ผลของสารสกัดมาตรฐานบัวบก (อีซีเอ 233) ต่อเมแทบอโลมในตับหนูแรทที่ได้รับโรทิโนน

นางสาวธิดารัตน์ อินทรรุจิกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECT OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* (ECA 233) ON LIVER METABOLOME OF ROTENONE-TREATED RATS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
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Thesis Title	EFFECT OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* (ECA 233) ON LIVER METABOLOME OF ROTENONE-TREATED RATS
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ตับเป็นอวัยวะที่มีความสำคัญในร่างกายซึ่งมีหน้าที่หลายอย่าง รวมทั้งการเมแทบอลิซึมของสารอาหารและการกำจัดสารพิษ โรคตับเป็นปัญหาสำคัญทั่วโลก ในปัจจุบันจึงมีการศึกษาผลของสารสำคัญจากพืชต่าง ๆ ที่มีฤทธิ์ในการปกป้องตับอย่างกว้างขวาง ซึ่งจากการศึกษาที่ผ่านมาพบว่าบัวบกมีฤทธิ์ในการปกป้องตับ บัวบกเป็นพืชสมุนไพรที่ใช้กันโดยทั่วไปในหลายประเทศในทวีปเอเชีย องค์ประกอบของสารสำคัญที่สกัดได้จากบัวบกนั้นมีปริมาณที่แตกต่างกันไปในแต่ละพื้นที่ ดังนั้น คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยจึงได้พัฒนาวิธีการสกัดเพื่อควบคุมปริมาณสารสำคัญ ได้แก่ triterpenoids ให้มีปริมาณมากกว่าร้อยละ 80 โดยมีอัตราส่วนของสาร asiaticoside ต่อ madecassoside เท่ากับ 1.5(±0.5):1 ซึ่งผลการศึกษาพบว่าสารสกัดมาตรฐานบัวบก อีซีเอ 233 มีฤทธิ์ช่วยเรื่องความจำ คลายกังวล และรักษาบาดแผล อย่างไรก็ตามยังไม่มีการศึกษาผลการปกป้องตับของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อเมแทบอโลมในตับของหนูแรทซึ่งถูกเหนี่ยวนำด้วยสารโรทิโนน โดยศึกษา metabolomics ด้วยวิธี GC-MS เพื่อศึกษากลไกที่เกี่ยวข้อง รวมทั้งศึกษาผลต่อเอนไซม์ตับ ผลต่อการเกิด lipid peroxidation และผลต่อ antioxidant enzyme ในตับ หนูแรทเพศผู้สายพันธุ์วิสตาร์ถูกแบ่งเป็น 3 กลุ่ม (กลุ่มละ 6 ตัว) ได้แก่ กลุ่มควบคุม กลุ่มโรทิโนน และกลุ่มอีซีเอ 233 โดยหนูแรทในกลุ่มอีซีเอ 233 ได้รับสารสกัดมาตรฐานบัวบก อีซีเอ 233 ในปริมาณ 10 มิลลิกรัมต่อกิโลกรัมทางปาก เป็นระยะเวลา 20 วัน จากนั้นจึงได้รับการฉีดสารโรทิโนน 2.5 มิลลิกรัมต่อกิโลกรัมเข้าทางช่องท้องเพื่อเหนี่ยวนำให้เกิดความเป็นพิษ ผลการศึกษา metabolomics แสดงในเห็นว่าการให้สารสกัดมาตรฐานบัวบก อีซีเอ 233 ช่วยป้องกันความเป็นพิษที่เกิดจากโรทิโนนได้ โดยมี pipecolinic acid เป็นเมแทบอไลต์สำคัญ ซึ่งพบว่า pipecolinic acid ในหนูกลุ่มโรทิโนนมีปริมาณลดลงเมื่อเทียบกับกลุ่มควบคุม การได้รับสารสกัดมาตรฐานบัวบก อีซีเอ 233 ก่อนได้รับโรทิโนนมีผลให้ระดับ pipecolinic acid เพิ่มมากขึ้นจนมีปริมาณอยู่ระหว่างกลุ่มควบคุมและโรทิโนน นอกจากนี้ยังพบว่าสารสกัดมาตรฐานบัวบก อีซีเอ 233 ช่วยยับยั้งการเกิด lipid peroxidation และช่วยเพิ่มการทำงานของ catalase ในตับได้อีกด้วย ข้อมูลที่ได้จากผลการศึกษาครั้งนี้ อาจใช้เป็นแนวทางในการพัฒนาสารสกัดจากบัวบก มาใช้เป็นผลิตภัณฑ์เสริมอาหารหรือยาที่ใช้สำหรับบำรุงและรักษาตับต่อไปในอนาคต

ภาควิชา	อาหารและเภสัชเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา	อาหารเคมีและโภชนศาสตร์ทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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THIDARAT INTARARUCHIKUL: EFFECT OF THE STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* (ECA 233) ON LIVER METABOLOME OF ROTENONE-TREATED RATS. ADVISOR: ROSSARIN TANSAWAT, Ph.D., CO-ADVISOR: ASST. PROF. RATCHANEE RODSIRI, Ph.D., 74 pp.

Liver plays an important role in the body's metabolic processes, with many functions including nutrient metabolism and detoxification of xenobiotics. Liver diseases cause serious public health problems worldwide. Several studies of hepatoprotective activities from natural plant extracts have been revealed during recent years. The protective effects of *Centella asiatica* on the liver have been reported. *Centella asiatica* (Apiaceae) is a medicinal herb that has been commonly used in many countries in Asia. However, the compositions of the main compounds extracted from the herb were varied across geographical regions. Therefore, the standardized extract of *Centella asiatica* (ECa 233) contained >80% triterpenoids with a ratio of madecassoside to asiaticoside kept at 1.5(±0.5):1 was developed by the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Previous studies shown therapeutic effects of ECa 233 to various conditions such as memory improvement, anxiolytic and wound healing effect. However, the protective effect of ECa 233 on the liver has not yet been determined. The aim of this study was to investigate the possible protective effect of ECa 233 on liver metabolome of rotenone-induced rats by using GC-MS based metabolomics. Metabolomic analysis was performed to better understand the underlying mechanisms in the liver tissue. Liver enzymes, lipid peroxidation and antioxidant enzymes in liver were analyzed. Adult male Wistar rats were randomly divided into three groups (with six rats/group): control, rotenone and ECa 233 group. Rats in the ECa 233 group received 10 mg/kg of ECa 233 orally for 20 days, followed by 2.5 mg/kg of intraperitoneal rotenone injection to induce toxicity before being sacrificed. Metabolomic analysis showed that supplementation of ECa 233 protected rat liver against rotenone toxicity. Pipecolinic acid appeared to be the most important metabolites; its level was decreased in the rotenone group as compared to the control. Supplementation with ECa 233 before administration of rotenone raised pipecolinic acid to levels intermediate between controls and rotenone alone. Antioxidant tests revealed that ECa 233 inhibited lipid peroxidation and increased catalase activities in liver tissue. The outcomes of this study provide further evidence in support of using *Centella asiatica* as a value-added component in new dietary supplements or medicines for liver disorders in the future.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
Field of Study:	Food Chemistry and Medical Nutrition	Advisor's Signature	.....
		Co-Advisor's Signature	.....
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LIST OF ABBREVIATIONS

α	alpha
β	beta
>	more than
<	less than
/	per
%	percentage
±	plus-minus sign
°C	degree Celsius
mL	milliliter (s)
mU	milliunit (s)
mg	milligram (s)
µg	microgram (s)
µL	microliter (s)
µM	micromolar (s)
AFLD	alcohol fatty liver disease
Akt	protein kinase B
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance

APAP	acetaminophen group
ASP	*Angelica sinensis* polysaccharides extract
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BSA	*N,O*-bis (trimethylsilyl)acetamide
CAT	catalase
CCl_4	carbon tetrachloride
COOH	carboxyl
CMC	carboxymethyl cellulose
Cu	copper
Cu^{2+}	copper (II) ion
$CuCl_2$	copper (II) chloride
CuZn SOD	cytosolic copper zinc superoxide dismutase
DMSO	dimethyl sulfoxide
e^-	electron
ECa 233	standardized extract of *Centella asiatica*
EC SOD	extracellular superoxide dismutase
EDTA	ethylenediamine tetraacetic acid
ERK1/2	extracellular signal-regulated kinases 1/2
ESI	electrospray-ionization
Fe^{2+}	ferrous

Fe^{3+}	ferric ion
g	gram (s)
GC	gas-chromatography
GEO	ginger essential oil
GPx	glutathione peroxidase
GPx-1	cytosolic and mitochondria glutathione peroxidase
GPx-2	gastrointestinal glutathione peroxidase
GPx-3	plasma glutathione peroxidase
GPx-4	phospholipid hydroperoxide glutathione peroxidase
GSH	glutathione, reduced glutathione
GSSG	oxidized glutathione
GTE	green tea extract
^1H NMR	proton nuclear magnetic resonance spectroscopy
HNO_2	nitrous acid
H_2O	water
H_2O_2	hydrogen peroxide
HOBr	hypobromous acid
HOCl	hypochlorous acid
HRP	horse radish peroxidase
INR	international normalized ratio
i.p.	intraperitoneal

kDa	kilodalton
kg	kilogram (s)
L	liter (s)
LC	liquid chromatography
$LOO^{\bullet}$	lipid radical
LOOH	lipid hydroperoxide
M	molarity
MCME	*Muntingia calabura* extract
min	minute (s)
MDA	malondialdehyde
MetPA	Metabolomics Pathway Analysis
Mn SOD	manganese superoxide dismutase
MS	mass spectrometry
MSTFA	N-methyl-trimethylsilyltrifluoroacetamide
Na_2CO_3	sodium carbonate
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NH_2	amine
nm	nanometer (s)
NMR	nuclear magnetic resonance spectroscopy
N_2O_4	dinitrogen tetroxide

N_2O_3	dinitrogen trioxide
$NO^{\bullet}$	nitric oxide radical
NO^{+}	nitrosyl cation
NO^{-}	nitroxyl anion
$NO_2^{\bullet}$	nitrogen dioxide
NO_2^{+}	nitronium (nitryl) cation
NO_2Cl	nitryl chloride
O_2	oxygen
$O_2^{\bullet -}$	superoxide anion radical
O_3	ozone
OH	hydroxyl
$OH^{\bullet}$	hydroxyl radical
OPLS	orthogonal partial least squares
p.o.	per os, oral administration
PUFAs	polyunsaturated fatty acids
Q-TOF	quadrupole time-of-flight
$R^{\bullet}$	alkyl radical
RH	fatty acid molecule
RNS	reactive nitrogen species
$ROO^{\bullet}$	peroxyl radical

ROOH	hydroperoxide
ROONO	alkyl peroxynitrites,
ROS	reactive oxygen species
s	second (s)
SDS	sodium dodecyl sulfate
SH	thiol
SOD	superoxide dismutase
SOD1	cytosolic copper zinc superoxide dismutase
SOD2	manganese superoxide dismutase
SOD3	extracellular superoxide dismutase
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TEP	1,1,3,3-tetraethoxypropane
TLC-UV	thin-layer chromatography-ultraviolet densitometric
TMCS	trimethylsilylchlorosilane
U	unit (s)
UPLC	ultra-performance liquid chromatography
Zn	zinc

CHAPTER I
INTRODUCTION

1.1. Background and Rationale

The liver plays an important role in the body's metabolic processes, with many functions including nutrient metabolism and detoxification of xenobiotics (Steadman, Braunfeld, and Park, 2013). Excessive toxins can damage the liver cells, leading to liver diseases. Liver diseases cause serious public health problems worldwide. Oxidative stress is one of the mechanisms that can cause liver damage.

Rotenone is a natural pesticide derived from the root of plants from the Leguminosae family, including Derris (*Derris elliptica*), Barbasco (*Lonchocarpus utilis*), and Rosewood (*Tephrosia spp.*). In Thailand, rotenone extracted from *Derris elliptica* is widely used in agriculture and aquaculture. Its residue can be found in the environment, including air, soil, and water. The first fatal case of rotenone toxicity in Thailand was revealed by Narongchai, Narongchai, and Thampituk (2005). A number of studies have reported neurotoxicity of rotenone by inhibition of electron transport chain complex I of the respiratory chain in mitochondria, resulting in diminishing of ATP production and increased levels of ROS (Li et al., 2003; Sherer et al., 2003; Testa, Sherer, and Greenamyre, 2005) that lead to parkinsonism in an animal model. Liver pathology changes were reported as well. Lapointe et al. (2004) showed that liver necrosis and degeneration were found in male Lewis rats after infusion of 2 mg/kg per

day of subcutaneous rotenone for 21 days. Narongchai et al. (2005) reported that rotenone treatment caused microscopic haemorrhage in human liver. Hepatic sinusoidal dilatation of Sprague-Dawley rats that received 3 mg/kg body weight rotenone per day for 30 days was documented by (Radad, Hassanein, Moldzio, and Rausch, 2013).

Several studies of hepatoprotective activities from natural plant extracts have been revealed during recent years (Pari and Amudha, 2011; Shaker, Mahmoud, and Mnaa, 2010; Shapiro et al., 2006; Wang, Zhao, Sun, and Yang, 2014). *Centella asiatica* (Apiaceae) is a medicinal herb that has been commonly used in many countries in Asia. This herb has been used for a variety of conditions such as wound healing and memory improvement (Manyam, 1999; Rosen, Blumenthal, and McCallum, 1967; Shukla et al., 1999; Veerendra Kumar and Gupta, 2003). Triterpenoids including asiaticoside, madecassoside, asiatic acid and madecassic acid are the major constituents found in *Centella asiatica* (Brinkhaus, Lindner, Schuppan, and Hahn, 2000). Previous studies have reported a hepatoprotective effect of *Centella asiatica* extract (Antony, Santhakumari, Merina, Sheeba, and Mukkadan, 2006; Sharma and Sharma, 2005; Zhang et al., 2010). However, the compositions of the main compounds extracted from the herb were varied across geographical regions. Therefore, standardization of the extract is now generally performed for quality control of the major components.

Standardized extract of *Centella asiatica* (ECa 233) was developed by the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The

standard extract ECa 233 used in the present study contained >80% triterpenoids with a ratio of madecassoside to asiaticoside kept at 1.5(±0.5):1 (Wanasuntronwong, Tantisira, Tantisira, and Watanabe, 2012). The study by Kam-eg, Tantisira, and Tantisira (2009) reported that pre-treatment of ECa 233 was effective in ameliorating cognitive deficits in mice. ECa 233 also presented anxiolytic properties in chronic immobilization stressed mice (Wanasuntronwong et al., 2012) and exhibited burn wound-healing effects in rats (Wannarat, Tantisira, and Tantisira, 2009). However, the protective effect of ECa 233 standard extract on the liver has not yet been determined.

1.2. Objectives

The aim of this study was to investigate the effect of ECa 233 on liver metabolome of rotenone-treated rats.

1.3. Scope

Blood samples were collected for hepatic enzyme tests, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Lipid peroxidation inhibition of the liver tissue was evaluated by thiobarbituric acid reactive substances (TBARS) assay. Antioxidant enzyme activities including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were determined. Metabolomic analysis was performed to better understand the underlying mechanisms in the liver tissue. This technique was formerly used to demonstrate protective effects of many plant extracts, such as *Angelica sinensis* (Ji et al., 2014), and *Muntingia calabura* (Rofiee

et al., 2015). In *Centella asiatica*, a metabolomics approach was applied to determine the effects of growth-lighting on metabolite content in three varieties of *Centella* (Maulidiani et al., 2012), predict the antioxidant activity in the extract of *Centella* varieties (Maulidiani et al., 2013) and investigate metabolic alterations in obese diabetic rats treated with *Centella asiatica* extract (Maulidiani et al., 2016).

1.4. Benefits of the study

This study provides the information of liver metabolome and protective effects of ECa 233 against rotenone toxicity. This information could be used to develop ECa 233 as dietary supplementation for improving liver health in the future.

CHAPTER II
LITERATURE REVIEW

2.1. Liver

Liver is the largest internal organ (approximately 1.0-1.5 kg) of the body. It is located in the right upper quadrant of abdominal cavity below the diaphragm. Liver composes of two sections, large right lobe and two left lobes (Gyamfi and Danquah, 2016). Liver has multiple functions such as nutrient metabolism and storage, xenobiotic detoxication and excretion.

2.1.1. Liver functions

2.1.1.1. Nutrient metabolism

Liver plays important role in nutrient metabolisms including carbohydrates, proteins and lipids. In the carbohydrate metabolism, liver is responsible for monitoring and regulating blood glucose levels by storing glucose as glycogen through glycogenesis in feeding state and converting glycogen to glucose as well as synthesizing glucose from amino acids and triglycerides via glycogenolysis and gluconeogenesis in fasting state. In lipid metabolism, liver uptakes and synthesizes fatty acids. Cholesterol, phospholipids, and lipoproteins are also synthesized by the liver. Besides, fatty acids are oxidized by the liver. In protein metabolism, liver synthesizes plasma protein albumin and nonessential amino acids. Moreover, amino acids

deamination and transamination are taken place in the liver (Crawford and Burt, 2012; Steadman et al., 2013).

2.1.1.2. Nutrient storage

Liver stores glycogen and vitamins such as vitamin B12, vitamin D and vitamin A. Ferritin, the main storage form of iron, is stored in the liver (Steadman et al., 2013).

2.1.1.3. Xenobiotics detoxification and excretion

Liver plays a major role in drugs and xenobiotics metabolisms because it has high volume blood via hepatic artery and portal vein, and comprises of many hepatic enzymes.

2.1.2. Causes of liver injury

There are many factors that can induce liver injuries including infections (from viruses, bacteria, fungi or parasites), immune-mediated responses, drugs and toxins, metabolic diseases, and environment (Table 1).

Table 1 Causes of liver injury (Crawford and Burt, 2012)

Causes of liver injury
Infections
Viral hepatitis – hepatotropic
Viral hepatitis – opportunistic
Bacterial
Fungal
Parasitic
Helminthic
Immune-mediated responses
Autoimmune hepatitis
Primary biliary cirrhosis
Primary sclerosing cholangitis
Transplant rejection
Graft-versus-host disease
Drug- and toxin-induced hepatotoxicity
Alcoholic liver disease
Therapeutic agents (including complementary medicines and drugs of abuse)
Metabolic diseases
Inherited metabolic disease
Acquired metabolic derangement
Non-alcoholic fatty liver disease
Mechanical factors
Obstructive cholestasis
Vascular disorders
Environmental factors
Environmental toxins
Heat stroke

2.2. Rotenone

Rotenone is a natural toxin derived from the root of plant in Leguminosae family such as Derris (*Derris elliptica*), Barbasco (*Lonchocarpus utilis*) and rosewood (*Tephrosia* spp.). Its chemical structure is shown in Figure 1. Rotenone is widely used as insecticide, pesticide and piscicide (fish killing agent) (Gupta, 2012). Rotenone is mitochondrial complex I inhibitor that inhibits electron transfer in the electron transport chain from Fe-s center in complex I to ubiquinone (Figure 2). This process results in inhibiting of oxidative phosphorylation, following by the generation of reactive oxygen species (ROS). Rotenone also causes mitochondria dysfunction as well as cell apoptosis. Several studies have demonstrated that rotenone can develop Parkinsonism symptoms in rats (Gupta, 2012; Ling, 2003; Sherer et al., 2003).

Nevertheless, previous studies have demonstrated that not only neurotoxicity that occurred by rotenone exposure, but also the other systemic toxicity. Liver necrosis and degeneration have been documented in rotenone treated rats (subcutaneous injection, 2.5 mg/kg/day) for 20 days (Lapointe et al., 2004). A study conducted by Radad et al. (2013) showed that the hepatic sinusoid dilation was observed in rat treated with rotenone (3 mg/kg/day) for 30 days. Lipid peroxidation in the liver tissue was increased by rotenone (Terzi et al., 2004).

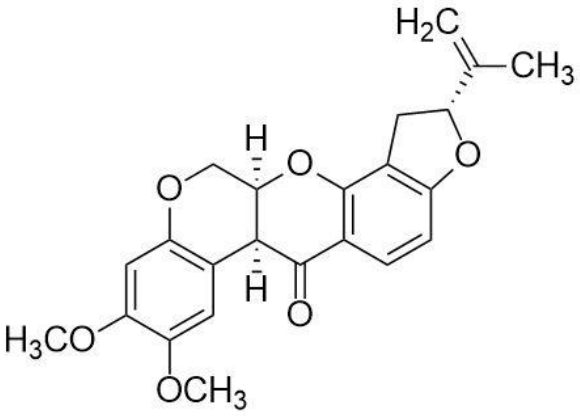


Figure 1 Chemical structure of rotenone

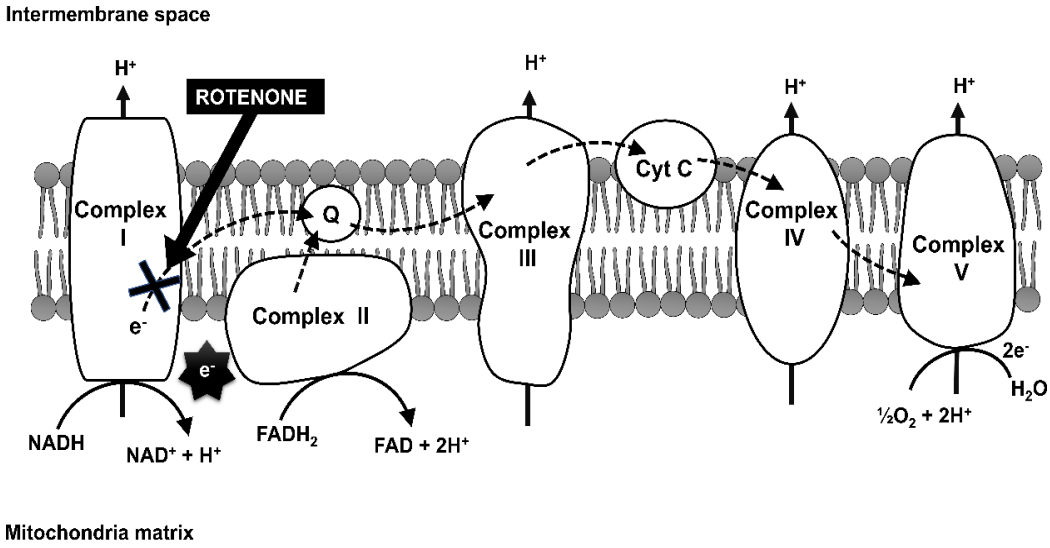


Figure 2 Mitochondria complex I inhibition of rotenone in electron transport chain

Although rotenone is less toxic in human and reports of toxicity are rare, fatal case was documented. The postmortem report of a 47-year-old woman with type 2 diabetes, consuming 200 mL of 0.8% rotenone solution, was observed multiple organ failure, pulmonary edema, congestion of heart, spleen and kidneys. An icteric with centrilobular necrosis and general disintegration of the liver were reported (Wood, Alsahaf, Streete, Dargan, and Jones, 2005). In addition, the first fatal case of rotenone toxicity in Thailand was revealed by Narongchai et al. (2005). The examination report also indicated microscopic hemorrhage in human liver.

2.3. Standardized extract of *Centella asiatica* (ECa 233)

Centella asiatica (L.) Urban, commonly known as Asiatic pennywort, Indian pennywort, Gotu Kola, or Bua-Bok (Thai) is a plant belong to Apiaceae (Umbelliferae) family. *Centella asiatica* is a perennial herbaceous creeper plant. Its leaves are round or kidney shape (Figure 3). It typically grows in moist areas in tropical and subtropical countries such as India, Sri Lanka, Madagascar, Australia, China, South Africa, America and Thailand. *Centella asiatica* is a medical plant that has been used in traditional medicine of Asian countries for a long period of time. According to the Thai National List of Essential Medicines: Herbal Medicines, *Centella asiatica* is used as wound healing cream and antipyretic herbal infusion and capsules (National Drug System Development Committee, 2012).

Figure 3 *Centella asiatica* (L.) Urban (Chandrika and Prasad Kumara, 2015)

The major chemical constituents of *Centella asiatica* are triterpenoids including asiaticoside, madecassoside and their aglycone forms; asiatic acid and madecassic acid. The chemical structure is presented in Figure 4. Other triterpenoids such as braminoside, brahminoside, thankuniside and isothankuiside are also found in this herb. In addition, *Centella asiatica* contains essential oils, flavonoid derivatives, alkaloids, sterols, fatty acids and amino acids (Brinkhaus et al., 2000). The main bioactive compounds of *Centella asiatica* are different depending on the origin of the plants.

ECa 233 was established by the Faculty of Pharmaceutical Sciences, Chulalongkorn University. ECa 233 is a white to off-white powder consisted of triterpenoids from *Centella asiatica* not less than 80%. The ratio of madecassoside to asiaticoside is controlled at 1.5(±0.5):1 (Wanasuntronwong et al., 2012).

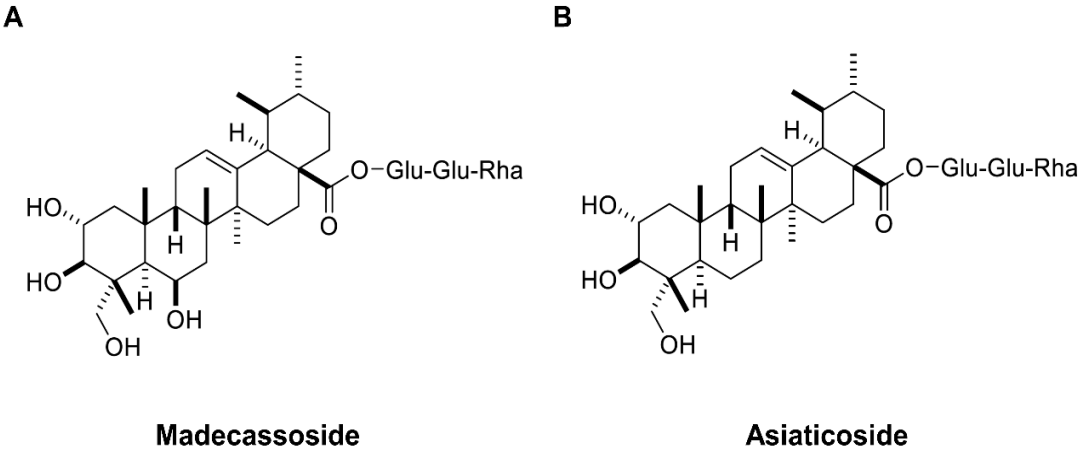


Figure 4 Chemical structure of triterpenoids in the standardized extract of *Centella asiatica* ECa 233, Madecassoside (A) and Asiaticoside (B); Glu = Glucose, Rha = Rhamnose.

Several studies of biological activities of ECa 233 have been conducted. Wannarat et al. (2009) demonstrated that gel containing 0.05% ECa 233 had wound healing effects on second degree burn wound in rats. Ruengprasertkit, Hongprasong, Tantisira, and Tantisira (2010) showed that the oral paste containing 0.05% ECa 233 had an efficacy of reducing pain, ulcer size and erythema in minor aphthous ulcers. Moreover, a study by Kam-eg et al. (2009) showed that pretreatment of ECa 233 (10 mg/kg p.o. twice daily) for 7 days significantly improved memory and learning of mice previously intracerebroventricular injected with β-amyloid peptide.

Previous study indicated that ECa 233 (10-100 μg/mL) enhanced neuronal process of human neuroblastoma IMR-32 cells by upregulated the levels of extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (Akt) (Wanakhachornkrai et al., 2013). Furthermore, Wanasuntronwong et al. (2012) reported

that ECa 233 exhibited anxiolytic effects on acute and chronic stress mice. Acute and sub-chronic toxicity studies of ECa 233 were studied by Chivapat, Chavalittumrong, and Tantisira (2011). The results showed that giving an oral administration of ECa 233 (10 g/kg) to mice for 14 days did not cause acute toxic sign and death. In addition, no sub-chronic toxicity was observed in rats administrated with ECa 233 (10-1,000 mg/kg) for 90 days.

2.4. Free radicals and oxidative stress

Free radical is defined as an atom or molecule containing one or more unpaired electron in the outer shell. Free radical is unstable, highly reactive, and can gain the electron from other compounds to be a non-radical state. Therefore, the molecules losing their electron become a free radical and can start the reactions over and over again. The radical reaction chain composes of three steps; initiation, propagation and termination.

In the initiation step, hydrogen is removed from the fatty acid molecule (RH) to form an alkyl radical ($R^{\bullet}$) (Equation 1). Then, in the propagation step, the alkyl radical quickly reacts with molecular oxygen to form peroxyl radical ($ROO^{\bullet}$) (Equation 2). Peroxyl radical can react with another fatty acid molecule and generate hydroperoxide (ROOH) (Equation 3). Free radical can react with another molecule and start a new chain reaction. The radical chain reaction will stop in the termination step that free

radical will share the electron with another free radical (Equation 4-6) (Kiokias, Varzakas, Arvanitoyannis, and Labropoulos, 2009; Schaich, Shahidi, Zhong, and Eskin, 2013).

(I) Initiation

$$X^{\bullet} + RH \longrightarrow R^{\bullet} + XH \qquad \text{-------- (1)}$$

(II) Propagation

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} + XH \qquad \text{-------- (2)}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \qquad \text{-------- (3)}$$

(III) Termination

$$R^{\bullet} + R^{\bullet} \longrightarrow R\text{-}R \qquad \text{-------- (4)}$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR \qquad \text{-------- (5)}$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \qquad \text{-------- (6)}$$

There are many types of radicals. Reactive oxygen species (ROS), radicals derived from oxygen, is the most important free radical in the biological systems that can be generated from normal cellular metabolisms. ROS includes superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$), and lipid radical ($LOO^{\bullet}$). Hydrogen peroxide is not free radical but can react with metal in Fenton reaction to form more ROS, a hydroxyl radical. Reactive nitrogen species (RNS) is free radicals that contain nitrogen in the molecule such as nitric oxide radical ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). Some reactive species (ROS and RNS) are shown in Table 2.

Table 2 Reactive Species (Halliwell, 2001)

Reactive Species	
Reactive Oxygen Species (ROS)	
Radicals	*Nonradicals*
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO^{\bullet}$ (e.g. lipid peroxyl)	Hypobromous acid, HOBr
Alkoxyl, $RO^{\bullet}$	Ozone, O_3
Hydroperoxyl, $HO_2^{\bullet}$	Single oxygen
Reactive Nitrogen Species (RNS)	
Radicals	*Nonradicals*
Nitric oxide (nitrogen monoxide), $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4,
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+
	(e.g. as nitryl chloride, NO_2Cl)
	Alkyl peroxynitrites, ROONO

2.4.1. Important reactive oxygen species

2.4.1.1. Superoxide anion radical ($O_2^{\bullet-}$)

Superoxide anion is considered as primary reactive oxygen species that can further interact with other molecules to generate secondary reactive oxygen species. Superoxide anion is mostly produced in mitochondrial electron transport chain. Superoxide anion is generated by one-electron reduction of molecular oxygen (O_2) (Equation 7) and then converted to H_2O_2 by superoxide dismutase enzymes (SOD). This radical is also formed by other enzymatic processes such as xanthine oxidase, lipoxygenase, cyclooxygenase and NADPH dependent oxidase (Phaniendra, Jestadi, and Periyasamy, 2015; Valko et al., 2007).

$$O_2 + e^- \longrightarrow O_2^{\bullet-} \qquad \text{----- (7)}$$

2.4.1.2. Hydroxyl radical ($OH^{\bullet}$)

Hydroxyl radical is a neutral form of hydroxide ion. It is highly reactive radical that can interact with other molecules, resulting in cellular damages. Hydroxyl radical can be produced by various reactions; for example, one-reduction of H_2O_2 by ferrous (Fe^{2+}) in Fenton reaction (Equation 8). It is also generated from the reaction between $O_2^{\bullet-}$ and H_2O_2 in Haber-Weiss reaction (Equation 9) (Phaniendra et al., 2015).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad \text{----- (8)}$$

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^- \qquad \text{----- (9)}$$

2.4.1.3. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is formed during dismutation reaction by SOD enzyme and can be formed from two hydroxyl radicals as well (Equation 10, 11). Hydrogen peroxide is not a free radical but this molecule can damage the cells at the concentration of 10 μM (Phaniendra et al., 2015). Hydrogen peroxide can react with metal (Fe^{2+}or Cu^{2+}) to form $OH^{\bullet}$. Hydrogen peroxide can be eliminated by endogenous antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Phaniendra et al., 2015).

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{----- (10)}$$

$$OH^{\bullet} + OH^{\bullet} \longrightarrow H_2O_2 \qquad \text{----- (11)}$$

2.4.2. Sources of reactive oxygen species

ROS can be generated from endogenous and exogenous sources. Endogenous sources include mitochondrial respiration, autoxidation, enzymatic reaction as well as some metal ions. External sources of ROS result from air pollution, smoking, medication, and radiation (Dasgupta and Klein, 2014).

2.4.3. Oxidative stress and diseases

Oxidative stress is an imbalance between free radical production and antioxidant defense system. Thus, the excessive free radicals can damage biological molecules including nucleic acids, lipids and proteins. Hydroxyl radical can react with

nucleic acids, resulting in several types of damages such as DNA bases damages, deoxyribose sugars damages, protein-DNA crosslink, single and double strand breaks. Cell membranes consist of lipid and protein, which phospholipids are the most component of the membranes. Since the polyunsaturated fatty acids (PUFAs) residues of phospholipid are sensitive to oxidation; therefore, cell membranes are usually the target of free radicals. ROS attacks to the cell membranes, resulting in lipid peroxidation. Lipid hydroperoxide (LOOH) is a primary product and malondialdehyde (MDA) is the main secondary products from lipid oxidation reaction. Protein can also be attacked by the ROS, leading to the generation of protein carbonyl derivatives (McGill and Jaeschke, 2013). The oxidative damages cause various pathological conditions such as inflammation, atherosclerosis, cancer, ageing, etc. (Table 3).

Table 3 Oxidative stress related diseases (Pham-Huy, He, and Pham-Huy, 2008)

Oxidative stress related diseases
Cardiovascular diseases
Coronary heart diseases
Atherosclerosis
Stroke/Ischemia-Reperfusion
Neurodegenerative diseases
Alzheimer's disease
Parkinson's disease
Renal diseases
Liver diseases
Pulmonary diseases
Rheumatoid arthritis
Ageing
Cancers

2.5. The antioxidant defense system

Antioxidant defense system consists of enzymatic and non-enzymatic antioxidants. The endogenous antioxidant enzymes include SOD, CAT and peroxidase. Examples of non-enzymatic antioxidants are glutathione, vitamin C and vitamin E.

2.5.1. Enzymatic antioxidants

2.5.1.1. Superoxide dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) is an important enzyme that converts $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen. There are three isoforms of SOD in human. Cytosolic copper zinc superoxide dismutase (CuZn SOD, SOD1) is a 32 kDa homodimer containing Cu and Zn in the active sites, and is mainly localized in cytosol. Manganese superoxide dismutase (Mn SOD, SOD2) is a homotetramer (96 kDa) containing one manganese per subunit (Matés, 2000) that generally found in mitochondria matrix. Extracellular superoxide dismutase (EC SOD, SOD3) is CuZn SOD that found in extracellular matrix. EC SOD is a homotetramer (135 kDa) composed of two disulfide-linked dimers. In dismutation reaction, $O_2^{\bullet-}$ can be oxidized or reduced into form O_2 or H_2O_2 (Equation 12-14) (Fukai and Ushio-Fukai, 2011; McGill and Jaeschke, 2013; Villamena, 2013).

$$O_2^{\bullet-} \xrightarrow{SOD} O_2 + e^- \quad \text{(oxidation)} \qquad \text{----- (12)}$$

$$O_2^{\bullet-} + e^- + 2H^+ \xrightarrow{SOD} H_2O_2 \quad \text{(reduction)} \qquad \text{----- (13)}$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \text{----- (14)}$$

2.5.1.2. Catalase (CAT)

Catalase (EC 1.11.1.6), also known as H_2O_2 oxidoreductase, is tetrameric enzyme (240 kDa) that contains ferriprotoporphyrin group in the structure. Catalase presents in peroxisome and converts H_2O_2 to water and molecular oxygen (equation 15) (Matés, 2000).

$$2H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \qquad \text{----- (15)}$$

2.5.1.3. Glutathione peroxidase (GPx)

Glutathione peroxidase (EC 1.11.1.19) is a selenium containing enzyme that transforms H_2O_2 to water and molecular oxygen by using glutathione (GSH) in the reaction (Equation 16). Glutathione peroxidase also catalyzes the reduction of hydroperoxide (Equation 17). There are many isoforms of GPx found in animal such as cytosolic and mitochondria (GPx-1), gastrointestinal GPx (GPx-2), plasma GPx (GPx-3) and phospholipid hydroperoxide GPx (GPx-4) (Dasgupta and Klein, 2014; McGill and Jaeschke, 2013). Mechanism of an important enzymatic antioxidants are summarized in Figure 5.

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O \qquad \text{----- (16)}$$

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \qquad \text{----- (17)}$$

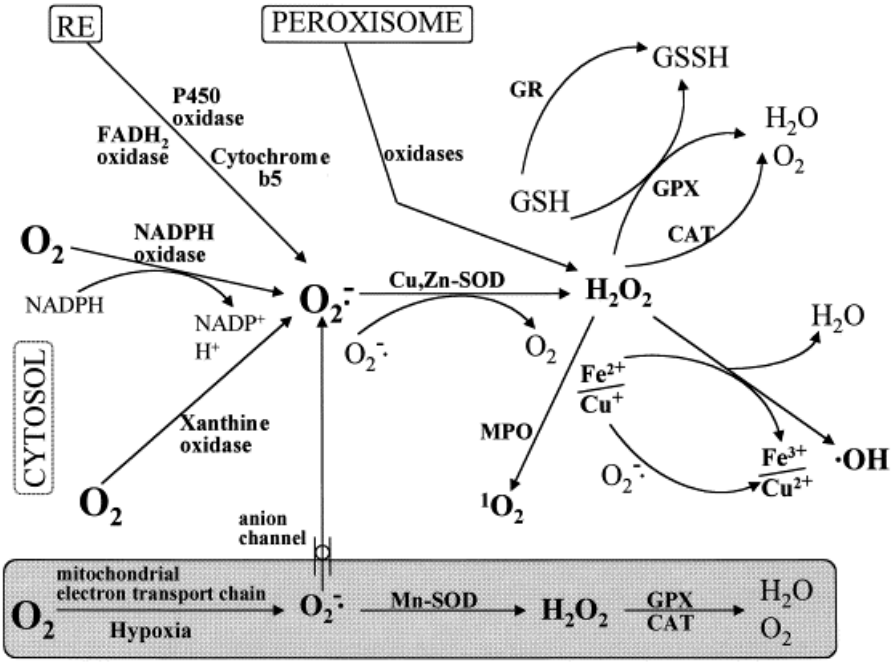


Figure 5 Generation of reactive oxygen species and the defense mechanisms (MatÉs, Pérez-Gómez, and De Castro, 1999).

2.5.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants are the antioxidants that are not functioned as enzyme; for example, food vitamins, plant polyphenols and other synthetic antioxidants, low molecular weight antioxidants, e.g., glutathione. Vitamin E (tocopherols and tocotrienols) is a fat-soluble vitamin that acts as chain-breaking antioxidant. The most active form of vitamin E is α-tocopherols. Vitamin E prevents lipid peroxidation of cell membrane and inhibits the autoxidation. Vitamin E functions as peroxyl radical scavenger by donating hydrogen from hydroxyl group to lipid peroxyl radicals. The antioxidant ability of vitamin E can be regenerated by other antioxidants such as vitamin C (Nimse and Pal, 2015).

Vitamin C or ascorbic acid is water-soluble antioxidant that can be obtained from diets such as cherry, strawberry, citrus fruits, etc. Vitamin C acts as free radical scavenger by donating its electron to the free radicals (Nimse and Pal, 2015; Padayatty et al., 2003). Glutathione is a tripeptide composed of three amino acid including L-glutamate, L-cysteine and glycine. Its thiol group in cysteine residue is a reducing agent, which can interact with the free radicals (Pastore, Federici, Bertini, and Piemonte, 2003; Townsend, Tew, and Tapiero, 2003). Glutathione can be found in both of thiol form (reduced glutathione, GSH) and disulfide forms (oxidized glutathione, GSSG). Reduced glutathione is used as a cofactor of GPx in the detoxification of H_2O_2. Subsequently, GSH is converted into GSSG form. GSSH can be reduced back to GSH by glutathione reductase enzyme together with the NADPH. Besides the protection against the ROS and RNS, glutathione has other cellular functions including xenobiotic detoxification and immune booster (Lushchak, 2012; Perricone, De Carolis, and Perricone, 2009). Moreover, glutathione can convert vitamin C and E back into their active forms (Birben, Sahiner, Sackesen, Erzurum, and Kalayci, 2012).

2.6. Metabolomics

Metabolomics is one of the omics that described a systemic identification and quantification of metabolome of biological system such as cell, tissue, biological fluid, and organism (the omics cascade is shown in Figure 6). Metabolome is defined as all small molecule metabolites (<1000 Da), e.g., amino acids, small peptides, sugars, and fatty acids. Changes in metabolites are resulted from gene, mRNA, protein functions, as well as the environments. Metabolomics represents the depiction of an organism's phenotype and how metabolites respond to genetic or environment at a specific time point (Nielsen and Oliver, 2005). There are many definitions used in the analysis of the metabolites; for example, metabolite targeting analysis and metabolomic profiling. Metabolite targeting analysis is a quantitative analysis of a few specific metabolites. Metabolomic profiling is a semi-quantitative analysis of a group of specific metabolites such as amino acids, carbohydrates, and lipids classes.

Metabolomics workflow consists of several steps including biological question, experimental design, sample preparation, data acquisition, data processing, data analysis, and biological interpretation (Figure 7). Generally, the high-throughput analytical techniques such as nuclear magnetic resonance spectroscopy (NMR), liquid chromatography (LC) and gas-chromatography (GC) coupled with mass spectrometry (MS) are used in metabolomics approach to identify and quantify the metabolites from a biological sample as much as possible.

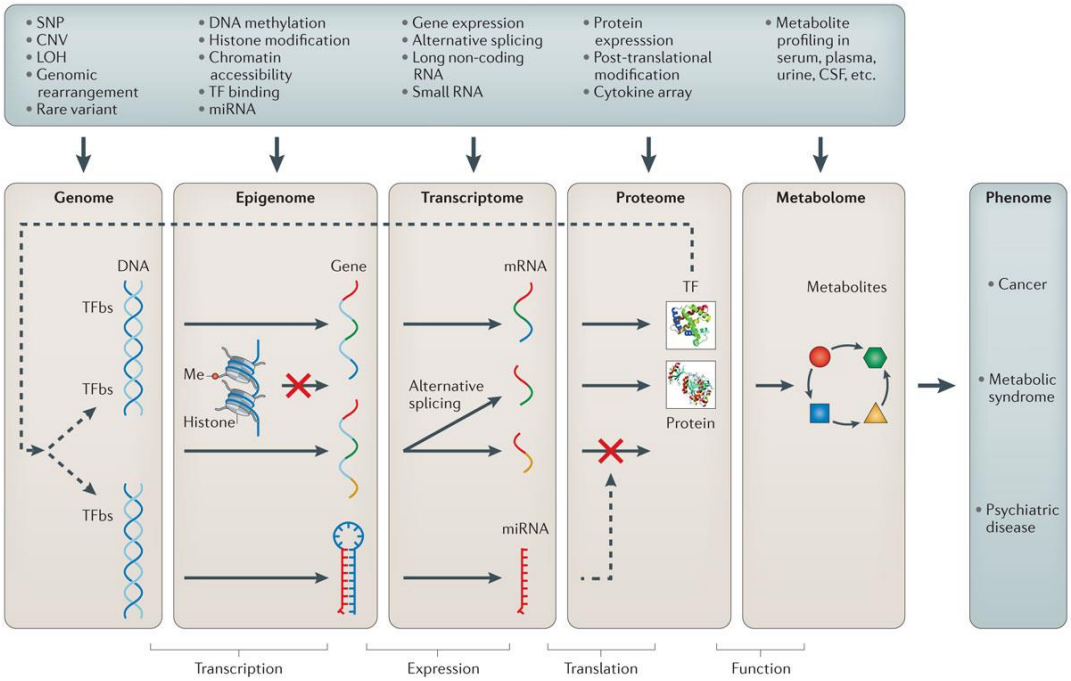


Figure 6 The relationship between different -omes (Ritchie, Holzinger, Li, Pendergrass, and Kim, 2015).

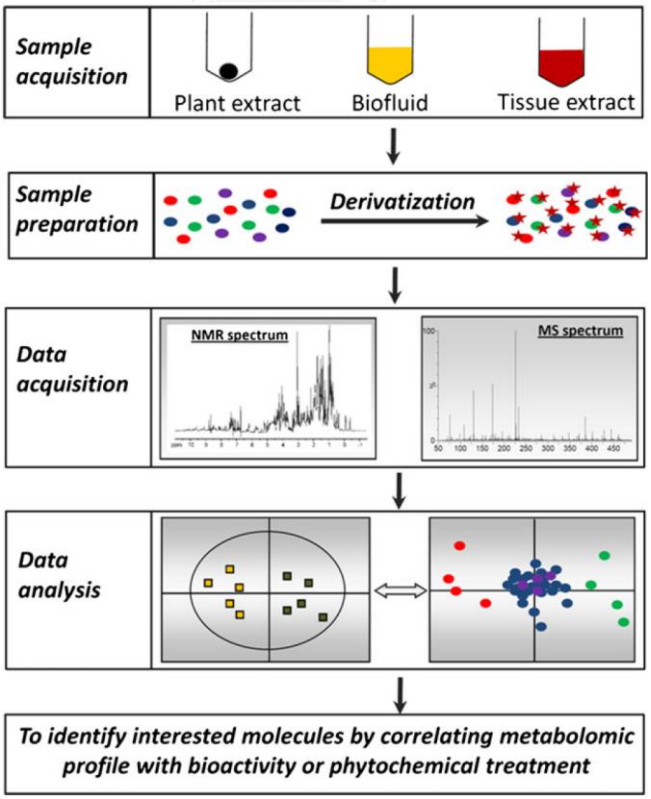


Figure 7 Metabolomics workflow (Wang and Chen, 2013)

Table 4 Analytical methods used in metabolomics approach (Verpoorte, Choi, Mustafa, and Kim, 2008)

	HPLC or TLC-UV	GC-MS	LC-MS	MS	NMR
Sample preparation	++	**–**	**–**	+	+++
Reproducibility	**–**	+	**–**	+	+++
Absolute Quantitation	**–**	**–**	**–**	**–**	+++
Relative Quantitation	+	++	+	++	+++
Identity	+	++	++	++	++
Compound number	Ca. 30	Ca. 1000	Ca. 200	Ca. 1000	Ca. 200
Sensitivity	+	++	++	+++	**–**

Scale from **–** to +++ for disadvantages to major advantages. HP: High Performance; LC: Liquid Chromatography; TLC-UV: Thin-Layer Chromatography-UV Densitometry; GC: Gas Chromatography; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance Spectroscopy.

Currently, there is no single method can be able to analyze all the metabolites at one time. Thus, a combination of analytical instruments is usually required. The comparison of each analytical method used in metabolomics is summarized in the Table 4. GC-MS technique is often used in metabolomics study because of high chromatographic separation power, high reproducibility as well as more availability of mass spectrum databases (Scalbert et al., 2009). However, the compound must be volatile or can be derivatized and thermally stable. Hence, this technique is less suitable for low volatility and highly polarity compounds (Wang, Liu, Hu, Li, and Wan, 2015). In sample preparation step of GC-MS based metabolomic analysis, volatile sample such as breath can be directly analyzed. Nevertheless, some metabolites, e.g., sugars, sugar alcohols, amines, amino acids, and organic acids that contain high polar

functional groups, thermally unstable, and less volatility. Therefore, derivatization step is needed to reduce their polarity and change the compounds to more volatile.

Methoxymation and silylation are usually performed in derivatization step. For methoxymation, carbonyl group is converted to oximes to prevent sugar ring formation (aldehyde and ketone groups). *O*-methoxyamine hydrochloride in pyridine is mostly used in this step. In the silylation step, active hydrogen atom in functional group such as hydroxyl (-OH), carboxyl (-COOH), amine (-NH_2) and thiol (-SH) is substituted by an alkylsilyl group; hence, metabolites are less polar and more volatile. There are many silylation reagents widely used in metabolomics study, for example, N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) and *N,O*-bis (trimethylsilyl)acetamide (BSA). Trimethylsilylchlorosilane (TMCS) can be added in MSTFA and BSA as a catalyst (Dettmer, Aronov, and Hammock, 2007; Garcia and Barbas, 2011; Grimm, Fets, and Anastasiou, 2016; Koek, Jellema, van der Greef, Tas, and Hankemeier, 2011).

Nowadays, metabolomics has been applied in various fields such as pharmacology, toxicology, medical and pharmaceutical sciences, food and nutrition, as well as plant biotechnology. Metabolomics has been used as a tool in diseases diagnosis or biomarker discovery (Chen et al., 2009; Ikeda et al., 2012; Nishiumi et al., 2010; Wu et al., 2009), to evaluate efficacy and toxicity of drugs and herbal plants (Craig et al., 2006; Ji et al., 2014; Lu et al., 2013; Scalbert et al., 2009; Yin et al., 2016) and to determine the effects of specific dietary consumption (Lloyd et al., 2011; Stella et al., 2006).

2.6.1. Metabolomics studies on hepatoprotective plants or plant extracts.

Currently, metabolomics approach is widely used to identify metabolite profiles of medicinal plants or the plants extracts. Metabolomics is also used to evaluate toxicity of the herbs, assess the therapeutic effects of the bioactive on various diseases, and explore more mechanisms and pathway involved. There are several metabolomics studies of hepatoprotective effects of natural products or plant extracts have been reported (Table 5).

A study of the effects of green tea extract (GTE) on acetaminophen-induced hepatotoxicity in mice by using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) and NMR were conducted by Lu et al. (2013). The animals were divided into a control group, acetaminophen group (APAP) (200 or 300 mg/kg), GTE group (500 or 1000 mg/kg), GTE (500 mg/kg)/APAP (200 or 300 mg/kg) group, and GTE (1000 mg/kg)/APAP (200 or 300 mg/kg) group. The effect of GTE pre-dose administration was studied 3 hours and 3 days before APAP treatment. The post-dose of GTE was investigated after the animal were treated with APAP for 6 hours. Liver tissue and blood sample were collected at 24 hours after APAP treatment for metabolomic analysis. The results revealed that the metabolites pattern change in GTE pretreatment was less than APAP treatment alone. Metabolic pathways that possibly involved were metabolisms of fatty acids, glycerophospholipids, glutathione, and energy.

Table 5 Metabolomics studies of hepatoprotective effects of plants or plant extracts.

Studies	Plants or extracts	Subjects	Samples	Analytical Instruments
Bao et al. (2017)	Shuihonghuazi formula *(Polygoni orientalis fructus*, *Ophicalcitum*, *Coicis Semen* and *Imperatae Rhizoma)*	Rats	Liver	HPLC/ESI-TOF-MS
Wang et al. (in press)	Glycyrrhizin and glycyrrhetinic acid	Mice	Serum	UPLC/ESI-TOF MS
Wu et al. (2017)	*Corydalis saxicola* alkaloids	Rats	Urine	^{1}H NMR
Xing, Sun, Jia, Qin, and Li (2017)	*Radix Bupleuri*	Mice	Liver	GC-MS
Liang et al. (2016)	*Corydalis saxicola*	Rats	Serum	^{1}H NMR
Rofiee et al. (2015)	*Muntingia calabura*	Rats	Serum	LC/ESI-Q-TOF MS
Wang, Su, Chen, Bai, and Pei (2015)	*Curcuma longa* and curcumin	Rats	Urine and serum	^{1}H NMR
Wang, Luo, Chen, Zha, and Pan (2015)	*Dendrobium huoshanense* polysaccharide	Mice	Serum	UHPLC/LTQ Orbitrap MS
Ji et al. (2014)	*Angelica sinensis* polysaccharides	Mice	Plasma and liver	GC-MS
Hua, Xue, Zhang, Wei, and Ji (2014)	*Angelica sinensis* polysaccharides	Mice	Liver	GC-MS
Lu et al. (2013)	Green tea extract	Mice	Liver	UPLC/Q-TOF MS and ^{1}NMR
Liu et al. (2013)	Ginger essential oil	Mice	Serum	HPLC/Q-TOF MS

HP: High performance; UHP: Ultra-high performance; LC: Liquid chromatography; ESI: Electrospray-ionization; Q: Quadrupoles; TOF: Time of flight; MS: Mass spectrometry; ^{1}H NMR: Proton Nuclear Magnetic Resonance; GC: Gas Chromatography

In another study, metabolomics was used to investigate the effect of ginger essential oil (GEO) and citral in alcohol fatty liver mice (Liu et al., 2013). Six-week-old C57BL/6 mice were divided into six groups: normal control, alcohol fatty liver disease (AFLD), two groups of GEO treatment and two groups of citral treatment. Mice in an AFLD and all treatment groups were fed with ethanol-containing diet (Lieber DeCarli diet), whereas those in control group were fed with normal liquid diet. The treatment groups were fed with either GEO (2.5 or 12.5 mg/kg) or citral (0.375 or 1.875 mg/kg) for 4 weeks. Mice in control and AFLD groups were treated with only vehicle in the same volume as the treatment groups. Serum was collected for metabolomic analysis. The metabolites profiling was determined by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF MS). The results showed that GEO and citral exhibited protective effect against AFLD. Metabolomics results revealed changes in some metabolites after alcohol administration. The levels of metabolites restored back to normal levels (comparable to the control) when treated with GEO and citral. The study indicated that GEO has the hepatoprotective effect to AFLD.

A study on hepatoprotective effects of *Angelica sinensis* polysaccharides extract (ASP) in mice using GC-MS metabolomics approach was carried out by Ji et al. (2014). Mice were divided into five groups: control, liver injury, and three doses of ASP. The animals were treated by normal saline or ASP (60, 120 and 240 mg/kg/day, respectively) for 3 days. Subsequently, liver injury was induced by carbon tetrachloride

(CCl_4) by intraperitoneal injection. After that, mice in ASP groups were administrated with ASP. Thirty-six hours after CCl_4 treatment, mice were sacrificed. Blood sample and liver tissue were collected for the analyses. The hepatoprotective effect of ASP was exhibited, and the dose of ASP 120 mg.kg/day showed the most protective effect. Ten metabolites in the plasma and nine metabolites in the liver were revealed as potential biomarkers.

Rofiee et al. (2015) evaluated the protective effects of *Muntingia calabura* extract (MCME) on CCl_4 induced liver injured rat by using LC-MS Q-TOF. Rat were randomly divided into six groups: normal control, negative control (normal saline), positive control (10 mg/kg silymarin), and pretreatment treated with three doses of MCME (100, 200 and 400 mg/kg, respectively). Animals were received test solutions for seven days, followed by hepatotoxicity induced by CCl_4 at 24 hours after the last administration. Then, 48 hours later, serum samples were collected for analysis. Metabolomics fingerprinting was performed using LC-MS Q-TOF and network and pathway analysis was performed by Metabolomics Pathway Analysis (MetPA). The results showed that MCME exhibited the protective effect from CCl_4-induced hepatotoxicity, and two pathways involved in hepatoprotective of MCME was revealed by MetPA were bile acid biosynthesis and arachidonic acid metabolism.

CHAPTER III
MATERIAL AND METHODS

3.1. Bioactive

Standardized extract of *Centella asiatica* (ECa 233) developed by the Faculty of Pharmaceutical Sciences, Chulalongkorn University was obtained from Siam Herbal Innovation Company Limited (Samutprakan, Thailand). ECa 233 was prepared by a patent-pending procedure (Saifah et al., 2009). Total triterpenoids of the ECa 233 used in this study were 89%, which composed of 51% madecassoside and 38% asiaticoside as quantitatively verified by Hengjumrut, Anukunwithaya, Tantisira, Tantisira, and Khemawoot (2017), using UHPLC 100 liquid chromatograph (Eksigent ekspert™, Montreal, Canada) connected to QTRAP 6500 mass spectrometer (AB Sciex, Pte. Ltd., Framingham, MA, USA) for the analysis.

3.2. Chemicals and Reagents

Deoxycholic acid sodium salt (extra pure, 99%) used as internal standard was purchased from Acros organic (Geel, Belgium). Rotenone, 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA), acetic acid, sodium dodecyl sulfate (SDS), n-butanol, pyridine, superoxide dismutase from bovine erythrocytes, xanthine, xanthine oxidase, sodium carbonate (Na_2CO_3), ethylenediamine tetraacetic acid (EDTA), nitroblue tetrazolium (NBT), bovine serum albumin, copper (II) chloride ($CuCl_2$), sodium chloride, acetone, pyridine (anhydrous, 99.8%), methoxyamine hydrochloride, N-methyl-N-

trimethylsilyl- trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) and n-heptane (for HPLC, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used in this study were analytical grade. Catalase, glutathione peroxidase and Bradford assay kits were purchased from Abcam (Cambridge, UK).

3.3. Animals

Adult male Wistar rats weighing 300-350 g were obtained from the National Laboratory Animal Centre, Mahidol University (Nakhon Prathom, Thailand). All rats were housed under the controlled conditions of 25 ± 2 ℃ with proper humidity and 12-hour dark/light cycles. Standard rat food and tap water were freely available in their home cage during the entire experimental process. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Approval No. 1533-004).

3.4. Study design

The experimental design is shown in Figure 8. Rats were randomly divided into 3 groups (N = 18); control, rotenone and ECa 233. Rotenone was dissolved in dimethyl sulfoxide (DMSO) to prepare rotenone stock solution and then rotenone was diluted in sunflower oil to prepare working solution (2.5 mg/mL). The amount of DMSO in working solution was 2%. ECa 233 was freshly prepared by suspending in 0.5 %

carboxymethyl cellulose (CMC) for the concentration of 10 mg/mL. On day 1 – 20, rats in the control and rotenone groups received 1 mL/kg of 0.5 % CMC while rats in the ECa 233 group received 10 mg/kg ECa 233 in 0.5% CMC orally. On day 15 – 20, 2.5 mg/kg rotenone in sunflower oil was intraperitoneally injected in the rotenone and ECa 233 groups whereas 2% DMSO in sunflower oil was injected in the control animals. On day 21, all animals were euthanized under pentobarbital anaesthesia (50 mg/kg intraperitoneally). Blood samples were collected for hepatic enzyme tests. Liver samples were immediately removed, snap-frozen in liquid nitrogen and stored at -80 °C until further measurements of metabolome and antioxidant capacities.

3.5. Liver enzyme tests

Blood samples were collected in heparinized tubes and centrifuged at 3000 rpm for 10 min (Hettich® Mikro 120 centrifuge, Tuttlingen, Germany). Plasma was used for measuring liver marker enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Plasma samples were sent to the Professional Laboratory Management Corp. Co., Ltd. (Bangkok, Thailand) to perform the analysis.

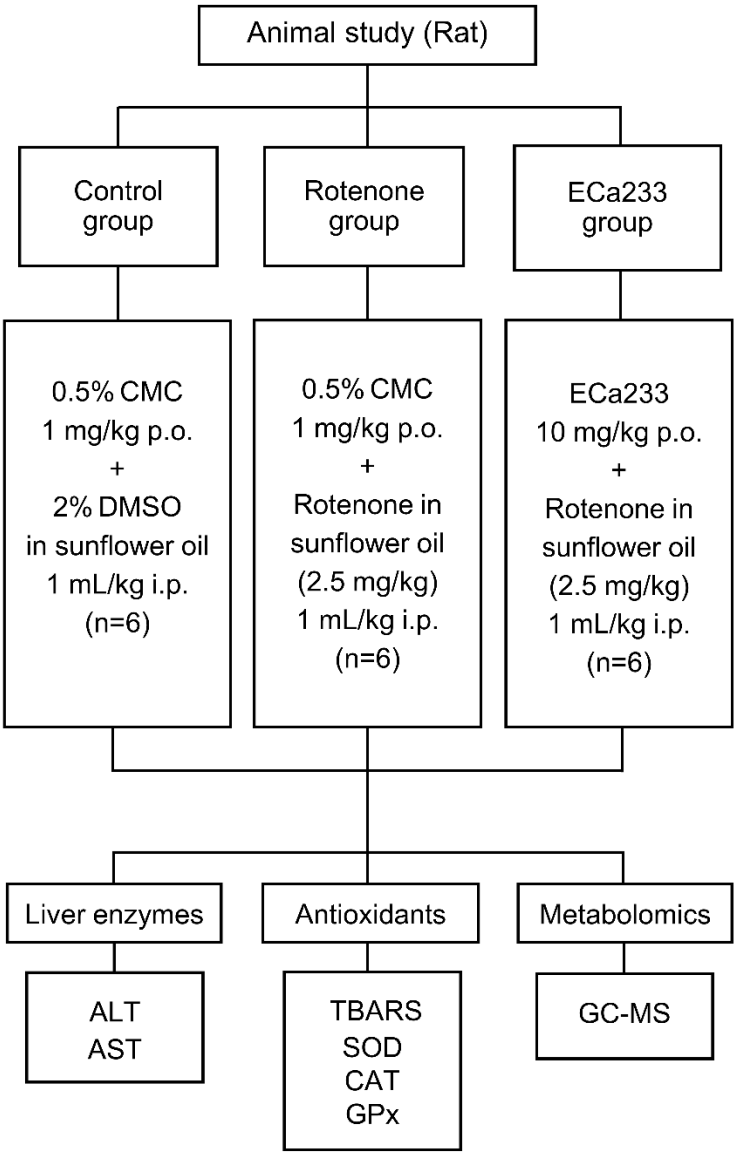


Figure 8 Experimental design of the study. CMC = Carboxy methyl cellulose

3.6. Antioxidant tests

Liver tissue was weighed and homogenized using a homogenizer (Glas-Col®, Terre Haute, IN, USA) in ice-cold 0.1 M phosphate buffered saline solution (pH 7.4, 1:10 w/v). Then, liver homogenate was centrifuged at 12,000 x g at 4 °C for 15 min (Sorvall™ Legend™ micro 21R, Thermo scientific™, Waltham, MA, USA). Supernatant was collected, snap-frozen in liquid nitrogen, and stored at -80 °C until further analysis. Thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) assays were carried out. The results were expressed per milligram of total protein.

3.6.1. Thiobarbituric acid reactive substances

Lipid peroxidation in liver samples was measured as the amount of malondialdehyde (MDA) by thiobarbituric acid reactive substances (TBARS) assay as described by the method of Ohkawa, Ohishi, and Yagi (1979). One-hundred µL of liver homogenate were mixed with 100 µL of 8.1% SDS, 750 µL of 20% acetic acid (pH 3.5) and 750 µL of 0.8% freshly prepared TBA. The volume of the mixture was made up to 2 mL with distilled water and heated at 95 °C in a water bath for 60 min. After that, the mixture was cooled at room temperature for 10 min. Five hundred µL of distilled water and 1 mL of n-butanol/pyridine (15:1) were added and vortexed. After centrifugation at 4,000 rpm for 10 min (HERMLE Z383K refrigerated centrifuge, Wehingen, Germany), the upper organic layer was taken and the absorbance was read

at 532 nm with a Spectramax® M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Lipid peroxidation was expressed as micromole of MDA equivalents/mg protein of the liver sample.

3.6.2. Superoxide dismutase

Superoxide dismutase (SOD) activity in liver samples were determined by the method of Sun, Oberley, and Li (1988). Initially, 25 µL of liver homogenate was mixed with 122.5 µL SOD reagent (a mixture of 0.3 mM xanthine, 400 mM Na_2CO_3, 0.6 mM EDTA, 150 µM NBT and 1 g/L Bovine serum albumin). Next, 2.5 µL of 167 units/L xanthine oxidase was added to start the reaction. The solution was then incubated at room temperature for 20 min. Subsequently, 50 µL of 0.8 mM $CuCl_2$ was added to stop the reaction. The absorbance of the solution was read at 560 nm with a Spectramax® M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was plotted using superoxide from bovine erythrocytes as a positive control. SOD activity was expressed as a percentage per mg protein of the liver sample.

3.6.3. Catalase

The activity of catalase (CAT) was carried out using a catalase assay kit following the manufacturer's instructions (Abcam, Cambridge, UK). One hundred mg of liver sample was homogenized in 100 µL cold assay buffer and centrifuged at 10,000 × g for 15 min at 4 °C to remove insoluble material. Then, 5 µL of supernatant was mixed with 45 µL assay buffer and 12 µL H_2O_2 in 96-well plates at 25 °C for 30 min. After that,

10 µL stop solution was added into each well to stop the catalase reaction. Fifty µL of the mixture of OxiRed™ probe, horse radish peroxidase (HRP) and assay buffer were added into sample wells and incubated at 25 °C for 10 min. The absorbance was measured at 570 nm with a CLARIOstar® microplate reader (BMG LABTECH, Ortenberg, Germany). Catalase activity was expressed as mU/min/mg protein of liver sample.

3.6.4. Glutathione peroxidase

The activity of glutathione peroxidase (GPx) was measured with a glutathione peroxidase assay kit (Abcam, Cambridge, UK). One hundred mg of liver sample was homogenized in 100 µL cold assay buffer and centrifuged at 10,000 x g for 15 min at 4 °C. The supernatant of liver homogenate was used for the assay. Five µL of supernatant was pipetted into 96-well plates and adjusted to a volume of 50 µL with assay buffer. Forty µL of the mixture, 40 mM of NADPH, glutathione reductase and glutathione were added into each well and incubated at room temperature for 15 min to deplete all oxidized glutathione (GSSG) in samples. Then, 10 µL of cumene hydroperoxide was added into each well and the plate was mixed to start the GPx reaction. The absorbance was measured with a CLARIOstar® microplate reader (BMG LABTECH, Ortenberg, Germany) at 340 nm. Subsequently, the plate was incubated at 25 °C for 5 min. The absorbance was measured again at 340 nm. Glutathione activity was expressed as U/min/mg protein of the sample.

3.6.5. Determination of liver protein content

Protein content of liver homogenate was determined with a Bradford assay kit following manufacturer's instructions (Abcam, Cambridge, UK). Ten µL of liver homogenate was mixed with 100 µL of protein assay reagent in 96-well plates and incubated at room temperature for 5 min. The absorbance was read at 595 nm with a Spectramax® M5 microplate reader (Molecular Devices, CA, USA).

3.6. Metabolomic analysis

Metabolomic analysis of rat liver samples were carried out using the validated method of Wang et al. (2009). Two-hundred mg of fresh liver sample was homogenized (Glas-Col® homogenizer, Terre Haute, IN, USA) in 1 mL of 1 M sodium chloride. A 250 µL volume of acetone was immediately added into 100 µL liver homogenate to stop enzymatic activity. Then, samples were spiked with an internal standard (10 µL of 2 mg/mL of sodium deoxycholate), vigorously shaken for 1 min and incubated on ice for 5 min. Next, the mixtures were centrifuged at 10,000 rpm for 10 min (Sorvall™ Legend™ micro 21R, Thermo Scientific™, Waltham, MA, USA). Two-hundred µL of supernatant was then transferred to a GC vial and evaporated under nitrogen. A 50 µL of 15 mg/mL methoxyamine pyridine, followed by 90 µL of MSTFA with 1% TMCS, were used for derivatization at room temperature for 1 h. Subsequently, 150 µL of n-heptane was added and filtered. Supernatant was used for further analysis.

Separation was performed by gas chromatography-mass spectroscopy with a GC-MS 7890B GC system/7000C GC/MS Triple Quadrupole (Agilent Technologies, Santa Clara, CA, USA) with HP-5ms column (30 m × 0.25 mm × 0.25 μm). One μL of derivatized samples was injected by the autosampler equipped with a 10-μL syringe with the split ratio 1:10 into a split, straight glass wool liner, using helium as a carrier gas at a flow of 1.0 mL/min. The syringe was washed at least 3 times with acetone between the adding steps. The mass spectrometer transfer line was kept at 300 ℃. The inlet temperature was set at 250 ℃. The column temperature was initially set at 85 ℃ for 5 min. The temperature of the ion source was 230 ℃. The acquisition was performed in electron impact mode (70 eV) at the rate of 2 scan/s. Full-scan mass spectra were acquired from 33 to 650 m/z with a scan rate of 5 spectra/s.

3.8. Statistical Analysis

For metabolomics measurements, peak picking and deconvolution were performed using MS-DIAL version 2.27 (Tsugawa et al., 2015) for the systematic detection of possible metabolites that were conserved across the samples. Metabolites were identified by a database search against the NIST 14 mass spectral library and the 2009 Fiehn metabolomics library. Data analysis and interpretation were performed by MetaboAnalyst 3.0 (Xia, Sinelnikov, Han, and Wishart, 2015) Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed to differentiate variables between classes. One-way ANOVA was applied for hepatic enzyme and

antioxidant measurements. SPSS version 22.0 (IBM, NY, USA) was used to identify differences among the treatments at 95% confidence level ($p < 0.05$).

CHAPTER IV
RESULTS

4.1. Liver enzyme tests

The AST and ALT levels of each group are shown in Figure 9A-B. The results showed that plasma levels of AST and ALT in the rotenone treated group were increased as compared to those in the control group; however, the change was not statistically significant. The level of AST and ALT in ECa 233 group were not significantly different from those in rotenone group.

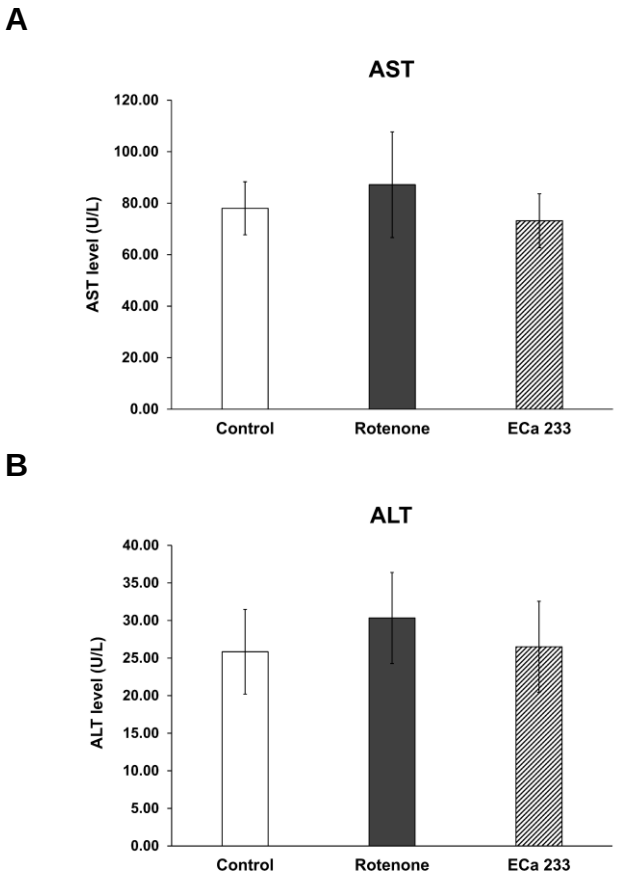


Figure 9 Effects of ECa 233 on the plasma levels of AST (A) and ALT (B). Data are reported as the mean ± SD of six animals in each group.

4.2. Antioxidant tests

TBARS assay was performed to evaluate lipid peroxidation that occurred in the liver tissues as shown in Figure 10A. TBARS level of the rotenone-treated group was increased compared to the control group, which indicated that rotenone induced lipid peroxidation in rat liver ($p < 0.05$). Supplementation of ECa 233 could reduce liver lipid peroxidation as decreasing the levels of TBARS were significantly decreased as compared to the rotenone-treated group ($p < 0.05$).

Antioxidant enzymes were determined as shown in Figure 10B-D. No significant difference was observed in hepatic SOD and GPx among the three groups (Figure 10B, Figure 10D). Nonetheless, a significant decrease in CAT activity was observed in the rotenone group as compared to the control group ($p < 0.001$). Moreover, ECa 233 pre-treatment statistically significantly reversed CAT activity back to normal compared to the rotenone group ($p < 0.001$; Figure 10C).

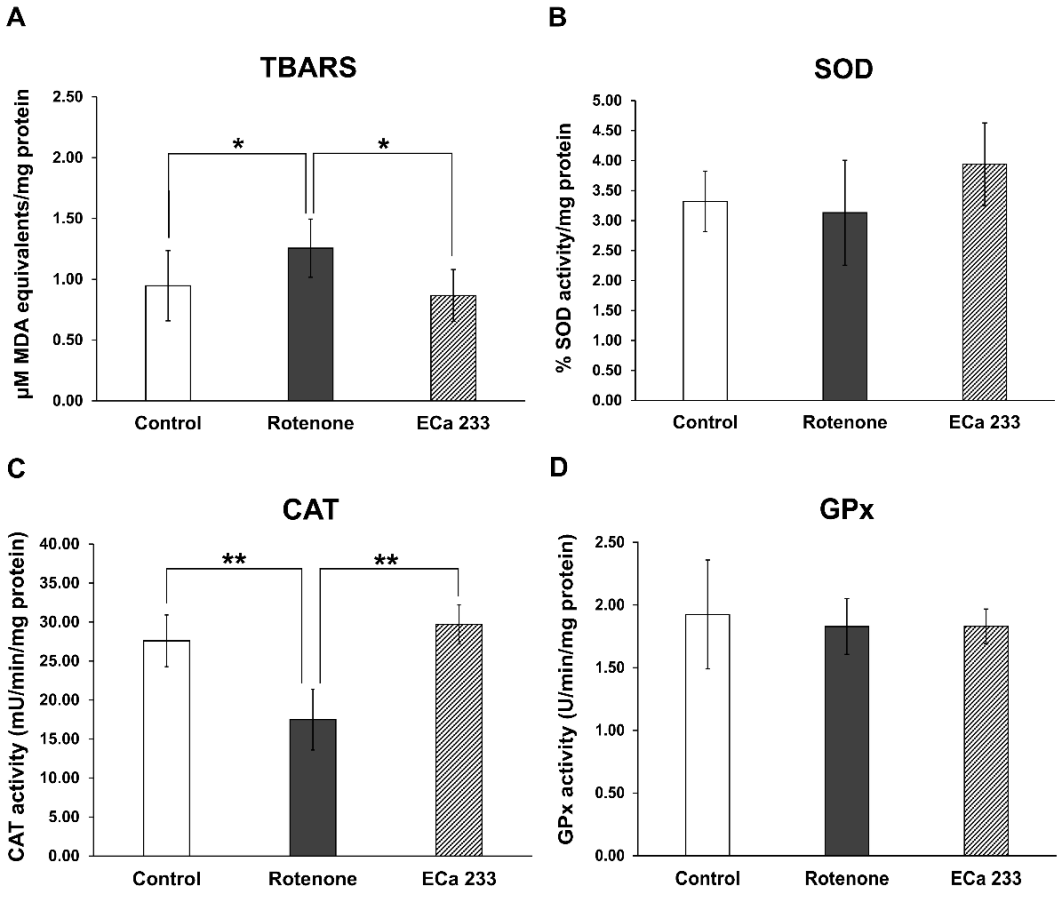
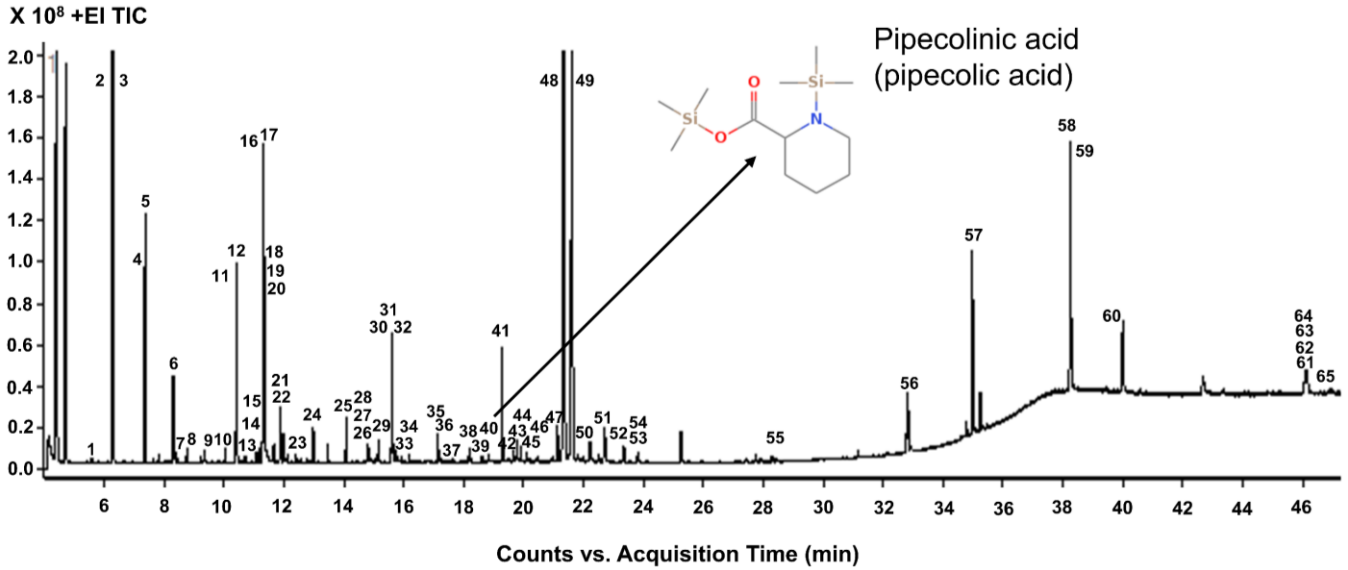


Figure 10 Effects of ECa 233 on TBARS level, SOD, CAT and GPx activity in rat liver after treatment of ECa 233 at 10 mg/kg. Data are reported as the mean ± SD of six animals in each group; * = $p < 0.05$, ** = $p < 0.001$. TBARS = thiobarbituric acid reactive substances as μM MDA equivalents/mg protein (A); SOD = superoxide dismutase as percentage inhibition/mg protein (B); CAT = catalase activity as mU/min/mg protein (C); GPx = glutathione peroxidase activity as U/min/mg protein (D).

4.3. Metabolomic analysis

One hundred and forty-three features were detected in this study, where 65 features were identified (Figure 11) and 78 features were unknown. Most of the identified metabolic features were involved in metabolic functions of the liver. Multivariate data analysis was performed using OPLS score scatter plot. Data were log transformed and pareto scaled. The model showed R^2X = 0.136, R^2Y = 0.566 and Q^2= 0.32. OPLS clearly showed complete separation between control and rotenone groups (Figure 12), which revealed that biochemical perturbation was apparent in rotenone-induced rats after exposure to rotenone for 6 days. Metabolic profiles of the ECa 233 group were located between the control and rotenone groups, and were similar to the control group. Hence, these results suggest that supplementation of ECa 233 at least partially protected the rat liver from rotenone toxicity.



1. 2-Hydroxypyridine
2. Lactic acid
3. 2-Hydroxybutanoic acid
4. Creatine
5. Sarcosine
6. 3-Hydroxynorvaline
7. 2-Ketobutyric acid
8. Beta-hydroxybutyric acid
9. Carnitine
10. Valine
11. Glycocyamine
12. Urea
13. DL-dihydrosphingosine
14. Noradrenaline
15. Norleucine
16. Phosphate
17. Glycerol
18. Isoleucine
19. L-allothreonine
20. Proline
21. Glycine
22. Succinic acid
23. Fumaric acid
24. Threonine
25. 5-Aminovaleric acid
26. Nicotinamide
27. Malonic acid
28. Iminodiacetic acid
29. Malic acid
30. Methionine
31. Oxoproline
32. 5-Methoxyindole-3-acetic acid
33. L-cysteine
34. D-alanine-D-alanine
35. Glutamate
36. Phenylalanine
37. Taurine
38. Ribose
39. Xylitol
40. Pipecolinic acid
41. Hypoxanthine
42. Ornithine
43. Hippuric acid
44. Myristic acid
45. Tyrosine
46. Lyxose
47. Glucose
48. Talose
49. Lysine
50. L-DOPA
51. Mannitol
52. Palmitic acid
53. Cysteine-glycine
54. D-erythro-sphingosine
55. Spermidine
56. Inosine
57. Sedoheptulose
58. Alizarin
59. Cholesterol
60. Thymine
61. Phytosphingosine
62. Glucose-1-phosphate
63. Maltotriose
64. Beta-mannosylglycerate
65. Thymol

Figure 11 Representative GC-MS chromatogram from metabolomic analysis of rotenone-treated groups. Peak 1-65: metabolites

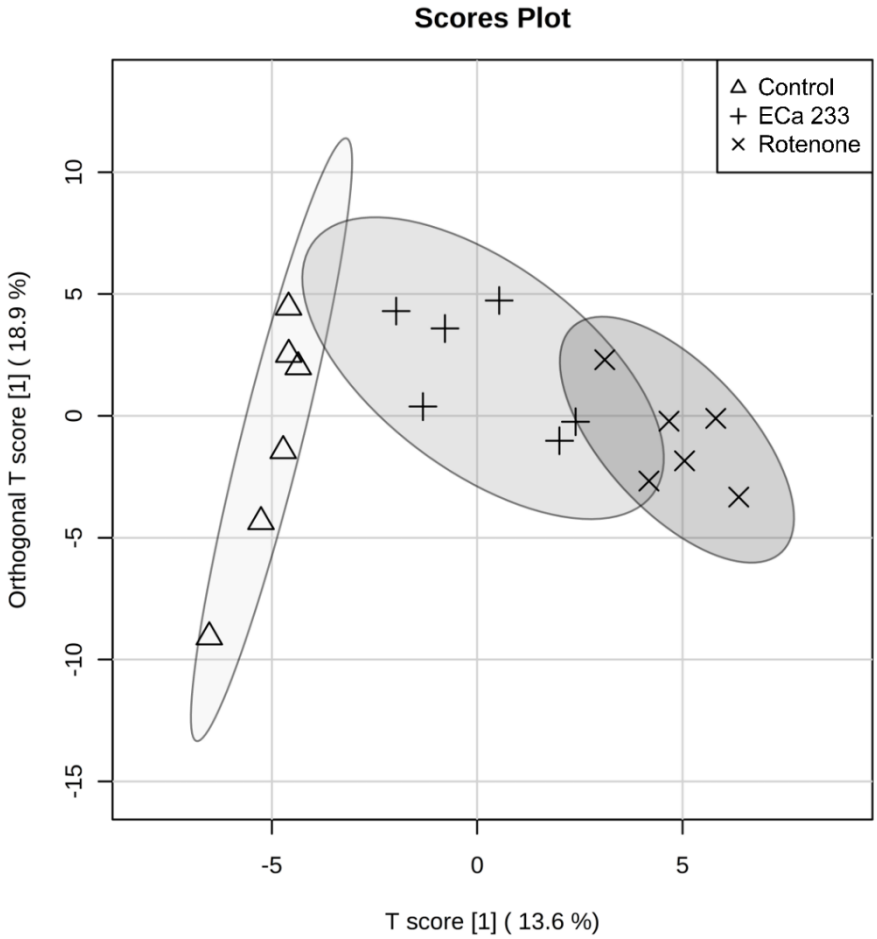


Figure 12 Orthogonal partial least squares (OPLS) score scatter plot showing cluster separation of mass spectra of control rats (Δ), rotenone-induced rats (×), and rotenone-induced rats that were pre-treated with ECa 233 (+).

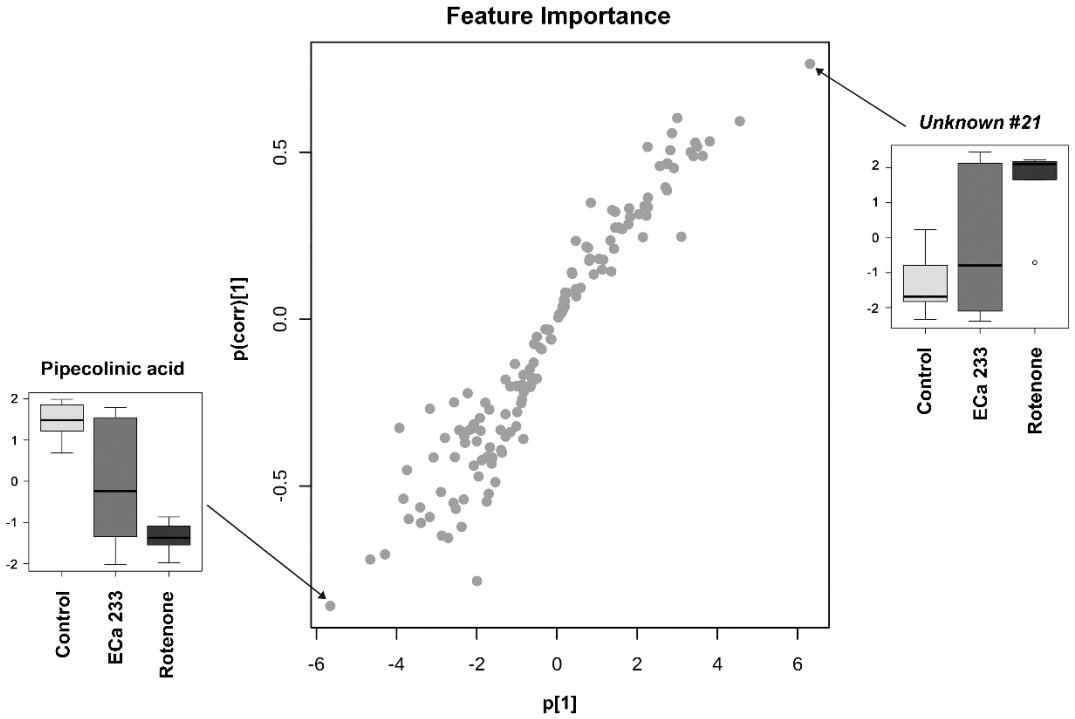


Figure 13 S-plot of metabolites observed from rotenone-treated rats. Box and whisker plots display the levels of pipecolinic acid and Unknown #21 in control (light grey), ECa 233 (grey), and rotenone (black) groups. The top and bottom of the box represent the 75th and 25th percentiles. The whiskers indicate the maximum and minimum points.

The S plot, which represented the covariance (*p*) against the correlation (*p*corr), exhibited the most relevant features among the three groups. Statistical analysis showed that pipecolinic acid and *Unknown #21* were the most important features (Figure 13). Pipecolinic acid was down-regulated while *Unknown #21* was up-regulated in the rotenone group as compared to the control. Administration of ECa 233 tended to reverse the levels of both metabolites back to a more normal level.

CHAPTER V
DISCUSSION

Liver enzymes is commonly used to evaluate hepatic functions. This liver function test includes alanine transaminase (ALT) and aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase, serum bilirubin, prothrombin time, international normalized ratio (INR) and serum albumin (Bedossa and Paradis, 2012). Liver function tests are used to screen for liver disease. Abnormal values of the tests can reflect liver status. Aminotransferases such as AST and ALT are marker enzymes of hepatocellular injury. AST is found in liver, kidney, brain, cardiac muscle, skeletal muscle, pancreas, lung, erythrocyte, and leukocyte, thus it less sensitive and specific indicator of liver damage or inflammation. ALT is mostly presents in liver and more specific to liver than AST. With hepatocellular damage, these aminotransferase enzymes are released into blood circulation. The blood levels of these enzymes are related to the intensity of the damages. In the present study, the levels of AST and ALT were slightly increased after 6 days of rotenone exposure, though the increase no change significant and the enzyme levels were in the range of normal value of rat. The slightly reduction of AST and ALT levels were observed in rats received ECa 233 (10 mg/kg) but no significances were observed.

Hepatocytes contain a high density of mitochondria, which are the main energy source of adenosine triphosphate (ATP) via the electron transport chain and oxidative

phosphorylation (Chinnery and DiMauro, 2005; Grattagliano et al., 2011). In this study, rotenone was used to generate ROS in the rats' liver. TBARS assay is used as the biomarker for estimation of lipid peroxidation. In this study, treatment with rotenone significantly increased MDA levels, which indicated that rotenone generated oxidative damage in the liver. Administration of ECa 233 significantly was found to reduce lipid peroxidation. Previous studies by Zhao et al. (2014) and Choi et al. (2016) also reported protective effects of *Centella asiatica* extracts on lipid peroxidation by decreasing TBARS levels in hamster and rat livers, respectively. Lipid peroxidation inhibition of ECa 233 against rotenone toxicity possibly resulted from free radical scavenging properties of the triterpenoids madecassoside and asiaticoside (Lin et al., 2014; Luo et al., 2015; Luo et al., 2014; Shukla et al., 1999).

The antioxidant defense system is essential to maintain redox balance in the body. Excessive ROS can cause oxidative stress, leading to dysfunction of many organs. Free radicals can damage biomolecules such as protein, lipids and DNA. The enzymatic antioxidant defense systems SOD, CAT and GPx are responsible for eliminating the free radicals. SOD is the primary enzyme that defends against oxidation, by converting superoxide radical anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). Superoxide radicals are produced by various mechanisms in the body, especially from the mitochondrial respiratory chain. Consequently, H_2O_2 produced by the reaction of SOD is converted to water and oxygen by CAT and GPx.

Although the increase of SOD and GPx activities was observed in many previous studies (Choi et al., 2016; Jayashree, Kurup Muraleedhara, Sudarslal, and Jacob, 2003; Zhao et al., 2014), rat liver pre-treated with ECa 233 did not show significantly higher SOD and GPx levels in the present study. However, significantly lower CAT activities were observed in the rotenone treated group. The reduction of CAT activity was likely associated with the scavenging of H_2O_2 that was generated from the dismutation of superoxide ions by SOD. Administration of ECa 233 prior to exposure to rotenone toxicity significantly enhanced the activity of CAT in rat liver. This finding was similar to the results by Choi et al. (2016) that hepatic CAT activity was increased in dimethylnitrosamine-induced liver injured rats treated with *Centella asiatica* extract (200 mg/kg) for 5 days. In addition, the activity of CAT in liver tissue of lymphoma-bearing mice was increased after treatment of *Centella asiatica* extract (50 mg/kg/day) for 14 days (Jayashree et al., 2003). Furthermore, Sainath, Meena, Supriya, Reddy, and Reddy (2011) reported that hepatic CAT activity was elevated in rats exposed to lead co-administered with aqueous extract of *Centella asiatica* (200 mg/kg/day) for 70 days.

For metabolomic analysis, pipecolinic acid (also known as pipecolic acid) was one of the most important metabolites in this study. Pipecolinic acid level was decreased in the rotenone treated group; however, supplementation with ECa 233 brought its concentration up to levels comparable to the controls. Pipecolinic acid is an intermediate metabolite of lysine. In general, lysine degradation occurs via two pathways; saccharopine and pipecolinic acid (Broquist, 1991). In this study, the amount

of lysine did not significantly change among the three groups. Thus, possible underlying mechanisms in the rats' liver were probably related to the increasing of lysine degradation via saccharopine pathway by rotenone.

Peroxisomal disorder might be another possible explanation of diminishing level of pipecolinic acid in rotenone-treated group. Typically, pipecolinic acid catabolism is metabolized by a peroxisomal enzyme, pipecolinic acid oxidase. In the liver, pipecolic acid oxidation occurs in mitochondria and peroxisomes, where 83% of pipecolinic acid oxidase inhibition by rotenone was reported (Rao, Tsai, Pan, and Chang, 1993). Both organelles cooperate in metabolic processes such as fatty acid β-oxidation and ROS metabolism. In particular, ROS are mainly produced by mitochondria, endoplasmic reticulum and peroxisomes. Boveris, Oshino, and Chance (1972) reported that approximately 35% of H_2O_2 was produced by peroxisomes in rat liver. Likewise, the antioxidant enzyme CAT that used to decompose H_2O_2 is mainly localized in peroxisomes. This enzyme has been used as a peroxisomal marker (Schrader and Fahimi, 2006). Therefore, peroxisomal disorders might affect cellular redox balance, resulting in alteration of the levels of pipecolinic acid. Nevertheless, further studies are needed to clarify clearly the relationship between pipecolinic acid and CAT.

CHAPTER VI
CONCLUSION

The outcomes of this study provide further evidence in support of using *Centella asiatica* as a value-added component in new dietary supplements or medicines for liver disorders in the future. GC-MS based metabolomic analysis revealed for the first time that supplementation of the standard extract of *Centella asiatica* ECa 233 protected against rotenone toxicity in rotenone-induced rats, where pipecolinic acid and *Unknown #21* were the most two important features in the present study. ECa 233 supplementation prior to exposure to rotenone inhibited lipid peroxidation in liver tissue, perhaps due to the antioxidant properties of triterpenoids contained in this herb, as well as boosting the activity of the CAT antioxidant enzyme. Since pipecolinic and CAT are mainly found in peroxisomes, this organelle is probably associated with the underlying mechanism of ECa 233 in the liver tissue. However, further investigations are required to elucidate the mechanisms involved.

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APPENDICES

APPENDIX A

Liver enzyme tests, TBARS and antioxidant enzyme activities

Table 6 Liver enzyme tests

Groups	AST (U/L)	ALT (U/L)
Control	78.00 ± 10.30	25.83 ± 5.64
Rotenone	87.17 ± 20.52	30.33 ± 6.06
ECa 233	73.17 ± 10.48	26.50 ± 6.06

Data are reported as the mean ± SD of six animals in each group. U/L = Units/L

Table 7 TBARS and antioxidant enzyme activities

Groups	TBARS	SOD	CAT	GPx
Control	0.947 ± 0.288	3.320 ± 0.503	27.590 ± 3.320	1.925 ± 0.435
Rotenone	1.256 ± 0.240*	3.130 ± 0.876	17.480 ± 3.890**	1.829 ± 0.222
ECa 233	0.867±0.213$^{\#}$	3.940 ± 0.690	29.690 ± 2.500$^{\#\#}$	1.830 ± 0.138

Data are reported as the mean ± SD of six animals in each group.

* $p < 0.05$, ** $p < 0.01$ compared with control group.

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with rotenone group.

TBARS = thiobarbituric acid reactive substances as μM MDA equivalents/mg protein

SOD = superoxide dismutase activity as percentage/mg protein

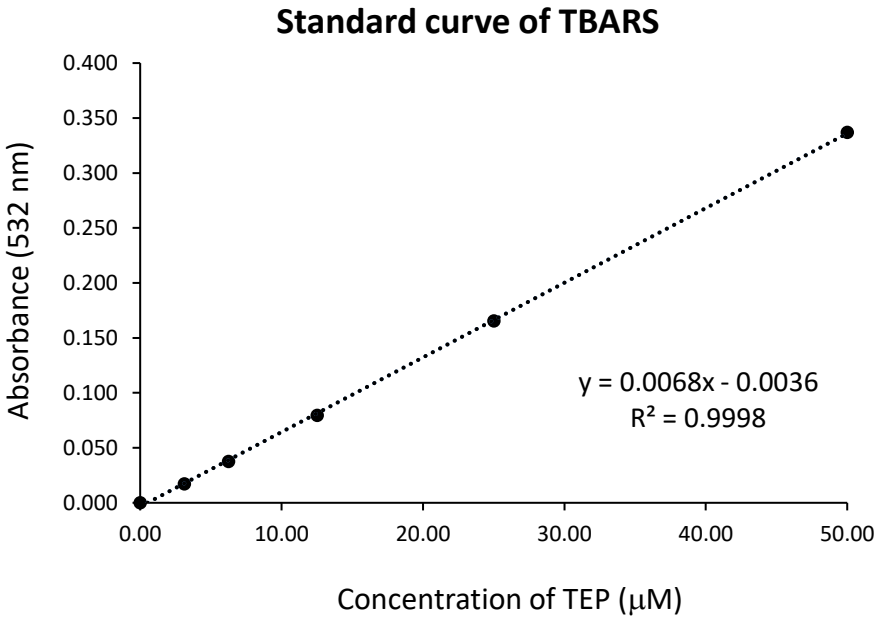
CAT = catalase activity as mU/min/mg protein

GPx = glutathione peroxidase activity as U/min/mg protein

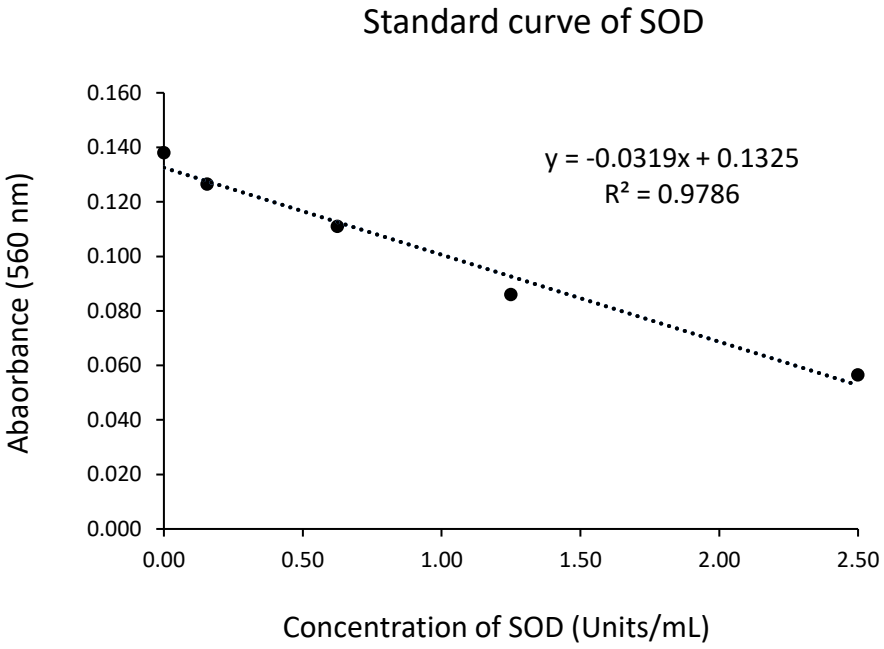
APPENDIX B

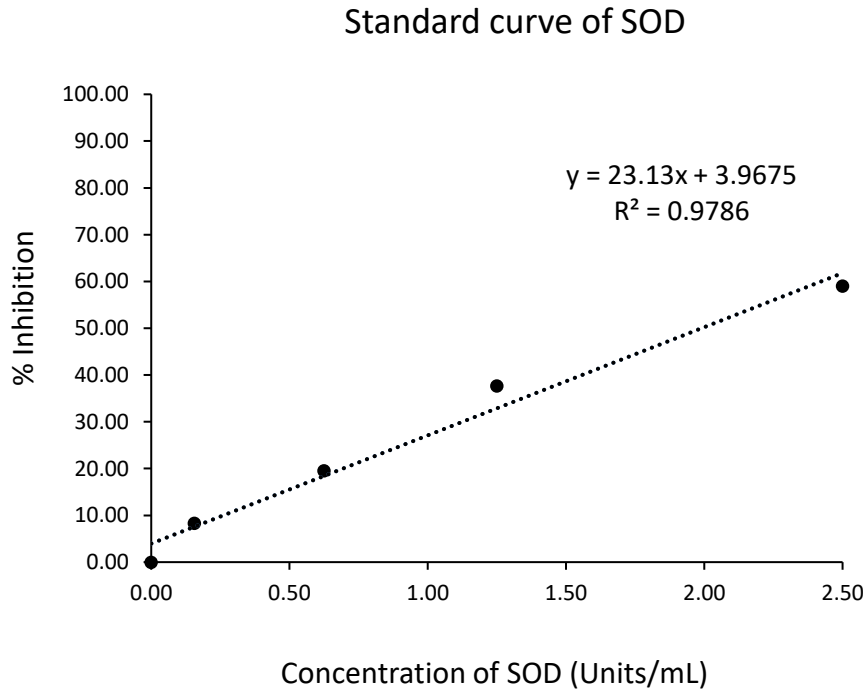
Standard calibration curves

TBARS

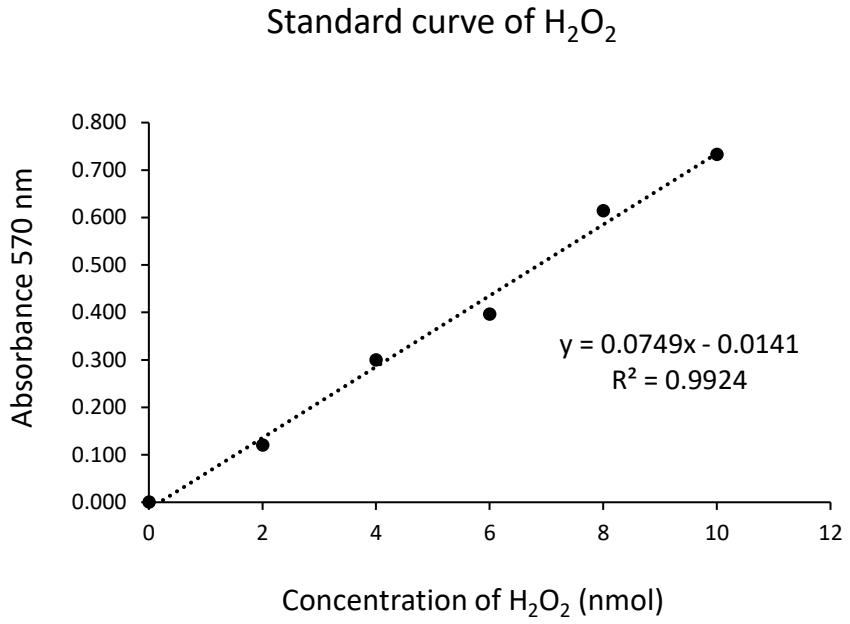


SOD

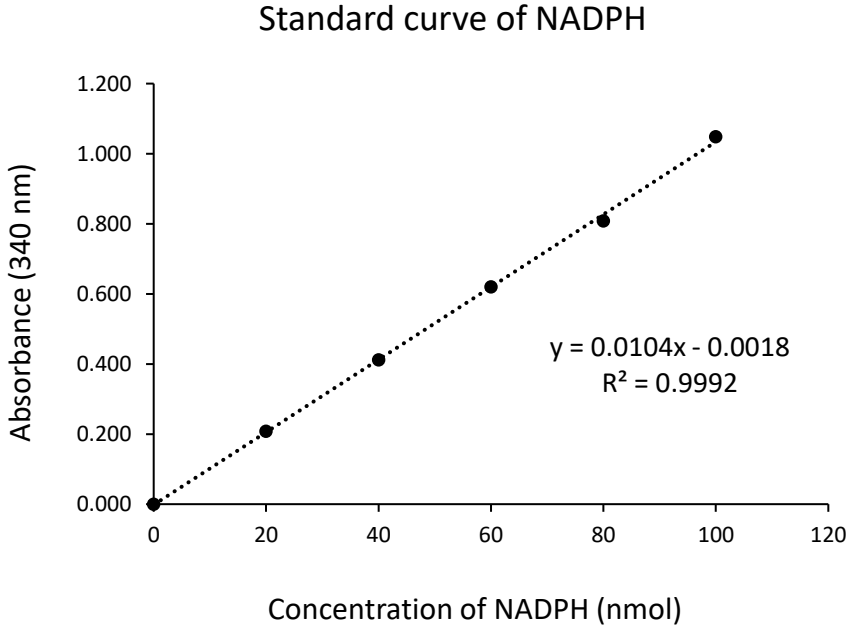

Standard curve of SOD
y = -0.0319x + 0.1325
R² = 0.9786
Abaorbance (560 nm)
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0.00
0.50
1.00
1.50
2.00
2.50
Concentration of SOD (Units/mL)


Standard curve of SOD
y = 23.13x + 3.9675
R² = 0.9786
% Inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.00
0.50
1.00
1.50
2.00
2.50
Concentration of SOD (Units/mL)

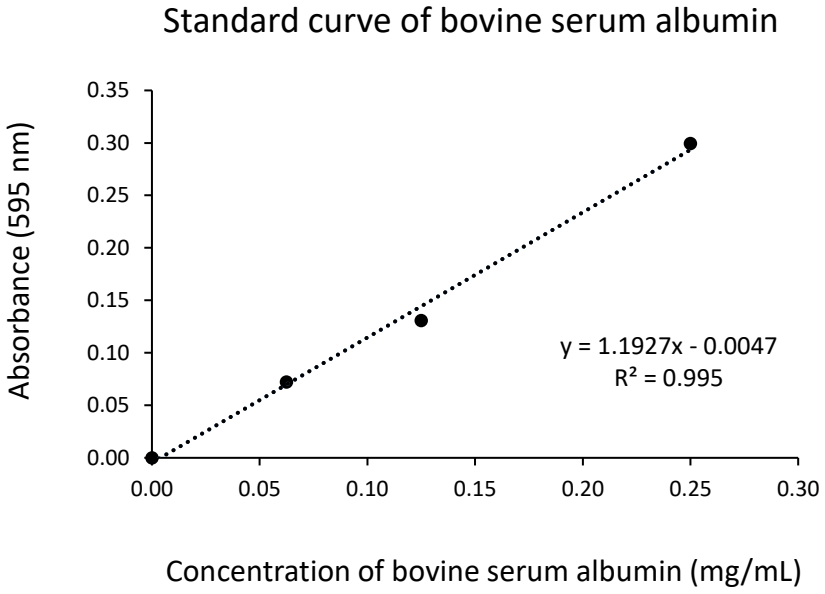
CAT


Standard curve of H_2O_2
Absorbance 570 nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
2
4
6
8
10
12
y = 0.0749x - 0.0141
R² = 0.9924
Concentration of H_2O_2 (nmol)

GPx


Standard curve of NADPH
Absorbance (340 nm)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
20
40
60
80
100
120
y = 0.0104x - 0.0018
R² = 0.9992
Concentration of NADPH (nmol)

Protein (Bradford Assay)



APPENDIX C

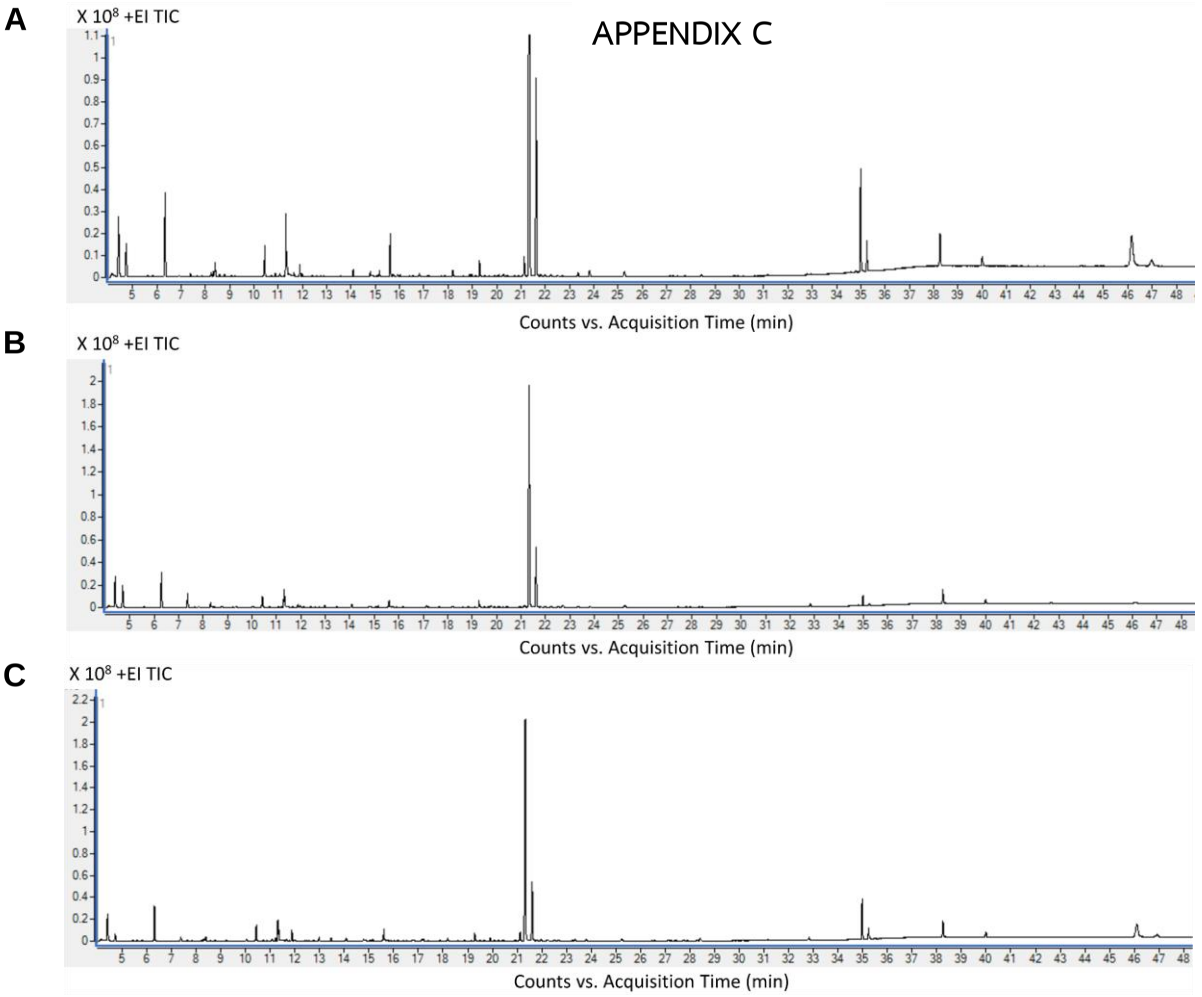


Figure 14 GC-MS chromatograms of control (A), rotenone (B) and ECa 233 (C) groups.

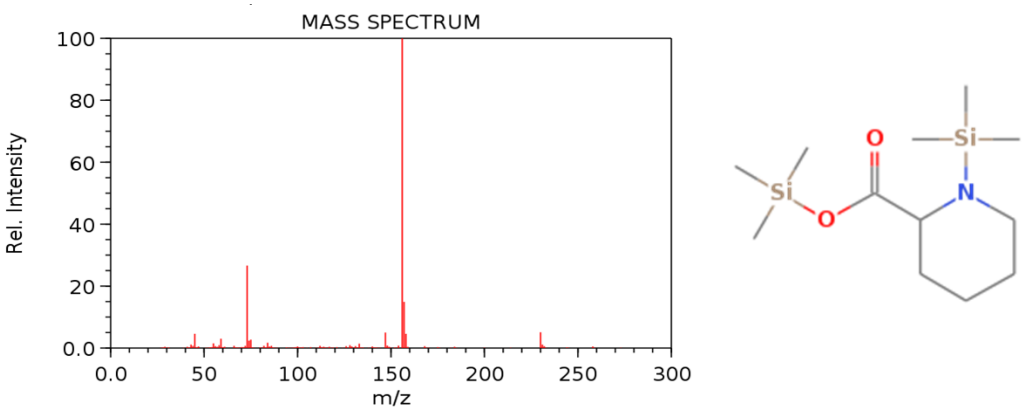


Figure 15 Mass spectrum of pipecolinic acid (NIST Mass Spec Data Center and Stein, 2017).

APPENDIX D

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 15-33-004	**Approval No. 15-33-004**

Protocol Title
NEUROPROTECTIVE EFFECT OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* Eca233 IN ROTENONE-INDUCED PARKINSONISM RAT MODEL

Principal Investigator

RATCHANEE RODSIRI, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	**Date of Expiration**
May 12, 2015	May 12, 2018

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title **THONGCHAI SOOKSAWATE, Ph.D.** **Chairman**	Name and Title **PORNCHAI ROJSITTHISAK, Ph.D.** **Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

VITA

Miss Thidarat Intararuchikul was born on November 13, 1983 in Bangkok, Thailand. She received a Bachelor of Sciences in Pharmacy from the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand in 2007. After graduated, she worked as a pharmacist at Phyathai Hospital 2, Bangkok, Thailand for 3 years. In addition, she was a full-time community pharmacist at Health Up drug store, Lampang, Thailand in 2013 and she also worked as a part-time pharmacist at a number of drug store in Lampang and Phrae Provinces, Thailand in 2011-2013.