Bio-Rad Protein Assay

The Bio-Rad Protein Assay, based on the method of Bradford (1976), is a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye to protein solution and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration.

Protein procedure

1. Prepare dye reagent by diluting 1 part dye reagent concentrate with 4 parts distilled water.

2. Prepare three dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.1 to 0.45 mg/ml.

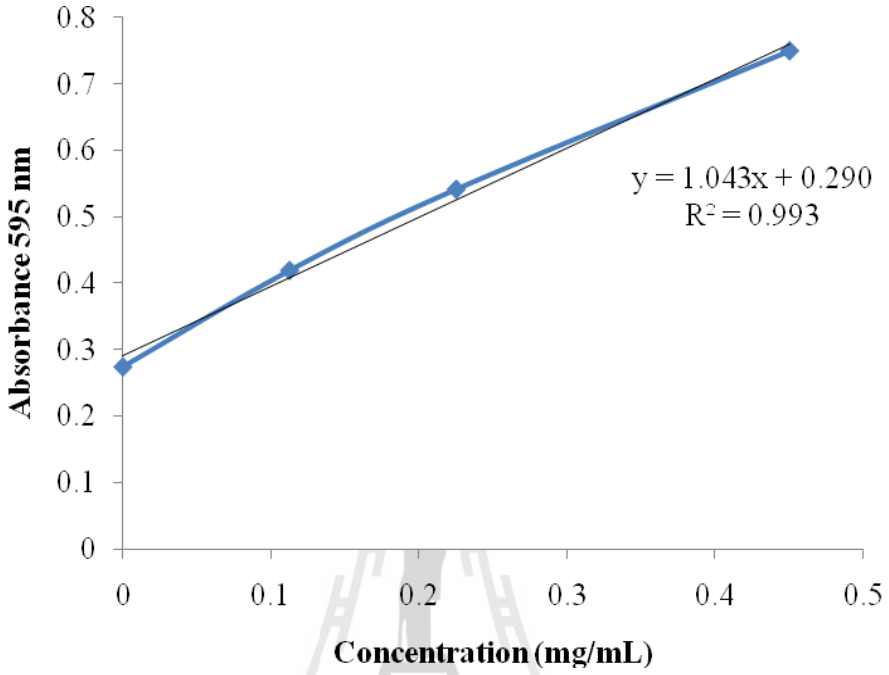
3. Pipet 200 µl of diluted dye reagent to each well.

4. Pipet 10 µl of each standard and sample solution into each well and resuspend. Protein solutions are normally assayed in duplicate.

5. Incubate at room temperature for at least 5 minutes.

6. Measure absorbance at 595 nm.

Typical standard curve (bovine serum albumin) for the Bio-Rad Protein



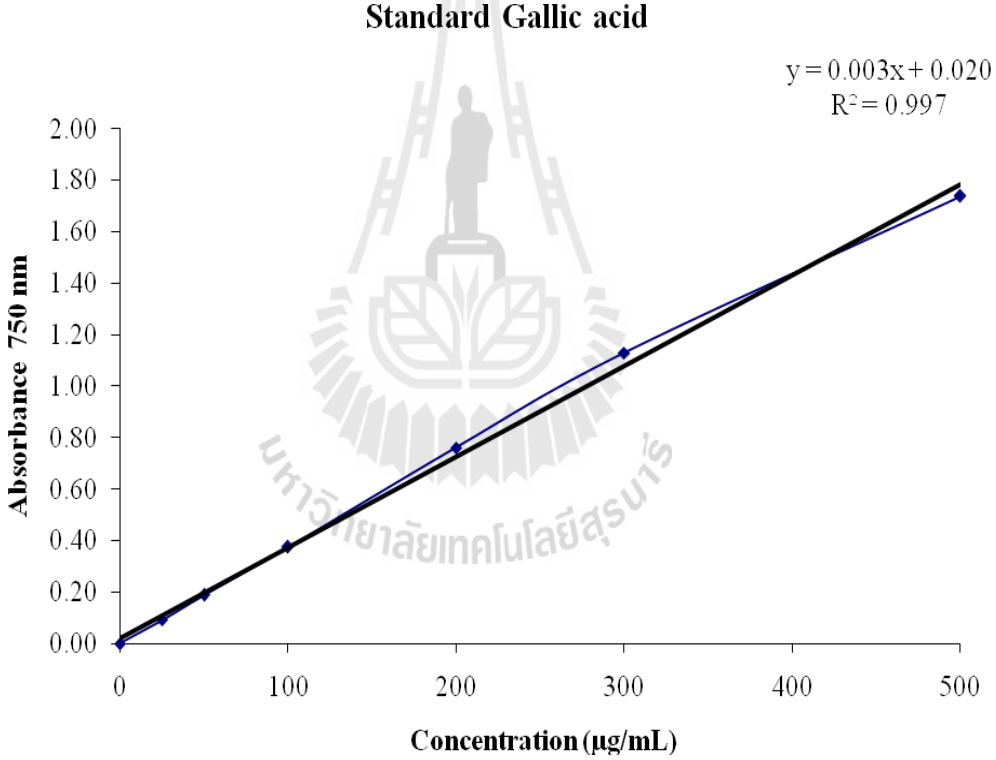
APPENDIX C

STANDARD CURVE

APPENDIX C

STANDARD CURVE

Total phenolics by Folin-Ciocalteu assay



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2. Suranaree University Teacher Assistantship for Graduated Student, 2005.

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