ฤทธิ์ของสารสกัดสมุนไพร Thunbergia laurifolia Linn., Moringa oleifera Lam. ต่อการแสดงออกของยีนที่ใช้ในการถอนพิษและเมทาบอลิซึมของพลังงานในเซลล์ตับ

นางสาวอฑิตยา โรจนสโรช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2556

THE EFFECTS OF THUNBERGIA LAURIFOLIA LINN, MORINGA OLEIFERA LAM EXTRACTS ON HEPATOCELLULAR DETOXICATION AND ENERGY METABOLISM GENE EXPRESSION

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Department of Clinical Chemistry

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Thesis Title	THE EFFECTS OF THUNBERGIA LAURIFOLIA LINN, MORINGA OLEIFERA LAM EXTRACTS ON HEPATOCELLULAR DETOXICATION AND ENERGY METABOLISM GENE EXPRESSION
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Field of Study	Clinical Biochemistry and Molecular Medicine
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ออกซิเดทีฟสเตรสเกิดจากการเสียสมดุลระหว่างกระบวนการรับส่งอิเล็กตรอนจนทำให้มีอนุมูลอิสระ ส่วนเกิน ซึ่งสร้างความเสียหายแก่องค์ประกอบต่าง ๆ ของเซลล์ เช่น ไขมัน โปรตีน และดีเอ็นเอ จนนำไปสู่การสูญเสีย โครงสร้างและการทำงานของเซลล์ สารต้านอนุมูลอิสระเป็นระบบที่ร่างกายใช้ในการป้องกันและซ่อมแซม เพื่อลดความ เสียหายที่เกิดจากอนุมูลอิสระ ปัจจุบันพืชสมุนไพรได้รับความนิยมและมีการใช้อย่างแพร่หลาย เนื่องจากพืชดังกล่าวเป็น แหล่งของสารต้านอนุมูลอิสระและสามารถหามาใช้ได้ง่าย โดยเชื่อว่ายาสมุนไพรปลอดภัยมากกว่ายาแผนปัจจุบัน ในขณะนี้ข้อมูลเรื่องคุณภาพ ความปลอดภัย และ การตอบสนองของเซลล์ต่อสมุนไพรเหล่านี้ยังมีน้อย และไม่แพร่หลาย การศึกษานี้จึงถูกออกแบบมาเพื่อตรวจสอบผลของสมุนไพรที่นิยมใช้กันอย่างแพร่หลายในประเทศไทย นั่นคือ รางจืด และ มะรุม โดยตรวจผลการออกฤทธิ์ และความเป็นพิษของพืชสมุนไพร เพื่อใช้เป็นแหล่งข้อมูลในการใช้ยาสมุนไพรให้ เกิดประโยชน์ได้และมีประสิทธิภาพสูงสุดต่อไป จากงานวิจัยพบว่าสารสกัดรางจืดและสารสกัดมะรุม มีความสามารถใน การต้านปฏิกิริยาออกซิเดทีฟ และสามารถยับยั้งอนุมูลอิสระที่พบมากในเซลล์ เช่น อนุมูลอิสระซูเปอร์ออกไซด์ อนุมูล อิสระไฮดรอกซิล อนุมูลอิสระไนตริกออกไซด์ และไฮโดรเจนเปอร์ออกไซด์ ซึ่งฤทธิ์ต้านอนุมูลอิสระ ดังกล่าวมีความ เชื่อมโยงกับสารโพลีฟีนอลที่ตรวจพบในสารสกัด โดยเชื่อว่า เป็นสาระสำคัญในการออกฤทธิ์ของสารสกัดสมุนไพร สาร สกัดสมุนไพรทั้งสองชนิดสามารถลดอัตราการเสื่อมสลายของ ไขมัน โปรตีน ดีเอ็นเอ และ สามารถกระตุ้นการทำงาน ของระบบต้านอนุมูลอิสระภายในเซลล์ นอกจากนี้ยังพบประโยชน์ของยาสมุนไพรในการต่อต้านโรคเบาหวาน แก้พิษจาก สารฆ่าแมลง ซึ่งสารสกัดสมุนไพรรางจืดสามารถยับยั้งเอนไซม์สำคัญสองชนิดที่เกี่ยวข้องกับภาวะน้ำตาลในเลือดสูง นั่น คือ แอลฟาอะไมเลสและแอลฟากลูโคซิเดส และยังสามารถยับยั้งการเกิดปฏิกิริยาไกลเคชัน ซึ่งเป็นส่วนสำคัญในการเกิด พยาธิโรคเบาหวาน ส่วนสารสกัดสมุนไพรมะรุมพบว่า มีผลป้องกันพิษยาฆ่าแมลงจำพวกออแกโนฟอสเฟสที่มีต่อ เอนไซม์อะซิทิลโคลินเอสเตอเรส เมื่อทำการทดลองฤทธิ์ของสารสกัดกับเซลล์ตับเพาะเลี้ยง (HepG2 cells) พบว่าความ เข้มข้นของสารสกัดที่สูงกว่า 1000 ไมโครกรัมต่อมิลลิลิตร เป็นพิษต่อเซลล์ เมื่อทดสอบด้วย วิธีนิวทรัลเรด และเอ็มทีที ความเป็นพิษของสารสกัดสมุนไพรเหล่านี้พบว่ามีความเชื่อมโยงกับไมโตครอนเดรียที่ทำงานบกพร่อง ผลการทดลองบ่งชี้ ว่าสารสกัดสมุนไพรมีผลรบกวนการสันดาปพลังงานและภาวะสมดุลแคลเซียม การศึกษาสารสกัดสมุนไพรในการรักษา ภาวะสมดุลในเซลล์โดยทดสอบด้วยการย้อมสีจำเพาะ และตรวจหาปริมาณกลูธาไทโอน พบว่า ทั้งสารสกัดจากรางจืด และมะรุมสามารถลดระดับออกซิเดทีฟสเตตสภายในเซลล์ และเฉพาะสารสกัดรางจืดเท่านั้นที่สามารถป้องกันผลกระทบ ที่เกิดจากการที่เซลล์รับสารออกซิเดชันจากภายนอกมากเกินไป สุดท้ายในการทดสอบสารสกัดสมุนไพรที่มีผลต่อระบบ การเมทาบอลิซึมของตับ พบว่า สารสกัดรางจืดมีผลต่อระบบการสันดาปเพื่อเปลี่ยนแปลงสารชีวเคมี (ไบโอทรานฟอร์เม ชัน) อย่างมีนัยสำคัญโดย พบว่า สามารถเปลี่ยนการแสดงออกและการทำงานของเอนไซม์เฟสหนึ่ง คือ ไซโทโครมพี450 หลายไอโซฟอร์ม นอกจากนี้ยังเพิ่มการขับสารพิษออกจากเซลล์ด้วย พี-ไกลโคโปรตีน ผลการทดลองเหล่านี้สอดคล้องกับ การใช้สมุนไพรแต่ดั้งเดิมที่ว่ารางจืดเป็นยาขับพิษ ในขณะที่สารสกัดมะรุมจะมีผลต่อกระบวนการสันดาป สังเคราะห์สาร มากกว่ามีผลต่อระบบไบโอทรานฟอร์เมชัน ซึ่งพบว่าสามารถลดระดับการแสดงออกของเอนไซม์ที่ใช้ในการสังเคราะห์ ไขมัน HMG-CoAR PPARα1 และ PPAR☐ ผลการทดลองทั้งหมดบ่งชี้ว่า พืชสมุนไพรเหล่านี้มีประโยชน์ สามารถ รับประทานเพื่อเสริมสุขภาพ แต่ก็ควรมีความระมัดระวังในการใช้พืชสมุนไพรด้วยเช่นกัน ผลการศึกษานี้จะสามารถใช้ เป็นแหล่งข้อมูลเพื่อเพิ่มความระมัดระวังและหลีกเลี่ยงอันตรายอันเกิดจากการรับประทานอาหารเสริมสมุนไพรร่วมกับ ยาแผนปัจจุบัน

ภาควิชา	เคมีคลินิก	ลายมือชื่อนิสิต	....
สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	....
ปีการศึกษา	2556	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	....

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ATITTAYA ROCEJANASAROJ: THE EFFECTS OF THUNBERGIA LAURIFOLIA LINN, MORINGA OLEIFERA LAM EXTRACTS ON HEPATOCELLULAR DETOXICATION AND ENERGY METABOLISM GENE EXPRESSION. ADVISOR: ASSOC. PROF. DR. WARIN SANGKITIKOMOL, Ph.D., CO-ADVISOR: ASST. PROF. DR. TEWIN TENCOMNAO, Ph.D., 190 pp.

Oxidative stress is a redox imbalance arises from the excess of free radicals which potentially lead to damage of cellular components. Oxidative stress has been implicated in the pathogenesis of many human diseases, as well as to the aging process. Antioxidants are one way that the body uses to defense and repair in order to minimize damage. Herbal plants become increasing use because they are the excellent sources of antioxidants and easy availability. However, quality, toxicological data and the cellular responses for these plants are rarely known and remained very little data. This present study was designed to investigate the most popular herbs widely used in Thailand: Thunbergia laurifolia (TL) and Moringa oleifera (MO) in the proposed understanding their pharmacological activity and toxicity. It was found that TL and MO extracts possessed high antioxidant levels and ability to scavenging some of the most common reactive species in cellular system including; superoxide radicals, hydroxyl radicals, nitric oxide radicals and hydrogen peroxide. Their antioxidant activities are associated with the phenolic compounds contain in the extracts. Also, both herbal extracts were significantly lowering the oxidative damage of lipid, protein, DNA molecules and could induce endogenous antioxidant molecules. Their other beneficial effects such as anti-diabetic and antidote against pesticide were observed. TLE significant inhibit α-amylase and α-glucosidase – enzymes related to hyperglycemia and lowering AGE formation while MOE is significantly protective effects against organophosphate-induced acetylcholinesterase activity inhibition. The crude extracts were subsequently investigated for the effects in HepG2 cell culture system. The data was indicated at high concentrations over 1000 μg/ml for both extracts induced cytotoxicity. The toxicity of these herbal extracts was evidently associated with mitochondrial dysfunction, partially, by interrupted energy production and calcium homeostasis. Both TLE and MOE could reduce intracellular oxidative levels but only TLE could maintain redox balance when cell face oxidative injury. Finally, the data showed that TLE significantly altered biotransformation system. TLE significantly changed phase I, cytochrome P450 isoenzymes (CYP450s) gene expression profile, enzyme activities and induced excretion process though up-regulated P-glycoprotein activity. These results support the traditional medicinal use of TLE for detoxification. Unlike TLE, MOE would rather to modulating lipid metabolism than the effect on transformation of xenobiotic. MOE was significantly found to modulated lipid biosynthesis by down-regulated mRNA expression of HMG-CoAR, and it regulatory transcription factor, PPARα1 and PPAR$\square$. Our results would be the index for further use by exploration for the molecular evidences about their toxicological information. Caution must be taken when mixing herbal supplement with drugs in order to assure the safety of these plants and avoid the adverse effect of the herb-drug interaction.

Department:	Clinical Chemistry
Field of Study:	Clinical Biochemistry and Molecular Medicine
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Student's Signature _____

Advisor's Signature _____

Co-Advisor's Signature _____

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CONTENTS

Chapter I

Introduction

Literature reviews

1. Free radical: The Reactive species

Oxygen (O_2) is an essential molecule for life and it plays a vital role in diverse biological phenomena. Because survival of aerobic organisms are depends on their ability to release energy from the oxygen–food combination that is used in many biological processes. However, Oxygen has double-edged properties, besides being part in a number of physiological processes; it can also aggravate the damage within the cell by transformed into a variety of potentially toxic reactive intermediates known as free radicals[1]. Free radicals are unstable forms of any molecules containing one or more unpaired electrons delocalized within their outer orbit. Free radicals are electron-deficient species. Once free radicals are formed, they react rapidly with any molecules in the immediate vicinity[2,3] via a variety of reactions including: hydrogen abstraction (capturing), electron donation or electron sharing. These free radicals exert their toxic effect by taking electrons from cell constituents such as lipid, protein and DNA. Free radicals may give rise to more free radicals, therefore; causing progressively more damage. Varieties of free radicals can be formed within our body[4]. Free radicals are derived from oxygen and nitrogen characterized as the most important class of radical species generated in aerobic living system[5] The terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) are often described both free radicals and other nonradical reactive derivatives. They are superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), hydroperoxyl radical ($HO_2\bullet$), lipid peroxyl radical ($LOO\bullet$), nitric oxide radical ($\bullet NO$), nitrogen dioxide ($NO_2\bullet$), peroxyl radical ($\bullet ROO$), and alkoxyl radical ($\bullet RO$). Hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), lipid peroxide ($LOOH$), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), ozone (O_3), peroxynitrite anion ($ONOO-$), and singlet oxygen ($^1O_2$)[6] are nonradical species. They easily lead free radical reactions and mediate damage to biological molecules.

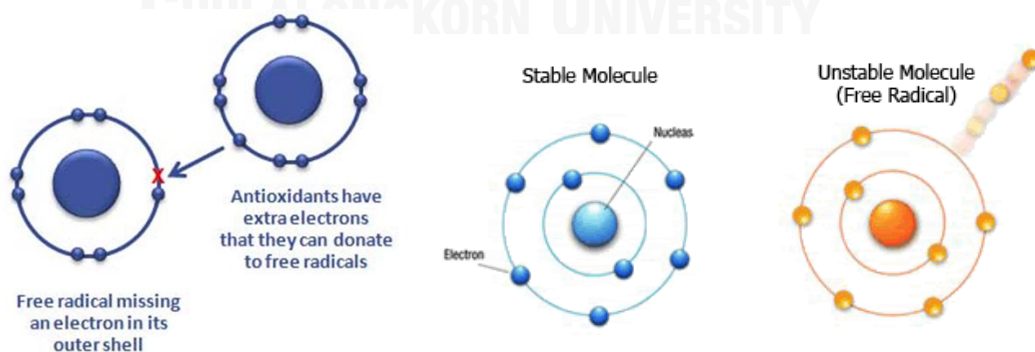Figure 1.1: **Free radical structure.** Free radical is a highly active species, usually with an unpaired electron. Photo from http://www.aquahealthproducts.com/free-radicals-and-antioxidants.
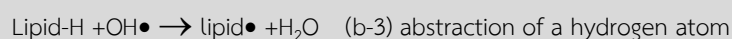
Table1.1: Definition and nomenclature of reactive species

Radicals	Nonradical
Reactive oxygen species (ROS)	
Superoxide, O_2^-	Hydrogen peroxide, H_2O_2
Hydroxyl, OH^-	Hypobromous
Hydroperoxyl, HO_2^-	Hypochlorous acid, HOCl
	Ozone O_3
Lipid peroxyl, LOO●	Singlet oxygen ($^1O_2$)
Lipid alkoxyl, LO●	Lipid peroxides, LOOH
	Peroxynitrite, ONOO-
Reactive Chlorine Species (RCS)	
Atomic chloride, Cl●	Hypochlorous acid, HOCl
	Nitryl (nitronium) chloride,NO_2Cl
	Chloramines
	Chlorine gas (Cl_2)
Reactive Nitrogen Species (RNS)	
Nitric oxide, NO●	Nitrous acid, HNO_2
Nitrogen dioxide, NO_2●	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, ONOO-
	Nitronium (nitryl) cation, NO_2^+
	Arkyl peroxynitrites, ROONO
	Nitryl (nitronium) chloride,NO_2Cl

1.1 Free radical classification[7]

These radicals are generated by either though (a) loss of one electron from a non-radical (X) (b) gain of one electron either by homolytic cleavage of a chemical bond (splitting of the electron pair forming the bond so as to leave one electron on each of the originally bonded atoms), or electron transfer reactions from hydrogen atom.

$$X \rightarrow X^{•+} + e^- \qquad \text{(a) loss of one electron}$$

$$X + e^- \rightarrow X^• \qquad \text{(b-1) gain of one electron}$$

$$X\text{-}Y \rightarrow X● + Y● \qquad \text{(b-2) homolytic fission of a covalent bond}$$

$$\text{Lipid-H} + OH● \rightarrow \text{lipid●} + H_2O \quad \text{(b-3) abstraction of a hydrogen atom}$$
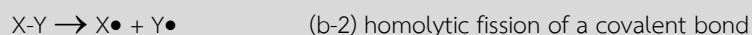
Reactive type of ROS and the most commonly produced free radicals in humans including:

Superoxide radical ($O_2^•$): It is usually generated during mitochondrial electron transport reaction by auto-oxidation of O_2. Alternatively, $O_2^{•-}$ is also produced enzymatically by xanthine oxidase and

cytochrome P450[8]. Once $O_2^{\bullet-}$ is produced, it triggers many cascade of events that creates other free radicals such as hydroxyl radicals and singlet oxygen. $O_2^{\bullet-}$ radical is responsible for intracellular protein and lipid peroxidation that lead to structural changes and loss of enzyme activities such as catalase (CAT)[9], superoxide dismutase and glutathione peroxide (GPx)[10]. $O_2^{\bullet-}$ causes DNA mutation by breaking DNA single strand and chromosomal shortening[11]. Intracellular $O_2^{\bullet-}$ is terminated by reacts with proton in water to form hydrogen peroxide (equations 1-3). These reactions are catalyzed by superoxide dismutase (SOD).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \text{ (equation 1)}$$

$$HO_2\bullet + O_2^{\bullet-} + H+ \longrightarrow H_2O_2 + O_2 \text{ (equation 2)}$$

$$2HO_2^{\bullet} \longrightarrow H_2O_2 + O_2 \text{ (equation 3)}$$
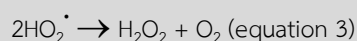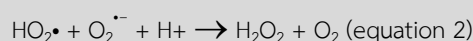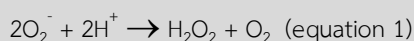
Superoxide can reduce Fe^{3+} to Fe^{2+}. These two reactions are known as Haber-Weiss reaction (equations 4-5). The superoxide anion can also react with nitric oxide (NO•) to form peroxynitrite ($ONOO^{\bullet-}$).

$$\text{Haber-Weiss: } O_2^{\bullet-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \text{ (equation 4)}$$

$$H_2O_2 + O_2^{\bullet-} \longrightarrow O_2 + OH^- + \bullet OH \text{ (equation 5)}$$

Hydroxyl radical (•OH): The protonated form of $O_2^{\bullet-}$ is resulted $HO_2\bullet$. •OH radical is most reactive chemical species, highly electrophilic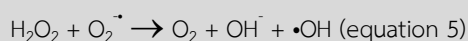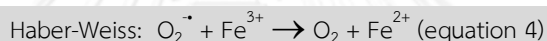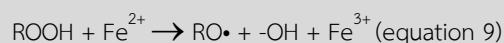 and consequently short-lived. This short-lived •OH molecule unspecifically attacks biomolecules nearby such as lipids, polypeptides, polysaccharides, proteins and nucleic acids located less than a few nanometers from its site of generation[12]. When •OH abstracts electrons from biomolecules would resulting in initiation of radical chain reaction. Beginning with the formation of carbon centered free radicals (R•) (equation 6). Then, carbon centered free radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical (ROO•) and alkoxy radical (RO•) (equation 7-9)[12].

$$RH + {}^{\bullet}OH \longrightarrow R^{\bullet} + H_2O \text{ (equation 6)}$$

$$RO^{\bullet} + O_2 \longrightarrow ROO\bullet \text{ (equation 7)}$$

$$ROOH + Fe^{3+} \longrightarrow ROO\bullet + Fe^{2+} + H^+ \text{ (equation 8)}$$

$$ROOH + Fe^{2+} \longrightarrow RO\bullet + {}^-OH + Fe^{3+} \text{ (equation 9)}$$
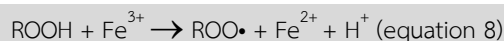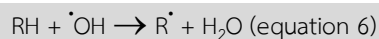
In the presence of metals such as Fe^{2+} or Cu^{2+}, hydroxyl radical also generate through Fenton reactions[13] by reaction of H_2O_2 with metals ions, Fe^{2+} is oxidized to Fe^{3+} and H_2O_2 is converted to •OH and OH- (equation 10).

$$\text{Fenton: } H_2O_2 + Fe^{2+} \longrightarrow {}^-OH + \bullet OH + Fe^{3+} \text{ (equation 10)}$$
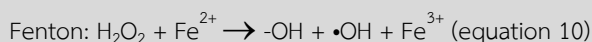
Hydrogen peroxide (H_2O_2): Hydrogen peroxide is generated through a dismutation reaction from superoxide anion using superoxide dismutase. Other enzymes such as monomeric oxidases located in the outer mitochondrial membrane and xanthine oxidase also produce H_2O_2 from superoxide anion. Although H_2O_2 is the least reactive molecule among reactive oxygen species, it is very damaging because H_2O_2 is highly diffusible and easily crosses the plasma membrane resulting in membrane disruption[14], DNA damage[15], and disrupted cellular calcium homeostasis[16]. Moreover, it can be converted to hydroxyl radical via Fenton reaction. Once H_2O_2 is generated, it is metabolized to water by catalase (in the peroxisomes) and glutathione peroxidase (both in the cytosol and mitochondria).

Singlet oxygen ($^1O_2$): Singlet oxygen has a non-radical and electrophilic character. Singlet oxygen is mostly generated from H_2O_2 during interaction with hypochlorite (OCl-) (equation 12-13). Singlet oxygen is a less toxic when compare with other reactive oxygen species. Thus, $^1O_2$ is high reac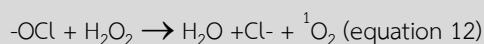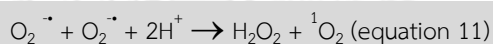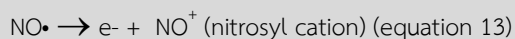tive that can induce oxidative reactions with biological macromolecules in its electron-rich moieties without the participation of free radicals. Singlet oxygen has ability to damage guanine components and nucleic acids[17] and oxidize of PUFA to initiate lipid peroxidation[18].

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2 \text{ (equation 11)}$$

$$-OCl + H_2O_2 \longrightarrow H_2O + Cl- + {}^1O_2 \text{ (equation 12)}$$

Peroxyl radical (ROO•): Peroxyl radical is formed by a direct reaction of oxygen with alkyl radical (R•). Decomposition of alkyl peroxides (ROOH) also results in peroxyl (ROO•) and alkoxyl (RO•) radicals. Peroxyl and alkoxyl radicals are good oxidizing agents which are involved in the propagation stage of lipid peroxidation.

Nitric oxide (NO•): Nitric oxide is a short-lived gas and reactive mediator produced by various body cells (endothelial cells, neurons, monocytes, macrophages etc). Nitric oxide is usually formed from L-arginine in biological system by nitric oxide synthase. Physiological production of NO plays an important role by act as oxidative biological signaling molecule in blood pressure regulation, defense mechanism against pathogens, smooth muscle relaxation and immune regulation[19]. But overproduction of NO and its metabolites, however, have been implicated in the pathogenic of conditions such as cancers[20], ischemia/reperfusion[21], neurodegenerative[22] and chronic inflammatory diseases[23].

$$NO\bullet \longrightarrow e- + NO^+ \text{ (nitrosyl cation) (equation 13)}$$

$$NO\bullet + e- \longrightarrow NO^- \text{(nitroxyl anion) (equation 14)}$$

Peroxynitrite (OONO–): When free radicals react together, there can also be a net loss of radicals. Radical–radical termination reactions are often beneficial by disposing of reactive radicals. However, this is not always true; sometimes the products are dangerous. For example, peroxynitrite can be generated by the reaction of NO• and superoxide anion (equation 15). Peroxynitrite is highly produced under

inflammatory[24] and ischemia/reperfusion condition[21]. Peroxynitrite (OONO−) is potent cytotoxic oxidative agent that could rapid oxidize protein, thios, lipid and DNA base and causes tissue injury.

$$O_2^{-\bullet} + NO\bullet \longrightarrow ONOO\text{- (equation 15)}$$
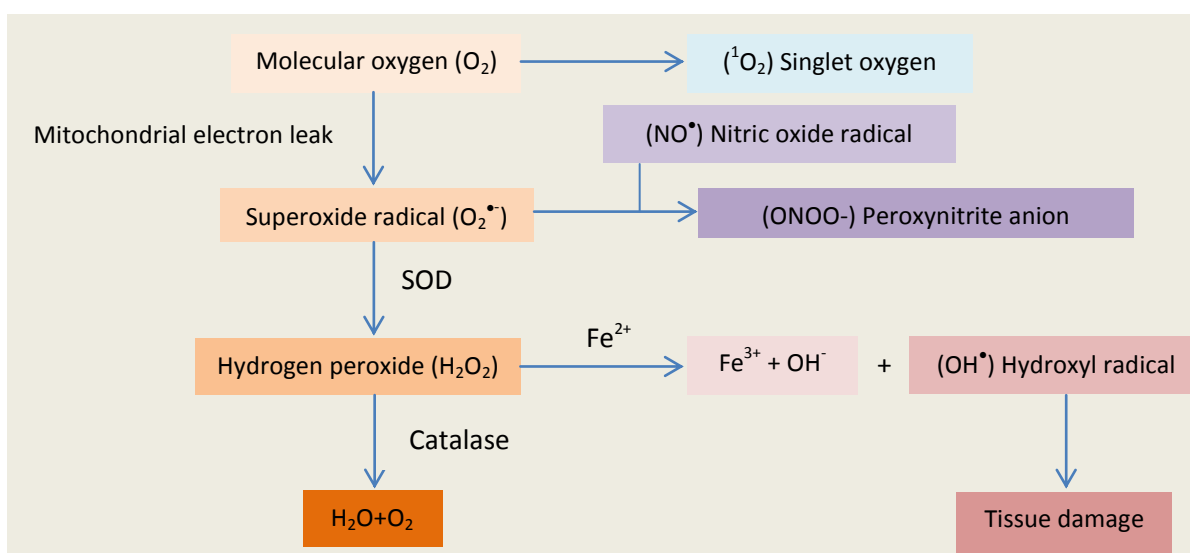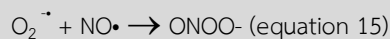
Figure 1.2: **Free radicals generation cascade.** Some free radicals arise normally during metabolism. These free radicals are reduced into water with the cooperation of the three main antioxidant enzymes: SOD, Catalase, and GPx. Metal induced generation of hydroxyl radicals from hydrogen peroxide in produces the development of oxidative cellular components and leads to cellular death.

1.3 Free radical sources

Free radicals represent the crucial part for aerobic life. Indeed, not all free radicals are bad. Free radicals are necessary for produce vital energy and various substances that the body requires. They are continuously produced in all cells, normally used for maintaining physiological functions[19]. Basically, they are naturally generated via enzymatic and non-enzymatic reaction as a result of the biochemical processes (during respiratory chain reaction and biosynthetic of complex biochemical compounds such as prostaglandin or hormones), the phagocytosis, cytochrome P450 system and oxidative phosphorylation in the mitochondria. Moreover, the body have evolved a defend system that uses ROS against invading infectious agents[19]. These ROS are generated by enzymes such as NADPH-oxidase, xanthine oxidase, and lipoxygenase[25] and multiplied by the catalytic role of transition metal ion-catalyzed redox reactions. At low or moderate-generating concentration, some of the reactive species plays beneficial physiological role *in vivo* this include function as an intracellular mediator in many molecular events. They are involve in energy production, cell growth, driven normal physiological activities, maturation process of cell structures, function in different cellular signaling mechanisms, induction of the immune system and activating enzymes that are needed for life.

Free radicals are normally present in the body in a tiny amount. Under normal circumstances the body can keep them in check. But when the body produces more free radicals beyond the threshold for normal physiological function in pathophysiologic condition by exposure to certain risk factors[26] can responsible for tissue injury[11]. Overproduction of the free radicals are found intracellularly and extracellularly and may be produced endogenously or arise from exogenous sources.

Important sources of endogenous reactive species include intracellular generated from activate immune cell, inflammation, infection, ischemia, mental stress, excessive exercise, cancer, aging, the catalyze and/or auto-oxidation of some drugs, pollutants, other chemicals and toxins by pro-oxidative enzyme systems[3]. Recent study found that hepatic CYPs450 were shown to contribute significantly to the total cellular production of ROS in rat liver[27]. Many evidences suggest that major source of cellular ROS production is mitochondrial respiration[28]. Because of more than 90% of the oxygen in aerobic living organisms are consumed by mitochondria and its contents varies metabolic processes that could already generate free radical fluxes. Approximately 1%-5% of the molecular oxygen consumed during normal physiological respiration is reduced and converted into superoxide radicals[29]. Exogenous sources of free radicals are result from environment pollutants, smoke, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), organic solvents, absorption of radiation (ionizing, sunlight ultraviolet [UV], visible, or thermal) anesthetics and pesticides[11,19]. ROS can also be formed in cooking food particularly smoked meat or used oil frying foods. Large numbers of free radicals produced within these foods through lipid oxidation and exposed to heat or light[30].
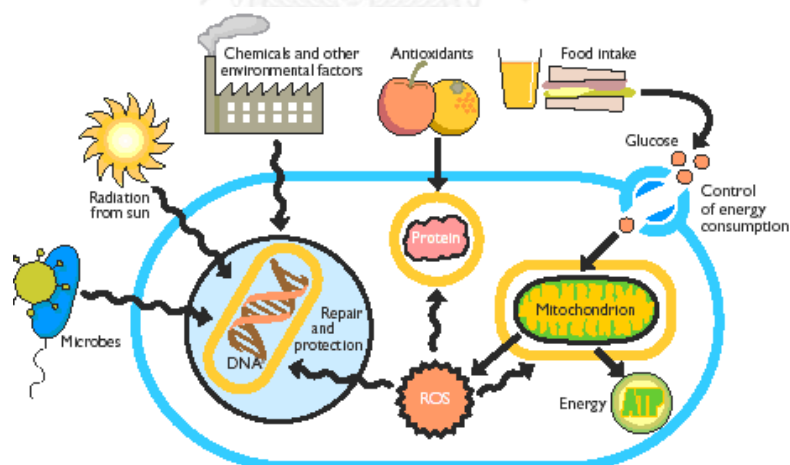
Figure 1.3: **Free radicals production and their adverse effect.** Free radicals attack cellular components involving DNA , protein and polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. The damage is consequently leading to decline in cellular functions as seen in the aging process as well. Photo from http://withfriendship.com/user/mithunss/oxidative-stress.php

1.4 The Chain reaction and Oxidative stress

Although each free radical may exist for only a tiny fraction of a second, but once formed, radical reactions are often sets up chain reactions[11], a series of self-propagation reactions leads to regenerates a

radical that can begin a new cycle of reactions. The damage it leaves behind can be irreversible. All chain reactions have three steps: 1. Chain initiation step: formation of radicals. 2. Chain propagation step: free radicals have the capacity to react with other molecules resulting in repeatedly regenerated free radicals 3. Chain termination step: destruction of radicals[11].

Free radicals could attack any site of cellular constituents then start the chain reaction. Thus, lead to detrimental to the integrity of cellular structure and functions. Disruption in cellular homeostasis and accumulation of damaged molecules[31]. ROS–induced oxidation of proteins can lead to changes in the protein's three-dimensional structure, their conformation as well as to fragmentation, aggregation, or cross-linking of the proteins[32]. Studies indicated that the side chains of all amino acid residues of proteins, particularly cysteine and methionine are susceptibility to oxidative reaction produce from free radicals[11]. ROS also induced lipid peroxidation, particularly the polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation, destroys cell membrane, organelles structures. Damaging cell membranes lead to retention of fluid in cells release intracellular components that leading to further cell death. Moreover, oxidative damage to carbohydrates can alter any of the cellular receptor function including those associated with hormonal and neurotransmitter responses[33]. In addition to damaging cell structure, free radicals can destroy genetic material. The free radicals cause DNA fragmentation and modulation of gene expression, leading to faulty translation of genetic material[34]. Eventually, ROS are leading to the disruption of cellular redox signal transduction networks. Modulation of signaling pathways such as MAPKs, PI3Ks and the subsequent activation of downstream redox sensitive transcription factors like Nrf-2, NF-kB, HIF-1, AP-1 results in alterations in gene and protein expression that significantly found in neurodegerative disease[35], chronic metabolic disease[36] and cancers[37]. Also, Peroxynitrite are found to involve in PARP activation of several cell types[38]. Excess ROS generation that considered being the potential agents that cause biological damage are termed oxidative stress and nitrosative stress[39]. Sies[40] has been proposed in 1991 that oxidative stress is a harmful condition causing on mostly by serious imbalance exists between reactive species (ROS/RNS) production and the antioxidant defense which tipped in the production of free radical overwhelms antioxidant defenses. This may cause tissue damage in different pathophysiological conditions that lead to causes diseases.

The idea about antioxidants would scavenge these free radicals and restore redox cellular status are behind antioxidant supplementation research, however, many reports shown that the intervention with antioxidant supplements did not show the results expected[41,42]. Some studies have indicated that antioxidants supplements could also have deleterious effects on human health depending on dosage and bioavailability[43]. Most pathologies are multi-factorial and oxidative stress is just one of many contributing factors and the mechanism of antioxidants action is more complex than just intercepting reactive free radicals[44,45]. In most human diseases, oxidative stress might be a secondary phenomenon, not the primary cause of the diseases[46]. Damaged tissues undergo more free radical reactions than healthy ones. Therefore, a new concept of oxidative stress was emerging, not limited to free radical damage of the macromolecular machinery but to perturbation of cellular redox status[47]. Jones re-

defined oxidative stress in 2007 as "a disruption in redox signaling and control"[48] means that redox cellular network is finely regulated and its perturbation provokes oxidative stress[47].
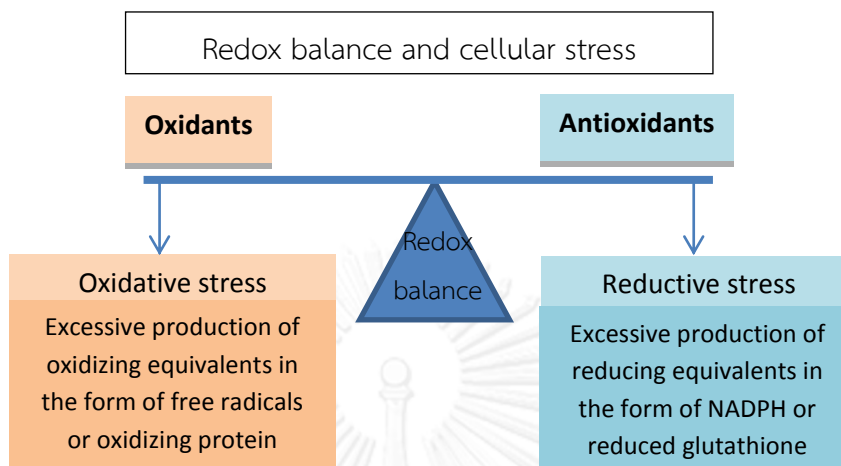
Figure 1.4: **Balance between oxidant and antioxidant molecules.** Cell functions can be altered by the buildup of oxidizing equivalents or reducing equivalents within a cell.

1.5 Oxidative stress and human health

Oxidative stress results in a series of events which deregulate the cellular functions as well as impacting on the signal transduction systems and plays a key role in the etiology and development of several pathologies both acute and a chronic nature. Oxidative stress is currently suggested it is implicated in the pathogenesis of over 200 clinical conditions[4,11] including:

♦ Cardiovascular malfunction[49] such as atherosclerosis, ischemic heart disease, cardiac hypertrophy, hypertension, shock and trauma

♦ Pulmonary disorders[50] such as asthma and chronic obstructive pulmonary disease

♦ Neurodegenerative disorders[51,52,53] such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, memory loss and depression.

♦ Metabolic diseases[54,55] such as diabetes and diabetic complications, liver disease, pancreatitis

♦ Immune dysfunction[56] such as rheumatoid arthritis, Immuno-depression

♦ Renal disorders[57] such as glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria, uremia

♦ Gastrointestinal diseases[58] such as peptic ulcer, inflammatory bowel disease and colitis.

♦ Infectious disease such as influenza[59] and HIV infection[60].

♦ Many types of cancer[61,62] such as liver cancer, lung cancer, leukemia, breast, ovary, colon cancer, etc.

♦ Ageing process and other age related diseases.

1.6 Oxidative stress biomarker

The use of oxidative stress biomarkers may help establish pathogenic stages of disease and provide further evidence of a causal relationship between these pathologic condition and oxidative damage[63]. Thus, wide variety of functional assays both *in vivo* and *ex vivo* are performed including:

1. Measurement of diminished levels of antioxidants defense enzymes such as CuZnSOD, MnSOD, catalase and glutathione peroxidase [64].

2. To determine the increasing production of reactive species such as the metabolized xenobiotic to generate reactive species (e.g., paraquat or cadmium)[65], or excessive activation phagocytes in chronic inflammatory diseases[66].

3. To examined the damaged macromolecules and tissue injury. This can involve damage to any or all molecular targets: lipids, DNA, carbohydrates, nucleotide and proteins[67]. Measurement of DNA oxidation (oxidized DNA bases such as 8-OHdG, autoantibodies to oxidized DNA, comet assay), lipid oxidation (thiobarbituric acid-reactive substances, exhaled pentane/ethane, low-density lipoprotein resistance to oxidation, isoprostanes, hemolysis), and protein oxidation (protein carbonyls, AGEs, Hienz's body formation) are the most general biomarker used because they are early targets of damage.

4. Detection of adaptation response of the cells against damage such as enhancing cell tolerance against the forthcoming induced oxidative insults[68].

5. Detection of cellular pathology such as the rupture of membrane blebs, autophagy formation [69].

Table 1.2: Biomarkers of oxidative damage associated with some human diseases[67]

Diseases	Biomarker
Diabetes mellitus	MDA, GSH/GSSG ratio, F2-isoprostanes, AGE, NO2-Tyr
Atherosclerosis	MDA, HNE, F2-isoprostanes, NO2-Tyr, Acrolein
Cardiovascular disease	HNE, GSH/GSSG ratio, F2-isoprostanes, AGE, NO2-Tyr, Acrolein
Alzheimer's disease	MDA, HNE, GSH/GSSG ratio, F2-isoprostanes, NO2-Tyr
Parkinson's disease	HNE, GSH/GSSG ratio, Carbonylated proteins, Iron level
Rheumatoid arthritis	F2-isoprostanes, GSH/GSSG ratio
Cancer	MDA, GSH/GSSG ratio, NO2-Tyr, 8-OH-dG

Abbreviation: 8-OH-dG= 8-hydroxy-20-deoxyguanosine; AGE= advanced glycation end productions; HNE= 4-hydroxyl-2-nonenal; GSH= reduced glutathione; GSSG= oxidized glutathione; MDA= malondialdehyde; NO_2-Tyr= 3-nitro-tyrosine.
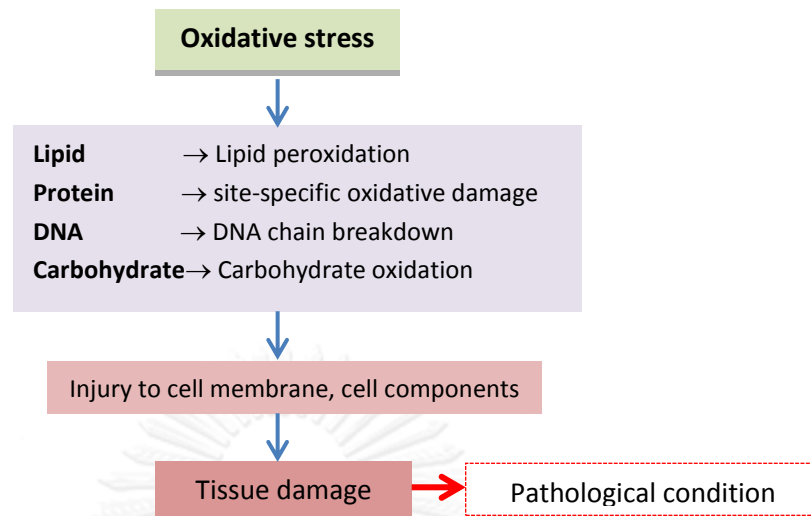
Figure 1.5: **Pathogenesis of tissue damage from oxidative stress.** Stressors induce breakdown of the cellular components, and subsequent deleterious oxidative stress damage. This results in a alterations in normal cellular function.

2. Anti-oxidants

In order to defend ourselves against the harmful effects of the free radicals and to maintain redox homeostasis, The body has several mechanisms to keep free radicals in check through an antioxidant network to keep free radicals in check and neutralize overwhelming production of reactive intermediates by action of free radical scavengers term 'antioxidants'. The antioxidant activity can be effective through various ways: The primary mechanism which the antioxidants could get rid of free radicals is though donation of electrons to oxygen species. Thus, reactive species transformed into non-radical species stable products, while the antioxidants become 'antioxidant-radicals', which are much less reactive[70]. Next, antioxidants may act as suppressor of free radical oxidation reactions by chelating metal ions (preventive oxidants) or served as chain breaking antioxidants by interrupting the propagation of the autoxidation and terminating the chain reaction. Finally, antioxidants act as inhibitors of pro-oxidative enzymes such as cyclooxygenases, clearing the damage out of the cells. Some antioxidants were reported that they could induce the biosynthesis of other antioxidants[71,72]. The human antioxidant system encompasses a vast array of substances which are classified into two major groups, enzymatic antioxidants and non-enzymatic oxidants. Either naturally generated in situ (endogenous), or externally supplied through foods (exogenous)[73]. These include protection afforded by:

◆ Endogenous enzymatic antioxidants, certain enzymes serve this vital function: Intracellular efficient enzymatic antioxidants involve catalase (CAT), glutathione reductase (GRx), glutathione peroxidase (GPx), and superoxide dismutase (SOD).

◆ Non enzymatic antioxidants: metabolic and nutrient antioxidants.

a. Metabolic antioxidants (endogenous): glutathione, L-arginine, CoQ10, melatonin, uric acid, transferring protein, metal binding proteins such as albumin, ceruloplasmin, ferritin, and lactoferrin.

b. Nutrient antioxidants (exogenous): carotenoids, vitamin C, vitamin E, omega 3 and omega 6 fatty acids, trace elements (selenium, zinc, manganese), phytoconstituents and phytonutrients.
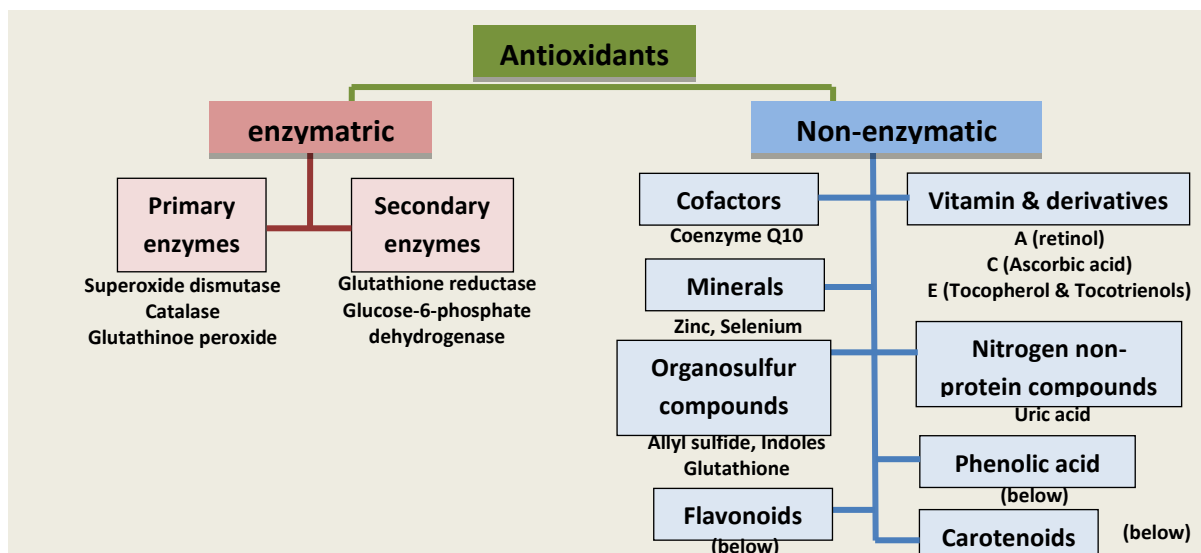
Figure 1.6: **Enzymatic and non-enzymatic classification of antioxidants.** Adapted from Pietta et al[74].

2.1 Enzymatic antioxidants[73]

The cells contain important antioxidant enzymes defense systems against free radicals that play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. Regarding enzymatic antioxidants they are divided into primary and secondary enzymatic defenses. About the primary defense, three of them are the main enzymatic scavengers responsible for prevention the formation or neutralization free radicals are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). SOD acts as the first line defense system against ROS particularly superoxide radicals. SOD catalyzes the dismutation of superoxide to hydrogen peroxide and prevents further generation of free radicals. There are two types of SOD, each of these enzymes works to protect a particular part of the cell including: copper/zinc SOD (Cu/Zn-SOD) and manganese SOD (Mn-SOD). Cu/Zn-SOD protects the cytoplasm, where free radicals are produced as a result of various cellular activities. Mn-SOD belongs to the mitochondria, protecting the mitochondria genetic information and maintenance cellular energy production process. Hydrogen peroxide from the dismutation reaction is subtracted for catalase and glutathione peroxidase. Both enzymes catalyze the formation of H_2O_2 and detoxify it to water and molecular oxygen. Each of these enzymes works to protect a particular part of the cell, GPx presents in the cytoplasm and catalase in the mitochondria. GPx is responsible for eliminates of peroxides as potential substrate for the Fenton reaction and could remove other peroxides such as hydroperoxides (ROO-) by coupling its reduction to H_2O with oxidation of GSH. The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Their functions are not to neutralize free radicals directly, but supporting the other endogenous antioxidants by regeneration of co-enzyme. Glutathione reductase regenerates glutathione (GSH) from its oxidized form (GSSG) to its reduced form (GSH), in the presence of NADPH. Thus it's recycling GSH supported the continuously

neutralizing more free radicals by GPx. NADPH is regenerated by glucose-6-phosphate creating a reducing environment.

Table 1.3: Different types of free radicals and their defense system[73]

Types of free radical (or) oxidants	Defense system
Superoxide anion ($O_2^{\bullet}$)	Superoxide dismutase
Hydroxyl radical (OH●)	SOD, Mn-SOD, Cu/Zn-SOD, glutathione
Peroxyl radical (ROO●)	Tocopherols, Ubiquinone
Singlet oxygen ($^1O_2$)	Carotenoid
Hydrogen peroxide (H_2O_2)	Catalase, Se Glutathione peroxidase
Hydroperoxides (ROO-)	Glutathione peroxidase, Glutathione reductase
Transition Metals (Fe^{3+}, Cu^{2+})	Metal chelators

2.2 Non-enzymatic antioxidants

Considering the non-enzymatic antioxidants, there are quite a number of them. Some endogenously produced such as cofactors of enzymes, nitrogen compounds, and peptides [75] and some provided through dietary intake (vitamins, mineral and phytochemicals). These non-enzymatic antioxidants also can be classified into metabolic and nutrient antioxidants. Metabolic antioxidants are the endogenous antioxidants that produced by metabolism in the body. While nutrient antioxidants cannot be produced in the body but externally supply to the body through diet. Mostly nutrient antioxidants are belonging to exogenous antioxidants.

The metabolic antioxidants such as lipoic acid, glutathione, NADH, carnosine, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin etc. These antioxidants are arrangement effort with each other as the network to protect cells and organ systems against free-radical-damage. The most abundant metabolic antioxidants in our body are GSH, CoQ10 and uric acid. GSH is a tripeptide and the major non-protein thiol compound (NPSH) present within the cytosol. GSH protects the cells against free radicals either by donating a hydrogen atom or an electron from its SH groups. GSH is capable of scavenging ROS directly or enzymatically via GPx and regeneration of other antioxidants such as vitamin C[76]. Coenzyme Q10 is co-factor in the cellular metabolism and presents in the membranes. Coenzyme Q10 has ability to prevent lipid peroxidation[77] and regenerate vitamin E through vitamin C[78]. Uric acid is the byproduct of purine nucleotide metabolism. Evidence shown that it has ability to prevent the overproduction of oxo-hem oxidants that resulting from the reaction of hemoglobin with peroxides[79]. Uric acid also prevents hemolysis by attenuated the peroxidation reaction and acted as potent scavenger of hydroxyl radicals and singlet oxygen[80].
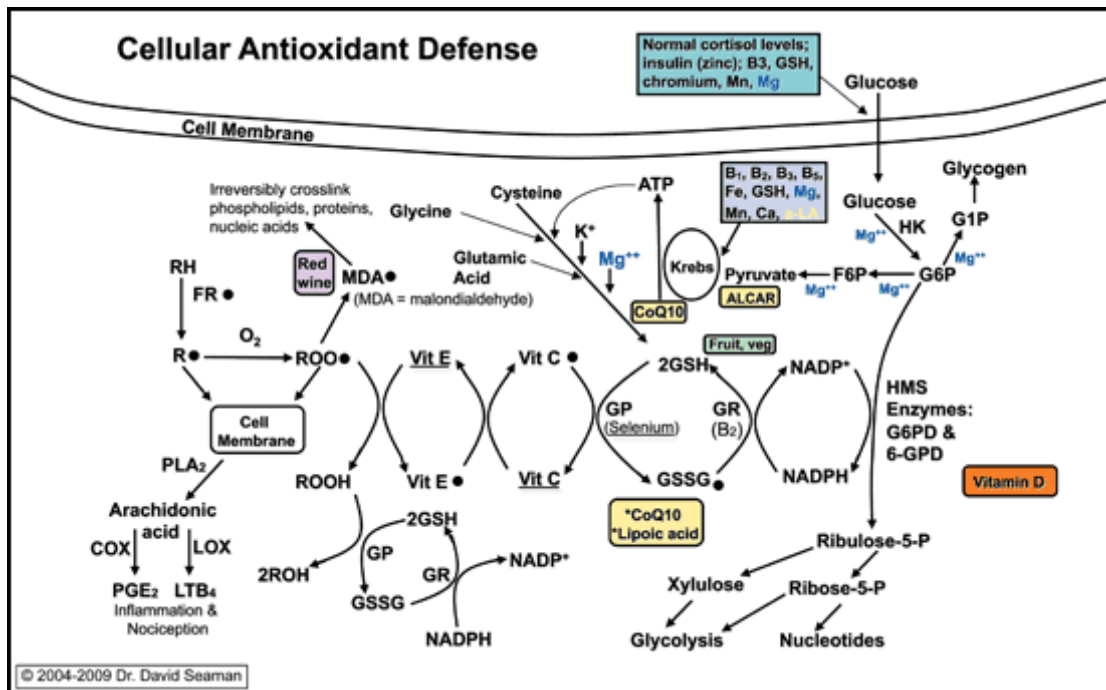
Figure 1.7: **cellular antioxidant defense system**. The body has developed several endogenous antioxidant defense systems to fight against oxidative stress. 1) The enzymatic defense system includes different endogenous enzymes such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD). 2) Non enzymatic defense system included glutathione (GSH), vitamin E, vitamin C and vitamin D. Photo from http://www.dynamicchiropractic.com.

Both enzymatic and non-enzymatic antioxidants work synergistically, and in combination with each other to protect cells and organ systems against free-radical-damage. However, these endogenous antioxidant defenses are not 100% efficient. Under oxidative stress, endogenous antioxidants may not be sufficient, then dietary antioxidants can help to prevent, diminish the cumulative effects of oxidative damage on cellular components[81], and may be required to maintain optimal cellular functions. Vitamin E and C are the major nutrient antioxidants exist within normal cells as well as they can be supplied through diet[11].

Vitamins C and E are generic names for ascorbic acid and tocopherols. They are non-enzymatic endogenous antioxidants that play a synergistic behavior with each other to neutralize free radicals. Vitamin E quenches radicals and turns them into less active than the radicals form. Then, vitamin C reduced tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential of vitamin E[82]. They break radical chain reactions by trapping peroxyl radicals found in plasma, red cells and tissues, allowing them for protection the integrity of membranes structures, prevention against many cancers, cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders[83]. Vitamin C is the potent scavenger for hydroxyl radicals, hydrogen peroxide, singlet oxygen, superoxide anions, and reactive nitrogen oxide[82]. Two forms of them have efficient antioxidant activity: L-ascorbic acid and L-dehydroascorbic acid which are absorbed through the gastrointestinal tract. Vitamin E has high antioxidant potency. It is composed of eight stereo-isomers: with four tocopherols (α, β, γ, δ tocopherol)

and four tocotrienols (α, β, γ, δ tocotrienol). Alpha-tocopherol is the most potent and abundant stereo-isomer in biological systems. It safeguards cell membranes from lipid peroxidation damage by donating its phenolic hydrogen to the peroxyl radicals forming tocopheroxyl radicals that are less reactive and unable to continue the chain reaction. Other important carotenoid is β-carotene, it is considered as pro-vitamin as it can be converted into vitamin A by the liver. It is the best quencher of singlet oxygen and known to have beneficial impacts on the body[82]. Selenium (Se) is a trace mineral that forms the active site of several antioxidant enzymes including glutathione peroxidase. And, essential long-chain polyunsaturated fatty acids which are found in fat fish or flex-seed, omega-3- and omega-6 fatty acids.
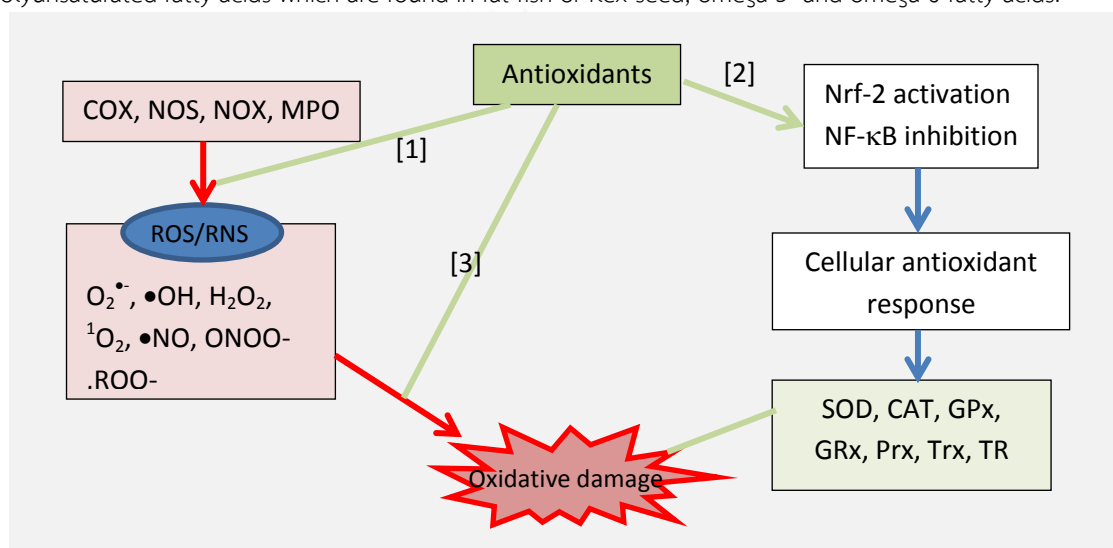
Figure 1.8: **Summary of action of antioxidants.** Three different mechanisms [1] inhibition of oxidant enzymes, that involve in the cellular free radical generation such as NOS (nitric oxide synthase), NOX (NADPH oxidase), COX (cyclooxygenase), MPO (myeloperoxidase), [2] interaction with redox signaling pathways that initiated cellular antioxidant response to the insult such as enhance gene and protein expression of antioxidant enzymes: CAT (catalase), SOD (superoxide dismutase), Prx (peroxiredoxin), GPx (Glutathione peroxidase), Trx (thioredoxin), TR (thioredoxin reductase, GRx (glutathione reductase), [3] direct interaction with free radicals result in formation of less toxic/reactive byproduct.

2.3 Phytochemical antioxidants

Plant resources are not just providing the basic needs of life such as food, fuel, and shelter. For thousands of years, Plants have been used for therapeutic purposes in traditional and folk medicine around the world and continue to provide new remedies to mankind. The Ayurveda, an ancient Indian system of medical description about the treatment is derived from medicinal plants. Medicinal plants have been reported to provide a large number of phytoconstituents which are important sources of antioxidants and capable to reduce the incidence of many diseases related to oxidative stress[84].The free radicals neutralizing property of several plants was reported by previous studies[85,86,87]. And several authors have reviewed the beneficial uses of these plant species[88]. Nowadays, herbal 'renaissance' is happening all over the world because of its better compatibility with the human body, safety and lesser side effects. The potential health beneficial effects of vegetable or fruit-rich diet have been account by many epidemiological studies[89]. For example, consumption of fruits and vegetables rich in antioxidants

may decrease or delay incidence of chronic degenerative diseases such as retard the progression of Alzheimer's disease[90,91]. These attributed to the specific ingredients such as vitamins, dietary fiber, carotenoids, minerals and other bioactive compounds[92]. Other than the healthy promoting micronutrients, plants also contain bioactive non-nutrient substances called phytochemicals. Phytochemicals are secondary plant metabolites that play central role in the interaction between the plant and its environment. Several thousand phenolic compounds occur widely throughout the plant kingdom and present in almost plant materials. All among of these phytochemicals, phenolic acids and flavonoids are the most important[93].
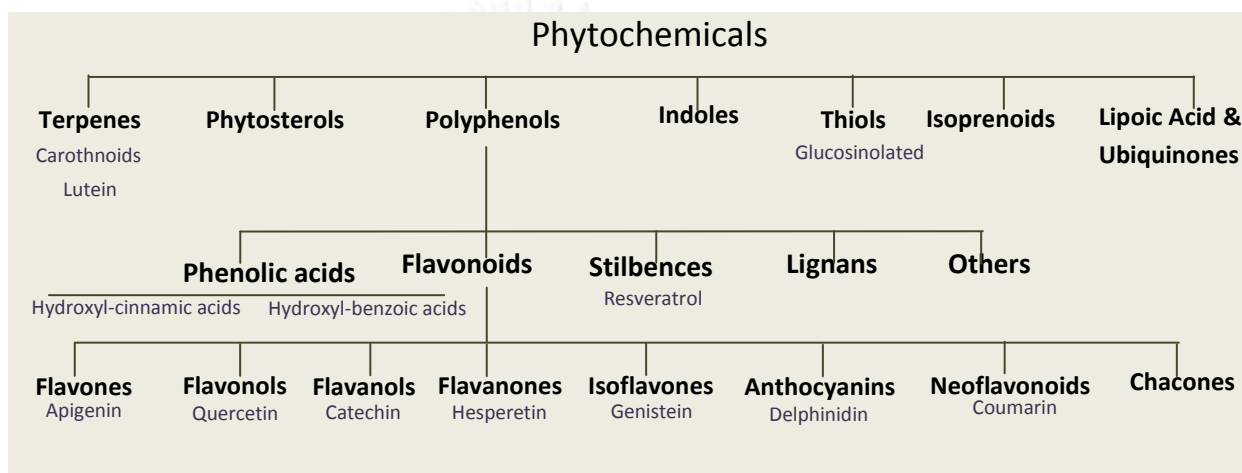
Figure 1.9: **Major classes of phytochemicals with antioxidant activity.**

Polyphenols represent one of the most numerous and ubiquitously groups of secondary metabolites present in fruits and vegetables and are part of human diet. Natural polyphenols could range from simple structure such as quinones or phenolic acids, to highly polymerized complex structures such as lignins or tannins, with theses, flavonoids representing the most common and widely distributed sub-group[94]. In addition, nowadays, they have received much attention because of their potential health benefits and rarely toxic[95]. They are well-known to act as a potent antioxidants[96] and have been shown to possess pharmacological effects on human health such as anti-inflammation, anti-toxic, antibiotic, anti-carcinogenic, anti-proliferative, anti-viral infection, improved metabolic function, and cytoprotective effects[97]. Several studies demonstrated that flavonoids and polyphenolics act by shifting the cellular redox potential to a more reduced state, which consequence modulated a wide range of cell signaling pathways. Indeed, it is generally assumed that these bioactive compounds are the primary candidates to be claimed as protective effects of medicinal plants.

Phenolic acids can be distinguished into two different classes, derivatives of benzoic acid, hydroxybenzoic acids (HBA) and derivatives of cinnamic acid, hydroxycinnamic acids (HCA). Examples of phenolic acids are caffeic acid, ferulic acid, gallic acid and chlorogenic acid. Moreover, complex forms of hydroxybenzoic acids such as hydrolyzable tannins were reported in many fruits such as gallotannins in green teas, red wine[98] and ellagitannins in red fruit such as berries, and pomegranates[99]. Phenolic acids are widely represented in fruits, usually present as esters and glycosides and can act as endogenous

precursors for many of the other phenolic molecules found in plants[100, 101]. Their distribution may vary ubiquitous to plant materials such as HBA found abundant in only a few red fruits, black radish, and onions eaten by humans. Tea is considered to be the important source of gallic acid[102] with turn to be one of the most studied and promising polyphenol compounds. Phenolic acids are of great interest to man because they have antioxidant activity as free radical scavengers or metal chelators with special impact over superoxide anions[103], hydroxyl and peroxyl radicals[104], and hydrogen peroxide[105]. A large number of reviews have been published concerning their antioxidants and roles[106,107].

Another polyphenolic compounds which are present in most plants are flavonoids. Flavonoids are found generally in fruits and vegetables as well as in food products and beverages derived from plants such as olive oil, tea, and red wine. Over 4000 different flavonoids have been discovered, and the number is continually increasing[108]. These are classified into flavanols, flavonols, flavanones, flavones, iso-flavones, anthocyanins, and proanthocyanidins. These entire compounds share the common diphenylpropane (C_6-C_3-C_6) skeleton i.e. two benzene rings (A and B) are linked through a heterocyclic pyran or pyrone ring (C) in the middle. Like phenolic acid, flavonoid possess potent antioxidant activity[74, 109], effective scavengers of reactive oxygen species[74, 109], activate antioxidant enzymes[110], inhibit oxidases[111], mitigate nitrosative stress[112], and even as metal chelators[113]. Several flavonoids had been reported about their anti-inflammatory, anti-toxic, anti-tumour, anti-microbial and anti-viral properties[114]. Flavonoids are considered to be the active ingredients in medicinal plants[115]. They have been extensively investigated because flavonoid content in fruits and vegetables are very high as 300 mg/kg fresh weight[116] and humans often consume between 20 and 80 mg flavonoids per day[117] which are higher than that for vitamin E[109]. Studies show that ingested flavonoids enter the blood circulation to enhancing the body's antioxidant defenses system or to maintenance the redox homeostasis[118]. Suggested that the effects of flavonoids may be physiologically significant[119], possible to reduce the risk of chronic diseases and prevent disease progression. Flavonoids can be classified according to their biosynthetic origins. The major classes of flavonoids, with specific examples, are summarized below.
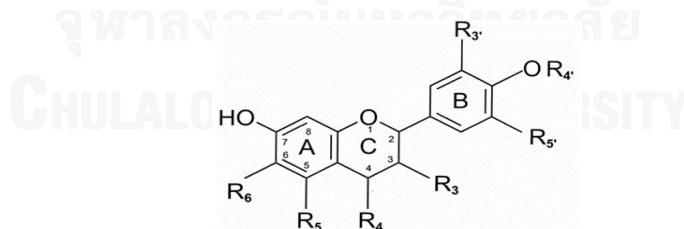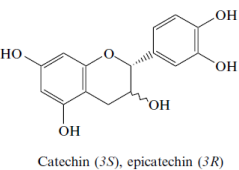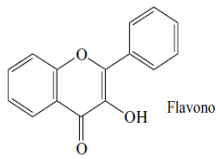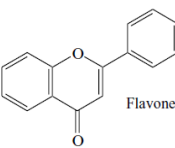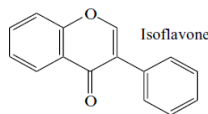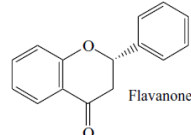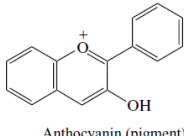
Figure1.10: **Flavonoid structure**. Photo from http://www.lurj.org/vol3n2/flavonoids.

Table1.4: The main flavonoid classes and examples of common dietary flavonoids.

Flavonoid subgroup	Representative flavonoid	Food source	Flavonoid subgroup	Representative flavonoid	Food source
Flavanols Catechin (3S), epicatechin (3R)	EGCG Epigallocatechin Catechin Epicatechin	Chocolate, beans, tea, red wine, apple, cherry, apricot	Flavonols Flavonol	Quercetin Myricetin Kaempherol Rutin	Onion, apple, cherry, broccoli, tomato, berries, tea, red wine, leek
Flavones Flavone	Luteolin Apigenin Chrysin	Capsicum pepper, thyme, Celery, Parsley	Isoflavonoids Isoflavone	Genistein Daidzein	Soya beans, legumes
Flavanones Flavanone	Naringenin Hesperidin Eriodictyol	Orange, Grapefruit	Anthocyanidins Anthocyanin (pigment)	Pelargonidin Cyanidin Malvidin	Rhubarb, cherry, stawberry, red wine, black rice, red rice

In human foods, most dietary flavonoids generally occur as glycosylated and sulfated derivatives. Plants often contain variety flavonoids between 5 to 10 different glycosides forms[120]. Flavanones are present in high concentrations in citrus fruit but also found in tomatoes and certain aromatic plants such as mint. Isoflavones are flavonoids with structural similarities to estrogens and has ability to bind to estrogen receptors which consequently classified as phytoestrogens. Isoflavones are found almost exclusively in leguminous plants such as soy. Flavanols are found in many types of fruits, red wine but green tea and chocolate are by far the richest sources. They exist in 2 forms: the monomer forms (catechins) and the polymer forms (proanthocyanidins). Anthocyanins are pink, red, blue, or purple pigments of flowers and fruit. In the human diet, anthocyanins are found in red wine, cereals and leafy and root vegetables such as cabbage, beans, onions, radishes, but they are most abundant in fruits. Flavones are much less common than other flavonoids in fruits and vegetables. Parsley and celery are only edible sources of flavones that had been report. However, the therapeutic value of most flavonoid medicinal plants rests are not on the one flavonoid fraction alone, but on a complex mixture of chemically different compounds. This aspect is common to many phytomedicines, whose activity cannot be assigned to specific constituents, since other components many either directly contribute or play the adjuvant role which strengthens the action of the active principles[118,121].

2.4 Phytochemical and their non-antioxidant effect on human health

Although, The antioxidant properties of flavonoids present in fruits and vegetables are thought to contribute to their preventative effect against many diseases[114]. But bioavailability of most dietary antioxidants generally reported to be relatively low. Bioavailability basically refers to the amount of a given nutrient which is available for normal physiological functions and storage within the body[122]. However, many natural antioxidants still have beneficial impact on the general public. Besides quenching ROS generation/propagation and interfere with cellular redox homeostasis and disturbed cell signal transduction, another effect of "antioxidant compounds" has recently emerged that is independent of antioxidant properties i.e. direct chemical interaction of the "antioxidant" with signaling enzymes and transcription factors[123]. As a result studies have been developed and focus to investigate effects of bioactive plant food components which are not related to traditional direct antioxidant action.

2.5.1. Effects on enzyme activity

2.5.1.1 Diabetes related enzyme: α-Amylase and α-glucosidase

Carbohydrates are normally converted into simple sugars which can be absorbed through the intestine. Digestive enzymes, especially alpha amylase and alpha glucosidase are the key enzymes responsible for carbohydrate hydrolysis[124]. Inhibitors of these enzymes systems preventing the digestion of carbohydrates such as starch, thus help to slow down glucose absorption rate. These enzyme inhibitors are used as oral anti diabetic drugs for treating type 2 diabetes mellitus. The presently used synthetic enzyme inhibitors caused gastrointestinal side effects such as diarrhea, flatulence, abdominal bloating etc[125]. Therefore α-amylase and α-glucosidase inhibitors from the natural resources are useful for treating postprandial hyperglycemia with minimal side effects. Plants are the potential source of these enzyme inhibitors. *Medicinal plants such as Lithocarpus polystachyus* Rehd[126], *Bergenia ciliata* Haw[127], *Acacia pennata*[128], some certain traditional Chinese herbs[129], guava leaves[130], or even cranberry-enriched cheese[131] had been report to be the effective enzymes inhibitors.

2.5.1.2 Cytochrome P450 Systems

CYP450 enzymes are key determinants in drug toxicities, reduced pharmacological effect and adverse drug reactions. Studies on the influence of natural antioxidants on CYP450 enzymes are discussed below. The effect of flavonoids on CYP450-dependent activity was found to link with their adverse effect and many herb-drug interactions. The extracts of *Acorus calamus*[132], *Kaempferia parviflora*[133], traditional remedy from Chinese medicine and Ayurveda medicine[134] are evidences to modulating CYP450 enzymes activity.

2.5.2 Antidotes for poisoning

Poisoning is used to describe the adverse toxic effects from substances – also called a toxin – (both natural and synthetic) produced[135]. Examples of some adverse effects are inflammation, cellular

dysfunctions, oxidative stress, enzyme inhibition, signaling interference, biochemical uncoupling, immune-interaction and neoplasmia. The nature and magnitude of a toxic effect of compounds depend on several factors such as their physicochemical properties, pharmacokinetic profile, exposure conditions required for their induction and the presence of defense mechanisms. For pharmacologically active and therapeutic agents, undesired toxic effects are described by different terms: side-effects, overdosage, intolerance, idiosyncrasy, secondary effects, and adverse drug interactions. Antidotes are the agents that limit or reverse the toxic effect of the toxin and exert their beneficial effects by a variety of mechanisms such as forming an inert complex with the toxin, accelerating toxin detoxification, attenuate the conversion of the toxin to a more potent compound, blocking certain essential receptors through which the toxic effects are mediated, and bypassing the effect of the toxin[136]. There are specific antidotes for only a small number of toxins and only few antidotes are employed in clinical practice[137]. Medicinal plants have been focus on searching for effective protections against these toxins.

2.5.2.1 Antidotes for alcohol toxicity

The isoflavonoids extract from *Pueraria lobata*[138] and *Radix puerariae*[139], herb long-used in traditional Chinese medicine, were shown inhibiting human aldehyde and alcohol dehydrogenase. Silymarin has been shown to have protective effects against ethanol *in vivo*[140] by stabilizing cellular membrane, preventing liver glutathione depletion and thus inhibiting lipid peroxidation induced by ethanol [141]. Therefore, these natural compounds represent a new class of compounds offering promise as safe and effective therapeutic agents for alcohol addiction and intoxication[142].

2.5.2.2 Antidotes for organic insecticides

Over 200 organic insecticides, aimed to kill insects without excessive toxic to humans and animals, are presently in use. Two of the largest-class of insecticides, act on the enzyme acetylcholinesterase (AChE) of nerve synapses, are organophosphate and carbamate. AChE is found primarily in the blood and neural synapses. Organophosphorus compounds react with active site serine residue of cholinesterase, thus overexposure to these insecticides can result in cholinesterase inhibition. Some symptoms of poisoning from cholinesterase inhibiting pesticides are excessive salivation, miosis (papillary constriction), muscle weakness, tachycardia, seizures, liver failure, coma and lethargy. *Satureja khuzestanica* (Lamiaceae) was the only herb that had been reported to increasing AChE activity and protect against toxicity of malathion, a commonly used organophosphorus[143].

2.5.2.3 Biological poisoning: poisonous animal and plants

The extract of *Aristolochia indica*[144], *Artemisia absinthium* L.[145], *Crinum jagus*[146], *Parkia biglobosa*[147], *Annona senegalensis* Pers.[148] had been shown to neutralize the effects of snake venoms and protect animal from death. Moreover, fruit of *Ziziphus jujuba* Mill. was shown the inhibitory effects against poisonous Euphorbiaceae plants on the inflammatory cells activation and protect the gastrointestinal tissue from irritant inflammatory injury[149].

3. Mitochondrial and cellular redox homeostasis
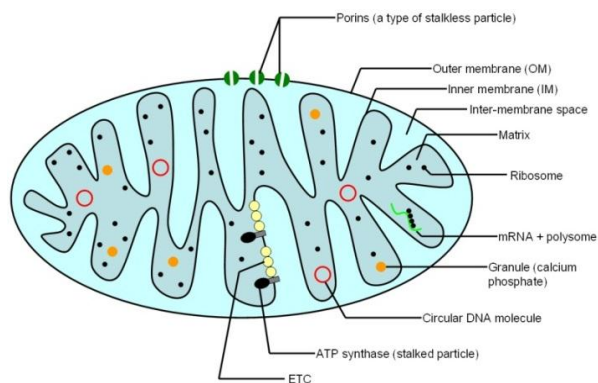
Figure 1.11: **Mitochondria structure.** Mitochondria consist of the matrix, an outer membrane, intermembrane space and an inner membrane. Electron transport occurs at inner membranes and produced ATP thought H^+ gradient between inner membrane and intermembrane space. Whereas the Krebs Cycle generally occur in the Matrix. Photo from http://cronodon.com/BioTech/Respiration.html

Mitochondria are unique dynamic organelles with highly complex organization. Mitochondria primary role is providing cells with energy in form of ATP, which is produced by the coupling of the ATP synthase with the electron transport chain. Mitochondria encompass the important metabolic pathways of Krebs cycle and fatty acid β-oxidation. Most cells contain hundreds of mitochondria[150]. Organs with high energetic requirements e.g. liver, heart, and muscle contain much more mitochondria to cope with their situation. Mitochondria generally consist of two compartments, the matrix, circumvented by the inner membrane, and the intermembrane space, which is delineated by the outer membrane. The inner membrane is folded forming so-called cristae, which clearly enlarge the surface, and is hardly permeable under physiological conditions. The protein complexes of the respiratory chain are embedded in this inner membrane. The outer membrane is much more permeable for low-molecular-weight solutes. This tightness of mitochondria membrane ensures the maintenance of the electrochemical gradient, comprising a membrane potential (negative inside) and a pH gradient (basic inside), which is the basis of the coupling of the respiratory chain to oxidative phosphorylation. In the other way, drawback of the production of ATP is the generation of reactive oxygen species (ROS). Major source of intracellular ROS are produced from the respiratory chain especially pronounced at complexes I and III. Even under ideal conditions, some electrons "leak" from the electron transport chain. These leaking electrons interact with oxygen to produce superoxide radicals, so that under physiological conditions, about 1-3% of the oxygen molecules in the mitochondria are converted into superoxide[151]. Thus, mitochondria have a central role in regulation the generation of reactive oxygen species, ion homeostasis and maintenance cellular redox potential[152]. Mitochondria are also involvement in mobilization of calcium as well[153,154].

Mitochondria dysfunction

Excessive production of these ROS from respiratory chain is leading to mitochondrial stress and dysfunction [155]. The primary site of oxygen radicals damage from superoxide radical is mitochondrial DNA (mtDNA)[156]. Oxidative damage modifies the genetic material permanently, and cell cannot fix mitochondrial DNA damage as much as it can fix nuclear DNA damage. Hence, extensive mtDNA damage accumulates over time and shuts down mitochondria. Oxidative stress also modulates mitochondria signaling pathways[150,155,156], finally induces a cellular redox imbalance. To date a number of calcium-regulated mitochondrial function have been identified that they may mediate the potentiating effect of calcium on metabolism[157,158]. Ca^{2+} generally, is important signaling mediator, regulates many intracellular processes including enzyme activity, gene transcription and apoptosis[159].The role of mitochondria on Ca^{2+} homeostasis was considered essential. Mitochondria can act as localized cytosolic calcium buffering organelles. The capacity of mitochondria to accumulate Ca^{2+} was enormous and could exceed 1,000 nmol/mg mitochondrial protein[160]. Upon accumulation, mitochondria could release calcium back to the cytosol via the antiporters[160], modulating several events of Ca^{2+} feedback inhibition or activation[158]. Within mitochondria itself, increase of mitochondrial calcium ($[Ca^{2+}]_m$) activates mitochondrial metabolism. Calcium increases the activity of several enzymes that have a crucial role on the Krebs cycle and ATP production, such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+-isocitrate dehydrogenase[161] as well as the ATP synthase[162].Thus, resulting in increasing the supply of ATP for a number of energy-consuming processes. Oxidative stress induced mitochondrial calcium handling defects[163]. The mitochondria Ca^{2+} dysregulation also sustained increase in cytosolic Ca^{2+} concentrations[164]. Raising cytosolic Ca^{2+} level triggers Bcl-2 family protein activation and open of the mitochondrial permeability transition (MPT) pore. Several molecules from the intermembrane space, including Apaf-1, cytochrome c, apoptosis inducing factor (AIF) are release[165] and initiate caspase cascade, intrinsic apoptosis pathway. Increasing lines of evidence pointed that perturbed cellular calcium (Ca^{2+}) homeostasis plays a key role in many degenerative diseases pathogenesis. Free radicals also increasing mitochondria dysfunction contributes to the decline of ATP production process as well as loose of mitochondrial integrity (usually used mitochondria membrane potential, $\Delta\psi_m$, as an indicator). Oxidative stress associated with the collapse of $\Delta\psi_m$[166]. Resulting in increase in $\Delta\psi_m$ then inhibits the electron transport through the cytochromes, thus leading to interruption of mitochondria respiration and to accumulation of NADH, swelling of the matrix, and, eventually, rupture of the outer mitochondria membrane[152]. However, in some instances, a mild insult occurring in the mitochondria, such as low levels of oxidative stress, results in the cell increasing a protective retrograde cellular response to manage the insult. The net outcome is actually beneficial to the cell, and termed this effect as "Mitohormesis"[167].
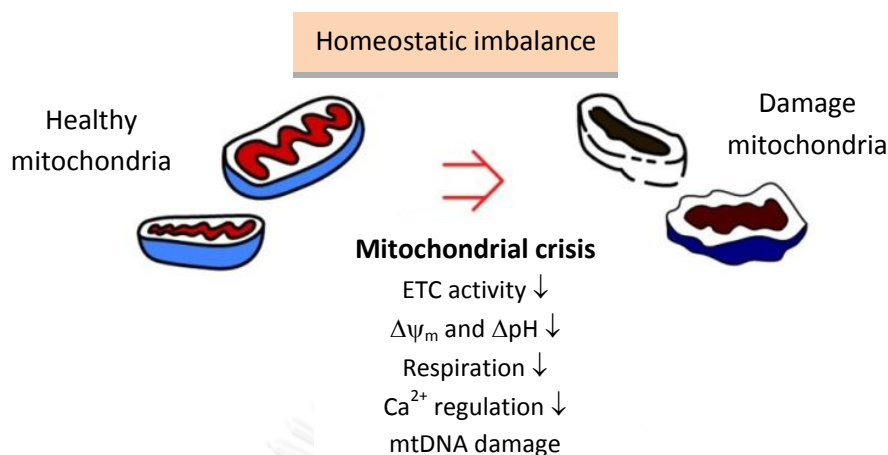
Figure 1.12: **Mitochondrial damage by ROS.** Oxidative stress exerts deleterious effects on mitochondria function by directly impairing mtDNA, mitochondria membrane potential and oxidative phosphorylation. As a result, decreasing ATP synthesis, increasing ROS production and cellular Ca^{2+} dysregulation were observe.

4. Adverse effect of herbal interaction

Unlike synthetic drugs, herbs are attractive because they are natural origin, cost-effective, easy to accessibility and had been long-time used as folk medicines. The usage of medicinal plants over the synthetic ones in the treatment of a wide range of acute and chronic conditions has accelerated in the last decade[168]. Many herbal products are categorized as dietary supplements and therefore are available in the market. Their regulatory requirements such as standard food safety tests and quality assurance along the manufacturing process are inadequately controlled and do not correspond as standards as conventional drugs[169]. However, herbal medicines are usually the mixtures of quite amount of pharmaceutically active ingredients, all effects of these plants are lying on the combination of several phytochemicals and they are not easy to denominate the actually side effect-causing constituent. The problem is that people often use herbal drugs as self-medication and take them not only as exclusive medication but also in combination with other – practitioner-prescribed – drugs. The concern has been raised about the potential of herbs to interfere with other chemicals. Although considered natural, most of the herbal medicine can interact or share the pharmacological mechanisms of action with already existing prescription or over-the-counter drugs[170] leading to either potentially dangerous side effects or decrease therapeutic benefits of the drugs[171]. Out of this, Studies usually focus on the efficacy and mechanism of action of botanicals, but the safety aspect has been overlooking because their pharmacokinetic have not been fully elucidated, might probably unrecognized and under-reported[169]. Herbal adverse effects after the consumption of herbal products situate the clinical pharmacologic interest in the safety and efficacy of such herbal remedies because of the realization that many people self-medicate using these agents[172]. Often, herbal toxicity is becoming known through case reports. Herbal toxicity is elicited only few is known and investigated so far. In these 5 years, numerous reported about herbal adverse effects[173] such as hemolytic anemia, chronic diarrhea, nephropathy, gastric upset and hypersensitivity reactions can be associated with their use[174]. Herbal use has been associated with

organ toxicity such as heart, liver, blood, kidneys, central nervous system, and skin[175,176]. The summary of adverse events of some herbal supplement is shown in table 1.5[177]. Table 1.6 indicates that potentially serious interactions exist between some common herbal remedies and widely used conventional pharmaceuticals. These several typical problems in connection with botanicals may arise due to adulteration, inappropriate formulations, and contamination than toxicity of themselves. Contaminations can occur unintentionally as well as deliberately, whereas adulterations are committed fraudulently. Examples for adulterants reported in traditional Chinese medicines are acetaminophen, caffeine, diazepam, amfepramone, sibutramine, midazolam, fluoxetine, sertraline, furosemide, chlortalidone, amiloride, and glibenclamide[178]. Misidentification, or a lack of understanding of herb-drug interactions or herbal-uses led to adverse effects that were sometimes life-threatening or lethal[179]. Recently, some of the investigations suggested that the modulation of CYPs450 mediated drug elimination is the major mechanism responsible for such types of interactions[180, 181].

Table 1.5: Possible adverse effects of some specific herbal supplements

Herbal agent	Organ	Most frequently reported adverse effects	Possible/more dangerous complications
Alfafa	Allergic/immunologic	Systemic lupus erythematosis	-
Burdock	Renal	Diuresis,hypoglycemia	-
Comfrey	Gastrointestinal	Hepatotoxicity	Hepatic insufficiency or fulminant hepatic failure
Dandelion	Gastrointestinal	Nausea/vomiting, Diarrhea, diuresis	-
Ephedra	Central Nerve system Cardiovascular	Seizures, psychosis, hypertension, coronary spasm, tachycardiaand coma	-
Garlic	Endocrinologic	Nausea/vomiting, Diarrhea, inhibit of iodine uptake	-
Ginkgo biloba	Allergic/immunologic Renal	Gastric upset, headache, dermatitis; sometime erythema, edema, papules, vesicles complicated by pruritus	Seizures, temporally related hemorrhages, hemorrhagic stroke
Ginsang	Central Nerve System Cardiovascular Endocrinologic system	Manai/euphoria, diarrhea, skin eruptions, nervousness, hypertension, edema, vaginal bleeding, hypertension, falsely elevated digoxin levels	Contains estrogen
Jimson weed	Central nerve system	Cholinergic toxicity	Contains atropine, scopolamine, hyoscyamine
Kava Kava	Central Nerve System	Dermatologic ("kava dermopathy"), neurologic, hallucinogenic, and liver dysfunction	Hepatitis, cirrhosis, and liver failure
Licorice	Renal	Hypertension, sodium and water retention, hyperkalemia	-
Propolis	Allergic/immunologic	Contact dermatitis	-
St. John's wort	Endocrinologic system	Photosensitivity, gastrointestinal complaints, dizziness, and headache	Serotonin syndrome
Salvia	Central nerve system Cardiovascular	Hypotension, sedative	-
Valerian	Central Nerve System	CNS sedation, Hallucinogenic,	Hepatotoxicity

Adapted from Biloba G. et al.[177]

Table 1.6: The Examples of drugs inducing herb-drug reaction

Herbal agent	Interacting drugs	Clinical effect
Betel nut	Flu	-
Danshen (salvia miltiorrhiza)	Warfarin	Bleeding
Dong quai	warfarin	Bleeding, Increase INR and widespread bruising
Eleuthero or Siberian ginseng	Digoxin	Increase blood levels
Ephedra	Caffeine, decongestants	Sympathomimetic toxidrome 9hypertension, tachycardia, CNS, CVS stimulation
Garlic	warfarin	Lowers blood levels, Increase INR
	Chlorpropamide	Hypoglycemia
Ginkgo biloba	Aspirin, clopidogral, dipyridamole, ticlopidine, warfarin, heparin	Bleeding
	Paracetamol,caffeine	Bilateral subdural haematoma
	Thiazide diuretic	Elevated blood pressure, hypertension
	Trazodone	coma
	Morphine	Lack of effect
Ginseng	Warfarin, ethanol	Lowers blood levels
	Phenelzine	Induces mania
	Alcohol	Increase alcohol clearance
Kava	Bezodiazepines, sedative-hypnotics	CNS depression
	levodopa	Increased "off" period
St. John's wort	Antidepressants	Serotonergic stimulation (theoretical)
	Cyclosporin	Decreased effect (cytochrome P450 inducer)
	Digoxin	Decreased serum level
Valerian	Anxiolyrics	CNS sedation

5. Herbal supplement should be consider as xenobiotics

5.1 Biotransformation

Xenobiotics are foreign compounds that are not nutrient (cannot be used to produced energy) either be natural in origin, or may have been synthetically produced by humans. Pharmacologic and recreational drugs are also xenobiotic compounds. The body is constantly taking up these xenobiotics from diet or through contact with the environment, via the skin and lungs. They are usually lipophilic in nature and hence tend to be poorly excreted. Many of these substances are toxic which, unless excreted, could accumulate to harmful levels. The body has many different types of detoxification or bioinactivation reactions for inactivating and facilitates the removal of toxic chemicals out of the body. However, most of these chemicals undergo metabolic conversion reactions *in vivo* through biotransformation. Biotransformation is biochemical process that is mediated by enzymes and result in the conversion of the parent chemicals to more polar and readily excretable metabolites. This process occur in all organs and tissues of an animal but mainly take place in the liver which highest concentrations of the xenobiotic metabolizing enzymes are found. The liver also synthesizes the precursors that are required for conjugation and inactivation reactions. For example, sulfate for sulfation

is used to clear steroid hormones from the circulation[182]. Many extrahepatic tissues, notably kidney, intestine and lung, however, also contribute significantly to xenobiotic metabolism.

Beside plays a significant role in energy metabolism and protein synthesis, Hepatocytes also contain large amounts of endoplasmic reticulum (ER). Some of the more important biotransformation enzymes are localized (incorporates) in these organelle's membranes, particularly the smooth endoplasmic reticulum (SER). In addition, hepatocytes are organized into plates separated by vascular channels or sinusoids. This structure is important in directing the excretion of the products of biotransformation away from the hepatocytes into bile and blood. Hepatocytes are highly expressed a suite of regulatory biotransformation nuclear receptors (FXR, PXR, CAR, PPAR, NR, Nf-ΚB and AHR), commonly called xenosensors[183,184,185,186].These orphan receptors are member of the nuclear receptors superfamily coordinate the expression of several genes encoding for drug and xenobiotic metabolism such as cytochrome P450 superfamily (CYP)[185]. They bind specific types of endogenous and exogenous molecules for example food components, drugs or natural compounds like flavonoids and thus initiate the modulation of biotransformation enzymes and the transporters.
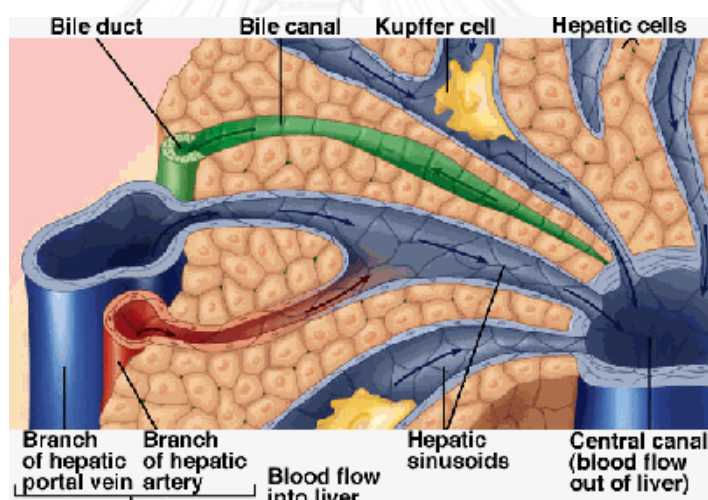
Figure 1.13: **Microscopic structure of the human liver.** Photo from http://www.daviddarling.info/encyclopedia/L/liver.html

Various food continents and plant-derived polyphenols treatment induced activity and nuclear binding of the transcription factors nuclear factor erythroid 2-related factor (Nrf)1 and Nrf2 to the antioxidant regulatory element (ARE) sequences contained in their promoters[187,188,189,190]. It has been reported that the Nrf2/ARE mediated expression of most CYP450[191] as well as other enzymes such as HO-1 and SOD[192]. Apart from the antioxidant capacity, these compounds appear to target detoxification system by impinging on Nrf/ARE pathway. Furthermore, polyphenolics also modulate activity of NF-ΚB or SIRT1 exerting hepatoprotective effects[193].

Oxidative stress, xenobiotic compounds Activates Oxidative Stress-Responsive transcription factor such as Nrf1, Nrf2, NFkB, ATF3 or FOXO in cytoplasm

Detoxifying, conjugating xenobiotic compounds & enhancing clearing toxicant out of the cell

Enzymes

Binding protein

Transcription factor

Transcription factor

Cytoplasm

Nucleus

Transcription factor

Antioxidant enzymes: e.g. HO-1
Biotransformation enzyme: e.g.
Phase I, II, and III enzymes

Gene expression

Figure 1.14: **The molecular mechanism of a regulatory transcription factor action in detoxifying and induce defense system against cell damage.**

The biotransformation pathways are classified into 3 categories: phase I (oxidation, reduction and hydrolysis), phase II (conjugation) and phase III (excretion)[194]. The biotransformation of xenobiotics is mediated by phase I and phase II. Phase I reactions are oxidation reactions that convert lipophilic xenobiotics to more chemically reactive molecules by introduce polar groups into the molecule through oxidative, hydrolytic and reductive processes[195]. The cytochrome P450 (CYP) system is pivotal to the phase I system. The phase II enzymes are characterized by their ability to conjugate the parent drug or phase I metabolite with endogenous cofactors such as glucuronic acid, sulphuric acid, acetic acid or an amino acid. to further increase the polarity of the compounds. Phase II reactions further increase the polarity of the compounds, resulting is more water solubility and chemically inreactive. The most common metabolites are the glucuronidation or sulfation products. Phase II metabolism often, but not always, occurs after metabolism by the CYP450 system. In the other hand, sometimes, the phase I and II reactions are backfire. Many xenobiotics and drugs are converted to toxins or potent chemical carcinogens such as epoxides, that can covalently bind to tissue macromolecules including DNA and protein[195]. Thus, biotransformations can affect overall therapeutic and toxic profile of a drug. It should be the key importance consideration in the food and pharmaceutical research field, and knowledge of the catalytic properties of enzymes is crucial essential.

Xenobiotic or waste metabolite in the diet or peripheral circulation

Phase I reactions

Reduction
Oxidation
Hydroxylation
Hydrolysis

Primary metabolite

Phase II reactions

Conjugation
Sulfation
Methylation
Glucuronidation

Secondary metabolite, suitable for excretion
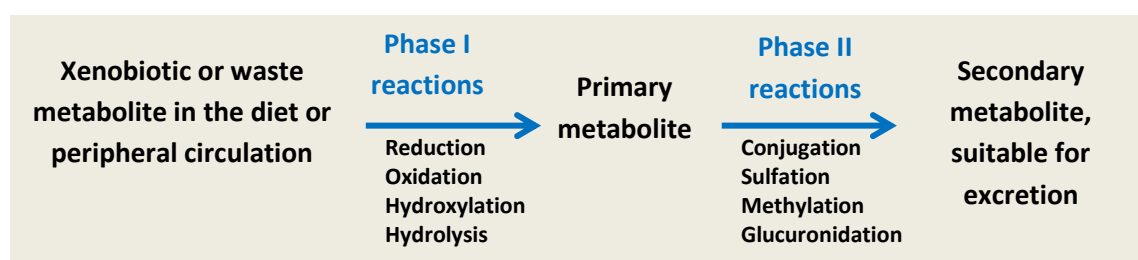
Figure 1.15: **The xenobiotic detoxification in the liver.**

5.2 Phase I biotransformation: CYP450

Although several enzymes acting in concert may be required for xenobiotic detoxification in the liver, the initial reaction usually involves a microsomal phase I enzyme catalyzing a predominantly oxidations, reductions, and hydrolysis reaction. During the first phase of biotransformation, compounds are enzymatically introduced functional groups into inert, a polar molecules or alter functional groups that are already present[194]. This makes it possible for foreign substances to be conjugated with polar substances via phase II reactions (see below). Oxidative reactions are the most important category of Phase I reactions. The key enzymatic constituents of this system are catalyzed primarily by the microsomal cytochrome P450 monooxygenase system (CYP450) and flavin protein oxido-reductase (also referred to as mixed-function oxygenase system : MFOs).

The human CYP450 (EC 1.14.14.1) is a superfamily of heme-thiolate containing enzymes[196]. CYP450 system gets its name from its unique spectrophotometric characteristics that absorbance maximum at 450 nm when form the complex with carbon monoxide. The reddish-brown color of the liver is mainly due to the large amounts of CYP450 that the liver contains. CYP450 enzymes are located mainly in the endoplasmic reticulum. These enzymes mediate many different types of oxidation reactions (see below) but the major role of the CYP450 enzymes is to oxidize substrates and insert an oxygen atom into the compound structure using heme as the redoxactive coenzyme. It metabolizes xenobiotic into more hydrophilic metabolites, using nicotinamide adenine dinucleotide phosphate (NADPH) as energy source[197]. Similar reactions can be carried out by MFOs but not often found (uncommon). These monooxygenases are induced by their substrates and show wide specificity. CYP450s have been shown to be involved in numerous metabolism of a wide range of endogenous compounds e. g., the biosynthesis of steroid hormones, bile acids, and eicosanoids, retinoids, as well as the formation of unsaturated fatty acids[194] and are determinants in biological degradation of foreign exogenous compounds including therapeutic drugs, nutrients, carcinogens, and pollutants[198]. Of the wide variety of enzymes that are involved in phase I xenobiotic metabolism, only the CYP450 is discussed here. Some common characterized of enzymes involved are described in Table 1.7.

Table 1.7: The cytochrome P450 isozymes all have certain features in common.

CYP450s general character include:
◆ Oxidize the substrate, and reduce oxygen.
◆ Have a flavin-containing reductase subunit that uses NADPH, and not NADH, as a substrate.
◆ Embedded in the phospholipid matrix of the endoplasmic reticulum.
◆ Bound to the lipid portion of the membrane, probably to phosphatidylcholine.
◆ Inducible by the presence of their own best substrate and somewhat less inducible by the substrates for other P450 isozymes.
◆ Generate a reactive free radical compound as an intermediate in the reaction

Adapted from Koolman J et al.[199]

5.2.1 CYP450s Reaction mechanism[200]

Phase I metabolism primarily serves to increase hydrophilicity of a parent drug. Initially, the substrate binds to oxidized heme iron (Fe^{3+}) of CYP450 to form the "CYP450-drug" complex. The necessary reducing equivalents are transferred to the actual monooxygenase by an FAD-containing auxiliary enzyme from the coenzyme $NADPH+H^{+}$. An electron from $FADH_2$ reduces the iron to the divalent form that is able to bind an O_2 molecule. Reductive cleavage of molecular oxygen (O_2) resulting in one of the two oxygen atoms is subsequently transferring to the "CYP450-drug" complex. The activated oxygen atom inserts itself into a C–H bond in the substrate, thereby forming a polar functional group such as -OH -NH_2 –SH or -CO_2H, while the other is released as water. Dissociation of the product returns the enzyme to its initial state and the creation of a more polar product.
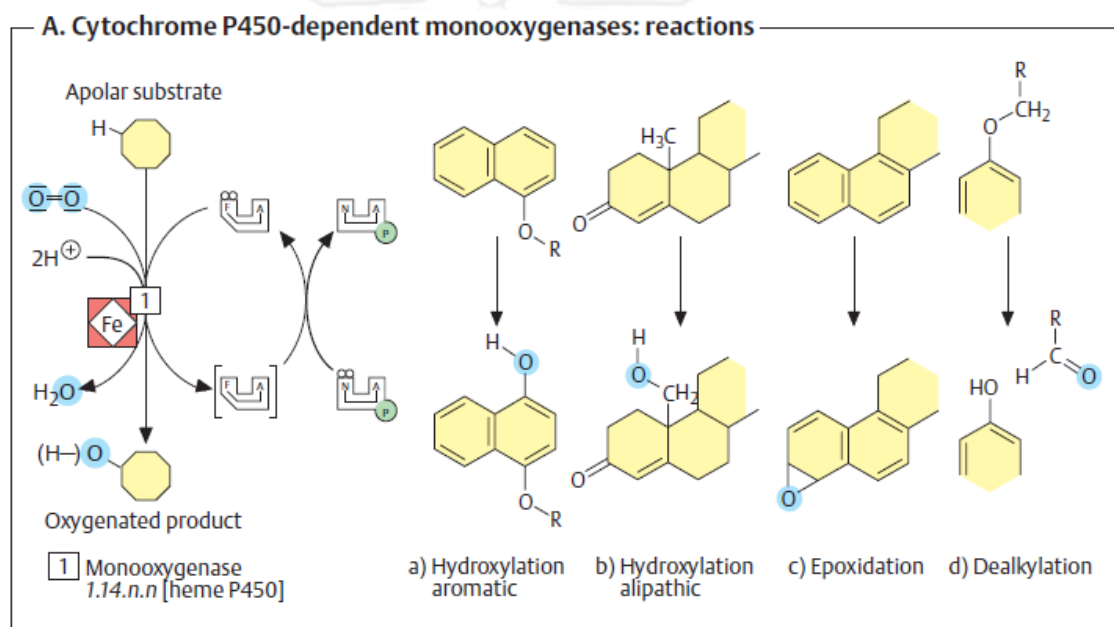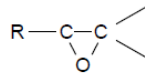
Fig 1.16: **Reaction mechanisms of cytochrome P450 monooxygenases (CYPs).** The reaction starts with the transfer from ferric ion (Fe^{3+}) to the substrate resulting in a highly reactive complex that cleaves the two oxygen atoms, one oxidizing the substrate, the other being released as water. Photo from Koolman J. et al.[199]

Table 1.8: some phase I reactions that occur in biotransformation

Biotransforma-tion process	Reaction	Some known substrates	Comment
Oxidations			
Oxidation	R-N→R-OH	Chlorpromazine	For example, benzene is oxidized into phenol, and toluene (methylbenzene) is oxidized into benzoic acid.
Hydroxylation	$R\text{-}H + O_2 + NADPH + H^+$ →R-OH + NADP+ + H_2O	Mycinamicin	Hydroxylation of aliphatic and aromatic compounds. Hydroxylation of aromatic rings plays a central part in the metabolism of medicines and steroids. Aliphatic methyl groups can also be oxidized to hydroxyl groups
Epoxide formation	$R\text{-}C\text{=}CH_2$→ $R-C-C$ with O bridge	Aldrin, benzo[a]pyrene	Epoxidation of aliphatic and aromatic compounds. Epoxidation of aromatics by CYP450 yields products that are highly reactive and often toxic. For example, the mutagenic effect of benzo[a]pyrene
Sulfoxide formation	R-S-R' → R-S=O-R'	Parathion	Desulfurations. The reactions take place in the hepatocytes on the smooth endoplasmic reticulum.
Dealkylation	$R\text{-}NH\text{-}CH_3$ → $R\text{-}NH_2 + CH_3OH$	Methylmercaptan, atrazine	N-dealkylation of amines, and O-dealkylation of ethers. Dealkylations. alkyl substituents of O, N, or S atoms are released as aldehydes.
Deamination	$R\text{-}NH_2$ → R-NHOH	Oxanosine	-
Reductions			
Reduction	R-C=O-R → $R\text{-}CH_2O\text{-}R$	Carbon tetrachloride, Chloroform	Reduction of carbonyl, azo-, or nitro-compounds, dehalogenation.
Methylations	$R\text{-}O + CH_3$ → $R\text{-}CH_3$ OH	-	Methylations such as the inactivation of the catecholamine norepinephrine by methylation of a phenolic OH group.
Hydrolysis			
Hydrolytic cleavages	R-R' + H_2O → R-OH + R'H	Acetylsalicylic acid	Hydrolytic cleavages of ether, ester, and peptide bonds.

The conjugation reactions add a negatively charged group such as glycine or sulfate to the molecule. Many xenobiotic compounds will be transformed through several different pathways.

5.2.2 CYP450 isoenzymes

The human enzymes are generally divided into six major subfamilies, and each of these is further subdivided. These isozymes have different but overlapping specificities, share at least 40% sequence homology. Fifty seven CYP450 different isozymes have been identified in the human and 47 contributing to the metabolism of xenobiotics[201]. The CYP1, CYP2 and CYP3 subfamilies are responsible for the metabolism of >90% of commercially available drugs[202]. Of these, CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2E1, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7[203] are considered to be the major hepatic

CYP450s which responsible for these metabolism and the main pathway to eliminate xenobiotics. Hepatic CYP450s can metabolize almost all organic xenobiotics. Individual CYP450 iso-enzymes metabolize specified substrates based on the chemical and structural features of these compounds.
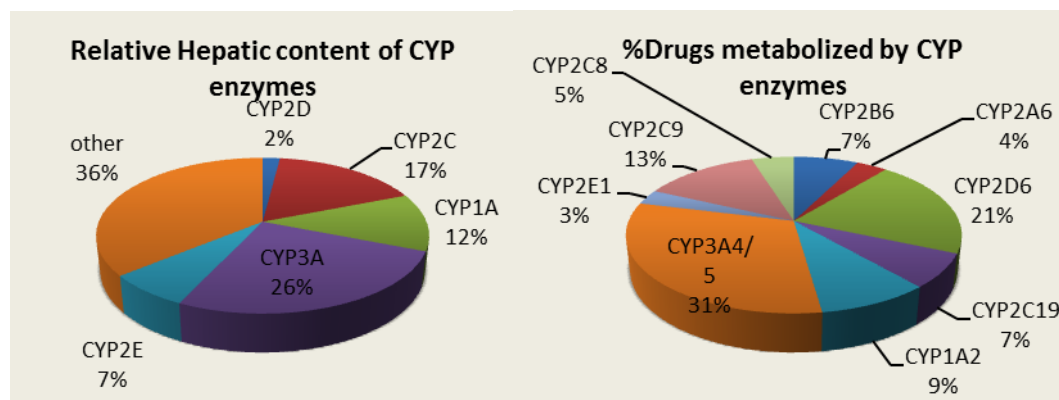
Figure 1.17: **The relative amount of the CYP450 enzymes in the liver**. The largest amount present in the liver belong to the 1A, 2C, 2E and 3A family. However, the abundance of the enzymes does not translate directly to the relatively importance of the enzyme with respect to the metabolism of pharmaceuticals. Adapted from Venkatakrishnan K. et al.[204]

CYP3A4 is the most abundant CYPs in hepatocytes, accounting for 60-70% of total CYPs. CYP3A4 is reported to responsible for metabolizing lipophilic substrates and known to metabolize over 50% of prescription drugs[205], food constituents and herbal remedies. CYP2C9 represents the second largest expressed CYPs in the hepatocytes and comprises about 10% of total CYPs[206] which are responsible for the metabolism of more than 15% of clinical drugs, particularly lipophilic and weak acids with the anionic site molecules such as warfarin and phenytoin. Most compounds with a basic nitrogen and/or positive charge such as arylalkyl amines are metabolized by CYP2D6. Complex phenols such as poly-aromatic hydrocarbons are metabolized by CYP1A2, while small and relatively soluble organics such as ethanol and nitrosamines are metabolized by CYP2E1[196]. Induction or inhibition of biotransformation enzymes is only two factors that can change the normal course of xenobiotic metabolism. Interestingly, the expression of some CYPs is clearly sex dependent. CYP3A4 is more expressed in females, whereas CYP2D6 is more expressed in males[207]. Other factors that may enhance or delay drug elimination are summarized in Table 1.9.

Table 1.9: Other factors altering normal biotransformation

Factor	For example
Diet	Some metal deficiencies mat decrease metabolism
Gender	More expressed CYP3A4 in females, more expressed CYP2D6 in males
hormones	Growth hormone or thyroxine increases metabolism
Age	Newborns and geriatric individuals usually have slower metabolisms
Genetic	Enzyme levels are, to some degree, genetically determined
Disease	Multifactorial, especially hepatic disease

Adapted from Ogu CC et al.[208]

Table1.10: CYP450 Substrates

CYP450 Isozyme	Substrates	Products that modify CYP450's expression
1A1	Caffeine, Testosterone, Warfarin	Omperazole
1A2	Acetaminophen, Caffeine, Cyclobenzaprine, Febuxostat, Flutamide Imipramine, Ranitidine, Roflumilast, Tacrine, Theophylline, Tizanidine, Zileuton Zolpidem	furafyllin
2A6	17β-Estradiol, Testosterone	Pyrazole
2B6	Bupropion,Cyclophosphamide,Clopidogrel,Efavirenz,Ifosfamide,Ketamine,Methadone,Sertraline Erythromycin, Testosterone	
2C-family	Acetaminophen, Tolbutamide (2C9); Hexobarbital, S- Warfarin (2C9,19); Phenytoin, Testosterone, Warfarin, Zidovudine (2C8,9,19);	Rifampicin, quercetin
2E1	Acetaminophen, Disulfiram, Halothane, Theophylline	ethanol
2D6	Amphetamine, Asenpine, Bifuralol, Chlorpheniramine, Chlorporamzine, Codeine, Cyclobenzaprine, Diphenhydramine, Donepezil, Fluphenazine, Fentanyl, Galantamine, Hydroxyzine, Lidocaine, Methadone, Morphine, Quinidine, Sertraline, Tamoxifen, Tramadol, Trazadone, Venlafaxine	quinidine
3A4	Atazanavir, Atorvastatin, Caffeine, Cannabinoids, Clindamycin, Cocaine, Codeine, Colchicine, Cyclophosphamide, Cyclosporine, Dextromethorphan, Diazepam, Doxorubicin, Erythromycin, Ethinylestradiol, Etonogestrel, Flutamide, Galantamine, Hydrocodone, Imatinib, Indinavir, Ketamine, Ketoconazole, Lovastatin, Methadone, Nifedipine Omeprazole, Quinidine, Quinine, Rifampin, Simvastatin, Steroids, Tamoxifen, THC, Verapamil, Vinblastine, Vincristine, Warfarin (r)	Rifampicin, ketoconazole

Adapted from: U.S. Food and Drug Administration publications

In addition to the liver, CYPs have been found in small and large intestines, kidney, lung and nervous tissue. Of these extra hepatic tissues, intestinal CYP is reported to be the most important contributor in drug biotransformation[209] because most therapeutic drugs and food substances are taken through oral route. Other important Phase I oxidative enzymes is flavin-containing monooxygenase, requiring NADPH and oxygen the same as CYP450s and exists as multiple isoforms in various tissues. However, FMO, unlike CYP, catalyzes only oxygenation reactions, has more specific substrate requirements. The FMO is found in highest levels in the liver, but is also found in significant levels in the lung and kidney. The microsomal FMO was known for a number of years as an amine oxidase which catalyzes the oxygenation of nitrogen and sulfur compounds and the epoxide hydrolases which hydrolyze various epoxides to diols. Other Phase I enzymes are epoxide hydrolases which catalyzed epoxide rings of certain alkene and arene compounds. Prostaglandin synthetase synthesis prostaglandins via a reaction sequence starting with arachidonic acid as substrate. Aldehyde oxidase, hydrolases, DDT-dehydrochlorinase and amidases play a role in the metabolism of neonicotinoid insecticides[210].

5.2.3: The modulation of CYP450s activities

Induction of either phase I or phase II enzymes are considered to be a detoxification process which increases in polarity usually facilitate excretion. But in some cases, CYPs450 oxidative reactions have the potential to form reactive intermediates/metabolites through their process. These unstable chemically reactive electrophilic metabolites are found to be much greater toxicity[211]. and carcinogenicity[212]. These reactive metabolites covalently bind to cellular proteins and DNA, leading to toxicity via multiple mechanisms such as direct cytotoxicity, oncogene activation, and hypersensitivity

reactions. Beside of their ability to generate reactive species, a number of naturally occurring compounds have been shown to modulate different effects on CYPs450 activity *in vitro* and *in vivo*, including the induction of specific CYPs450 isozymes, and the activation or inhibition of these enzymes[213].

Several important therapeutic agents have been identified as CYPs substrates as shown in Table 1.10. Some of these have potential to induce or inhibit the activity of CYPs. The concomitant ingestion of two CYP450s substrates could potentially induce competition for the binding site, which, in turn, could alter the blood levels of these two agents. The drug with the highest affinity for the enzyme would be preferentially metabolized, whereas the metabolism (and degradation) of the other drug would be reduced. The latter drug's concentration in the blood would then rise. Moreover, many substances or drugs impair or inhibit the activity of the CYP450s enzyme, thereby impairing the body's ability to metabolize these drugs. The importance of CYPs in the metabolism of these drugs leads to increased pharmacokinetic interaction potential with other substances. Numerous clinically significant drug interactions associated with these agents have been reported, for example, the interaction between lipid-lowering agents known as the Statins (HMGCoA reductase inhibitors) and grapefruit juice. Grapefruit juice is a potent inhibitor of CYP3A4-mediated drug metabolism which Statins require CYP3A4 for its degradation. Evidence suggests that if a Statin is regularly taken with grapefruit juice, its level in the blood may increase as much as 15-fold leads to the muscle and liver toxicity. Another example is co-administering anti-hyperglycemia Sulfonylurea drugs (mainly metabolized by CYP2C9) with other CYP2C9 inducers or inhibitors. Decrease in the anti-hyperglycemia effect of Sulfonylureas drugs was reported when they were combined with Rifampicin (CYP2C9 inducers)[214].

5.3 Phase II biotransformation: conjugate formation

Other enzyme systems detoxify potentially toxic compounds is phase II biotransformation system. For example, benzoic acid is conjugated with glycine to form the more soluble and less toxic hippuric acid (N-benzoylglycine). The enzymes involved couple ester or amide bonds are transferases, and their products are known as conjugates. In contrast with unconjugated compounds, the conjugates are much more water-soluble and capable of being excreted. Phase II reactions consist primarily of conjugation of a chemical metabolites or the products of phase I with one of several endogenous electron-rich molecules (nucleophiles) such as glucuronic acid, methionine, sulfate or glutathione to form water-soluble and hence eliminated from the liver either by the biliary route or by the renal route. Conjugations may be just simple but often they are more complicated processes in which the final product is derived by several steps[215]. Phase II enzymes play important role against oxidative stress, they are considered to be antioxidant enzymes. As we know, sometimes a highly reactive electrophile or free radical is formed during a phase I reaction. Glutathione conjugation, glucoside formation, glucuronic acid formation, sulfate formation, and conjugation with amino acids are used to neutralize these reactive intermediates. If the phase II conjugation reaction is impeded, these reactive intermediates may react directly with hepatocyte macromolecules, resulting in damage to the hepatocyte[194].

The most common of conjugate formation reactions for the elimination of xenobiotics from the body is glucuronidation[216]. The coupling with glucuronide catalyze by uridine diphosphate glucuronosyl transferease (UGTs), with one of a number of possible functional groups, such as R-OH, R-NH$_2$, R-COOH, and others[217], such as the glucuronidation of tetrahydrocortisol, a metabolite of the glucocorticoid cortisol, is the underlying cause for one important drug-drug interaction between gemfibrozil (fibric acid derivatives) and several statins[218]. While The UGTs are localized in the microsomes, the sulfotransferase and glutathione S-transferase are soluble enzymes primary present in the cell cytosol. Conjugation with glutathione is mediated by the glutathione S-transferase (GSTs). This enzyme is an abundant family of dimeric proteins that have the capacity to conjugate glutathione with a variety of compounds containing electrophilic centers, consequently leading to a mercapturic acid. The major hepatic cytosolic GSTs in human can be classified into three classes: Alpha (**α**), Mu (**μ**) and Pi (**π**) based on sequence similarity and catalytic activity[219]. GST is believed to be an important predisposing factor in the interaction between environmental and diseases. This enzyme is reported to detoxify carcinogenic chemicals. Several pesticides are metabolized by GST, particularly organophosphorus compounds, DDT, **γ**-HCH, and organothiocyanates. Moreover, GST is an important protective agent against aflatoxin induced liver cancer or smoking inducted lung cancer. Another important phase II enzyme is sulfotransferase (SULT) and sulfatase enzyme superfamilies that catalyze sulfation and sulfate conjugate hydrolysis reaction especially the conjugation of 3'-phosphoadenosine 5'-phosphosulphate (PAPS) with an O-, N- or S-acceptor. O-sulfation represents the dominant cellular sulfonation reaction. The process first begins with the sulfation reaction which transfers the "active sulfate" from sulfate donor, PAPS to the xenobiotic molecules. Next step is hydrolysis of these sulfate esters by sulfatases and let the products formed the amide bond with other functional groups such as glycine and glutamine. SULT with various xenobiotics generally result in the production of water-soluble sulfate esters, which are then eliminated. SULTs has a significant role in the biotransformation of steroids, catecholamines, serotonin, iodothyronines, eicosanoids, retinol, 6-hydroxymelatonin, ascorbate, vitamin D and procarcinogens[220]. Other Phase II Enzymes include Methyltransferases which performed N-, O-, and S-methylation either xenobiotics or endogenous metabolites with methionine. Cysteine Conjugate **β**-Lyase conjugates substrates such as original xenobiotic, ammonia, and pyruvic acid, the thiol derivative with cysteine. Acylation enzymes are involving in transfer of an acetyl group form acetyl-CoA to the xenobiotic. Phosphate conjugation is uncommon in mammalian, only has been described only in insects.
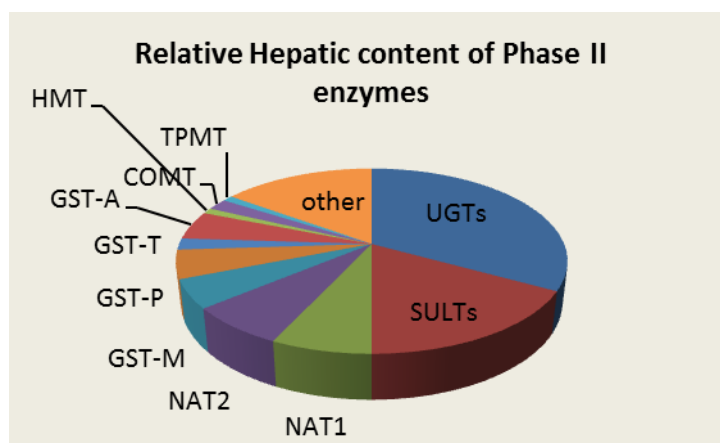
Figure 1.18: **The Phase II biotransformation enzymes.** UDP-glucuronyltransferases (UGT) , Sulphotransferases (SULT), N-Acetyltransferases (NAT), Glutathione S-transferases (GST), catechol O-methyltransferase (COMT), thiopurine S-methyltransferase(TPMT) and histamine methyltransferase (HMT). Adapted from Evans WE. et al.[221]

Table 1.11: Summaries of Major enzyme systems involved in the biotransformation of chemicals

Phase I reactions
Oxidation
Cytochrome P-450 monooxygenase*
Flavin-containing monooxygenase*
Alcohol dehydrogenase
Aldehyde dehydrogenase
Amine oxidases
Prostaglandin endoperoxide synthetase (co-oxidation)
Reduction
Nitroreductase
Azoreductase
Hydrolysis
Epoxide hydrolases*
Esterase
Phase II reactions
UDP-glucuronosyl transferase*
Sulfotransferase*
Glutathione transferases*

* =Most important.

5.4 Phase III biotransformation: drug efflux transporters

After biotransformation process by phase I and II enzymes, metabolites are produced and concentrated within cells. Even the metabolite that harmless non-reactive, when reaches its final threshold over a period of time, it can develop toxicity effect. To protect the cell from these xenobiotics metabolites, active transporters on cell membrane pump these compounds out to the extracellular space so they can be excreted. Transporters are membrane-bounded proteins regulating the influx and efflux of drugs across the plasma membrane. They are classified into two families, namely solute carrier (SLC) and ATP-binding cassette (ABC) transporter. The ABC transporters include P-glycoprotein (P-gp, also

known as multidrug resistance proteins MDR1), multidrug resistance associated proteins (MRPs), and breast cancer resistance protein (BCRP)[222]. These transporters are localized either in the apical surface or basolateral surface of hepatocytes. Among these efflux transporters, P-gp has been the most extensively studied transporter in modulating drug disposition. P-gp serves to eliminate metabolites and a wide range of hydrophobic foreign substances, including drugs, from hepatocytes by acting as an efflux transporter either to bile acid or back into blood circulation. They are involved in limiting drug absorption and disposition with broad range of xenobiotic specificities and the ability to transport structurally unrelated compounds[223].

P-glycoprotein is a member of the ATP-binding cassette (ABC) superfamily of proteins. It is a 170 kDa glycosylated transmembranous protein expressed in various tissues including the liver, adrenal gland, blood-brain barrier, small intestine, colon, pancreas, kidney and testis[224,225]. This protein forms a pore in the cell membrane and actively pumps drugs out of the cell, using ATP as an energy source (ATP dependent efflux pumps). The substrate specificity of P-gp is very broad, and a large number of compounds have been identified as P-gp substrates. In general, these substrates are small molecules with either positively charged or neutral compounds[222]. Like CYPs, P-gp is susceptible to induction and inhibition by many drug substances. Many drug interactions mediated by P-gp inhibition have been reported. Because of the importance of P-gp in drug absorption and disposition, alterations of its function have been associated with a number of clinically significant drug interactions. Evidences from *in vitro, in vivo* and clinical studies have indicated that P-gp expression in healthy tissues plays important role in drug interactions[225] and is involved in adverse effect of many herbal products[226]. For instance, co-administering certain macrolides antibiotics (a P-gp inhibitor) with digoxin (a well-known P-gp substrate), resulted in increasing serum digoxin level in a clinical study[227]. In terms of P-gp induction, the increasing P-gp pumps the drug out of the cell before the drug can exert its effects such as in case of impaired digoxin absorption resulted from drug interaction with rifampicin (a P-gp inducer)[228].
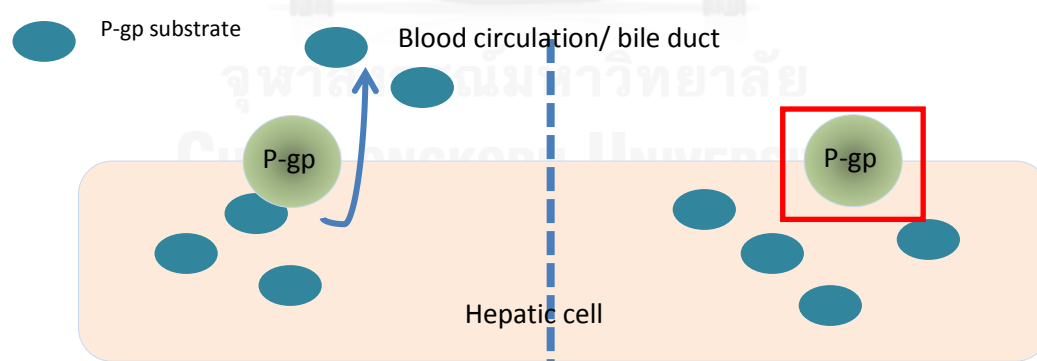
Figure 1.19: **P-glycoprotein efflux in absence and presence of P-gp inhibitor.** P-gp actively effluxes drug metabolites or incoming parent drugs out of hepatocytes back to blood circulation or bile duct in an ATP dependent manner. Changing in P-gp activity will alter systemic disposition of drugs. For example: inhibited P-gp activity by its inhibitors lead to an increased concentration of a drug system.

Cell response	Normal	Antioxidant defense	Cytokine,chemokine response	Cell death
Step	Resting state	Step I	Step II	Step III
Signaling pathway		Nrf-2	MAP Kinase JAK/STAT NF-KB cascade	Mitochondria, PT pores
Genetic response		Antioxidant response element (ARE)	AP-1 and NFkB	N/A
Proteins		Phase II, antioxidant enzymes	Cytokine, chemokine	Bcl-2 family
outcome		cytoprotection	Inflammation response	cytotoxicity

Figure 1.20: **Cellular response to xenobiotic.** These responses depend on the pro/antioxidant balance. At step I, ROS generation from xenobiotic metabolism induces activation of cellular antioxidant systems in order to restore redox homeostasis. If cell cannot eliminated xenobiotic insult and its defense system insufficient, the stress triggers an inflammatory response through redox sensitive pathways (step II). At a last stage, stress overwhelming, and the defense systems could not cope with this insult lead to cell death. Adapted from Nel A. et al.[229]

5.5. Biotransformation: Modulation of CYP450s and phase II and P-gp by medicinal herbs.

One important consideration about phytochemicals is their alternation of biotransformation enzymes functions. Evidences from *in vitro, in vivo* and clinical studies have indicated that various herbal products have the potential to modulate activities of human drug metabolizing CYP450s and P-gp[230]. Herbal extracts also exert beneficial by lowering the formation of toxic metabolites and thus inhibit carcinogenesis, as CYPs450 metabolically activate procarcinogens to reactive intermediates that trigger carcinogenesis. On the other hand, modulation of this enzyme system by plant substances can influence the metabolism of other xenobiotics, leading to accumulation of co-administration drugs and having the impact on the drug pharmacokinetic and -dynamic profile. Because of frequently use, herbal medication has a potential to interact with other medications. Interactions between herbs and drugs may increase or decrease the pharmacological or toxicological effects of either component. These interactions could cause problems ranging from insufficient therapeutic effect to fatal toxic consequences. Decreased or increased plasma levels of these drugs could result in a lower therapeutic efficacy or a higher risk of toxicity[231], especially for narrow therapeutic windows drugs, pharmacokinetic interactions could easily lead to clinically relevant effects. The previous literatures reviews a lot about potential roles in drug-

herb interactions[232, 233,234], some well-known examples are St. John's wort, grapefruit juice and *Ginkgo biloba*. St. John's wort is a powerful inducer of both CYP3A4 and P-gp and better known for its potential drug interaction than its medicinal properties. Consequently, it accelerates the clearance of many prescription drugs including alprazolam[235] Studies have also demonstrated that St. John's wort are potent ligands induces the orphan nuclear receptor which regulates CYP3A4 and P-gp gene expression[236]. Adverse effects of St. John's wort with some anti-HIV drugs due to the presence of it would induce CYP3A4[237]. Moreover, a few publications have report that *G. biloba* extracts induce CYP2C9 activity, in both animal models and in clinical studies. *G. biloba* extract significantly attenuated the hypoglycemic effect of tolbutamide[238], as well as some report about interaction between grapefruit juice and some cardiovascular drugs[239].

While the mechanisms of drug-herb interactions of many western medicinal plants are well studied, there is limited information regarding the interactions between the medicinal plants and therapeutic drugs. Investigation of interaction between Thai herbal medication and drug-metabolizing enzymes including metabolic behavior and inhibitory potential of compounds should be given more attention from both experimental research and clinical point[240].
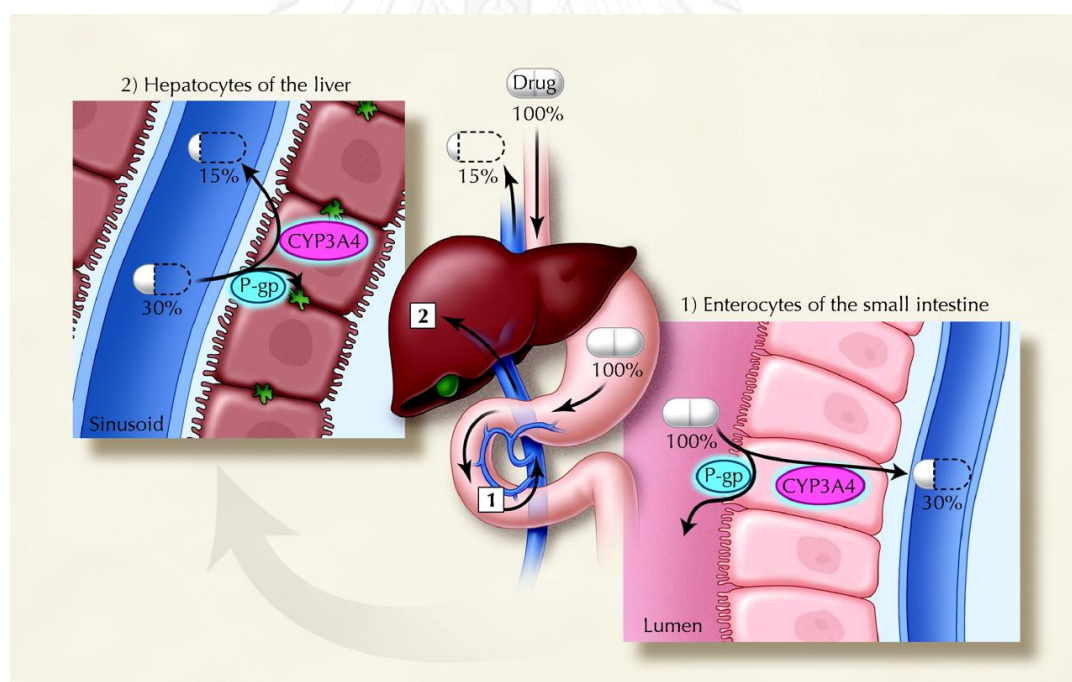
Figure 1.21: Example of elimination of xenobiotic through metabolism by CYP450 incorporation with transporter P-glycoprotein (P-gp). Drugs were first-pass metabolized in enterocytes of the small intestine and then hepatocytes of the liver. Thus, the modulation of CYP450 represents an important mechanism for the enhanced or reduced bioavailability of coadministered drugs. Even if the drug is fully absorbed but only few percent were left after pass the biotransformation process. Photo from Bailey DG. et.al.[241]

6. Energy metabolism in the liver

Beside metabolized xenobiotic, the liver also plays a significant role in metabolism of endogenous molecules. The liver is major metabolic site responsible for supplying whole-body energy needs. The liver is capable to induce a number of changes in terms of stored and released energy in the body under the different nutritional situations. When there is a good supply of nutrients in the fed state, the liver converts glucose to pyruvate (via glycolysis), which is oxidized to provide energy. The excess dietary nutrients are stored in the liver as glycogen as a small part while most of it channeled into pathways for synthesis of fatty acids and triglycerides (via lipogenesis) for storage. In the fasted or starvation state, the liver began to breakdown of stored hepatic glycogen and synthesis glucose from amino acids, glycerol, and fatty acid via fatty acid oxidation and ketogenesis to supply energy for extrahepatic organs[242]. In these conditions, the lipid metabolism is readjusted. Adipose tissue releases fatty acids which taken up by the liver. The liver is the most important site for the metabolism of fatty acids, triacylglycerols, ketone bodies, and cholesterol. Alterations in hepatic lipid metabolism affect whole-body metabolism, energy homeostasis and hormonal changes in the body. Lipid dysmetabolism is deranged with very markedly elevated concentrations of triacylglycerol and fatty acids in the blood. The increasing blood lipid profiles are found to underlie the development of cardiovascular disease[243], metabolic syndrome[244] and inflammation[245]. It was observed that phytoestrogens and flavonoids hesperidin and naringin[246] affected lipid metabolism in the liver. Studies in animal showed that changing in plasma lipid parameters after animal received green tea[247], black tea[248], grape polyphenols[249], apple pectin[ap1860],and Persimmon[250] leading to improved metabolic diseases.

7. Interesting plants in this study

Two Thai medicinal plants which have recently gained widespread popularity are *Thunbergia laurifolia* Linn. (TL), and *Moringa oleifera* Lam. (MO).

Thunbergia laurifolia Linn. (Thunbergiaceae), Thai known as Rang-Jued and its English name is Babbler's Bill. This plant is widely used in the Thai traditional herbal medicine for detoxification of toxic for food and environmental toxicants. This plant is rich in phytochemicals such as polyphenolic, alkaloid, carotenoid and chlorophyll[251]. Most of these polyphenolics are caffeic acid, apigenin, casmosiin, delphinidin-3-5-di-O-β-D-glucoside and chlorogenic acid. Those are capable to terminate free radical reactions and prevent oxidative damage[252]. The extract of dried leaves, dried root, fresh leaves, and bark of TL can be effectively used as an antidote. The antidotal activity of TL was used against pesticides, arsenic, strychnine[253,254] and lead poisonings[255]. TL was also used to treat drug addiction[252,256], diabetes mellitus[257], ethanol-induced hepatotoxicity[258] and anti-inflammatory[259] had been reported. Recent studies show that TL extracts can prevent parasite invasion and fungal infection[260]. Besides, it is powerful inducer for Phase II xenobiotic detoxification enzyme quinone reductase[251].

Moringa oleifera Lam. (Moringaceae) is commonly known as drumstick or horseradish tree. This plant is widely available in the tropics and subtropics. It is also known as multipurpose tree to serve as nutritional supplement due to the significant source of minerals, amino acids, vitamins, and phytochemicals such as carotenoids, sterols, glycosides, alkaloids, flavonoids and polyphenolics[261]. In folk medicine, the medicinal values of these herbs have long been recognized. Various parts of MO are reported to possess numerous pharmacological properties especially leaves. Leaves are used in the indigenous systems of human medicine for the treatment of a variety of ailments such as hypertension, hypocholesterolemic and hypoglycemic agents[262], anti-tumor[263,264], antioxidant[264], radio-protective[265], anti-inflammatory[266], anti-microbial[267], ant-ihepatotoxic[268], antiurolithiatic[269], anti-ulcer activities[270], regulate thyroid status[271] and use for improve wound healing process[272].

So far, there is no information available regarding the effects of these herbal effects on hepatic CYP gene expression and activity. In this study, we selected these herbs to investigate their antioxidant properties, effects on cell functions and to evaluate and predict the herb–drug interaction potential of herbal extracts mediated by CYP inhibition/induction. The selection of the parts of plant is based on the formal use of herbal remedies.

Statement of the Problem

Based on the proceeding literature review, it is apparent that consumption of these plants could leads to an increase in cellular antioxidant levels that provides a buffer against oxidative stress. Suggest that there are beneficial effects from these herbal medicines. However, toxicity profile and the role of these herbal medicines can induce or repress CYPs450 against adverse effects of toxic agents through biotransformation system are still not well documented. Hence it is worthwhile to estimate the effects of these herbs in view of their possible roles in antioxidant and capacity to detoxify chemical toxicants, during Phase I and Phase II of drug biotransformation may provide for therapeutic potential of many oxidative stress related diseases especially impaired energy metabolism.

Aims of the study

The general aim of the study is to find out 2 of Thai medicinal plants, *Thunbergia laurifolia* Linn. and *Moringa oleifera* Lam., as a potential therapeutic agent and to ensure safety and effective use of herbal medicines to prevent disease and improve health. The specific aims of our research are as following:

1. To investigate polyphenolic in herb extracts and demonstrate whether these polyphenolic are responsible for antioxidant capacity of herb extracts. The screening of their biological activities is employing various chemical tests such as antioxidant properties assays, scavenging activity assays, reducing power assays and enzyme activities assays.

2. To evaluate the possible protection of both plants using crude *extracts* and pure compounds against hepatotoxicity induced by toxicants and the mechanism involved in these hepatoprotective activities.

3. To evaluate the effects of both plants crude extracts on the biotransformation, enzymatic activity for detoxification and to estimate the possible adverse effects of herb-drug interactions in human hepatic cell line.

4. To provide a scientific validate for folk medicinal usages of these plants in diseases arising from oxidative stress such as anti-diabetic, anti-inflammation and anti-dotes.

We hope that this study would provide a better understanding about antioxidant-based plants for their antioxidant properties and other functions for maintaining cellular homeostasis.

Hypothesis for research:

Thai herb extracts have potential antioxidant properties and can scavenging various types of free radicals. These antioxidant activities of these plants could protect lipids, proteins, and DNA structures from oxidative stress and well maintain cellular functions. Only optimal dosage of herbal extracts is beneficial to promote health. Improper misuse of herbal extracts seems to be ineffective and lead to several adverse effects.

Conceptual frameworks:

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┌─────────────────────────────────────────────────────────────────┐
│          Crude extracts from 2 Thai herbal medicines              │
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                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│                          Phytochemicals                           │
│   1. Total polyphenols content analysis by Folin-Ciocouteu assay  │
│   2. Total flavonoids content analysis by Aluminum chloride assay │
│   3. Total condense tannin content analysis by Vanillin assay     │
│   4. Total nitrate content analysis by Griess assay               │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│                      Antioxidant properties                       │
├─────────────────────────────────────────────────────────────────┤
│   I. Antioxidant capacity analysis by chemical tests              │
│   1. Antioxidant activity (ORAC, TEAC, Reducing power)            │
│   2. Free radicals scavenging capacity (superoxide anion,         │
│   hydroxyl radical, nitric oxide radical, hydrogen peroxide)      │
├─────────────────────────────────────────────────────────────────┤
│   II. Antioxidant capacity analysis on living cells               │
│   1. Inhibition of lipid peroxidation (Hemolysis test)            │
│   2. Inhibition of protein oxidation (Heinz body formation and    │
│      AGEs formation)                                              │
│   3. Inhibition of DNA oxidation (Comet assay)                    │
│   4. Effect on endogenous antioxidant system (ePMRS assay)        │
└─────────────────────────────────────────────────────────────────┘
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Toxicity and effects on cellular functions	Biotransformation system	Other biochemical actions
1. Effect on cell viability (MTT and Neutral red assay)	1. Phase I enzymes for deactivation of xenobiotic determination of CYPs450 expression and their activity (RT-PCR for gene expression, fluorometric method for enzymatic activity)	1. Anti-DM (Inhibition of α-glucosidase and α-amylase activity)
2. Effect on oxidative stress (DCFH-DA assay)		2. Anti-hyperlipidemia (lipid metabolism gene expression (RT-PCR method)
3. Effect on intracellular GSH level (DNTB assay)	2. Phase II enzymes for antioxidants, detoxification (RT-PCR method)	3. Anti-dotes for neurotoxin (Inhibition of cholinesterase activity)
4. Effect on mitochondrial membrane potential (TMRM staining)	3. Phase III enzymes activity for excretion (fluorometric method for enzymatic activity)	4. Anti-inflammation gene expression (NF-κB, TNFα, COX-1, COX-2 and 5-LOX) (RT-PCR method)
5. Effect on intracellular free calcium content (Rhod2-AM staining)		
6. Effect on intracellular ATP level (luciferase assay)		

Chapter II
Meterials and Methods

2.1 Materials

2.1.1 Materials and solvents: All reagents were analytical grade. Water was purified using a Millipore Q water system with filter through 0.22 μM pore size, one carbon cartridge followed by two ion exchange cartridges (Millipore, USA).

Chemicals	Companies, Countries
2,2'-azobis(2-amidinopropane) dihydrochloride AAPH	Sigma Aldrich, USA
2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS	Sigma Aldrich, USA
2,4,6-Tripyridyl-s-Triazine (TPTZ)	Merck, Germany
2',7'-Dichlorofluorescin diacetate	Sigma Aldrich, USA
2,3 Dihydroxybenzoic acid	Sigma Aldrich, USA
5,5' Dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma Aldrich, USA
Ascorbic acid	Sigma Aldrich, USA
Acetronitrile	Sigma Aldrich, USA
Acetphenyl hydrazide	General purpose reagent, UK
Ammonium thiocyanate	Sigma Aldrich, USA
Apigenin	Sigma Aldrich, USA
Bovine serum albumin	Sigma Aldrich, USA
Calcium chloride	Merck, Germany
Catechin	Sigma Aldrich, USA
Chlorogenic acid	Sigma Aldrich, USA
Carbonyl cyanide m-chlorophenyl hydrazine (CCCP)	Sigma Aldrich, USA
Crystal violet	Merck, Germany
Chloroform	Sigma Aldrich, USA
Curcumin	Sigma Aldrich, USA
Diethy-p-nitrophenylphosphate	Sigma Aldrich, USA
Digitonin	Sigma Aldrich, USA
4-(Dimethylamino)-cinnamaldehyd (DMACA)	Sigma Aldrich, USA
Dimethyl Sulphoxide (DMSO)	Merck, Germany
Dulbecco's modifies Eagle's medium (DMEM :with 4 mM/L Glutamine, 4500 mg/L Glucose without Sodium Pyruvate)	Sigma Aldrich, USA
Deoxyribonuclease I	Biobasic, Canada
Diethyl pyrocarbonate (DEPC)	Sigma Aldrich, USA
DNA Ladder 100 bp	Biobasic, Canada

Ethylenediaminetetraacetic acid (EDTA)	Merck, Germany
EDTA-Trypsin 0.25% (1X)	
Ethanol	Merck, Germany
Ethidium Bromide	Sigma Aldrich, USA
Formaldehyde	Merck, Germany
Fetal Bovine Serum (FBS)	Sigma Aldrich, USA
Ferric chloride-6H_2O	Sigma Aldrich, USA
Ferrous chloride	Sigma Aldrich, USA
Fluorescein	Sigma Aldrich, USA
Folin Ciocalteu's phenol reagent	Sigma Aldrich, USA
Gallic acid	Sigma Aldrich, USA
Glutathione reduced	Sigma Aldrich, USA
Guanidine hydrochloride	Sigma Aldrich, USA
Glucose	Merck, Germany
Hydrogen peroxide 30% w/v	Merck, Germany
Isoprep Ficoll-Hypaque 1077	Robbins Scientific corp., USA
Isopropanol	Merck, Germany
i-Tag DNA polymerase with dNTPs 10 mM	iNtron biotechnology, South Korea
Low temperature melting point agarose gel	Invitrogen, USA
Methanol	Merck, Germany
M-Mllv-Reverse transcriptase	Finnzymes, Finland
Neutral Red Dye	Sigma Aldrich, USA
Nicotinamide adenine dinucleotide phosphate (NADPH)	Sigma Aldrich, USA
Naphtyl ethylene diamine dihydrochloride (NEDD)	Sigma Aldrich, USA
Normal temperature melting point agarose gel	Invitrogen, USA
Nitro Blue Tetrazolium (NBT)	Sigma Aldrich, USA
Oligo-dT 20-mer	Biobasic, Canada
p-nitrophenyl acetate (Paraoxon)	Sigma Aldrich, USA
Penicillin-Streptomycin Solution	Biochrom AG, Germany
(10,000 units/ml Penicillin, 10,000 µg/ml Streptomycin)	
Phenyhydrazine hydrochloride	Merck, Germany
Potassium persulfate	Merck, Germany
Potassium phosphate monobasic KH_2PO_4	Merck, Germany
Potassium monohydrogen phosphate K_2HPO_4	Merck, Germany
Phosphate Buffered Saline	Merck, Germany
(PBS : without calcium without magnesium)	
Primer	Biobasic, Canada
Quercetin	Sigma Aldrich, USA

RNAse Inhibitor	Biobasic, Canada
Sodium azide	Sigma Aldrich, USA
Sodium acetate	Sigma Aldrich, USA
Sodium carbonate	Sigma Aldrich, USA
Sodium chloride	Sigma Aldrich, USA
Sodium hydroxide	Merck, Germany
Sodium phosphate	Merck, Germany
Sulfuric acid	Sigma Aldrich, USA
Triton-X 100	Sigma Aldrich, USA
Trichloroethanoic acid (TCA)	Merck, Germany
Tetramethylrhodamine, methyl ester (TMRM)	Invitrogen, USA
Tris base	Riedel-de Haen, Germany
Thiazolyl Blue Tetrazolium Bromide	Sigma Aldrich, USA
Trypan Blue Stain 0.4%	Invitrogen, USA
Thiobarbituric acid	Sigma Aldrich, USA
Trolox	Sigma Aldrich, USA
Vanillin	Sigma Aldrich, USA
Verapamil	Sigma Aldrich, USA
Vanadium trichloride	Sigma Aldrich, USA

2.1.2 Tool and Device:

Instruments	Companies, Countries
-20°C Freezer	Sanyo Electric, Japan
-80°C ULT Deep Freezer	Liofreeze, USA
4°C Refrigerator	Sharp, Japan
6,12,96 well cell culture plate flat bottom with lid	Nunc, Denmark
Analytical Balances	Mettler Toledo, Switzerland
Auto pipette	GILSON, France
Block heater	Wealtec Corp., USA
Cell Culture Flask (25,75 cm^2)	Nunc, Denmark
Centrifuge tube 15, 50 ml	Corning Inc., USA
CO2 incubator	Sheldon Manufacturing, USA
Confocal laser scanning microscopy	Carl Zeiss, Germany
Cryovial tube 2.0 ml	Nunc, Denmark
Differential interference contrast microscopy	Carl Zeiss, Germany
Disposable Serological pipette (5, 10 ml)	Corning Inc., USA
Electrophoresis power supply	Bio-Rad Laboratories, USA
Fluorescence microscope	Olympus Optical, Japan

Freeze dryer	Thermo Electron Corporation, USA
Gel documentation (gel doc) systems	Syngene, UK
Gel Electrophoresis Apparatus	Bio-Rad Laboratories, USA
Glassware	Pyrax, USA
Hem cytometer	Hausser Scientific, USA
High-Pressure Steam Sterilizer	Tomy Kogyo, Japan
Incubator	Memmert, Germany
Inverted microscope	Olympus Optical, Japan
Larminar Flow Cabinet	E.S.I. Flufrance, France
Light microscope	Olympus Optical, Japan
Liquid Nitrogen Tank	Taylor-Wharton, USA
Luminometer Plate	PerkinElmer, Finland
Microcentrifuge	Beckman Coulter, USA
Microcentrifuge tube (1 .5 ml)	BIO-RAD, USA
Micro Refrigerated Centrifuge	Vision Scientific, South Korea
Microscope slide (1"x3"), Cover glass	Sail Brand, China
Multichannel pipette	Brand, Germany
PCR tube	Bioscience Inc., USA
Pipette controller	Jencons (Scientific), UK
Pipette tips (10, 200, 1,000 μl)	Biobasic, Canada BIORAD, USA
Rotary Evaporator	Heidolph Instruments, Germany
Sonicator	Soniclean, USA
Sterile aerosol pipette tip (10, 200, 1,000 μl)	Labcon, USA
SUB CELL electrophoresis tray	BIO-RAD, USA
Thermal Cycler	MJ Research Inc., USA
UV Transilluminator	UVltec, UK
UV-Visible Spectrophotometer	PerkinElmer, Finland
UV-Visible Spectrophotometer for RNA	Beckman Coulter, USA
Vacuum Concentrator (DNA SpeedVacs)	Thermo Electron Corporation,USA
Vortex Mixer	FINEPCR, South Korea
Multilabel Counter flourometer	PerkinElmer,Finland
Water Bath	Memmert, Germany

2.2 Herbal samples

Two species of Herbs were used in this study. The voucher specimen was botanically identified and given herbarium number by Department of Botany, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand.

1. *Thunbergia laurifolia* Lindl. (TL) Its common names are purple allamanda, Laurel clock vine or Blue trumpet vine, known in Thai as Rang-Jued which belong to Acanthaceae family, and given a herbaium number was 013424 (BCU).

2. *Moringa oleifera* Lam. Its common name is Horse radish tree, known in Thai as Marum. which belongs to Moringaceae family and given a herbarium number was 013521 (BCU)
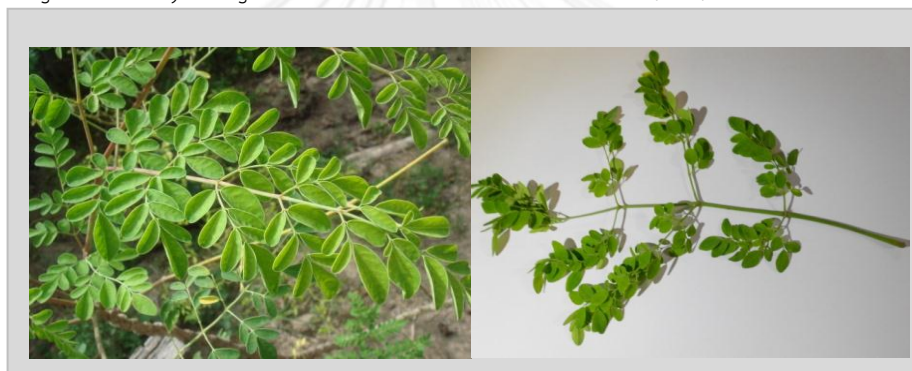
Herbal collection: The leaves of *Thunbergia laurifolia* Linn. and *Moringa oleifera* Lam. are collected from herbal garden in Bangkok, Thailand in May-June 2010. Taxonomic (botanical) identification is confirmed by Department of Botany in Science Faculty Chulalongkorn University, Thailand.

Herbal extraction: The leaves of *T. laurifolia* and *M. oleifera* were first washed with distilled water to remove salts and debris, then dried at $24^{\circ}C$ and pulverized into powder and filtered through 1 mm Ø pore size. Herbal extracted were prepared by extraction with 80% ethanol as follows: 10 grams of herbal powder were soaked in 1,000 ml 80% ethanol, shaken in ultrasonic bath for 30 min, cooled, let to stand at $4^{\circ}C$ for 7 days, removed the sediment using centrifuged at 3,000 *rpm* for 15 min and collected only supernatant. About 5 ml of clear supernatant was used to pre-analyze antioxidant activities using chemical methods and the other part of supernatant was concentrated by vacuum rotary evaporator at a low temperature ($<45^{\circ}C$). The crude extracts were finally concentrated using lyophilizer freeze-dried at $-50^{\circ}C$ 400 mbar into lyophilized form. Dried extracts were re-dissolved in water/culture medium or kept them at $-80^{\circ}C$ until further investigation.
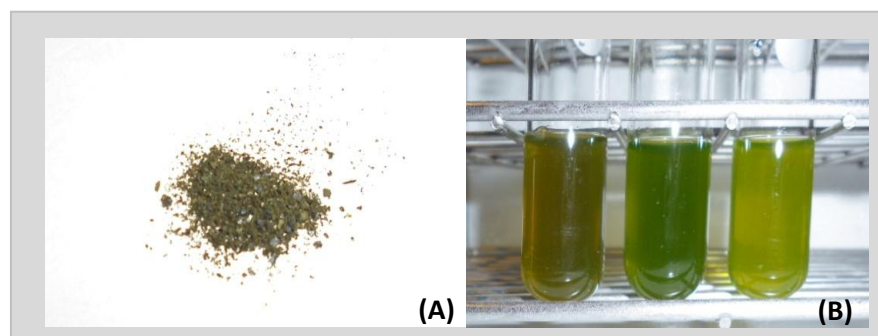
Figures 2.1: Lyophilized herbal powder (A), the maximum solubility of herbal extracts in water is 15 mg/ml, no precipitation were seen (B)

2.3 Human cell culture model

HepG2 (Human hepatoma) cell were a generous gift from Assoc. Prof. Dr. Parvapan Bhattarakosol, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. HepG2 were cultured in DMEM supplemented with 10% FBS and 1% penicillin/steptomycin (Doubling time=29±3 hr.). Cell cultures were maintained at 37°C under 5% CO_2 with 95% relative humidity. The culture medium was changed every 48-72 hr.
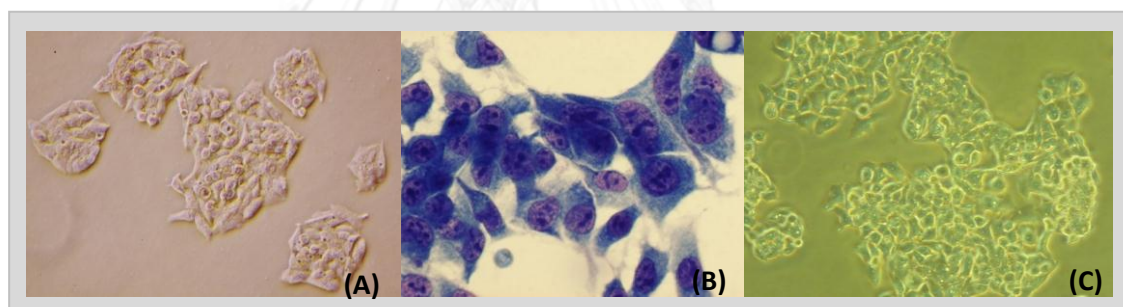
Figures 2.2: Morphological assessments of hepatoma cells (HepG2) by DIC microscopy 400X (A), Staining with Giemsha under light microscopy 1,000X (B), and by Invert microscopy 100X (C)

2.4 Experiment procedure

2.4.1. Determination of phytochemical contents in herbal extracts

Many active compounds were found in herbal plants[273]. Most of these phytochemicals presented in the plants were act as antioxidants[274]. In this study, the total phenolic content, total flavonoid content, total tannins content, total flavonol content and nitrate content of these herbal extracts were determined.

2.4.1.1 Analysis of phenol groups by UV-VIS spectroscopy scan spectrum

Difference active compounds in herbal extracts could absorb specific wavelengths[275]. The simple experiment to indicated flavonoid compounds in the herbal extracts is scanning their UV-VIS spectra.

Most flavonoids show their peaks in the 210 to 290-nm region and a second band at 320-380 nm. For anthocyanins, the latter peaks are in the visible region (490-540 nm).

Procedure

Individual flavonoids were dissolved in methanol (the concentration of flavonoids would absorb UV or visible light region between 0.05 and 1.00 Abs), and the basic spectrum were measured.

2.4.1.2. Determination of polyphenolics using Folin-Ciocateau Phenol assay (FCP assay)

Phenolic compounds, both phenolic acids and flavonoids, are the mainly antioxidant constituents of natural products. Polyphenolics are one of the most potent free radical terminators because of their many phenolic hydroxyl groups which acting by donating hydrogen radicals[276,277]. Folin Ciocalteau Phenol assay (FCP) is the method base on single electron transfer base (SET) used to measure reducing potential of antioxidant compound in sample. Total phenolics content in the extracts was determined using Folin–phenol reagent as described by Singleton et al[278]. Many studies are shown that herbal extracts contained a lot of polyphenolics which are the main reducing agents in the extracts. Polyphenolics could reduce yellow color of Folin-Ciocalteau Phenol reagent to blue solution.

Procedure

1. Mixed different concentrations of herbal extracts (500-10,000 µg/ml) or Gallic acid standard   with working 10% Folin–Ciocalteau's Phenol solution (w/w) *as shown in Table 3.1*.

2. The mixture was then allowed to stand for 25 min at room temperature and 350 µl 10 mM Na_2CO_3 were added to the mixture, let stand for another 20 min for turning blue of the solution.

3. The absorbance is measured at 750 nm against blank. Phenolic content was calculated using gallic acid as standard. The total phenolic content in sample was shown in gallic acid equivalent (GE) mM/kg dry wt.

Table 2.1: The procedure step to measure total polyphenols content by FCP assay

Reagents	Blank (µl)	Samples (µl)	Standards (µl)
1. Herbal extracts	-	500	-
2. Gallic acid solution	-	-	500
3. Deionized distilled water	500	-	-
4. Working Folin Ciocalteu's Phenol Reagent	500	500	500
Mixed, incubated at room temperature for 20 min			
5. Na_2CO_3 solution	350	350	350
Incubated at room temperature for 20 min than measure the absorbance of standard and samples at 750 nm against blank			

2.4.1.3. Determination of total flavonoids using aluminum chloride assay

The major class of polyphenol that had many reported to contribute directly to antioxidant actions is flavonoid[279].Flavonoids have important different functional properties in reactive oxygen species scavenging[280], free radical generation inhibitions and chain-breaking activity[281], stabilizing lipid oxidation[282] and metal chelation[283]. Total flavonoids contents in herbal extracts are established in the colorimetric reaction with aluminum chloride described by Zhishen et al[284].

Procedure

1. Each aliquot of diluted sample or standard (quercetin) 500 μl was mixed with 150 μl of 5% $NaNO_2$ solution (w/v). After 6 min, 150 μl of 10% $AlCl_3$ solution (w/v) was added and allowed to stand for 6 min.

2. Five hundred μl 1.0 M NaOH was added to the mixture. The final volume was adjusted to 2.5 ml with distilled water, thoroughly mixed and allowed to stand for another 15 min.

3. Absorbance was then determined at 510 nm against the water blank. Total flavonoid contents were expressed as Quercetin equivalents (QE) mM/kg dry wt.

Table 2.2: The procedure step to measure total flavonoid content by aluminum chloride assay

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	500	-
2. Quercetin standard	-	-	500
3. 5% $NaNO_2$ (w/v)	150	150	150
4. 10% $AlCl_3$ (w/v)	150	150	150
Mixed and let stand at room temperature in dark for 6 min, then added H_2O to achieved final volume 2.5 ml, let stand at room temperature for another 15 min, the pink color was developing at this time, and then measure the absorbance at 510 nm against blank.			

2.4.1.4. Determination of condensed tannins content using Vanillin assay

The active polyphenolics are contained in a variety of plants are in complex polymeric forms[285] called condense tannins. Tannins were more tolerant to degradation from gastic enzyme than monomer polyphenolics and shown to form complexes with proteins in digestive tract[286]. Tannin was detected with acidified vanillin reaction specific for estimating tannin content[287]. The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the meta-substituted ring of flavanols to red adduct which could measure at 500 nm.

Procedure

1. One ml sample of the extract was mixed with reagent *as shown in table 2.3* and then incubated at 25°C for 15 min.

2. After the incubation, the absorbance was read at 500 nm. The concentration of total tannins was estimated from a calibration curve, constructed by plotting known solutions of catechin (0-80

$\mu g/ml$).The concentration of the condensed tannin was expressed as Catechin equivalent (CE) mM/kg dry wt.

Table 2.3: The procedure step to measure total tannin contents by Vanillin assay

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	1,000	-
2. Catechin standard	-	-	1,000
3. Deionized distilled water	1,000	-	-
4. Working Vanillin reagent (1% w/v in 7.0 M H_2SO_4)	2,000	2,000	2,000
Mixed, let stand in the dark at 25°C at 15 min. Measured the absorbance at 500 nm against blank			

2.4.1.5 Determination of total flavanols content by DMACA-HCL reagent

Because the test for condense tannins in this study are nonspecific[288]. Both condense tannins and flavonol are able to detect by this method. We design to further confirm our condense-tannin-result and to determinations which form of these compounds are constituted in herbal sample. The total flavanol content was estimated using the p-dimethylaminocinnamaldehyde (DMACA) method[289]. This method has a great advantage because no interference by anthocyanins and provide higher sensitivity and specificity.

Procedure

1. Herbal extracts and standard catechin were introduced into a 1.5 ml microcentrifuge tube and added DMACA solution *as shown in Table 2.4*. The mixture was mixed and allowed to stand at room temperature for 10 min.
2. Read absorbance at 640 nm against blank that prepared similarly without DMACA. The concentration of total flavanols was estimated from a calibration curve, constructed by plotting known solutions of catechin (0–16 $\mu g/ml$). The concentration of the flavonols content was expressed as Catechin equivalent (CE) mM/kg dry wt.

Table 2.4: The procedure step to measure total flavonols content using DMACA-HCL reagent

Reagents	Reagent Blank (μl)	Sample Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	200	200	
2. Catechin standard	-	-	-	200
3. Deionized distilled water	200	1,000	-	-
4. DMACA solution (0.1% w/v in 1.0 M of HCl in MeOH)	1,000	-	1,000	1,000
Mixed, let stand in the dark at 25°C at 10 min. Measured the absorbance at 640 nm against blank				

2.4.1.6 Determination of nitrate content by Griess reagent

Nitrate usually found in green leaves. Vegetable provides high amounts of nitrate while most fruits are low in nitrate[290]. Therefore, Nitrate were shown biological effect such as mitochondria[291,292]. Because nitrate is absorbed 100% from digestive tract, we consider nitrate might be one of the factors that are health benefit. This assay was based on the principle that vanadium (III) chloride (VCl_3) in hydrochloric acid was used to convert nitrite and nitrate to nitric oxide (NO), which was then quantified by the Griess method[293,294].

Procedure

1. Herbal extracts and $NaNO_3$ standard were added into 96-well plate *as shown in Table 2.5.* The experiment was performed at room temperature.
2. To measure nitrite and nitrate (NOx) concentrations, addition of VCl_3 to each well was rapidly followed by Griess reagents, SULF and NEDD, respectively. Sample blank values were obtained by substituting diluting medium for Griess reagent.
3. For Nitrite measurement, regent was added in a similar manner except that samples and nitrite standards were only exposed to Griess reagents. No VCl_3 was added in nitrite measurement.
4. The mixture was mixed and allowed to stand at room temperature for 30-45 min. The absorbance was read at 540 nm against blank. These values were then subtracted to give the nitrate concentration.

Table 2.5: The procedure step to measure total nitric oxide and nitrite contents by using Griass reagent assay

Reagents	Total NOx			Nitrite		
	Blank(µl)	Samples (µl)	Standards NaNO₃ (µl)	Blank(µl)	Samples (µl)	Standards NaNO₂(µl)
1. Water	400	200	200	1,000	800	800
2. Herbal extracts	-	200	-	-	200	-
3. Std. solution (200–1600 µM)	-	-	200	-	-	200
4. VCl₃ in 1M HCl	200	200	200	-	-	-
5. 2% SULF(w/v)	100	100	100	100	100	100
6. 0.1% NEDD (w/v)	100	100	100	100	100	100
Total volume	800			1200		
Mixed, let stand in the dark at 25°C at 30-45 min. Measured the absorbance at 540 nm against blank						

It was advisable to determine the nitrite and total NOx concentrations for a particular sample in the same plate so that the conditions were identical for each measurement.

2.4.2. Determination of antioxidant activity of herbal extracts:

2.4.2.1 Determination of antioxidant activity of herbal extracts using chemical methods

2.4.2.1.1. Oxygen radical Absorbance Capacity (ORAC) assay

ORAC assay is directly measured the antioxidant activities of chain-breaking antioxidants against peroxyl radicals by using fluorescein as the fluorescent probe[295,296]. ORAC were based on hydrogen atom transfer-based reaction (HAT) which turns to be one of the advantages of the ORAC assay because it relates to biologically relevant free radicals. Our ORAC assay is according to Huang et al. method with some modification[297]. ORAC is used to determine the antioxidant capacity of herbal extracts by determination of ability to prevent quenching of fluorescence by $ROO^{\bullet}$ radical. This free radical is generated by hydrophilic 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) reacting with oxygen. $ROO^{\bullet}$ radical causes damaging in fluorescent probe structure lead to conformation change and lost their fluorescent intensity. Antioxidant in herbal extract distress these damaging effects therefore fluorescent are going on. The intensity of fluorescence will change along with quantity of antioxidant in the herbal extracts.

Procedure

1. In 96 well plate, 50 µl of different concentration of herbal extracts (in PBS, pH7.4) or standard Trolox solutions were mixed with 80 µl fluorescein solution (40 nM in 75 mM PBS, pH 7.4). The plate was equilibrated by incubating for a minimum at 37°C for 30 min. Reaction is initiated by added 20 µl freshly prepared AAPH (153 mM in 75 mM PBS, pH 7.4) for a final volume of 150 µl and followed by shaking for 10 sec. The fluorescent intensity is then monitored kinetically with data taken every minute for 35 min using 485 nm and 535 nm filters for excitation and emission, respectively. The ORAC values were calculated by using area under curve (AUC). The AUC and the Net AUC of the standards and samples were determined using regression software with the equations *shown in Table 2.6.*

2. The loss in fluorescence can be assessed by measuring the area under the curve (AUC) of the kinetic plot for each concentration. The greater the extent of florescent decay, the smaller the expected AUC value would be. When net AUC were calculated from these kinetic curves and plotted against Trolox concentrations a linear relationship was observed. The calibration curve was obtained by plotting the net AUC of different Trolox concentrations against their concentrations. The results were expressed as Trolox equivalents (TE) mM/kg dry wt.
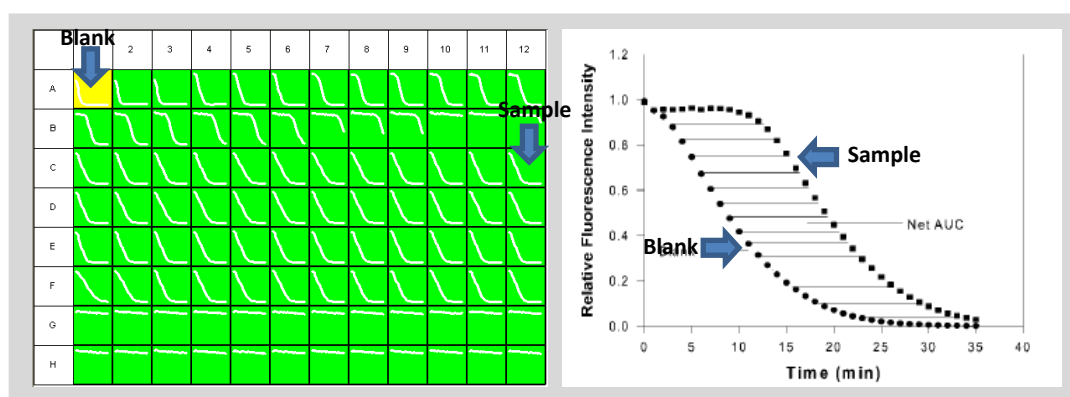
Figure 2.3: **The ORAC assay monitors the reaction between peroxyl radical and fluorescent substrate during the period about 35-40 min.** The antioxidant activity is determined from the net integrated areas under the fluorescence decay curves.

Table 2.6: The procedure step to measure antioxidant activity by ORAC assay

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	25	-
2. Phosphate buffer (pH 7.4)	25	-	-
3. Trolox standard solution	-	-	25
4. Working fluorescein dye	80	80	80
Mixed, incubated at 37 $^\circ$C 30 min			
5. AAPH solution	25	25	25

The fluorescent intensity was then monitored kinetically with data taken every minute for 35 min using 485 nm and 535 nm filters for excitation and emission, respectively. The ORAC values were calculated by using area under curve (AUC). The AUC and the Net AUC of the standards and samples were determined using regression software with the equations:

$$AUC = 0.5+(R2/R1)+(R3/R1)+(R4/R1)+....+0.5(Rn/R1)$$

R1 was the initially fluorescence light at 0 min, R2 is the 2nd fluorescence light at 1 min,... Rn was the final fluorescence intensity

$$\text{Net AUC}_{Standard} = \text{AUC}_{standard} - \text{AUC}_{blank}$$

$$\text{Net AUC}_{Sample} = \text{AUC}_{Sample} - \text{AUC}_{blank}$$

2.4.2.1.2. Trolox equivalent antioxidant capacity (TEAC) assay

Trolox equivalent antioxidant capacity (TEAC) assay is based on the reduction of the 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS$^{\bullet+}$) by electron-donating compounds. The TEAC assay is used to evaluate the capacity of an herbal extract to scavenge ABTS$^{\bullet+}$ radicals by comparing the radical scavenging activity of a compound with Trolox, a water soluble vitamin E analogue[298].

Procedure

1. The ABTS$^{\bullet+}$ was freshly prepared before experiment by mixing ABTS stock solution (7mM in PBS) with $K_2S_2O_8$ (2.45mM). This mixture has to remain for 24 hr until the reaction was completed and the

absorbance was stable. For measurements, the ABTS$^{\bullet+}$ solution is diluted with PBS to an absorbance of 0.70±0.02 at 734 nm.

2. One ml of the ABTS^{2+} solution was mixed with 20 μl of different concentrations of herbal extracts and measured after allow to stand at room temperature for 3 min at 734 nm against blank. The antioxidant activity of the herbal extracts was calculated by determining the decrease in absorbance by using the following equation:

$$\% \text{ Antioxidant activity} = \frac{(\text{Abs}_{(control)} - \text{Abs}_{(sample)})}{\text{Abs}_{(control)}} \times 100$$

Table 2.7: The procedure step to measure antioxidants activity by TEAC assay

Reagents	Reagent Blank (μl)	Sample Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	20	20	-
2. Trolox solution	-	-	-	20
2. ABTS•+ solution	1,000	-	1,000	1,000
3. Deionized distilled water	20	1,000	-	-
Mixed, stand in the dark for 3 min at 25°C. Measure the absorbance at 734 nm against blank				

2.4.2.1.3. Ferric reducing antioxidant power (FRAP) assay

Reducing power is often used to evaluate the ability of natural antioxidants to donate electrons. There are direct correlation between reducing power and their antioxidant activity[299]. The reducing power of herbal extracts was determined according to the method of Oyaizu et al[300]. This assay was based on ability to reduce ferric 2,4,6-tripyridyl-s-triazine complex to the ferrous form by antioxidants. The presence of reductants (antioxidants) in the sample would result in the reduction of the Fe^{3+}/ferricyanide complex to its ferrous form (Fe^{2+}). Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Procedure

1. Added 250 μl of different concentration of herbal extracts (500-10,000 μg/ml in 0.2M PBS, pH 6.6) plus 250 μl of 1% K$_3$Fe(CN)$_6$ solution (w/v) are mixed and incubated at 50°C for 20 min.

2. Then, 10% trichloroacetic acid (w/v) was added to terminate the reaction and the resultant mixture was centrifuge at 3,000 *g* for 10 min. An aliquot of 100 μl upper layer was diluted with equal volume of distilled water before added 20 μl 0.1% FeCl$_3$ solution (w/v), mixed and the absorbance are measured at 700 nm. Ascorbic acid was used as the standards. Increased absorbance of the reaction mixture indicated reducing power was increased.

Table 2.8: The procedure step to measure antioxidant activity by FRAP assay

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	250	-
2. Ascorbic acid standard	-	-	250
3. Deionized distilled water	250	-	-
4. 1% $K_3Fe(CN)_6$ solution (w/v)	250	250	250
Mixed, incubated at 50°C for 20 min.			
5. 10% Trichloroacetic acid (w/v)	250	250	250
Gently mixed and incubated at 20°C for 15 min then measured the absorbance of standards and samples at 500 nm against blank			

2.4.2.1.4. Determination of hydroxyl radical scavenging activity of herbal extracts

The hydroxyl radical is highly reactive radical usually generates hydroxyl radicals by the process 'Fenton chemistry'. The experiment is based on production of hydroxyl radical from Fenton reaction attack deoxyribose and degrades it. Deoxyribiose degradation is used as an indication of hydroxyl radical scavenging activity. The hydroxyl radical-scavenging activity assay is evaluated according to the method of Gutteridge et al[301] which is used to determine the hydroxyl radical scavenging activity in an aqueous medium. The hydroxyl radical scavenging of herbal extracts were measured by the competition between deoxyribose and herbal extracts for the hydroxyl radicals generated from the Fenton reaction (Fe^{3+}/ascorbate/EDTA/H_2O_2 system). Deoxyribose fragments then react with thiobarbituaric acid (TBA) upon heating at low PH to form a pink color compound which can be detected spectrophotometrically.

Procedure

1. The reaction mixture containing each 8 μl of 1 mM $FeCl_3$, 1 mM EDTA, 60 mM deoxyribose and 50 μl of different concentrations of herbal extracts (500-10,000 μg/ml in 20 mM PBS, pH7.4) was mixed with the reaction reagent *as shown in Table 2.9*, pre-incubated at 37°C before adding 8 μl of 10 mM H_2O_2 and 8 μl 2 mM ascorbic acid to initiated the reaction.
2. After incubation at 37℃ for 1 hour, the extent of deoxyribose degradation was measured using TBA method. TBA and TCA were added to the mixture followed by heated at 95°C for 15 min. Finally the reaction mixture was cooled on ice and centrifuged at 5,000 rpm for 15 min.
3. The pink color was developed and supernatant was transferred to the new 96 well plate and measured at 532 nm. All readings were corrected for any interference such as green color of the extract or antioxidant by using appropriate controls. The negative control without any antioxidant was considered 100% deoxyribose oxidation. The % hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control. The scavenging activity of herbal extracts was expressed as the percentage inhibition of the deoxyribose degradation and is calculated using the following formula:

$$\% \text{ Hydroxyl radical scavenging activity} = \frac{\text{Abs}_{(control)} - \text{Abs}_{(sample)}}{\text{Abs}_{(control)}} \times 100$$

The IC_{50} (defined as the concentration of sample at which 50% of hydroxyl radical is scavenged) is calculated for each sample.

Table 2.9: The procedure step to measure hydroxyl radical scavenging activity of herbal extracts

Reagents	Blank (μl)	Samples (μl)	Control (μl)
1. Herbal extracts (500-1,000 μg/ml)	-	50	-
2. Ascorbic acid (positive control)	-	-	50
3. 1 mM FeCl₃	8	8	8
4. 1mM EDTA	8	8	8
5. 60 mM Deoxyribose	8	8	8
6. 20 mM Potassium phosphate buffer pH 7.4	50		
Incubated at 37°C foe a few min			
7. 10 mM H₂O₂	8	8	8
8. 2 mM Ascorbic acid	8	8	8
Incubated at 37°C for 1 hour, final reaction volume was made 1.0 ml with PBS (20 mM, pH 7.4)			
9. 0.5% TBA in 0.025 M NaOH	80	80	80
10. 2.8% TCA (w/v)	80	80	80
Gently mixed and incubated at 95°C for 15 min then measure the absorbance of controls and samples at 532 nm against blank			

2.4.2.1.5. Determination of superoxide radical scavenging activity of herbal extracts

Superoxide anion is the most common free radicals which being a precursor for other ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen. Superoxide has the potential reactivity with biological molecules and inducing tissue damage. The experiment is based on Alkaline DMSO, used as a superoxide generating system, reacts with NBT to give colored diformazan. Superoxide scavenging activity of the herbal extracts is determined by alkaline DMSO method according to the method of Govindarajan et al. with slightly modification[302]. Alkaline DMSO is used as a superoxide generating system. The concentration of superoxide in alkaline DMSO system corresponds to the concentration of dissolved oxygen in DMSO.

Procedure

1. Added 30 μl of different concentration of herbal extracts (100-3,000 μg/ml) in the reaction mixture containing 100 μl 0.1% NBT (w/v, in DMSO) and 100 μl of alkaline DMSO (0.1 ml of 5mM NaOH in 1ml DMSO) to a final volume of 230 μl.

2. The absorbance is measured at 560 nm against DMSO as a control negative in which DMSO was added instead of alkaline DMSO. The decreased absorbance of herbal extracts at 560 nm indicated the consumption of generated superoxide. The percentage of superoxide radical scavenging by herbal extracts and standard were calculated using following formula:

$$\% \text{ Superoxide radical scavenging activity} = \frac{Abs_{(control)} - Abs_{(sample)}}{Abs_{(control)}} \times 100$$

Table 2.10: The procedure step to measure superoxide radical scavenging activity of herbal extracts

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	-	30	-
2. Catechin standard	-	-	30
3. DMSO	30	-	-
4. 0.1% NBT reagent (w/v in DMSO)	100	100	100
5. Alkaline DMSO	100	100	100
Gently mixed and incubated at 20°C for 15 min then measured the absorbance of standards and samples at 560 nm against blank			

2.4.2.1.6. Determination of nitric oxide radical scavenging activity of herbal extracts

Nitric oxide is an abundant essential reactive molecule that acts as an oxidative biological signaling mediator in a variety of physiological processes. But over production of NO nitrogen species lead to Nitrosative stress[303] that can alter the structure of protein and inhibit their normal function via nitrosylation reactions. Nitric oxide radical scavenging capacity of herbal extracts is analyzed according to the method of Green et al., which slightly adapted for 96-well microplates[304]. The principle is based on sodium nitroprusside (SNP) in aqueous solution at physiological pH; it is spontaneous generated nitric oxide. Nitric oxide interacts with oxygen to produce nitrite ions, which can be estimated by Griess reaction.

Procedure

1. Twenty mM SNP was mixed with different concentration of herbal extracts (500-10,000 µg/ml in PBS). The mixture is incubated under the light at 25°C for 180 min. Finally, to terminate reaction, the equal volume of Griess reagent (1% sulfanilamide, 2.5% phosphoric acid and 0.1% naphtyl ethylene diamine dihydrochloride) was added to each well.

2. The absorbance of chromophore was measured at 540 nm. The result is expressed as a percent of scavenged nitric oxide with respect to the negative control without added any antioxidant. Ascorbic acid was used as a positive control. An IC$_{50}$ values (defined as the concentration of sample at which 50% of nitric oxide radical is scavenged) is calculated for each sample.

Table 2.11: The procedure step to measure nitric oxide radical scavenging activity of herbal extracts

Reagents	Blank (μl)	Samples (μl)	Controls (μl)
1. Herbal extracts	-	250	-
2. Ascorbic acid	-	-	250
2. SNP 20mM in PBS, pH 7.4	250	250	250
3. Deionized distilled water	250	-	-
3. Griess reagent (1% sulfanilamide, 2.5% phosphoric acid and 0.1% naphtyl ethylene diamine dihydrochloride)	500	500	500
Gently mixed and incubated at 20°C for 15 min then measured the absorbance of control and sample at 500 nm against blank			

2.4.2.1.7. Determination of Hydrogen peroxide scavenging activity of herbal extracts

Hydrogen peroxide is a relatively unstable metabolic product by itself is not very reactive. But H_2O_2 could highly penetrate through cellular membrane; it tends to form hydroxyl radical on reacting with free heavy metal and initiated Fenton reaction[305]. Thus, the removing of hydrogen peroxide is essential for preserved normally cell function. The ability of the extracts to effectively scavenge hydrogen peroxide was determined according to the method of Sinha et al.[306] with slightly modification.

Procedure

1. Mixed 100 μl of different concentration of herbal extracts with H_2O_2, then added $K_2Cr_2O_7$ solution, let stand at room temperature for 1 hour. Heat the reaction mixture at 100°C for 15 min.
2. The absorbance of hydrogen peroxide at 560 nm was determined after ten min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity of hydrogen peroxide was then calculated using the following formula:

$$\% \text{ scavenged activity of } H_2O_2 = \frac{Abs_{(control)} - Abs_{(sample)}}{Abs_{(control)}} \times 100$$

Table 2.12: The procedure step to measure hydrogen peroxide scavenging activity of herbal extracts

Reagents	Blank (μl)	Samples (μl)	Standards (μl)
1. Herbal extracts	100	100	-
2. H_2O_2 (500-2,000 mM)	-	500	500
3. PBS, pH 7.4	1,000	1,000	1,000
4. Deionized distilled water	900	400	500
5. 5% $K_2Cr_2O_7$ solution in 1:3 glacial acetic acid(v/v)	500	500	500
Gently mixed and incubated at 25°C for 1 hour then heat at 100°C for 15 min. Measured the absorbance of standards and samples at 620 nm against blank			

2.4.2.2 Determination of protective effect of herbal extracts on macromolecules against oxidative damage

2.4.2.2.1. Measurement of DNA oxidative damage using alkaline single cell gel electrophoresis (Comet assay)[307]

To study DNA oxidative damage in human mononuclear cells by using Comet assay or single cell gel electrophoresis. The Comet assay is a simple, rapid, and sensitive technique for analyzing and quantifying DNA damage in individual mammalian cells. When DNA is damaged in the nucleus, genetic materials breakdown into fragment and migrated under subjection to electrophoresis. After staining, randomly selected mononuclear comet images are analyzed by fluorescence microscope. The characteristics of the comet tail may also be useful in assessing semi-quantitative differences in the type of DNA damage.

Procedure

A. Blood sample

Peripheral venous blood samples were collected into heparinized tube. Peripheral blood mononuclear cells were used in this study. Mononuclear cells in Ficoll reagent were isolated by a density gradient centrifugation (density of 1.077-1.080 g/mL at 20°C) at 2,000 rpm for 10 min. The buffy coat was obtained. The mononuclear cells in buffy coat were washed twice and re-suspended in PBS, pH7.4, and made a final concentration of 1-2 $\times 10^5$ cells/ml.

B. Experiment procedure

1. The procedure were done in 1.5 ml microcentrifuge tubes, 200 μM H_2O_2, herbal extracts with and without H_2O_2, was mixed with mononuclear cells for 2 hr at 4°C in the dark *as shown in Table 2.13*. Then, cells were wash twice with ice-cold PBS and re-suspend in PBS.

2. Added 10 μl cell suspensions in 75 μl 0.5% low melting-point agarose (w/v, 37°C), mixed and an aliquot was immediately spread on the pre-coated-slide 1% normal-melting-point-agarose, let stand for 5 min at 4°C for gel to solidify and covered slide with another layer of 100 μl 1% low melting-point-agarose (w/v).

3. Allow the gel to solidify, then immersed the slide in the lysis buffer solution [10 mM Tris–HCl, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 (v/v), and 10% DMSO(v/v)] for at least 1 hour at 4°C.

4. Put the slide in the electrophoresis tank filled with pre-chilled electrophoresis solution [300 mM NaOH, 1 mM EDTA and 0.2% DMSO, pH >13.0]. The slides were equilibrated in that buffer for 40 min at 4°C to allow the DNA for unwinding before subjected to electrophoresis for 25 min at 4°C with the current adjusted to 300 mA.

5. Slides were then washed with neutralization buffer, 0.4 M Tris–HCl, pH 7.5, three times after run for 5 min each at 4°C for removing any alkaline present.

6. One % Ethidium bromide solution (w/v) was applied to the slides and visualized under fluorescence microscope using excitation and emission wavelengths of 515 and 535 nm, respectively.

C. Analysis by fluorescence microscopy

When excited, the DNA-bound ethidium bromide emitted red light. In healthy cells the fluorescence is confined to the nuclei: undamaged DNA is super-coiled and thus did not migrate very far from the nuclei under the influence of an electric current (Figure. 2.4). In cells that have accrued damage to the DNA, the alkaline treatment unwinds the DNA, releasing fragments that migrate from the nuclei when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. The longer one implies severe damage of DNA. The degree of DNA damage was scored from 1,000 randomly selected cells per slide using a 20x objective. Tail length (TL), the percentage of fluorescence in the tail, was scored as followings; 0 = undamaged nucleus, 1 = nucleus with tiny tail, 2 = nucleus with dim tail, 3 = nucleus with a clear tail, and 4 = all DNA in tail. Each comet was given a value according to its classification to produce an overall score for each slide ranging from 0 to 4000 arbitrary units.

Scores are calculated using the following formula in which N_0, N_1, N_2, N_3, and N_4 represent the number of cells in each group from 0 to 4:

$$\% \text{ Total damage} = \frac{(N_0 \times 0) + (N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4)}{N_0 + N_1 + N_2 + N_3 + N_4} \times 100$$

Table 2.13: The procedure step to measure the oxidative DNA damage by Comet assay

Reagents	Negative Control (μl)	samples (μl)		Positive Control (μl)
		Without H_2O_2	with H_2O_2	
1. PBMCs	80	80	80	80
2. PBS, pH 7.4	920	-	-	-
3. Herbal extracts*	-	920*	460**	-
4. H_2O_2 200 μM	-	-	460**	920
Mixed, stranded at 4°C in the dark for 2 hr then collect the cells sediment to analysis				

* range concentration range from 600 to 1,000 μg/ml

** 2X concentration for finally made concentration equal 1X

DNA fragment are released from the supercoiling and migrate from the head of the nucleoid to form the comet tail over 80 - 100% (point), no clearly nucleoid. This teardrop shape comet term as Apoptotic comet. Mostly found in Apoptosis cell death

(A)

Long DNA fragment are released from the supercoiling and migrate into the gel but nucleoid comet head still exit. Usually grade by found DNA damage in tail over 50% to 80%

(B)

Modulated DNA fragment are released from the supercoiling and migrate into the gel about 10-50%. Nucleoid comet head were clearly seen.

(C)

Mild DNA fragment about 5-10 %were release from the supercoiling, generally found near the Nucleoid comet head **(D)**, No DNA fragment were seen after run gel electrophoresis **(E)**

(D)   **(E)**

Figures 2.4: **DNA fragments that migrate from the cell when subjected to an electric field**. Classified into 5 levels: level 4 = more than 80%-100% DNA in the tail, long comet tail, no DNA head were seen **(A)**, level 3 = more than 50% DNA in the tail, long comet tail, little DNA head **(B)**, level 2 = more than 10% DNA in the tail, moderate comet tail, have DNA head **(C)**, level 1 = 1-10% DNA in the tail, some DNA fragments were seen near DNA nucleus **(D)**, level 0 = no DNA at all in the tail **(E)**

2.4.2.2.2. Measurement proteins oxidative damage using Heinz body formation [308]

Oxidative damage of protein is indicated by the formation of Heinz bodies. Heinz bodies are precipitate of oxidized or denatured hemoglobin that adhere strongly to the red blood cell (RBC) membrane. The protective effect of herbal on oxidative damage to RBCs heme proteins induced by free radical initiators, APHZ, was studied.

Procedure

A. Blood sample

Peripheral venous blood samples were collected into heparinized tube. Whole blood was used in this study.

B. Experiment procedure

1. The procedure were done in 3 ml tubes, herbal extracts and positive control is mixed with fresh whole blood into PBS, pH7.4 *as shown in Table 2.14*. Then, the mixture was incubated in shaking water-bath for 2 hr at 37°C under aerobic condition. Negative control is done similarly except only no APHZ is added. The sample extract is done in 2 conditions, one was the same as positive control and the other was the same as negative control except only added herbal extracts at concentration of 100 – 1,000 μg/ml in reaction mixture.

2. Heinz bodies in RBCs were stained with 3 drops of 1% crystal violet solution (w/v in 0.73% of normal saline) and 3 drops of RBC suspension for 5 min at room temperature. Made blood smear on glass slides and count RBCs with Heinz bodies inside/1,000 of RBCs with light microscope. The results are reported in % of Heinz body formation.
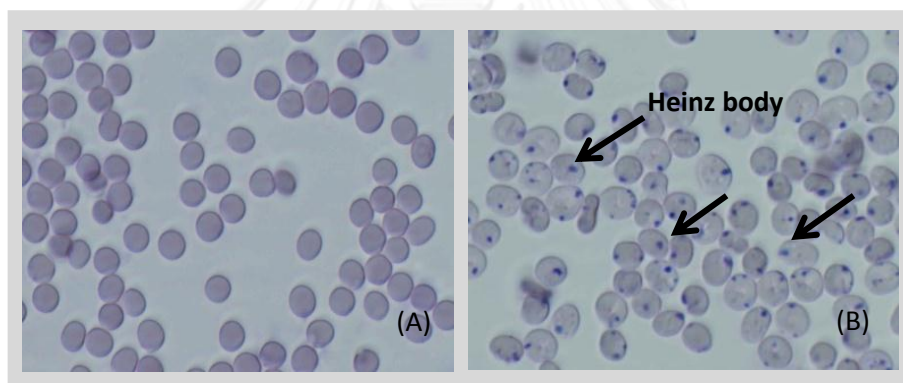

Figure 2.7: Normal red blood cells **(A)** and Heinz body formation from precipitation of oxidized hemoglobin **(B)**

Table 2.14: The procedure step to measure the protective effect of herbal extract on protein and lipid oxidation induced by free radicals using Heinz body inhibition assay and hemolysis inhibition assay

Reagents	Negative Control (µl)	Samples (µl)				Positive Controls (µl)		Hemolysis reference (µl)
		Hemolysis inhibition assay[a]		Heinz body inhibition assay[b]		Hemolysis inhibition assay	Heinz body inhibition assay	
		Without APPH	With APPH	Without APHZ	With APHZ			
1.Whole blood	100	100	100	100	100	100	100	100
2.PBS, pH 7.4(0.02% w/v glucose)	900[b] 1900[a]	-	-	-	-	-	-	-
3.Herbal extracts	-	900*	450**	1800*	900**	-	-	-
4.AAPH (200mM)	-	-	-	-	900**	-	900	-
5.APHZ(0.01% w/v)	-	-	450**	-	-	900	-	-
6.Milli-Q water	-	-	-	-	-	-	-	900
Gently mixed and left at 37°C at dark, for design incubation times, then centrifuge to get cells to analysis								

a= final volume= 1 ml

b= final volume=2 ml

* Herbal extracts concentration range between 100, 200, 400, 600, 800 and 1,000 µg/ml

** Double strength concentration to give the 1X final concentration

2.4.2.2.3. Measurement of lipids membrane oxidative damage using erythrocytes hemolysis assay[309]

Erythrocytes are high susceptibility to peroxidation because membrane of erythrocytes is rich in polyunsaturated fatty acids. erythocytes have been widely used as a model to investigate oxidative damage in lipid bilayer[310]. The erythrocyte hemolysis was induced by a peroxyl radical initiator, AAPH. The peroxyl radicals generated from AAPH can attack the polyunsaturated fatty acids on the membrane of erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. Therefore, oxidative damage of lipid is indicated by the extent of hemolysis which is directly resulted from erythrocyte membrane destruction. It is realistic and easy to use method to determine cell membrane damage.

Procedure

A. Blood sample

Peripheral venous blood samples were collected into heparinized tube. Whole blood was used in this study.

B. Experiment procedure

1. The procedure were done in 1.5 ml microcentrifuge tubes, herbal extracts in warm PBS (pH 7.4) was mixed with 100 µl fresh whole blood in a reaction volume of 1 ml *as shown in Table 2.14.* Briefly incubate at 37°C for 5 min. Thereafter, hemolysis of RBCs was carried out by mixing the suspension with 200 mM AAPH solution (in PBS; final concentration 100 mM). This suspension is shaken gently at 37°C under aerobic condition for 240 min. One hundred µl is withdrawn to 1.5 ml of ice cold PBS at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min after AAPH is added and centrifuged at 3500 rpm for 10 min.

2. The extent hemolysis is estimated spectrophotometrically by measuring absorbance of the hemoglobin released into the supernatant at 540 nm. Negative control is done the same as positive control except only no AAPH is added. The herbal extracts suspension alone without RBCs was used as blank. Complete hemolysate was done by treated RBCs with distilled water and was used as 100% reference hemolysis.
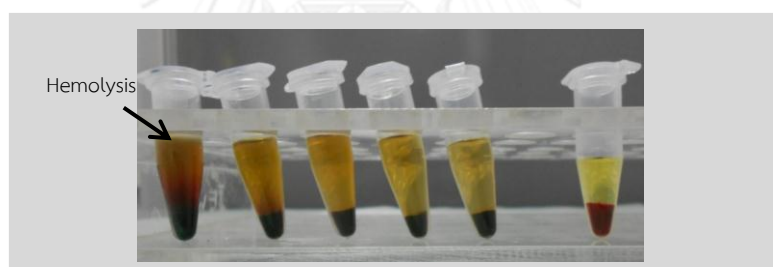
Figures 2.8: **Hemolysis reaction induced by AAPH as source for generating peroxyl radicals in in vitro systems.**

2.4.2.2.4. Measurement the inhibitory effect of herbal extracts on advanced glycation end products (AGEs) formation

Advanced glycation end products (AGE) are formed during the Maillard reaction where reducing carbohydrates react with lysine side chains and N-terminal amino groups of various macromolecules, particularly proteins. The advanced glycation end products can adversely affect the function of these macromolecules. One of the most prevalent advanced glycation end products, N-ε-(Carboxymethyl) lysine, has been implicated in oxidative stress and vascular damage. The non-enzymatic reaction of reducing carbohydrates with lysine side chains and N-terminal amino groups of macromolecules (proteins, phospholipids and nucleic acids) is called the Maillard reaction or glycation. The products of this process, termed advanced glycation end products (AGEs), adversely affect the functional properties of proteins, lipids and DNA. For example, N-ε-(carboxymethyl) lysine (CML), one of the prevalent AGEs, has been implicated in oxidative stress and vascular damage. Tissue levels of AGEs increase with age and the formation of AGEs is predominantly endogenous, though these products can also be derived from

exogenous sources such as food and tobacco smoke. AGEs modification of proteins can contribute to the patho-physiology of aging and long-term complications of diabetes, atherosclerosis and renal failure. AGEs also interact with a variety of cell-surface AGE-binding receptors (RAGE), leading either to their endocytosis and degradation or to cellular activation and pro-oxidant or pro-inflammatory events. Glycoaldehyde-AGE-BSA was prepared by reacting BSA with glycolaldehyde, and followed by extensive dialysis. Fluorescence of AGEs was measured with excitation and emission are 370 and 415 nm, respectively. The formation of AGEs is assessed by characteristic fluorescence according to a slightly modified method of Yamfaguchi et al[311].

Procedure

1. The procedure was done in 1.5 ml microcentrifuge tubes, 20 μl of different concentration of herbal extracts (500-10,000 μg/ml) was added to the reaction mixture *as shown in Table 2.15.*

2. The reaction mixture was incubated for at 60°C for 24, 48 and 72 hr; individual vials were removed at desired times and total protein are participated by 25 μl 100% trichloroacetic acid (w/v).

2. Protein were collected and dissolved in 1,000 μl of PBS buffer, pH 10. Transferred 200 μl test solution to 96 well plates. The fluorescence from the AGEs is measured. Aminoguanidine (33mM) were used as AGEs inhibitor control. The % inhibition has been obtained using the formula:

$$\% \text{ AGEs inhibition}= 1- \frac{(Abs_{(sample)} - Abs_{(sample\ blank)})}{(Abs_{(control)} - Abs_{(control\ blank)})} \times 100$$

Table 2.15: The procedure step to measure herbal extracts inhibitory effect on advanced glycation end products (AGEs) formation

Reagents	Blank Control(μl)	Controls(μl)	Samples(μl)	Blank unknown(μl)	Inhibitors(μl)
1.BSA 10mg/ml	400	400	400	400	400
2. 1 M Glucose	80	80	80	80	80
3. Herbal extracts[a]	-	-	20	20	-
4. 33 mM aminoguanidine	-	-	-	-	20
5. PBS, pH7.4	20	20	-	-	-
Mixed, incubate 60°C for 18 hr (Blank unknown and Blank control keep at 4 ℃)					
6. 100 % (w/v) TCA	25	25	25	25	25
Mixed centrifuge collected sediment for analysis					
7. PBS, pH 10	1,000	1,000	1,000	1,000	1,000
Mixed, measured fluorescence intensity with excitation and emission were 370 and 415 nm, respectively					

a) Dissolved in PBS, pH 7.4

2.4.2.2.5. Evaluate antioxidant potential of herbal extracts by activation of erythrocytes plasma membrane redox system.

Studies show that human erythrocytes contain a plasma membrane redox system (PMRS), which transfers electrons from intracellular substrates (NADH and/or ascorbate (ASC)) to extracellular electron acceptors. Activation of red cell (PMRS) by some polyphenols constitutes a mechanism whereby these compounds elicit their antioxidant effects and protect cell from oxidative stress. the ability of herbal extracts to donate electrons to the erythrocyte PMRS and to reduce extracellular ascorbate free radicals (AFR) to ASC via AFR reductase was determined to link the antioxidant potential assessed by *in vitro* assay and the biological activity observed *in vivo*. Erythrocyte trans-plasma membrane redox activity was estimated by following the reduction of ferricyanide, according to the method of Avron and Shavit[312].

Procedure

A. blood samples

Peripheral venous blood samples were collected into heparinized tube. Packed Red blood cells (pRBCs) were used in this study.

♦ *Preparation of pRBCs*

Whole blood was centrifuged at 1800g for 10 min at 4°C. The plasma and buffy coat was aspirated out and the pRBCs were subsequently washed three times with cold PBS, pH 7.4.

B. Experiment procedure

1. The procedure were done in 1.5 ml microcentrifuge tubes, different concentration of herbal extracts (500-10,000 μg/ml) was mixed with 300 μl pRBCs were suspended in PBS containing 5 mM glucose to a final volume of 1500 μl. The suspensions were incubated for 30 min at 37°C. After exposure to herbal extracts, RBCs were washed twice with PBS to remove any amount of the herbal extracts out.

2. Added 1 mM freshly prepared potassium ferricyanide solution was added in pRBCs. The suspensions were incubated for the next 30 min at 37°C and then centrifuged at 1800g at 4°C.

3. Collected the supernatants for assayed ferrocyanide content using 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid disodium salt (DPI) as an indicator and measuring absorption at 535 nm. Results are expressed in μM ferrocyanide/ml pRBC/30 mins. Quercetin was used as positive control of changes in erythrocyte PMRS.

Table 2.12: The procedure step to measure hydrogen peroxide scavenging activity of herbal extracts

Reagents	Blank (μl)	Sample blank (μl)	Samples (μl)	Positive controls (μl)
1. Herbal extracts[a]	-	1200	1200	-
2. Quercetin standard (5-500 μM)[a]	-	-	-	1200
3. PBS, pH 7.4 containing 5 mM glucose	1200	300	-	-
4. packed RBCs	300	-	300	300
Gently mixed and incubated for 30 min in the dark at 37ºC. Then, collected pRBCs and wash twice with PBS				
5. 1mM KCNFe solution	200	200	200	200
Gently mixed and incubated for 30 min in the dark at 37ºC. Then, collected supernatant 200 μl and then reacted with the following reagents.				
6. 3 M NaoAc	200	200	200	200
7. 0.2 M Citric acid	200	200	200	200
8.3.3mM FeCl3 in 0.1M acetic acid	100	100	100	100
9. Deionized Water	700	700	700	700
10. 6.2 mM DPI solution	100	100	100	100
Gently mixed. After 5 min incubation, immediately measure absorbance 535 nm against blank				

a) Dissolved in PBS containing 0.154 mM NaCl, 10 mM Na_2HPO_4 and 5 mM glucose, pH 7.4

2.4.2.3. Analysis the ability of herbal extracts for modulation activity of 3 enzymes: *α-glucosidase, α-amylase and acetylcholine esterase*

2.4.2.3.1. Inhibition effect of herbal extracts on *α-glucosidase activity*

Inhibitory effect on digestive enzymes, especially carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase are useful to retard glucose absorption rate, thus causing a reduction in postprandial hyperglycemia and decreasing the risk of developing diabetes. α-Glucosidase is function to hydrolyze maltose to glucose for use as a food. Aberrant activities have been implicated in diabetes[313,314]. Alpha-glucosidase inhibitory activity is performed according to the procedure described previously by Kim et al[315] with minor modifications. This assay used p–nitrophenyl–a–D–glucopyranoside (PNPG) as chromogenic substrate for α-glucosidase. Colorless glycoside was hydrolyzed specifically by α-glucosidase, releases the yellow product p–nitrophenol (PNP) which maximal absorbance at 405nm. The extent of the reaction could then be measured by determining the intensity of the yellow color which is, in turn, directly proportional to the concentration of PNP. The rate of the reaction is directly proportional to the enzyme activity.

$$\text{p-nitrophenyl } \alpha\text{-D-glucoside} \xrightarrow{\quad \alpha\text{-glucosidase} \quad} \alpha\text{-D-glucose} + \text{p-nitrophenol}$$
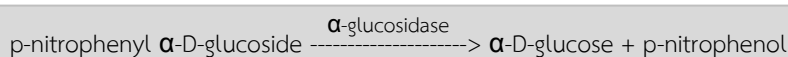
Procedure

1. The procedure were done in 96-well plate, different concentration of herbal extracts (500-10,000 µg/ml) was mixed with α-glucosidase (1 U/ml) to make 100 µl of final reaction volume, *as shown in Table 2.16* below.

2. The mixture is pre-incubated for 10 min at 37°C, and then 3 mM pNPG was added to the mixture as a substrate. After further incubation for 30 min at 37°C, the reaction was terminated by adding 0.1M NaCO$_3$. PNP absorbed maximally in alkaline solutions. The reaction should take the absorbance readings as soon as possible. The substrate is unstable in alkaline solutions.

4. Enzymatic activity was quantified by measuring the p-nitrophenol released from PNP-glycoside at 405 nm wave length. Read absorbance at 405nm (t = 0), and again after 20 min (t = 20 min) on a plate reader.

5. The % inhibition has been obtained using the formula:

$$\% \ \alpha\text{-glucosidase activity inhibition} = 1 - \frac{(Abs_{(sample)} - Abs_{(sample\ blank)})}{(Abs_{(control)} - Abs_{(control\ blank)})} \times 100$$

The concentration of the extracts required to inhibit 50% of α-glucosidase activity under the assay conditions is defined as the IC$_{50}$ value. Acarbose was used as α-glucosidase-inhibitor control.

Table 2.16: The procedure step to measure Inhibition effect of herbal extracts on α-glucosidase activity

Reagents	Blank (µl)	Control (µl)	Sample Blank (µl)	Samples (µl)	Inhibitor (µl)
1. 0.2 M PBS, pH 6.8	90	90	50	40	40
2. Herbal extracts	-	-	50	50	-
3. 10 ug/ml Acarbose (known inhibitor)	-	-	-	-	50
4. 1.67 U/ml α-glucosidase	10	10	-	10	10
Mixed, incubate for 5 min at 37°C					
5. 3 mM p-NPG	-	100	-	100	100
6. 0.2 M PBS, pH 6.8	100	-	100	-	-
Mixed, incubate for 15 min at 37°C					
7. 0.1 M Na$_2$CO$_3$	750	750	750	750	750
Mixed, measure the absorbance at 405 nm against blank					

a) Dissolved in PBS, pH 6.8

2.4.2.3.2. Inhibition effect of herbal extracts on α-amylase activity

Another important carbohydrate digestive enzyme is α-amylase. Amylase belongs to the family of glycoside hydrolase enzymes that break down starch into glucose molecules by acting on α-1,4-

glycosidic bonds. The **α**-amylases (EC 3.2.1.1) cleave at random locations on the starch chain, ultimately yielding maltotriose and maltose, glucose and "limit dextrin" from amylose and amylopectin[316].

Procedure

1. The procedure was done in 1.5 ml microcentrifuge tubes, mixed herbal extracts with reagent *as shown in Table 2.17*. The reaction mixture was incubated at room temperature for 10 min.

2. Added 1% soluble starch solution (w/v) in 0.05 M citrate buffer, pH 4.5.The reaction mixture was incubated at 37$^{\text{o}}$C for 10 min.

3. The reaction was quenched by adding 80 µl of 3,5-Dinitrosalicylic acid (DNS) in the reaction tube and then was immersed in boiling water bath (95-100$^{\text{o}}$C) for 10 min.

4. The absorbance was measured at 540 nm with spectrophotometer

$$\% \text{ α-amylase activity inhibition} = 1 - \frac{(Abs_{(sample)} - Abs_{(sample\ blank)})}{(Abs_{(control)} - Abs_{(control\ blank)})} \times 100$$

Table 2.17: The procedure step to measure Inhibition effect of herbal extracts on **α**-amylase activity

Reagents	Blank (µl)	Control (µl)	Sample Blank (µl)	Samples (µl)	Inhibitors (µl)
1. 20 mM PBS, pH 6.8	160	80	100	60	-
2. Herbal extracts[a]	-	-	20	20	-
3. 10 ug/ml Acarbose	-	-	-	-	80
4. Serum **α**-amylase	40	40	-	40	40
Incubated at room temperature for 10 min					
5. 1% potato Starch solution (w/v) in 0.05% citrate buffer,pH4.5	40	40	40	40	40
Immediately mixed and incubateed for exactly 10 min at 37℃					
6. DNS reagent	-	80	80	80	80
Mixed, incubated for 10 min at 95° C. Measured the absorbance of sample and inhibitor at 540 nm against blank					

a) Dissolved in PBS, pH 6.8

2.4.2.3.3. Modulating effect of herbal extracts on acetylcholinesterase activity against organophosphate pesticide

Intoxication with the paraoxon (POX) organophosphorus compound still is frequent in Thailand. Organophosphate pesticides is an inhibitor target on enzyme acetylcholinesterase (EC 3.1.1.7) with consequentially lead to excess acetylcholine accumulation and resulting in symptoms of cholinergic excess. This enzyme is responsible for the degradation of the neurotransmitter acetylcholine. Prevent the activity of AChE offer great potential for the treatment of insecticide poisoning. Also, Upregulation of AChE activity could be a therapeutic strategy for the cell proliferative conditions such as cancers. In toxicological field, inhibition of acetylcholinesterase activities by the herbal extracts were measured with minor adjustments of the spectrophotometric method developed by Ellman et al.[317] which was suitable for a 96 well plate. Acetylthiocholineiodide was employed as substrates of the reaction. This reaction

was relied on the hydrolysis of the substrate acetylthiocholine by AChE to thiocholine and acetate. Thiocholine combined with dithiobisnitrobenzoate (DTNB) and the yellow byproduct, 5-thio-2-nitrobenzoate was formed and then the absorbance was measured at 410 nm. The absorbance was proportion to the activity of the enzyme. Electric eel AChE (Type VI-S: Sigma) is used as source of acteylcholinesterase enzyme.

Acetylthiocholine -- $\xrightarrow{\text{acetylcholinesterase}}$ thiocholine + actate

Thiocholine + dithiobisnitrobenzoate-----------> yellow color
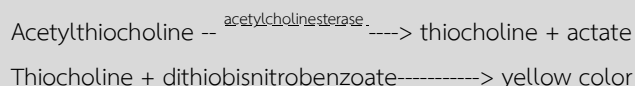
Procedure

1. The procedure was done in 96 well plate, herbal extracts (500-10,000 µg/ml) were added to the wells following by the reagent *shown in Table 2.18* then incubated for 5 min at 25°C.
2. The reaction was initiated by the addition of eel AChE solution (0.28 U/ml). The product from hydrolysis of acetylthiocholine reaction is monitored spectrophotometrically. Recording absorbance of the samples at 1-min intervals for a total of 5 min at a wavelength of 412 nm was done. Paraoxon acts as an acetylcholinesterase inhibitor was used as a reference inhibitor.
3. The percentage of cholinesterase activity inhibition was calculated using the following formula:

$$\% \text{ AChE activity inhibition} = 1 - \frac{(\text{Abs}_{(sample)} - \text{Abs}_{(sample\ blank)})}{(\text{Abs}_{(control)} - \text{Abs}_{(control\ blank)})} \times 100$$

Table 2.18: The procedure step to measure the effect of herbal extracts on acetylcholinesterase activity against organophosphate pesticide

Reagent	Blank (µl)	Control (µl)	Sample Blank (µl)	Sample (µl)	Inhibitor (µl)	Co-administration (µl)
1. 0.5 mM DTNB	125	125	125	125	125	125
2. 0.71 mM ATCI	25	25	25	25	25	25
3. 0.1M PBS, pH 8.0	100	75	75	50	50	25
4. Herbal Extracts	-	-	25	25	-	25
5. 90 nM paroexon (known Inhibitor)	-	-	-	-	25	25
Mixed, incubated for 5 min at 25 °C						
6. 0.28 U/ml AChE (immediately added before measurement)	-	25	-	25	25	25
Mix by inversion and transfer the solutions to suitable cuvettes. Record the absorbance of all at 1-min intervals for a total of 5 min at 412 nm against blank.						

Repeat the experiment on at least three separate occasions, with quadruplicate wells for each compound and condition tested.

2.4.3 The effect of herbal extracts on cell culture system

2.4.3.1 Routine Maintenance of HepG2 Cell Line protocol:

HepG2 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate) in 95% humidified air and 5% CO_2 at 37°C.

2.4.3.1.1 Subculture of HepG2 cells

These adherent cells were grown to near confluence and passaged as follows:

1. Medium was changed twice a week. Healthy passage of HepG2 cells was indicated by determined cells morphology and it's doubling time. HepG2 cells were back-cultured every 3-4 days using standard trypsinization procedures to maintain the cell line. All the experiments were carried out while the cells were in exponential growth phase. Seeding HepG2 onto fresh feeder layers at a density shown in Table2.19, about 3 $\times 10^3$ cells/cm^2 of surface area.

2. Allow the cells to grow overnight before initiation of experiments to make cells grow to sub-confluence ($\sim$70% to 80%).

Table 2.19: The density of HepG2 cells in culture plates

Cell plates/Times	4 hr	29 hr	58 hr
6 well plates (RT-PCR)	-	1,000,000	-
12 well plates (CYP450s activity)	25,0000	500,000	-
96 well plates (cytotoxicity determination)	60,000	30,000	15,000
Perish dice (confocal, DIC imaging)		500,000	-

2.4.3.1.2 Determine doubling time of HepG2[318]

The number of population doublings (PD) can be calculated by the formula:

$$PD = \frac{\ln\left(\frac{N_{finish}}{N_{start}}\right)}{\ln 2}$$

ln is the natural logarithm, N start is the number of cells seeded at the beginning of the growth cycle, and N finish the number of cells recovered at the end.

Check cell doubling time every 3 mounts, if cell doubling time change should discard and chose the new one by thrown new cell-stock from liquid nitrogen.

2.4.3.2. Exposure HepG2 cells to herbal extracts /pure polyphenolic compounds

1. Herbal extracts: lyophilized herbal extracts were freshly prepared immediately before used by directly dissolved in cell culture media then filtered thought 0.22 μM filter for sterilization. Concentration were ranged between 100, 200, 400, 600, 800, 1,000, 2,000, 3,000 μg/ml.

2. Pure polyphenolic compounds: all pure compounds were dissolved in 100% DMSO to get the stock concentration at 100 mM. Store at -20°C up to 2 mounts, diluted stock concentration with cell

culture media to get range the concentration between 5, 10, 25, 50, 75, 100 μM. Final concentration of DMSO is not above 0.01%

3. Both herbal extracts and pure polyphenolic compounds were diluted into fresh growth media and pre-treat cells for 4, 29, 58 hr. At the end of the treatments the cells were used for the experiments described below. All treatments were carried out in at least 3 independent replications. The inhibitory activity was calculated by comparing with a plotted standard response curve. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve.

2.4.3.3. The toxicity and effects of herbal extracts on cellular functions were evaluated in cell culture system:

2.4.3.3.1. Cytotoxicity of herbal extracts using 2 biomarkers: MTT and neutral red assay

2.4.3.3.1.1 Effect of herbal extracts on cellular viability using MTT assay

The MTT assay provides an easy, time- and cost-effective measurement of growth and survival in adherent cell lines[319]. This colorimetric assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tatrazolium bromide (MTT) by succinate dehydrogenase in metabolically active cells, to purple formazan crystals. These secreting enzymes from inner membrane of mitochondria and endoplasmic reticulum react with diffusing yellow solution of MTT into the cells using NADPH as electron carrier to produce insoluble dark-purple sediment. Amount crystal is varies and related with live cells.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, load each well with 20 μl 0.5% MTT solution (w/v, in PBS) and incubation for 4 hr at 37°C.

2. The MTT reaction was terminated by adding medium is carefully removed and 100 μl of 100% DMSO (v/v) added to lyses the cells and dissolve the formazan product.

3. Transfer 200 μl of supernatant to the new 96-well plate. Read absorbance at 550 nm using a spectrophotometer. An absorbance correction should be made to take into account the background value from wells containing DMSO alone.

4. Results were expressed as the percentage of cell viability compare with the corresponding control value. The % cellular viability is calculated according to the following formula:

$$\% \text{ Cellular viability} = \frac{(Abs_{(sample)} - Abs_{(blank\ of\ sample)})}{(Abs_{(control)} - Abs_{(blank\ of\ control)})} \times 100$$

2.4.3.3.1.2 Effect herbal extracts on cellular viability using neutral red assay [320]

Neutral Red dye (3-amino-7-dimethyl-2-methylphenazine hydrochloride, NR) is the weak cation soluble dye. Viable cells take up this dye and sequester it in the lysosomes. The dye binds with weak

acidic property polysaccharide on the surface of lysosome vesicle or moves to react with photon in lysosome and is trapped inside cells. Because cells need energy (ATP) to engulf NR dye, the accumulation of NR dye is related to total cellular activity. Damaging cell or dead cell has no more energy to accumulate dye. However, neutral red is not retained by nonviable cells. Uptake of neutral red is quantified by fixing the cells in formaldehyde and solubilizing the stain in acetic ethanol, and measuring absorbance on microplate reader at 550 nm. This assay does not measure the total number of cells, but it does show a reduction in the absorbance related to loss of viable cells.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, cells were washed with PBS and replaced with media containing 0.4% NR (w/v) for 3 hr at 37°C for allowing lysosomes of viable cells to take up the vital stain. This process requires metabolically active cells. Failure to take up NR, therefore, indicates that those cells have suffered damage.

2. The cultures were rapidly washed with washing solution (1% formaldehyde 1% calcium chloride) to remove the excess NR. A mixture of 1% acetic acid-50% ethanol is added to the cells to extract the NR from these cells at room temperature for 30 min.

3. The supernatants are transferred to new 96-well plates, and the absorbance at 550 nm is then measured. The % cell activity is calculated according to the following formula:

$$\% \text{ Cellular activity} = \frac{(\text{Abs}_{(sample)} - \text{Abs}_{(blank\ of\ sample)})}{(\text{Abs}_{(control)} - \text{Abs}_{(blank\ of\ control)})} \times 100$$

2.4.3.3.2. Effect of herbal extracts on cellular oxidative stress using DCFH-DA assay[321]

DCFH-DA assay is becoming popular as a means of visualizing 'oxidative stress' in living cells. Non-fluorescent Dichlorofluorescin-diacetate (DCFH-DA) compound is taken up by cells and tissues. Usually within cell, DCFH-DA is undergoing deacetylation by intracellular esterase enzymes. When DCFH lost acetyl group, it cannot cross through cell membrane and continually oxidized by several oxidant species within cell, probably including $RO^{\bullet}_2$, $RO^{\bullet}$, $OH^{\bullet}$, $HOCl$, $ONOO^-$, and lipid hydreperoxides but not $O_2^{\bullet-}$ or H_2O_2 and leads to fluorescence 2',7'-dichlorouorescein (DCF) which can easily be visualized (strongly emission at 525 nm with excitation at 485 nm) and recorded fluorescent intensity by flurometer.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, cells were wash with PBS.

2. Added 50 μM DCFH-DA, mixed and incubated for 30 min at 37°C. Media is then washed and replaced by pre-warmed PBS (pH7.4). The excess dye was removed by washing the cells with PBS twice.

3. The fluorescence intensity as a result of DCF fluorescence is monitored by the fluorometer with excitation at 485 nm and emission at 525 nm.

4. Intracellular ROS are confirmed by laser-scanning confocal Imaging. Culture the cells on coverslip and place on microscope slide. Image of DCF fluorescence is obtained by using laser-scanning confocal microscope with excitation at 488 nm and detection at emission, 520 nm. To measure the fluorescent intensity, 20 cells from each image are picked randomly, and their averages of fluorescent intensity are calculated.

5. The results were normalized using protein concentration. Values were calculated and expressed in percentage change in fluorescence relative compare with untreated control groups.

2.4.3.3.3. The effect of herbal extracts on intracellular glutathione level using dithiobis-nitrobenzoic (DTNB) acid assays

Glutathione (GSH) is the most abundant non-protein sulfhydryl in most cells. According to the method of Ellman et al.[322]. This is standard method to assay for glutathione is by using by 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB, Ellman's Reagent) which react with GSH to generate the 412 nm yellow colored byproduct, 2-nitro-5-thiobenzoic acid (TNB) and GS-TNB.

Procedure

1. The cells were grown in 12 well plates and after treatment with herbal extracts or pure polyphenolic compounds, or 0.1 mM H_2O_2 (positive control). To assess protective effect of herbal extracts on endogenous antioxidant molecules against oxidative stress, the cells were pretreated with herbal extracts before 12.5 mM H_2O_2 were added to the medium and incubated for another 2 hr. Then Remove the culture medium, washed the cells twice with iced-cold PBS.

2. Lyse the cells with 100 μl 10% trichloroacetic acid (w/v) in chilled PBS which is used for deproteination the high molecular weight proteins. Centrifuge the lysate at 13,000g for 5 min to remove debris if any, collected supernatant for GSH analysis.

3. About 50 μl of supernatant was mixed with 200 μl of reaction reagent (0.73 mmol/L DTNB, 0.24 mmol/L NADPH, 0.09% SSA in PBS, pH8.0). The mixture was incubated for 10 min at room temperature.

4. The absorbance was read at 412 nm against appropriate control samples that were processed in parallel without adding the DTNB by spectrophotometer.

5. The concentration of GSH was estimated from calibration curve containing 6 GSH concentrations to be generated for each series of analyses. The results were normalized using protein concentration. Values were calculated and expressed in percentage change in nM GSH per mg of protein compare with untreated control groups.
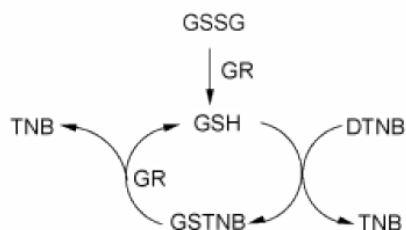
Figure 2.12: the GSH recycling mechanism reaction of Ellman's Reagent with reduced glutathione, give the yellow color product that could measure at 412 nM. Although GSH forms the majority of DTNB reactive thiols, the smallmolecular-weight thiols other than GSH too react with DTNB. Therefore, the total DTNB reactive thiols were also addressed as non-protein thiols content.

2.4.3.3.4. Effect of herbal extracts on mitochondrial membrane potential ($\Delta\Psi_m$) using TMRM assay[332]

It is known that changes in the mitochondrial membrane potential ($\Delta\Psi_m$) are a good indicator of mitochondria dysfunction. Loss of $\Delta\Psi_m$ triggers a cascade of reactions leading to cell apoptosis. Disrupted mitochondrial membrane dynamic is involved in execution of both necrosis and apoptosis[324]. Moreover, imbalance levels between intracellular ROS production and antioxidant defense system are known to cause mitochondrial membrane depolarization[325]. Mitochondrial membrane potential is indirectly estimated by tetramethyl rhodaminemethylester (TMRM), lipophilic cation fluorescent probe that accumulates in mitochondria. TMRM is sequestered preferentially in mitochondria and the amount is retained, reported by fluorescence intensity, is proportional to $\Delta\Psi_m$.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, or 25 µM CCCP (positive control). Remove the culture medium. Wash the cells twice with iced-cold PBS.

2. Added 20 µM TMRM in Ca^{2+}-free standard medium, incubated for 30 min at 37°C in the dark. After incubation, removed excess TMRM by rinsing the media and replaced by pre-warmed sterile PBS, pH7.4.

3. Then cells are immediately recorded fluorescent intensity by fluorometer. Fluorescent excitation and emission wavelengths were 525 and 585 nm, respectively. Uncoupled agent, 25 µM CCCP, is used as mitochondrial membrane potential collapsed control.

4. The results were normalized using protein concentration. Values were calculated and expressed in percentage change in $\Delta\psi_m$ per mg of protein compare with untreated control groups.

5. The mitochondrial membrane potential result was confirmed by confocal imaging. Cells were staining with 300 nM TMRM and DNA specific fluorescent dye, 2.5 µg/ml Hoechst 33342,for 30 min at 37°C in the dark, washed 3 times and left for 10 min until the fluorescence is equilibrated. The $\Delta\psi_m$ images are collected using laser scanning confocal microscope with excitation at 488 nm and emission at 560 nm. Approximately 10 images were randomly captured and analyzed. The results are expressed as relative fluorescent intensity.
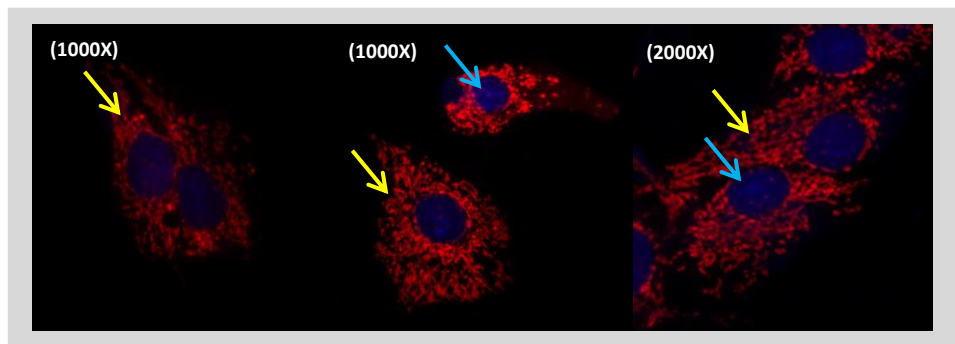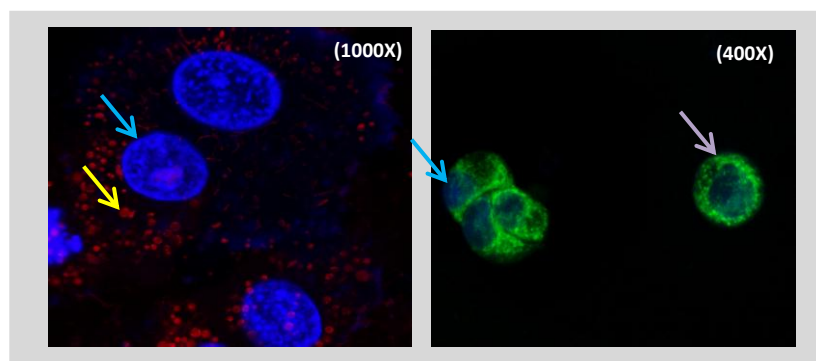
Figures 2.13: HepG2 cells staining with TMRM and Hoechst33342. Nucleic acid was stained by Hoechst 33342 (blue arrow) and active mitochondria were stained by TMRM (yellow arrow).

2.4.3.3.3.5. Effect of herbal extracts on intracellular calcium using Rhod2-AM staining

Many studies have identified a relationship between calcium signaling and mitochondrial bioenergetics parameters[326]. Evidence shown that pathological increases in intracellular calcium levels significantly[327]. Many studies propose that natural products have effects involvement in intracellular calcium signaling in various cell types[328,329,330,331]. The changes in mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) is assayed by using mitochondrial specific fluorescent indicator dye, Rhod2-AM. Its acetoxy-methyl ester is induced cellular permeable with a net positive charge and allowed sequestration into mitochondria. Stock of dye solution are first dissolved in DMSO and diluted with calcium free HBSS.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, or 50 μM digitonin (positive control). Cells were washed and re-suspended in calcium-free incubated media (200 mM glucose, 20mM Hepes in HBSS, pH 7.4).

2. Added 10 μM Rhod-2AM in calcium-free media, incubated for 40 min at 37°C to allow completely de-esterification of AM esters dyes. Then, cells are washed twice with incubated buffer to remove any excess dye.

3. The change of Rhod-2 fluorescent intensity was measured by fluorometer using a 488 nm excitation and 585 nm emission wavelengths. Concentrations of Ca^{2+} level are calculated from calcium chloride calibration curve. Values were calculated and expressed in percentage change in ($[Ca^{2+}]_m$) per mg of protein compare with untreated control groups.

4. Intracellular Ca^{2+} concentration is confirmed by confocal imaging. Cells are staining with Rhod2-AM and DNA specific fluorescent dye, Hoechst 33342, then incubated for 30 min at 37°C in the dark, washed 3 times and left for 10 min until the fluorescence is equilibrated. $[Ca^{2+}]_m$ images are collected using laser scanning confocal microscope (excitation, 488 nm; emission, 585 nm). Approximately 10 images were randomly captured and analyzed. The results are expressed as relative fluorescent intensity.

Figures 2.14: HepG2 cells staining with Rhod2AM and Hoechst 33342 **(A)**, HepG2 cells staining with Rhodamine 123 and Hoechst 33342 **(B)**. Nucleic acid was stained by Hoechst 33342 (blue arrow), calcium in mitochondria were stained by Rhod2AM (yellow arrow) and P-gp activity was determined by measure Rhodamine 123 (purple arrow) accumulation within cells.

2.4.3.3.3.6. Effect of herbal extracts on intracellular ATP level using bioluminescence assay

The cell energy, ATP, is majorly synthesized by the enzyme mitochondria ATP synthase using the free energy of an electrochemical gradient of protons. Disruption of mitochondria electron transport, oxidative phosphorylation is expected to decrease the flow of electrons through the ETC which ultimately will lead to a decrease in ATP level and consequently cell death. Intracellular ATP levels were quantified using luciferin and luciferase bioluminescent assay[332]. This assay based on the production of light is causing by the reaction of ATP with luciferase and D-luciferin as enzyme and substrate[333]. The generation of luminescent signal by luciferase reaction is proportional to the amount of ATP present within cell.

Procedure

1. The cells were grown in 96 well plates and after treatment with herbal extracts or pure polyphenolic compounds, or 100 µM ouabain (ATPase inhibitor). Remove the culture medium and wash the cells twice with iced-cold PBS.
2. Cells were treated with ATP-Lite™1step kit reagents (Perkin Elmer) as per manufacturer's instructions.
3. Light emission is quantified immediately in luminometer. Concentrations of ATP level are calculated from known ATP calibration curve. Values were calculated and expressed in percentage change in nM of ATP per mg of protein compare with untreated control groups.

2.4.3.3.3.7 Effects of herbal extracts on P-glycoprotein activity using rhodamine 123 efflux assay

P-glycoprotein (P-gp) is a primary, active efflux pump that uses the energy of ATP to transport its substrates out of cells[334]. Basically, it found physiologically expressed in normal excretory and barrier tissues such as liver, kidney and intestine. Experiments were performed to determine the effects of herbal extracts on P-gp using rhodamine 123-accumulations and -efflux as an indicator[335]. The assay is based on the principle of the reversal of drug resistance in modified HepG2 cells specifically altered to express high efflux protein activity. The addition of a plant extract containing a P-gp substrate or modulating molecules will alter the excretion of any xenobiotic and consequently interrupted the

detoxifying system. Rhodamine 123 is the well-known fluorescent substrate of P-gp.   The increased accumulation of rhodamine 123 in the cells was usually resulting from inhibition of rhodamine 123 efflux.

Procedure

1. The cells were grown in 96 well plates. Loaded cells with 20 µM rhodamine 123 in Hank's balanced salt solution was added to each well and incubated for 1 hour at 37$^{\text{o}}$C, removed and washed cells twice with ice-cold PBS.

2. Herbal extracts, pure polyphenolic compounds, or 50-150 μM Verapamil (P-gp inhibitor) was added and incubated for 4 hr at 37$^{\text{o}}$C, removed and washed cells twice with ice-cold PBS.

3. Then, 100 µL 0.1% Triton X-100 was added for cell lysis. The change of rhodamine-123 fluorescent intensity was then record by fluorometer at 485 nm excitation and 528 nm emission wavelengths. The results were normalized using protein concentration. Values were calculated and expressed in percentage inhibition in intracellular accumulation of rhodamine 123 per mg of protein compare with untreated control groups using the following equation:

$$RI[\%]_{test} = \frac{i.c.fluorescence_{test}}{i.c.fluorescence_{verapamil}} \times 100$$

The verapamil-treated positive control wells as 100% relative inhibition (RI).

4. The result was confirmed by confocal imaging. Cells were staining with Rhodamine123 and DNA specific fluorescent dye, Hoechst 33342, and then incubated for 30 min at 37$^{\text{o}}$C in the dark, washed 3 times and left for 10 min until the fluorescence is equilibrated. P-glycoprotein activity images are collected using laser scanning confocal microscope using excitation at 488 nm and emission at 585 nm. Approximately 10 images were randomly captured and analyzed. The results are expressed as relative fluorescent intensity.

2.4.3.3.3.8. Effects of herbal extracts on Cytochrome P-450 Enzyme Activity (phase I biotransformation) using fluorescence method

Cytochrome P450 enzymes is known to primary biotransformation system for a broad range of structurally diverse foreign chemicals and endogenous substances. A previous study found that herb-drug interaction effects are based on the modulation effect of herbal extracts on the activity of CYP450s[336,337,338]. Determination of CYPs450 activities are performed by directly incubated selected substrates with cells cultured according to the method described by Donato et al.[339] based on the formation of fluorescence product from non-fluorescence substrates which reflects the CYP450 activity of hepatocytes.

Procedure

1. The cells were grown in 12 well plates and after treatment with herbal extracts, inducer or inhibitor (reference control). Remove the culture medium. Wash the cells twice with iced-cold incubation medium (1 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4) twice.

2. The reaction was initiated by adding 600 µl of specific substrates and their experimental conditions for each CYPs450 subgroups *as shown in Table 2.20.* After reach the time point indicated by the particular experiment, the reaction was stopped by aspiration of incubation medium and wash cells with incubation medium.

3. Potential metabolite conjugates formed (by phase II enzymes) during the activity assays are hydrolyzed by incubation of supernatants with 100 **µ**l **β**-glucuronidase/arylsulfatase solution (150 Fishman units/ml and 1200 Roy units/ml, respectively) for 2 hr at 37℃ in the dark. Terminate **β**-glucuronidase reaction by adding 100 **µ**l of corresponding quenching solution *shown in Table 2.20.*

4. Transfer 100 **µ**l of supernatant from each sample to the appropriate wells of a 96-well plate and fluorescent metabolite formation was measured by fluorometer.

5. Enzymatic activity is normalized for cellular protein content. Values were calculated and expressed as percent of metabolite is formed compare with control (untreated) groups.

Table 2.20: Substrates and corresponding metabolites for the evaluation of drug-metabolizing enzyme activities for the evaluation of enzyme inhibition and enzyme induction potential

CYP 450	Substrates[a]	Concentration (µM)[b]	Metabolite	Incubation time(min)	Ex/Em	Quenching Solution	Inductor(ind) or inhibitor(inh)
1A	CEC	30	Cyano-hydroxycoumarin	90	408/455	0.1 M phosphate potassium buffer, pH 7.4	Furafylline (inh)
2A	Coumarin	50	7-hydroxy coumarin	45	355/460	0.1 M Tris, pH 9	Methoxalen (inh)
2B	EFC	30	HFC	60	410/510	0.25 M Tris in 60% (v/v) acetonitrile	Tranylcypromine (inh)
3A	BFC	100	HFC	60	410/510	0.25 M Tris in 60% (v/v) acetonitrile	Ketoconazole (inh), rifampin (ind)

AMMC, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; CEC, 3-cyano-7-ethoxycoumarin; EFC, 7-ethoxy-4-trifluoromethylcoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; MFC,7-methoxy-4-trifluoromethylcoumarin. a) The functionality of the preparation was characterized with CYP-specific substrates and reference inhibitors (fu748). b) Used concentration is sufficient for each specific CYP450s activity (mu123,cy130).

2.4.3.3.3.9. Total Protein Determination by the Lowry assay[340]

The Lowry reaction for protein determination is an extension of the biuret procedure. The first step involves formation of copper ion complex with peptide bonds, resulting in the formation of reduced copper ion in alkaline solution. The Cu(I):amide complex then reduces a phosphomolybdate/phosphotungstate-containing Folin Ciocalteu reagent to yield an intense blue color.

This assay is much more sensitive than the biuret method but is also more time consuming. These assay using bovine serum albumin as standard.

Procedure

1. The cells suspension about 50 μl were transfer to 96 well microplate. Add 60 μl of alkaline copper solution which containing 45 μl of 2% Na_2CO_3 (w/v) in 0.10 N NaOH and 15 μl of 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartrate. Mixed and allow to stand at 65^oC for 10 min.

2. Add 120 μl of 10% Folin-Ciocalteu phenol reagent and mix immediately. Allow to stand for 30 min or longer.

3. Read the tests and the BSA standard on a spectrophotometer or microplate reader at 750 nm against the reagent blank.

2.4.3.3.3.10. Effect of herbal extracts on gene expression that involve in cellular biotransformation, detoxification, antioxidant, inflammation and energy metabolism using RT-RCR method

Effect of herbal extracts on cellular biotransformation, detoxification, inflammation and metabolism are analyzed at mRNA expression level. The gene expression profile is evaluated by RT-PCR to determined mRNA expression between untreated HepG2 group and treated HepG2 group by using Reverse Transcriptase–Polymerase Chain Reaction assay. The process begins with converting total cellular mRNA to cDNA then amplified specific genes with design primers.

Procedure

A. Isolation of RNAs

1. The cells were grown in 6 well plates and after treatment with herbal extracts, remove the culture medium. Wash the cells twice with iced-cold PBS. Total RNA was prepared from cells using the TRI[®] reagent as per the manufacturer's recommendations. Briefly, Cells were disrupted by addition of Trizol reagent at ratio 1 ml per 0.1-1×10^{7} cells. Vigorous mixing and centrifuge at 12,000g for 10 min at 4°C.

2. Collect the supernatant (RNA is on the upper layer: lower layer are protein, DNA and debris). The supernatant were mixed with equal volume of 100% isopropanol to participant RNA. Centrifuge at 12,000g for 5 min at 4°C. White sediment of RNA was seen, purified by wash 2-3 times with 75% Ethanol. The total RNA was collected by centrifugation at 10,000g for 5 min.

3. The purity of total RNA was determined as a ratio of absorbance at 260 and 280 nm and yield was calculated using absorbance at 260 nm before RT-PCR or stored at -80^oC until use.

B. Formaldehyde Agarose Gel Electrophoresis: quality of total RNAs

1. Prepare RNA by mixed with RNA loading buffer, and heated the mixture at 65°C for 15 min.

2. Load RNA samples to each well, run on 1.5% MOPS gel for 30 min at 50 Volt (4 to 6 V/cm of gel) in MOPS buffer.

3. Photograph gel using the UV transilluminator to record positions of 28S and 18S rRNA.

C. Elimination of DNA contaminate

1. Each sample was incubated with DNaseI in a mixture of DNaseI Incubation buffer *as shown in Table 2.21* for 15 min at 25°C.

2. Then terminated DNaseI reaction by adding EDTA, heated for 10 min at 65°C.

Table 2.21: DNase step to eliminated contaminated DNA RNA

Reagents	concentration	Volume per 1 reaction (μl)
RNA	1 μg /μl	2
10x DNase I Reaction buffer	10x	1
DNase I	0.1 Unit/ml	1
DEPC treated water	-	6
Mixed, incubated for 15 min at 37°C		
EDTA, pH 8.0	25 mM	1
Mixed, incubated for 10 min at 65°C		

D. Reverse Transcription Reaction

1. In order to synthesize cDNA, Total RNA was reverse transcribed to cDNA using reverse transcriptase enzyme *as shown in Table 2.22*. First step, it would to allow RNA sample is primewith random oligo-dT$_{20}$mers

2. cDNA synthesis was carried out by mixing the RNA-primer mixture with reaction buffer and RNase inhibitor *as shown in Table 2.22*. Warm briefly for 1-2 min at 42°C before started the reaction by adding 200 units of M-MuLV-reverse transcriptase, incubated the samples for 1 hour at 42°C.

3. The reverse transcriptase reaction was terminated by heating for 3-5 min at 95°C. The cDNA product was diluted with DEPC water and stored at –20°C until the quantitative PCR amplification was carried out.

E. PCR Amplification and gel electrophoresis

1. To amplified cDNA, PCR technique was performed. cDNA was amplified by Taq-DNA polymerase in 25 μl PCR reaction master mix *as shown in Table 2.23*. The specific primers for each gene expression *are shown in Table 2.24*.

2. The PCR reaction condition is as followings: initial denaturation at 95°C (4min) followed by 35 cycles of 3 steps: denature step for 60 sec at 95°C, annealing step for 30 sec and extension step for 30-60 sec at 72°C. Post-PCR final incubation step were done by hold 10 min at 72°C to promote complete synthesis of all PCR products.

3. Run PCR product on 2.0% agarose gel in 0.5% TBE buffer in the presence of 0.01% EtBr (w/v), 80 Volt for 35-40 min. Bands were visualized under UV through a gel doc digital imaging system.

4. Band intensities were normalized to **β**-actin (an internal control) amplified in parallel and means were calculated from the triplicate reactions. Values were calculated and expressed in a ratio of interested genes over those of **β**-actin, compared with control (untreated) groups.

Table 2.22: The procedure step to convert RNA to cDNA

Reagents	concentration	Volume per 1 reaction (μl)
1. RNA	1 μg/μl	5
2. dNTPs	10 mM	1
3. Oligo-dT20mer	10 ρM/μl	2
4. DEPC treated water	-	7
Mixed, incubated for 5 min at 75°C to lose RNA coil and matched with primer, cooled rapidly by placing on ice to prevent RNA reforming structure		
5. 10x reaction buffer	500 mM Tris-HCl, 30 mM MgCl$_2$, 750mM KCl, 100 mM DTT	2
6. RNase inhibitor	40 Unit/μl	1
7. DEPC-treated water	-	0.5
Mixed, incubated for 1-2 min at 42°C		
8. Reverse Transcriptase	200 Unit/μl	1.5
Mixed, incubated for 60 min at 42°C, stopped the reaction by briefly heated for 2-3 min at 95°C, cDNA were than PCR or keep at -20°C		

All herbal extract samples were run in triplicate and were averaged prior to analysis.

Table 2.23: The procedure step for gene expression analysis

Reagents	concentration	Volume per 1 reaction (μl)
1. cDNA	0.5 μg/μl or 10^4-10^7 molecules	10
2. 10x Reaction buffer	100 mM Tris-HCl, 20 mM MgCl$_2$, 500 mM KCl, Enchancer solution	2
3. dNTPs	10 mM*	2
4. Specific Primer	10 ρM	2**
5. Milli-Q water	-	8.8
6. Taq-DNA polymerase***	5 Unit/μl	0.2
Mixed, amplified DNA by PCR techniques using ThermalCycler		

*the final concentration of dNTPs must be in between 20-200 μM, too highly dNTPs cause Mismatched base pairing of Taq DNA polymerase

** 1 pair of primer for PCR (Forward and Reverse) adding each 1 μl to get 2 μl

*** Half-life of Taq DNA polymerase = 40 min at 95°C

Table 2.24: Sequences of primers for RT-PCR products

Gene	Primer sequences (5'→3')	Annealing Temperature	PCR product size (bp)
LDLR sense primer	5'-CAA TGT CTC ACC AAG CTC TG-3'	58.5	258 bp
LDLR anti-sense primer	5'-TCT GTC TCG AGG GGT AGC TG-3'		
HMGCoA-red sense primer	5'-CTT GTG TGT CCT TGG TAT TAG AGC TT-3'	56.5	247 bp
HMGCoA-red anti-sense primer	5'-TTA TCA TCT TGA CCC TCT GAG TTA CAG-3'		
PPARα sense primer	5'-AGT CTC CCA GTG GAG CAT TGA ACA-3'	60.2	728 bp
PPARα anti-sense primer	5'-ATA CGC TAC CAG CAT CCC GTC TTT-3'		
PPARγ sense primer	5'-AGC CTC ATG AAG AGC CTT CCA ACT-3'	59.4	434 bp
PPARγ anti-sense primer	5'-TGT CTT TCC TGT CAA GAT CGC CCT-3'		
LXRα sense primer	5'-AAC CCA CAG AGA TCC GTC CAC AAA-3'	62.9	818 bp
LXRα anti-sense primer	5'-ATT CAT GGC CCT GGA GAA CTC GAA-3'		
β-actin sense primer	5'-ACG GGT CAC CCA CAC TGT GC-3'	58.0	656 bp
β-actin anti-sense primer	5'-CTA GAA GCA TTT GCG GTG GAC GAT G-3'		
CYP1A1 sense primer	5'-TTC GTC CCC TTC ACC ATC-3'	56.0	302 bp
CYP1A1 anti-sense primer	5'-CTG AAT TCC ACC CGT TGC-3'		
CYP1A2 sense primer	5'-TCG TAA ACC AGT GGC AGG T -3'	60.0	254 bp
CYP1A2 anti-sense primer	5'-GGT CAG GTC GAC TTT CAC G-3'		
CYP2B6 sense primer	5'-ATG GGG CAC TGA AAA AGA CTG A-3'	62.0	283 bp
CYP2B6 anti-sense primer	5'-AGA GGC GGG GAC ACT GAA TGA C-3'		
CYP2C9 sense primer	5'-CTG GAT GAA GGT GGC AAT TT-3'	59.0	308 bp
CYP2C9 sense primer	5'-AGA TGG ATA ATG CCC CAG AG-3'		
CYP2D6 sense primer	5'-AGC TGC TAA CTG AGC ACA GGA TGA-3'	62.9	341 bp
CYP2D6 anti-sense primer	5'-AAA GCG CTG CAC CTC ATG AAT CAC-3'		
CYP2E1 sense primer	5'-GAC TGT GGC CGA CCT GTT-3'	58.0	297 bp
CYP2E1 anti-sense primer	5'-ACT ACG ACT GTG CCC TTG G-3'		
CYP3A4 sense primer	5'-ATT CAG CAA GAA GAA CAA GGA CA-3'	64.0	314 bp
CYP3A4 anti-sense primer	5'-TGG TGT TCT CAG GCA CAG AT-3'		
CYP3A7 sense primer	5'-GGT TCA GTA AAA AGA ACA AGG ACA A-3'	66.0	306 bp
CYP3A7 anti-sense primer	5'-TGG GGC ACA GCT TTC TTA AA-3'		
COX-1 sense primer	5'-ACC TCG GCC ACA TTT ATG GAG ACA-3'	62.1	447 bp
COX-1 anti-sense primer	5'-CAA TGC GGT TGC GGT ATT GGA ACT-3'		
BAX sense primer	5'-ATC AGA ACC ATC ATG GGC TGG CAC-3'	62.8	382 bp
BAX anti-sense primer	5'-ACT TGA GCA ATT CCA GAG GCA GTG-3'		
BCL2 sense primer	5'-TTT GCA GTT GGG CAA CAG AGA ACC-3'	58.3	265 bp
BCL2 anti-sense primer	5'-TGG CCT CTC TTG CGG AGT ATT TGT-3'		

Gene	Primer sequences (5'→3')	Annealing Temperature	PCR product size (bp)
TNFα sense primer	5'-ACC CTC AAC CTC TTC TGG CTC AAA-3'	60.1	300 bp
TNFα anti-sense primer	5'-AGG CCT AAG GTC CAC TTG TGT CAA-3'		
NF-ΚB sense primer	5'-TGT AAC TGC TGG ACC CAA GGA CAT-3'	60.2	285 bp
NF-ΚB anti-sense primer	5'-AAA GCT GTA AAC ATG AGC CGC ACC-3'		
ALDH2 sense primer	5'-GGC AAC TGG AAA CGT GGT TGT GAT-3'	62.9	597 bp
ALDH2 anti-sense primer	5'-TGG ATG AAG TAA CCA CGG TCA GCA-3'		
HO-1 sense primer	5'-AGG AGA TTG AGC GCA ACA AGG AGA-3'	63.9	651 bp
HO-1 anti-sense primer	5'-TCG CCA CCA GAA AGC TGA GTG TAA-3'		
Nrf1 sense primer	5'-ACG GAG TGA CCC AAA CCG AAC ATA-3'	61.1	689 bp
Nrf1 anti-sense primer	5'-CCAGATGGGCTTGCAGCTTTCTTT-3'		
Nrf2 sense primer	5'-TGC CCA CAT TCC CAA ATC AGA TGC-3'	58.1	488 bp
Nrf2 anti-sense primer	5'-TTC TGT GGA GAG GAT GCT GCT GAA-3'		
GPX1 sense primer	5'-ACT TAT CGA GAA TGT GGC GTC CCT-3'	62.3	451 bp/ 730 bp
GPX1 anti-sense primer	5'-AGG CTC GAT GTC AAT GGT CTG GAA-3'		
SULT1A1 sense primer	5'-TCA GCA CAC GTC GTT CAA GGA GAT-3'	60.2	403 bp
SULT1A1 anti-sense primer	5'-TCT CAC TAT GTT GCC CAG GTT GGT-3'		
UGT1A1 sense primer	5'-ACT GGA ACC CGA CCA TCG AAT CTT-3'	57.9	207 bp
UGT1A1 anti-sense primer	5'-AGT CTC CAT GCG CTT TGC ATT GTC-3'		
MDR1 sense primer	5-TTG GAG CCT ACT TGG TGG CAC ATA-3'	59.6	849 bp
MDR1 anti-sense primer	5'-AGT CTG CAT TCT GGA TGG TGG ACA-3'		

Statistical Analysis and evaluation method

All result data were presented as mean ± S.E.M. of at least three determinations. SPSS version 16.0 for Windows is used to analyze the variance (ANOVA). Means are compared using the Duncan test at the 95% significance level. Tests for correlation coefficients between different antioxidant activities are run using Pearson analysis at the 99% significance level.

Chapter III

Results

3.1. Examination of quality and quantities of Herbal extracts

3.1.1. Extraction yield

To find the best condition to extract herbal sample, we considered many factors that might influence such as source of herbal sample, temperature of extraction, extraction duration, and solvent of used. Using total polyphenol level as an indicator for screening, we found that using 1500 ml 80% ethanol to extract 10 grams of herb and kept at 4^oC in the dark for 48 hr was the best condition because it gave the highest total polyphenolic contents for all herbal samples were tested as show in Table 3.1.

Table 3.1: Optimal condition to extract herbs

Solvent	60 min	48 hr	
	mmol/kg dry wt.	mmol/kg dry wt.	solution color
(A)[a]EtOH100%	20.3286	26.97378	green
(A)EtOH80%	86.20317	108.7251	green
(A)MtOH100%	49.50648	69.85516	green
(A)MtOH80%	89.69956	114.5348	green
(A)Water60^{o}C	135.2146	165.8541	Red-brown
(A)Water25^{o}C	100.0959	124.2177	Red-brown
(B)EtOH100%	42.16333	56.71409	green
(B)EtOH80%	**199.8969**	**265.4496**	green
(B)MtOH100%	137.1004	199.6059	green
(B)MtOH80%	183.8729	253.8302	green
(B)Water60^{o}C	157.1458	218.4184	Red-brown
(B)Water25^{o}C	164.1369	212.0554	Red-brown

a (A) Commercial herbal sample, (B) The herbal sample collect from the herbal garden.

The percentage yield of extract was calculated using the following formula:

$$\% \text{ yield of the extract} = \frac{\text{Mass of the extract obtained}}{\text{Mass of the dry material taken for extraction}} \times 100$$

The percentage yields of different herbal extracts were as follows: The % yields of T. laurifolia leaf, M. oleifera leaf were 2.0 grams/10 grams (20%) and 2.5 grams/10 grams (25%), respectively.

3.2. Determination of active compounds in herbal extracts

In this study, the total polyphenol content, total flavonoid content, total tannins content, total flavonol content and nitrate of these herbal extracts were determined. The total polyphenol, flavonoid, condense tennin, flavonol and nitrate contents of these herbal extracts were shown in Table 3.2.

3.2.1 Scan spectrum

Difference active compound in herbal extracts could absorb unique wave lengths and gave the specific absorption spectrum. It was the easiest way to predicted about the active compound in herbal sample. One thousand µg of herbal extract dissolved in 1 mL of water then scanned the absorption spectrum. The results were shown below.
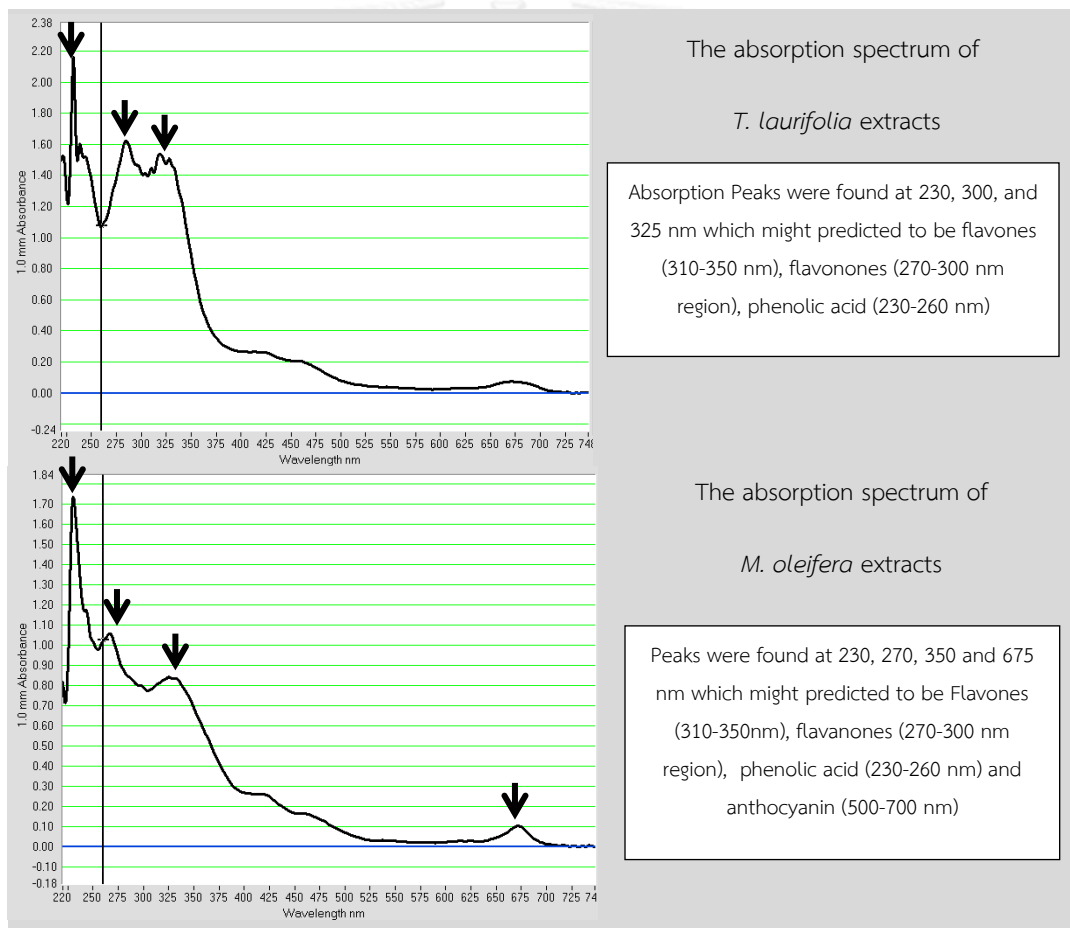
Figures 3.1: **The UV-visible spectra of** *T. laurifolia* **(upper) and** *M. oleifera* **(lower).** Polyphenolic compounds were typically detectable under UV light detectors which generally consisted of two absorption maxima in the ranges 240-285nm and 300-550 nm. The UV-vis spectrum provides a valuable aid to their identification since the wavelengths of the absorption maxima were often characteristic of a particular class of compounds.

3.2.2. Total polyphenol content

T. laurifolia, and *M. oleifera* extracts were found to possess total polyphenol 808.1 ± 27 and 218.3 ± 1 GE mM/kg dry wt., respectively and all were shown in Table 3.2

3.2.3. Total flavonoid content

T. laurifolia, and *M. oleifera* extracts were found to possess total flavonoid 2971.1 ± 23 and 285.7 ± 12 QE mM/kg dry wt., respectively and all were shown in Table 3.2

3.2.3. Total condense tannin content

T. laurifolia, and *M. oleifera* extracts were found to possess total flavonoid 5.1 ± 0.1 and 0.7 ± 0.2 CE mM/kg dry wt., respectively and all were shown in Table 3.2

3.2.4. Total flavonol content

T. laurifolia, and *M. oleifera* extracts were found to possess total flavonol 2.6 ± 2.0 and 0.6 ± 0.1 CE mM/kg dry wt., respectively and all shown in Table 3.2

3.2.5. Nitrate content

T. laurifolia, and *M. oleifera* extracts were found to possess nitrate content 0.68±0.01 and 0.02 ± 0.00 mM/kg dry wt., respectively and all were shown in Table 3.2

Table 3.2: Contents of phytochemicals: polyphenol, flavonoids, condense tennin, flavonol and nitrate contents from these study herbal extracts.

Herbs	Total polyphenol (GE mM/kg dry wt.)	Flavonoid content (QE mM/kg dry wt.)	Tannin content (CE mM/kg dry wt.)	Flavonol content (CE mM/kg dry wt.)	Nitrate content (mM/kg dry wt.)
T. laurifolia	808.1 ± 27	2971.1 ± 23	0.695 ± 0.24	2.557 ± 1.97	0.675 ± 0.01
M. oleifera	218.3 ± 1	285.7 ± 12	5.077 ± 0.12	0.588 ± 0.05	0.016 ± 0.00

All data were expressed as means± S.E.M. of triplicate test.

3.3 Assessment of antioxidant activity of herbal extracts in a cell-free system.

Although antioxidant activity was usually reported in all plants, it was still important to investigate the antioxidant properties of our herbal extracts in order to indicate the potential of the extracts as the source of nutraceuticals with potential application to diminish the oxidative stress with consequent to prevent or slow down the degenerative diseases. Antioxidants have been found to exhibit their activities by various mechanisms at different stages of oxidation such as antioxidant capacity, scavenging of the reactive oxygen species, and metal ion chelation. We separated the test of antioxidant properties of herbal extracts in two categories. Firstly, to determine their total antioxidant capacity; ORAC, TEAC and FRAP assay. Secondly, to investigate the free radical scavenging activity; superoxide radical, hydroxyl

radical, nitric oxide radical and hydrogen peroxide degradation. Finally, to calculate the active-compounds were possible related to antioxidant capacity and/or free radical scavenging properties.

3.3.1. Antioxidant capacity of herbal extracts using ORAC assay

The total antioxidant capacity of these herbal extracts was monitored by ORAC assay using the AAPH as the peroxyl radical generator. The result was shown in Table 3.3, *T. laurifolia,* and *M. oleifera* extracts were found to exhibit antioxidant activity 23163.9 ± 297 and 9306.7 ± 364 TE mM/kg dry wt, respectively. Compared to Trolox, *T. laurifolia* was stronger antioxidant capacities than *M. oleifera.*

3.3.2. Antioxidant capacity of herbal extracts using TEAC assay

The total antioxidant capacity of *T. laurifolia* and *M. oleifera* was also monitored by TEAC assay using the ABTS radical. This TEAC was used to confirm antioxidant activity of herbal extracts from ORAC result because the mechanism of TEAC assay is operated on the basis of single electron transfer, whereas the ORAC assays is a hydrogen atom transfer-based reaction. As shown in the Table3.3, *T. laurifolia,* and *M. oleifera* extracts were found to exhibit antioxidant activity 1,058.9 ± 77.1 and 574.0 ± 48.1 TE mM/kg dry wt, respectively. *T. laurifolia* was a stronger antioxidant which was in agreement with the ORAC assay results.

3.3.3. Antioxidant capacity of herbal extracts using FRAP assay

Natural antioxidants content in herbal extract was evaluated their ability to donate electrons using ferric reducing antioxidant power (FRAP) assay and compared with ascorbic acid, standards, was the reducing agent. As shown in the Table 3.3, *T. laurifolia*, and *M. oleifera* extracts were found to exhibit antioxidant power 1268.1 ± 24.0 and 784.0 ± 8.1 Vit.CE mM/kg dry wt, respectively. These results were in agreement with the ORAC and TEAC results.

Table3.3: Antioxidant activity of *T. laurifolia* and *M. oleifera* extracts[a,b].

Herbs	ORAC (TE mM/kg dry wt.)	TEAC (TE mM/kg dry wt.)	FRAP (Vit.CE mM/kg dry wt.)
T. laurifolia	23163.9 ± 297	1058.9 ± 77.1	1268.1 ± 24.02
M. oleifera	9306.7 ± 364	574.0 ± 48.1	784.04 ± 8.07

a) Activity was expressed as means % ±SEM of triplicate test.
b) A good correlation between radical scavenging activities as measured using TEAC, ORAC and reducing power assays was observed in Table3.6.

3.4 Assessment of in vitro free radical scavenging properties of herbal extracts

Reactive oxygen species have key roles in pathogenesis of many diseases. Most common free radicals that found in cells are those H_2O_2, OH•, NO• and superoxide radicals. Cell produced these free radicals very low and tightly control. Any disturbance in this regulation process results in oxidative stress eventually causing cell death and/or disease. However, the presence of antioxidants is helpful in restoring the normal balance against scavenging of free radicals and their production which are consequently preventing cellular damage and the oxidative pathological process.

3.4.1 Superoxide radical scavenging activity

Superoxide radical scavenging activity of these herbal extracts was assessed by the PMS-NADH-NBT method under alkaline DMSO condition. In this system, superoxide anion derived from dissolved oxygen and PMS–NADH coupling reaction which reacts with NBT to give colored diformazan. Superoxides scavenging activity was tested the herbal extracts in a concentration dependent manner. As shown in Table 3.4, *T. laurifolia* extract showed effective superoxide radical scavenging activity with 356.00 ± 4.08 Vit.CE M/kg dry wt. whereas, activity was observed in case of *M. oleifera* extract with 163.00 ± 1.67 Vit.CE M/kg dry wt. respectively. IC_{50} for scavenging of superoxide was 233 μg/ml for *T. laurifolia* extract, and could not determine for *M. oleifera*, respectively. The IC_{50} values for vitamin C were 0.1919 mM.

3.4.2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of these herbal extracts was assessed by following deoxyribiose degradation as an indicator. The hydroxyl radical was generated by the Fenton reaction would attack deoxyribose and degrades it. Attenuated hydroxyl radical generation would reverse deoxyribiose degradation. Both herbal extracts exhibited concentration dependent scavenging activity against hydroxyl radicals produced in Fenton reactions. Hydroxyl scavenging activity was obvious at all the tested herbal extracts in a concentration dependent. As shown in Table 3.4, *T. laurifolia* extract showed hydroxyl radical scavenging activity with 13.78 ± 3.38 Vit.CE M/kg dry wt. whereas, more potent activity was observed in case of *M. oleifera* extract with with 22.50 ± 11.30 Vit.CE M/kg dry wt., respectively. IC_{50} for scavenging of hydroxyl radical were 4537.8 μg/ml for *T. laurifolia*, and 2576.8 μg/ml for *M. oleifera*, respectively. The IC_{50} values for vitamin C were 3.29 mM.

3.4.3. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging ability of these herbal extracts was assessed by measuring H_2O_2 level directly after exposed with herbal extracts for 24 hr. It was shown in Table 3.4. The scavenging activity of *T. laurifolia* was 10,000 μg/ml which exhibited 23.33% (reduce from 1000 mM to 766.70 mM) scavenging activity on hydrogen peroxide. On the other hand, using the same amounts, *M. oleifera* exhibited only 9.52% (reduce from 1000 mM to 904.77 mM) hydrogen peroxide scavenging activity.

3.4.4. Nitric Oxide scavenging activity

Nitric oxide scavenging ability of these herbal extracts was assessed using Griess reagent assay. Both extracts have nitric oxide scavenging activity in a dose dependent manner. From Table 3.4, it was observed that *T. laurifolia* showed strong scavenging activity was 8.51 ± 1.96 Vit.CE M/kg dry wt. Whereas, less activity was observed in *M. oleifera* within 7.48 ± 0.83 Vit.CE M/kg dry wt. IC_{50} of nitric oxide scavenging were 3631.5 μg/ml for *T. laurifolia*, and 4473.8 μg/ml for *M. oleifera*, respectively. The IC_{50} value for vitamin C was 14.87 mM.

Table 3.4: Free radical scavenging activity of herbal extracts

Herbs	Hydrogen peroxide (mM)[a]	Superoxide (Vit.CE M/kg dry wt.	Nitric oxide (Vit.CE M/kg dry wt.)	Hydroxyl (Vit.CE M/kg dry wt.)
T. laurifolia	766.70 ± 45.51	356.00 ± 4.08	8.51 ± 1.96	13.78 ± 3.38
M. oleifera	904.77 ± 28.85	163.00 ± 1.67	7.48 ± 0.83	22.50 ± 11.30

a) Scavenging activity at 10,000 μg/ml, the values refer to reduce from the initiated H_2O_2 level (1,000 mM)

Table 3.5: IC_{50} values of the herbal extracts in free radical scavenging activity.

Herbs and positive controls	IC_{50} (μg/ml)		
	Hydroxyl radical	Superoxide radical	Nitric oxide radical
T. laurifolia	4537.8	233.4	3631.5
M. oleifera	2576.8	N/A	4473.8
Vitamin C	3.29 mM	0.1919 mM	14.87 mM

Each value presented the mean of IC_{50} values of at least three independent experiments. N/A =not available.

3.4.5. Relationship among the bioactive molecules, the total antioxidant power and free radical scavenging properties

The correlation coefficients (*r*) of the total antioxidant power, bioactive molecules and the free radical scavenging properties of the herbal extracts were shown in Table 3.6. All methods of antioxidant techniques showed high correlation with total phenolic content and total flavonoids content, but low correlation with total flavonol and nitrate content. In addition, antioxidant activities of both herbal extracts were strong and similar, even though their phenolic profiles differed. The result also showed the strong correlation between free radical scavenging property and antioxidant activity, and also with total phenolic content and total flavonoid content. Furthermore, high correlation between others content of bioactive molecules such as tannin, flavonol and nitrate with free radical scavenging property were observed. These results could indicate that polyphenols, particularly flavonoids class was important contributor to antioxidant activity in both herbal extracts and could attenuated varies types of free radicals.

Table3.6: Statistical analysis using Pearson's correlation coefficient (r)[b], to determine correlation with the findings of the polyphenolic contents, antioxidant activity and the free radical scavenging variables.

	Assay[a]	ORAC	TEAC	FRAP	OH	SO	NO	H_2O_2	TP	TF	TC	FC	NC
ORAC	Pearson Correlation	1	.978	.998*	-.999*	.844	.653	.604	.989	.998*	-1.000*	.118	.136
	Sig. (2-tailed)		.135	.037	.023	.360	.547	.587	.095	.042	.016	.925	.913
TEAC	Pearson Correlation	.978	1	.988	-.970	.938	.480	.758	.998*	.961	-.972	.324	.341
	Sig. (2-tailed)	.135		.098	.158	.225	.681	.452	.040	.177	.151	.790	.778
FRAP	Pearson Correlation	.998*	.988	1	-.996	.874	.609	.649	.996	.992	-.997	.175	.193
	Sig. (2-tailed)	.037	.098		.059	.323	.583	.550	.059	.079	.053	.888	.876
OH	Pearson Correlation	-.999*	-.970	-.996	1	-.825	-.680	-.575	-.983	-1.000*	1.000**	-.082	-.101
	Sig. (2-tailed)	.023	.158	.059		.383	.524	.610	.118	.020	.006	.947	.936
SO	Pearson Correlation	.844	.938	.874	-.825	1	.146	.937	.915	.807	-.830	.632	.646
	Sig. (2-tailed)	.360	.225	.323	.383		.907	.227	.265	.402	.376	.565	.553
NO	Pearson Correlation	.653	.480	.609	-.680	.146	1	-.208	.533	.702	-.673	-.675	-.661
	Sig. (2-tailed)	.547	.681	.583	.524	.907		.866	.642	.504	.530	.529	.540
H_2O_2	Pearson Correlation	.604	.758	.649	-.575	.937	-.208	1	.716	.550	-.583	.863	.872
	Sig. (2-tailed)	.587	.452	.550	.610	.227	.866		.492	.629	.603	.338	.326
TP	Pearson Correlation	.989	.998*	.996	-.983	.915	.533	.716	1	.977	-.985	.265	.282
	Sig. (2-tailed)	.095	.040	.059	.118	.265	.642	.492		.138	.112	.830	.818
TF	Pearson Correlation	.998*	.961	.992	-1.000*	.807	.702	.550	.977	1	-.999*	.052	.070
	Sig. (2-tailed)	.042	.177	.079	.020	.402	.504	.629	.138		.026	.967	.955
TC	Pearson Correlation	-1.000*	-.972	-.997	1.000**	-.830	-.673	-.583	-.985	-.999*	1	-.092	-.111
	Sig. (2-tailed)	.016	.151	.053	.006	.376	.530	.603	.112	.026		.941	.929
FC	Pearson Correlation	.118	.324	.175	-.082	.632	-.675	.863	.265	.052	-.092	1	1.000*
	Sig. (2-tailed)	.925	.790	.888	.947	.565	.529	.338	.830	.967	.941		.012
NC	Pearson Correlation	.136	.341	.193	-.101	.646	-.661	.872	.282	.070	-.111	1.000*	1
	Sig. (2-tailed)	.913	.778	.876	.936	.553	.540	.326	.818	.955	.929	.012	

*. Correlation was significant at the 0.05 level (2-tailed).

**. Correlation was significant at the 0.01 level (2-tailed).

a) ORAC=antioxidant activity based on ORAC assay, TEAC= antioxidant activity beased on TEAC assay, FRAP= Ferric reducing antioxidant power, TP= total phenolics, TF= total flavonoids, FC= flavonol content, TC= tennin content, NC= Nitrate content, OH= hydroxyl scavenging activity, SO= superoxide scavenging activity, NO= nitric oxide scavenging activity, H_2O_2= hydrogen peroxide scavenging activity.
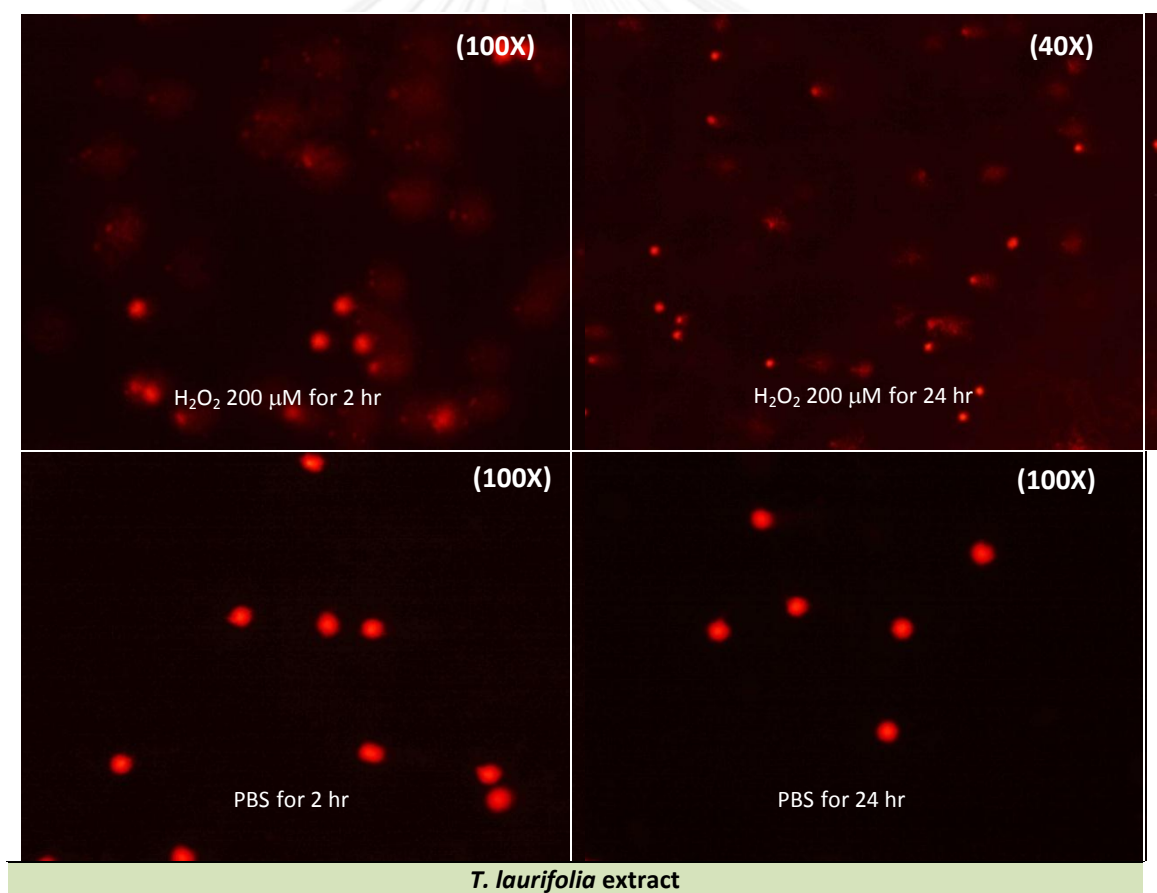
b) Using this test, r gives a measure of the strength of the relationship of the variables under examination (r =1 for a perfect straight line with upward slope, r = -1 for a perfect straight line with a downward slope).

3.5 Determination of antioxidant activity of herbal extracts using human living cells

3.5.1. Measurement of DNA oxidative damage using Comet assay

Broken DNA strand in peripheral blood mononuclear cells (PBMCs) was analyzed after exposured to 200 μM of H_2O_2 using comet assay. This method can be used to assess effects of herbal extracts against DNA damage. As shown in Figure 3.2, the maximal level of broken DNA strand was detected after treated H_2O_2 for 2 hr and then declining after 24 hr which closed to the control level. In non-pretreated PBMCs, the presence of H_2O_2 significantly increased the formation of broken DNA strand, showing 10% undamaged cells and 90% damaged cells. At all *M. oleifera* concentration tested, Mean values of Comet

score measured in all-treated samples were not significantly change when compared to PBMCs in PBS, the negative control. In *T. laurifolia* treatment alone at level 600 and 800 μg/ml, it seemed to be lower the mean values of comet scores than the negative control. After increased *T. laurifolia* level to 1,000 μg/ml, DNA damage did not decline in H_2O_2-non-pretreated cells, but it was even higher than the negative control. *T. laurifolia* at high concentration was generated slight damage to DNA by itself. The distribution of different grade of DNA damage in PBMCs treated with H_2O_2 and herbal extracts was analyzed as shown in Table 3.7, and Table 3.8. Figures 3.2 represented nuclei after electrophoresis showed a control group with normal nucleus (top left panel) and pretreated with herbal extracts (400-600 μg/ml TLE and 400-800 μg/ml MOE) with significantly decreasing degrees of broken-DNA migration. The best protective effects against oxidative DNA damage caused by hydrogen peroxide was achieved by 600 and 800 μg/ml *of T. laurifolia*. Comet assay showed antioxidant effect of *T. laurifolia* on PBMCs' DNA oxidative damage in a dose dependent manner. Co-treatement cells with 600, 800 but not 1,000 μg/ml of *T. laurifolia* significantly lower the comet scores up to 29.5, 37.8, and only 2.0%, respectively. In contrast, contrarily to what happened with *T. laurifolia*, no significant reduced DNA damage induced by H_2O_2 was seen in *M. oleifera* co-treated cells.

T. laurifolia extract

(40X)

Negative

(40X)

TLE 600 μg/ml + H$_2$O$_2$ 200 μM

(40X)

TLE 800 μg/ml + H$_2$O$_2$ 200 μM

(40X)

TLE 1,000 μg/ml + H$_2$O$_2$ 200 μM

M. oleifera **extract**

(40X)

Negative

(40X)

MOE 600 μg/ml + H$_2$O$_2$ 200 μM

(40X)

MOE 800 μg/ml + H$_2$O$_2$ 200 μM

(40X)
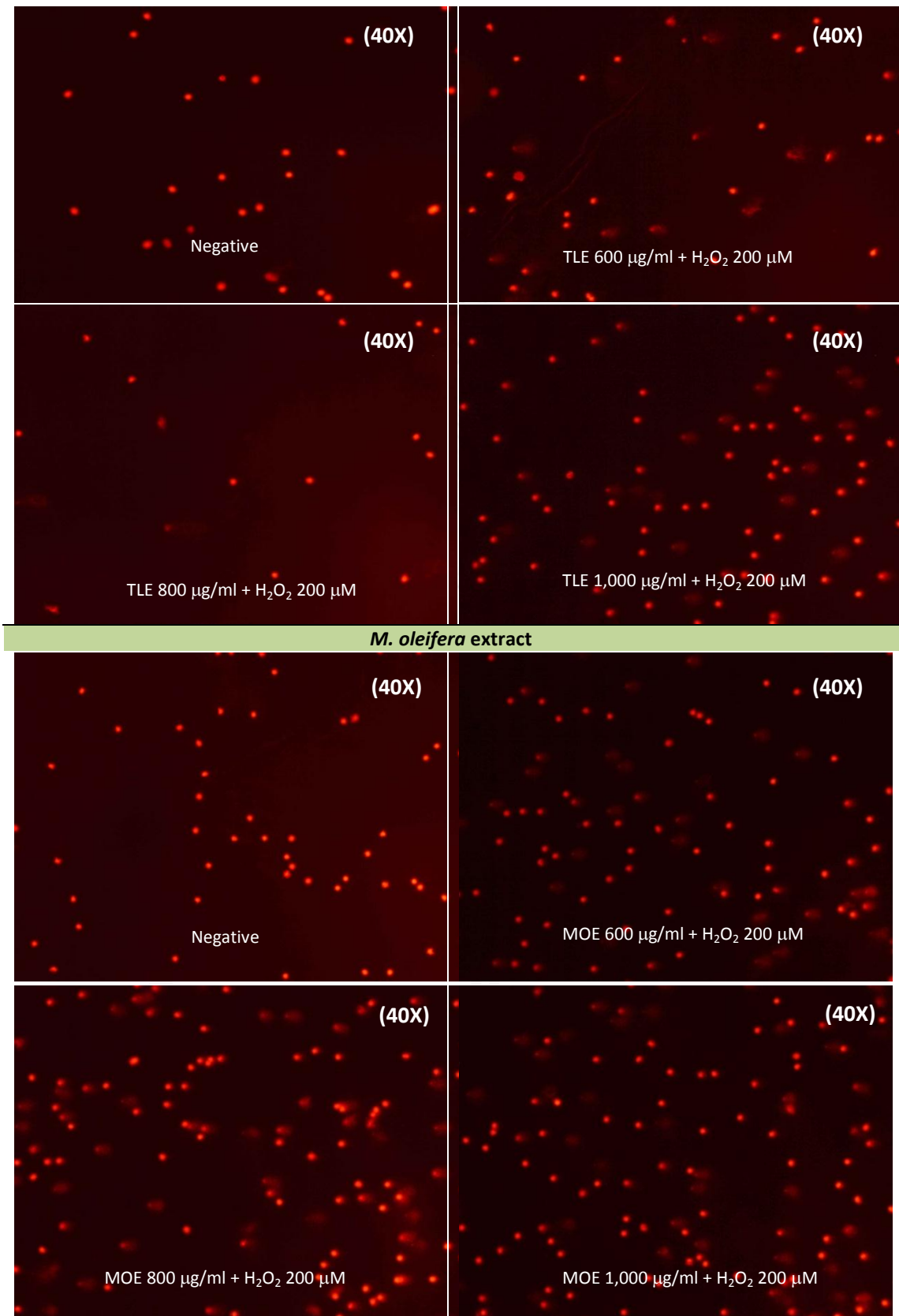
MOE 1,000 μg/ml + H$_2$O$_2$ 200 μM

Fig 3.2: **The ability of herbal extracts to protect DNA from H$_2$O$_2$ -induced oxidative stress to induce DNA strand breaks in PBMCs was assessed by the alkaline comet assay.** Human PBMCs were exposed to herbal extracts with/without

200 μM of H_2O_2 for 2 hr at 4^oC and following subjected to the appropriate measurement. Data represented the means± SEM of 3 independent experiments. The grade of damaged was assessed visually on a 5-point scale(0-4); (0) Appoximately 10% DNA in tail, (1) 20% DNA in the tail, (2) 40% DNA in the tail, (3) 70% DNA in the tail, and (4) more than 90% DNA in the tail.

Table 3.7: Protective effect of *T. laurifolia* against H_2O_2-induced DNA damage.

Treatment	Total cell count					Comet score
	0	1	2	3	4	
PBS,pH7.4 (neg)	896 ± 23.5	56 ± 21.7	19 ± 8.2	9 ± 4.3	20 ± 7.1	**202.7 ± 18.5**
TLE 600 μg/ml	993 ± 4.6	5 ± 4.0	1 ± 1.0	1 ± 1.5	0 ± 0.0	**11.0 ± 10.6[#]**
TLE 800 μg/ml	972 ± 10.4	24 ± 8.3	3 ± 1.0	1 ± 1.5	0 ± 0.0	**33.7 ± 14.9[#]**
TLE 1,000 μg/ml	741 ± 46.7	132 ± 41.7	43 ± 12.0	18 ± 10.6	68 ± 6.5	**438.0 ± 146.3**
TLE 600 μg/ml+H_2O_2	376 ± 9.1	85 ± 19.8	55 ± 30.1	83 ± 22.5	400 ± 63.7	**2045.7 ± 91.2***
TLE 800 μg/ml+H_2O_2	420 ± 25.3	95 ± 56.1	61 ± 34.9	106 ± 4.4	317 ± 85.0	**1805.8 ± 118.2***
TLE 1,000 μg/ml+H_2O_2	147 ± 84.9	100 ± 15.9	84 ± 49.4	99 ± 35.0	569 ± 8.1	**2842.3 ± 77.2**
H_2O_2 200 μM	177 ± 9.9	75 ± 10.1	52 ± 6.2	65 ± 7.2	633 ± 12.2	**2901.6 ± 14.6**

Each value presented the mean of comet score values ± SEM of at least three independent experiments. * Significantly different from PBMCs exposed to the H_2O_2 control at p≤0.05. [#] Significantly different from PBMCs exposed to the negative control at P-value≤0.05.

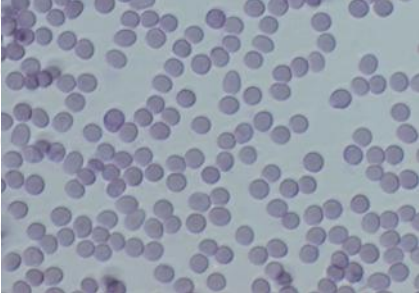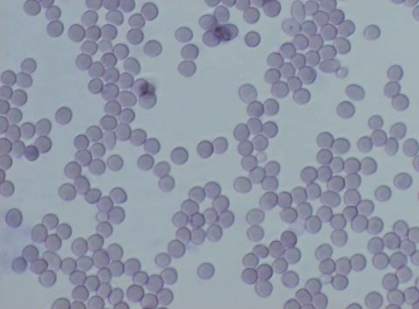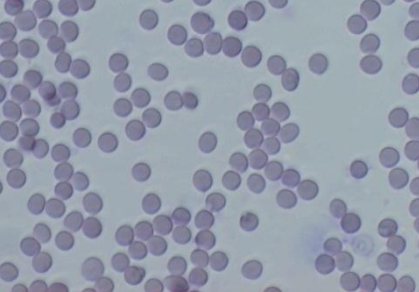Table 3.8: Protection by *M. oleifera* against H_2O_2-induced DNA damage

Treatment	Toxicity scale					Comet score
	0	1	2	3	4	
PBS,pH7.4 (neg)	889 ± 44.8	49 ± 20.7	16 ± 9.0	15 ± 6.4	31 ± 12.4	**249.5 ± 41.0**
MOE 600 μg/ml	977 ± 8.1	15 ± 4.0	8 ± 6.1	0 ± 0.6	0 ± 0.0	**32.3 ± 18.0[#]**
MOE 800 μg/ml	972 ± 8.1	21 ± 8.4	5 ± 3.2	1 ± 1.0	1 ± 0.6	**39.7 ± 20.1[#]**
MOE 1,000 μg/ml	961 ± 2.6	27 ± 2.3	8 ± 0.6	4 ± 1.73	0 ± 0.0	**54.7 ± 8.6[#]**
MOE 600 μg/ml+H_2O_2	126 ± 27.2	55 ± 8.7	55 ± 4.2	112 ± 13.3	652 ± 34.2	**2980.8 ± 122.9**
MOE 800 μg/ml+H_2O_2	191 ± 37.0	40 ± 4.4	46 ± 5.2	99 ± 18.5	624 ± 40.4	**2830.5 ± 121.3**
MOE 1,000 μg/ml+H_2O_2	77 ± 19.5	100 ± 11.9	84 ± 7.0	99 ± 29.0	569 ± 23.0	**3150.0 ± 33.9**
H_2O_2 200 uM	127±7.8	61 ± 3.0	47±6.4	87 ± 12.4	679 ± 20.6	**3128.0 ± 13.1**
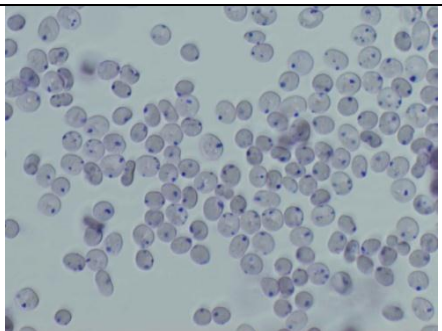
Each value presented the mean of comet score values ± SEM of at least three independent experiments. * Significantly different from PBMCs exposed to the H2O2 control at p≤0.05. # Significantly different from PBMCs exposed to the negative control at P-value≤0.05.

3.5.2. The effects of herbal extracts on oxidative damage to heme proteins of erythrocytes: Heinz body inhibition assay
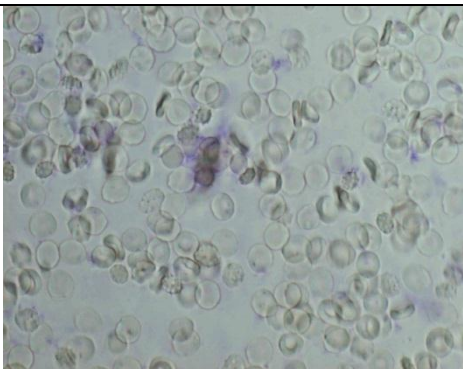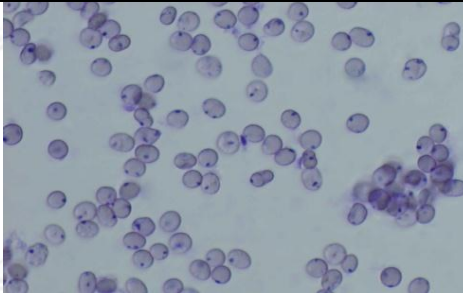
The protective effect of herbal extracts on oxidative damage to heme proteins in red bold cells (RBCs) induced by free radical initiator, APHZ, was studied. RBCs were taken from random normal subjects. The percentages of Heinz body inhibition of herbal extracts were shown in Table 3.9. Treated RBCs with herbal extracts could prevent Heinz body formation induced by APHZ in dose-dependent manner. Concentration between 1,000 to 3,000 μg/ml of both *T. laurifolia* and *M. oleifera* extracts did not significantly reduce Heinz body formation. When increasing concentration, Heinz body formation was significantly reduced to the baseline values. Heinz body formation in RBCs induced by APHZ was completely inhibited at 5,500 μg/ml of *T. laurifolia*, and 8,000 μg/ml for *M. oleifera*, respectively.
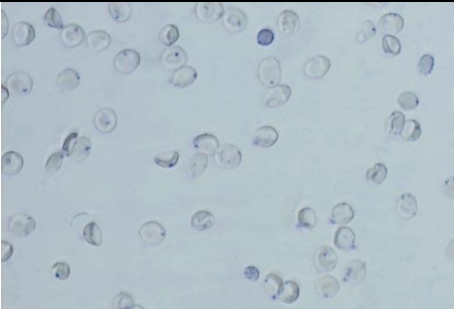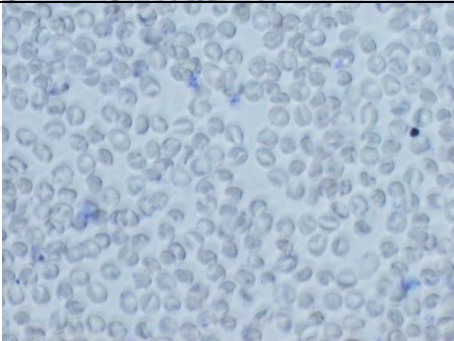
Table 3.9: The effect of herbal extracts on protein heme oxidation in normal Red Blood cells. RBCs were exposed to 2 hr then they were subjected to the appropriate measurement.

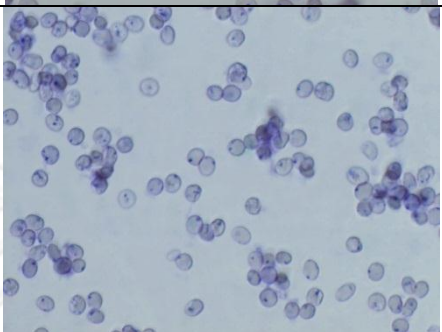| Treatment condition	Image	results
Negative		No Heinz body
TLE-treatment at all concentrations were used		No Heinz body
MOE- treatment At all concentrations were used		No Heinz body

100 mg% ATPZ-treatment (Positive)		Detect Heinz bodies at every RBCs (100%) No hemolysis
1,000 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=1.35% No hemolysis
2,000 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=1.95% No hemolysis
3,000 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=7.35% No hemolysis
5,000 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=85.8% No hemolysis

7,500 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=100% No hemolysis
10,000 μg/ml TLE+100 mg% ATPZ-treatment		Heinz bodies formation inhibition=100% No hemolysis
1,000 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=2.8% No hemolysis
2,000 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=4.35% No hemolysis
3,000 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=15% No hemolysis

5,000 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=18.3% No hemolysis
7,500 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=97.1% No hemolysis
10,000 μg/ml MOE + 100 mg% APHZ-treatment		Heinz bodies formation inhibition=100% No hemolysis

RBCs were exposed to 2 hr then they were subjected to the appropriate measurement. All images were taken under 400X microscopic.

a % Heinz body inhibition = 100-% Heinz body formation

b Red blood cells were obtain from normal subject (n = 3 in each group).

3.5.3. The effects of herbal extracts on oxidative damage to lipids membrane of erythrocytes: hemolysis inhibition assay

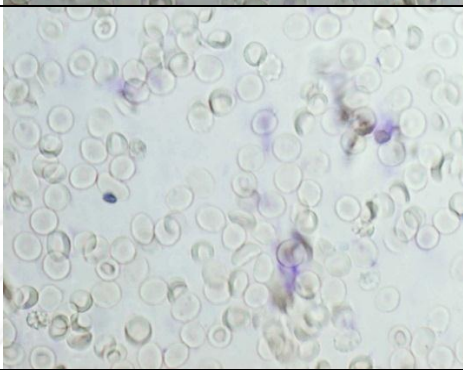The protective effect of herbal extracts on oxidative damage to polyunsaturated fatty acids of RBC-membrane induced by free radical initiator, APPH, was studied. Erythrocytes were used as the model because of high susceptibility to peroxidation. Lipid peroxidation could initially induced chain oxidation of other lipid and protein molecules of RBC-membrane, disturbing the membrane structure and eventually leading to hemolysis. Figure 3.4 and Figure 3.5 showed the inhibitory effect of different concentration range 100-3,000 μg/ml of *T. laurifolia* and *M. oleifera* extracts, respectively which AAPH induced hemolysis on RBCs. At high concentration 2,000-3,000 μg/ml, *T. laurifolia* extract alone could induce hemolysis which was dose and time dependent manner. At 240 min of incubation, treated RBCs with lower concentration of herbal extracts alone resulted in hemolysis, 2,000 μg/ml for *T. laurifolia*, and 100 μg/ml of *M. oleifera*. In the present of AAPH, incubation of RBCs with 2,000-3,000 μg/mL of *T. laurifolia* resulted in enhancing the induction of AAPH-mediated hemolysis, while the lower concentration 200-800 μg/ml of *T. laurifolia* resulted in completely inhibited hemolysis. *M. oleifera* range 200-2,000 μg/ml showed dose-dependent inhibited hemolysis and completely inhibited AAPH-

induced hemolysis at 600 μg/ml, while 3,000 μg/ml of *M. oleifera* shown reduced in inhibition efficiency. These results indicated that the herbal extracts had protective effect against hemolysis of RBCs *in vitro* conditions but differently in inhibition efficiency and at high concentration of both extracts might be inappropriate doses because they induced damage.
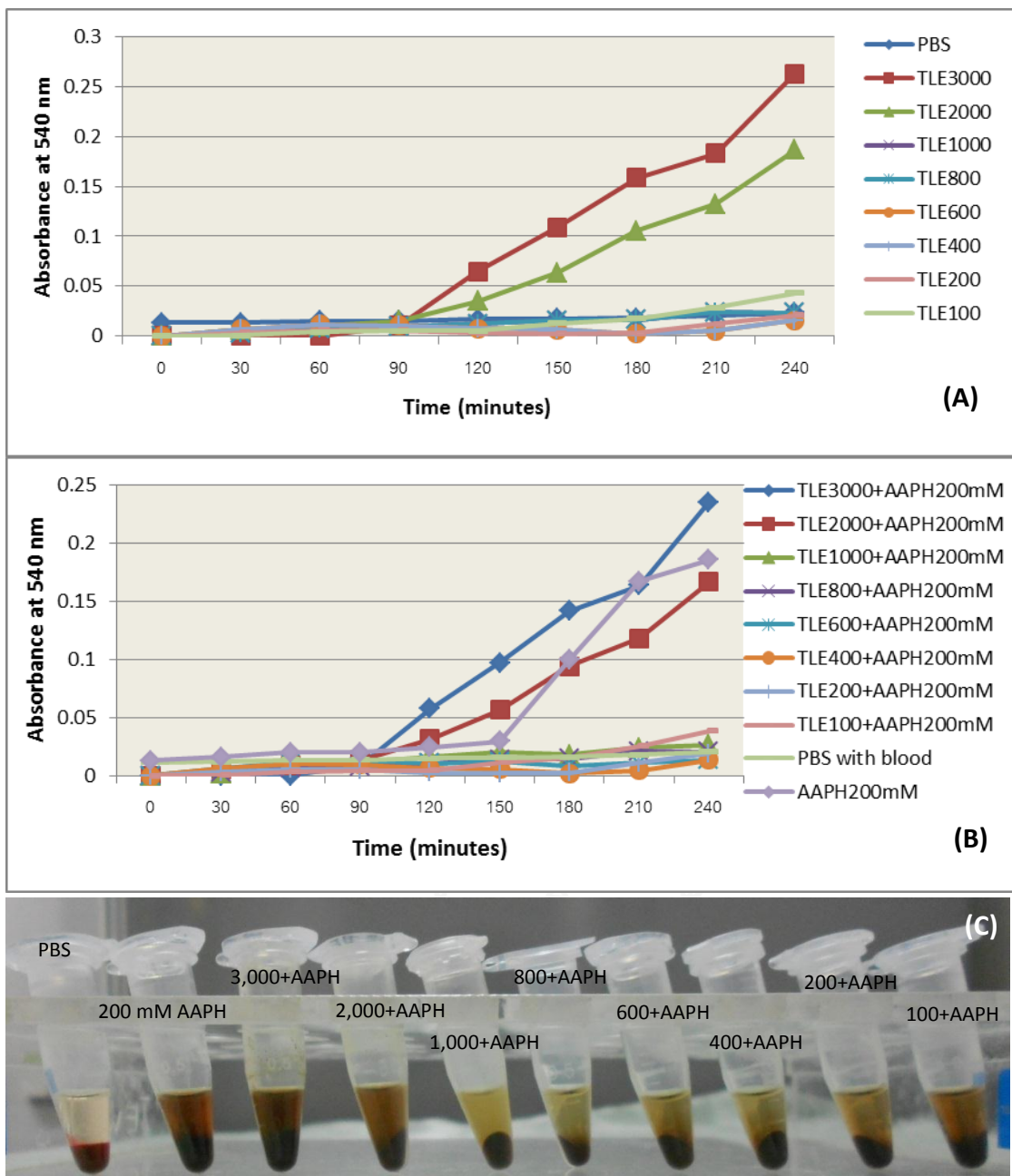
Figure 3.4: **The effect of *T. laurifolia* on lipid peroxidation measuring on red blood cell lipid bilayer.** The oxidation of lipid membrane disturbing the membrane organization and consequently and eventually leading to membrane break down. In the case of RBCs, membrane rupture called hemolysis and releaseed heme which could measure at 540 nm. Human erythrocytes exposed to *T. laurifolia* with/without 200 μM of AAPH for 3 hr at 37°C and following subjected to the appropriate measurement at every 30 minutes. Data represented the means ± SEM for 3 independent experiments. RBCs were incubated with *T. laurifolia* **(A)**. RBCs were co-incubated with *T. laurifolia* and AAPH**(B)**. Hemolysis inhibition effect of *T. laurifolia* at 240 mins **(C)**.
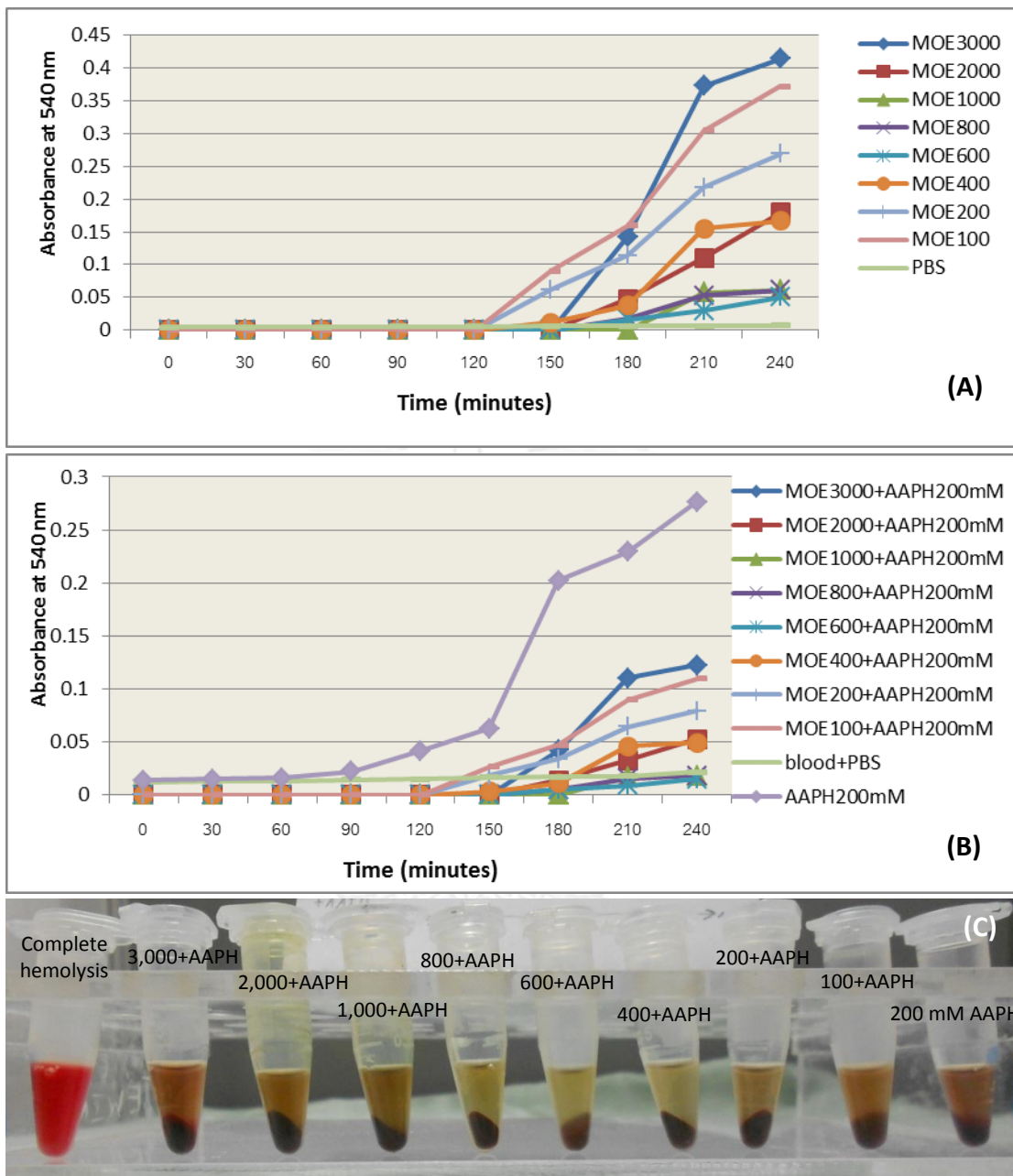
Figure 3.5: **The effect of *M. oleifera* on lipid peroxidation measuring on red blood cell lipid bilayer.** The oxidation of lipid membrane disturbing the membrane organization and consequently and eventually leading to membrane break down. In the case of RBCs, membrane rupture called hemolysis and released heme which could measure at 540 nm. Human erythrocytes were exposed to *M. oleifera* with/without 200 μM of AAPH for 3 hr at 37°C and following subjected to the appropriate measurement at every 30 minutes. Data represented the means ± SEM for 3 independent experiments. RBCs were incubated with *M. oleifera* **(A)**. RBCs were co-incubated with *M. oleifera* and AAPH**(B)**. Hemolysis inhibition effect of *M. oleifera* at 240 mins**(C).**

3.5.4. The effect of herbal extracts on erythrocyte plasma membrane electron transferring system

Plasma membrane redox system (PMRS) is the link mechanism whereby extracellular antioxidant compounds elicit their antioxidant effects by accept electrons transfer from intracellular substrates NADH and/or ascorbate (ASC) and protect cell from oxidative stress. In this study, the ability of herbal extracts to donate electrons to the erythrocyte PMRS were determined in order to find the relationship between antioxidant potential assessed by *in vitro* assay to the biological activity. Quercetin was used as an inducer reference compound to activate erythrocyte PMRS whereas H_2O_2 was used as an inhibitor reference to deactivated erythrocyte PMRS. Table 3.9 showed the activation of erythrocyte PMRS activity by herbal extracts and quercetin in time series experiment. The results show that quercetin activated erythrocyte PMRS activity in dose-dependent manner at 6, 12 and 24 hr but not at 3 hr suggesting that it was limited uptake quercetin by red blood cells at this time.

Among herbal treatment, higher induced erythrocyte PMRS activity was observed in *T. laurifolia* treatment. The activation of the PMRS activities by *T. laurifolia* was dose-and time dependent manner. At 24 hr, the effect of *T. laurifolia* on the activity of erythrocyte PMRS were similar to the quercetin activation. *M. oleifera* also caused significant change in erythrocyte PMRS activity in dose-dependent manner at 6, 12 and 24 hr treatment but in minor effect. However, at 3 hr treatment, dose dependent effect of *M. oleifera* was observed at only high concentration. No such significantly change in response to increasing incubation time was seen in *M. oleifera* treatment either.

Table 3.9: effect of herbal extracts on erythrocyte plasma membrane redox system (PMRS) *in vitro.*

Treament	% ePMRS at 3 hr (Mean ± SEM)	% ePMRS at 6 hr (Mean ± SEM)	%ePMRS at 12 hr (Mean ± SEM)	%ePMRS at 24 hr (Mean ± SEM)
Untreated	100.0 ± 0.9	100.0 ± 0.6	100.0 ± 0.9	100.0 ± 1.3
H_2O_2 12.5 mM	76.8 ± 2.2*	81.1 ± 0.8*	72.2 ± 1.4*	76.8 ± 1.0*
Quercetin 62.5 µM	205.6 ± 1.6*	123.2 ± 2.1*	162.5 ± 0.8*	174.0 ± 3.4
Quercetin 125 µM	243.3 ± 1.9*	182.0 ± 1.5*	198.5 ± 1.3*	188.7 ± 1.1
Quercetin 250 µM	244.0 ± 1.3*	235.4 ± 0.1*	222.2 ± 1.3*	253.8 ± 4.0
TLE 100 µg/ml	99.3 ± 0.5	101.8 ± 0.5	118.4 ± 1.1*	103.2 ± 1.4*
TLE 200 µg/ml	111.0 ± 0.7*	106.7 ± 0.7*	124.1 ± 0.8*	104.7 ± 0.7
TLE 400 µg/ml	114.2 ± 0.8*	105.2 ± 0.4*	129.8 ± 0.9*	136.5 ± 1.0*
TLE 600 µg/ml	113.7 ± 0.9*	118.8 ± 0.3*	131.1 ± 0.8*	141.0 ± 0.8*
TLE 800 µg/ml	125.2 ± 0.7*	129.9 ± 0.7*	132.3 ± 0.6*	141.0 ± 1.3*
TLE 1,000 µg/ml	133.0 ± 1.2*	141.0 ± 0.2*	132.8 ± 0.6*	158.1 ± 0.9*
TLE 2,000 µg/ml	139.4 ± 0.8*	147.4 ± 0.4*	148.2 ± 1.0*	195.0 ± 1.4*
TLE 3,000 µg/ml	150.4 ± 0.8*	176.5 ± 0.8*	177.2 ± 1.0*	255.0 ± 3.1*
MOE 100 µg/ml	96.5 ± 0.7	91.4 ± 0.8	92.0 ± 1.3	108.1 ± 0.6
MOE 200 µg/ml	94.3 ± 1.2	96.8 ± 0.8	105.7 ± 0.8	104.3 ± 0.7
MOE 400 µg/ml	96.6 ± 0.6	97.0 ± 0.4	106.6 ± 0.7*	107.8 ± 1.0
MOE 600 µg/ml	97.4 ± 1.1	101.4 ± 1.0	110.8 ± 0.8*	111.1 ± 0.9*
MOE 800 µg/ml	109.3 ± 0.6*	104.1 ± 0.8	113.4 ± 0.9*	118.7 ± 0.8*
MOE 1,000 µg/ml	115.3 ± 1.7*	110.7 ± 0.5*	112.4 ± 0.7*	131.4 ± 0.8*
MOE 2,000 µg/ml	117.0 ± 0.8*	112.5 ± 0.8*	111.3 ± 0.6*	163.0 ± 1.5*
MOE 3,000 µg/ml	119.0 ± 0.8*	115.6 ± 0.8*	128.5 ± 0.4*	151.0 ± 1.5*

Activity of erythrocytes PMRS activity was expressed in term of umol ferrocyanide/ ml PRBC/30 min and represents in % mean± SEM.* indicates significance of values compared to untreated group at P-value ≤0.05.

3.5.5. The Effect of herbal extracts on advanced glycated end product (AGEs) formation

The BSA-AGEs derived from the incubation of bovine serum albumin (BSA) with glucose at 60°C. The effect of herbal extracts on advanced glycated end products (AGEs) formation was shown in Figure 3.6, Figure 3.7 Figure 3.8, Table 3.10, Table 3.11 and Table 3.12. AGE-specific absorbance was detected at 360 nm. Control, non-modified BSA, showed no significant signal at 360 nm. Treatment of BSA with glucose resulted to increase the specific AGEs formation. Aminoguanidine was used as positive control

which reduced AGE formation more than 50% at 33 mM for 72 hr-incubation. Pretreatment of BSA with *M. oleifera* or *T. laurifolia* alone did not induced specific AGE formation at all concentration and both time points explored. Treatment of BSA with glucose and *T. laurifolia* resulted in a dose-dependent increase in specific AGEs formation and no increment was shown for *M. oleifera* at 24 hr. However, AGEs formation was down-regulated when continually incubated with herbal extracts for 48 and 72 hr, both herbal extracts produced a dose-dependent inhibition in AGEs formation. Only *T. laurifolia* could reach IC_{50} of AGEs formation which IC_{50} were 10 mg/ml for 48 hr-incubation and 4.6 mg/ml for 72 hr-incubation.
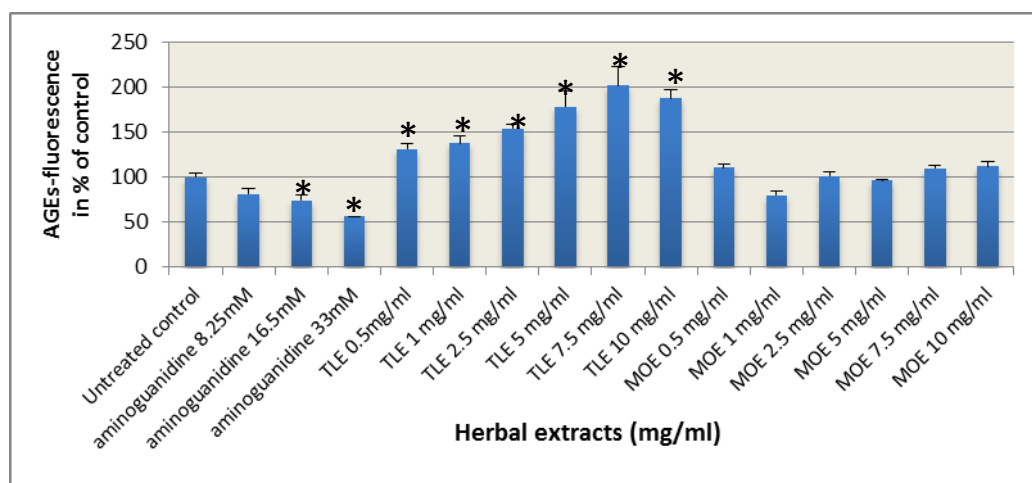
Figure 3.6: **The effect of herbal extracts on AGEs formation after treated for 24 hr.** The results were represents as % mean ± SEM. * indicated significance of values compared to untreated group at P-value≤0.05.
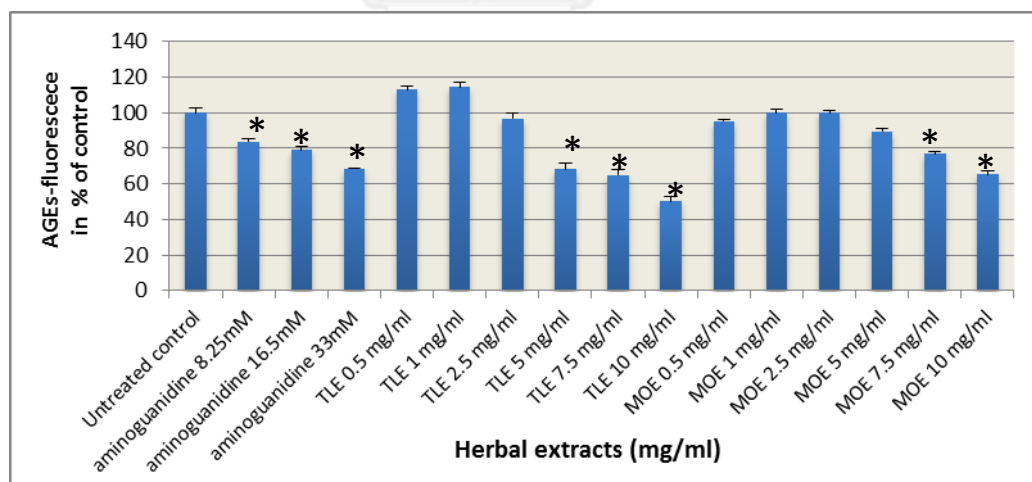
Figure 3.7: **The effect of herbal extracts on AGEs formation after treated for 48 hr.** The results were represented as % mean ± SEM. * indicated significance of values compared to untreated group at P-value≤0.05.
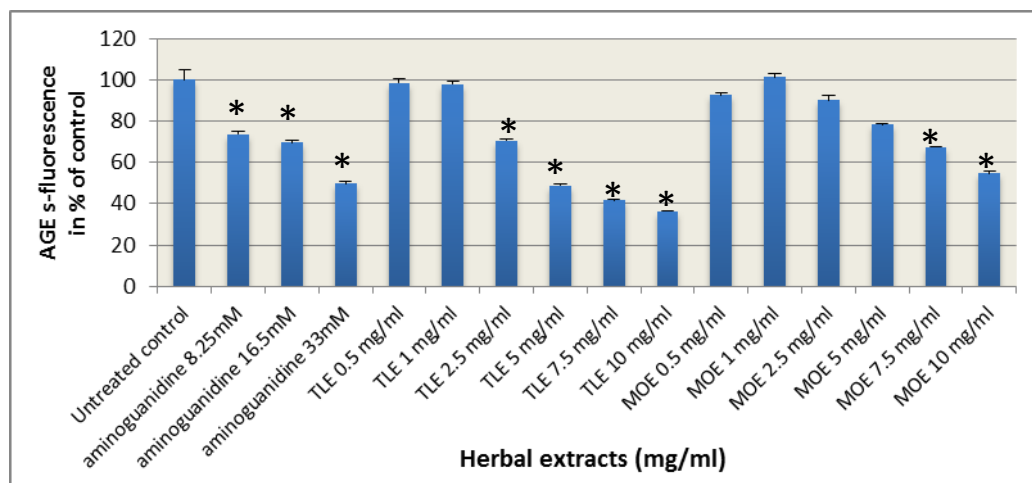
Figure 3.8: **The effect of herbal extracts on AGEs formation after treated for 72 hr.** The results were represented as % mean ± SEM. * indicated significance of values compared to untreated group at P-value≤0.05.

3.6. Bioactivities

The effect of herbal extracts on digestive enzymes, especially carbohydrate-hydrolyzing enzymes such as **α**-amylase and **α**-glucosidase and organophosphate targeting enzymes were determined to assess the potential of these herbal extracts for reduction of postprandial hyperglycemia and decreasing the toxicity of pesticide poisoning.

3.6.1. The effect of herbal extracts on α-Amylase activity

Several medical plants have been reported for their antidiabetic activity. However, the mechanism of action to control blood glucose level of these herbs has not been evaluated yet. Table 3.10, Table 3.11 and Table 3.12 showed activation/ inhibition **α**-amylase activity of both herbal extracts. Acarbose, a derivitized maltotriose antidiabetic drug, was used as the positive control. *M. oleifera* showed potential inhibition against **α**-amylase which its maximum inhibition was 36.2% and the minor percentage observed in *T. laurifolia* was 17.7%. Interestingly, 500-5,000 μg/ml of *M. oleifera* and *T. laurifolia* extracts enhanced **α**-amylase activity. However, no herbal treatment was reaching IC_{50}, indicating that the effectiveness of enzymatic inhibition of these herbal extracts were quite low. Acarbose displayed about 75.1% inhibition at 155 μM.

3.6.2. The effect of herbal extracts on α-glucosidase activity

Evaluation biological properties of herbal extracts that could inhibit carbohydrate digestive enzymes are worth to find additional therapeutic resources for diabetes control. The **α**-glucosidase inhibitory activity of the herbal extracts was shown in Table 3.10, Table 3.11 and Table 3.12. The potential inhibition against **α**-glucosidase of herb extracts varied from 9.0% to 72.7%. IC_{50} were calculated to evaluate the effectiveness of the herbal extracts which was 2,930 μg/ml *for T. laurifolia* and more than 10,000 μg/ml *for M. oleifera*. *T. laurifolia* extract inhibited **α**-glucosidase activity in dose-dependent manner. *M. oleifera* could not reach IC_{50} even in the highest concentration, 10,000 μg/ml, used. The positive control, acarbose, for **α**-glucosidase inhibitory activity which was significantly inhibited

α-glucosidase activity in dose-dependent manner. Acarbose displayed about 42.1% inhibition at 155 μM. When comparing the potential inhibition against α-amylase and α-glucosidase, no correlation was observed.

3.6.3. The effect of herbal extracts on acetylcholinesterase activity

Effect on acetylcholinesterase (AChE) activity of herbal extracts was analyzed and the results were shown in Table 3.10, Table 3.11 and Table 3.12. Acetylcholinesterase activity increased with additional herbal extracts. *T. laurifolia* extract showed a little effeced on acetylcholinesterase activity, while *M. oleifera* extract exhibited more induction than *T. laurifolia* extract. The protection of AChE activity against paraoxon poisoning of the herbal extracts were further studied. Paraoxon, the positive control, showed strongly inhibited AChE, about 30% at 90 nM. *M. oleifera* extract with concentration range 1,000-2,500 μg/ml was significantly protecting against paraoxon induced AChE degradation. Moreover, 1,000 μg/ml *M. oleifera* extract showed high improved AChE activity which almost 15 % improved paraoxon inhibitory effect. In the other hand, no significant protection was seen in *T. laurifolia* treatment at all.

Table3.10: Effect of *Thunbergia laurifolia* extract on inhibition of AGEs formation, α-glucocidase activity, α-amylase activity and amelioration of acetylcholinesterase activity

TLE Treatment (μg/ml)[a]	% Inhibition of AGEs formation at 72 hr (Mean ± SEM)	% Inhibition of α-Glucocidase activity (Mean ± SEM)	% Inhibition of Amylase activity (Mean ± SEM)	% Enhance ACHE activity when added TLE (Mean ± SEM)	% Enhance ACHE activity when added TLE & paroxon 90 nM (Mean ± SEM)
0	0 ± 0.3	0.0 ± 0.06	0 ± 1.6	0 ± 1.7	0 ± 3.1
500	1.6 ± 1.1	9.0 ± 0.5*	-0.55 ± 0.2	7.8 ± 1.3	11.0 ± 2.61
1,000	2.1 ± 0.8	11.8 ± 0.8*	-0.8 ± 1.2	14.0 ± 1.3*	11.1 ± 1.83
2,500	29.5 ± 0.5*	35.4 ± 1.0*	-13.3 ± 0.2	16.4 ± 6.6*	14.3 ± 6.08
5,000	51.3 ± 0.6*	70.8 ± 1.3*	-6.6 ± 5.5	5.3 ± 1.7	9.8 ± 2.17
7,500	58.2 ± 0.1*	70.3 ± 4.4*	3.6 ± 5.0	2.7 ± 3.9	4.9 ± 3.96
10,000	63.6 ± 0.1*	72.7 ± 1.1*	17.7 ± 0.7*	-2.9 ± 3.2	10.4 ± 7.86

a) Zero μg/ml represent the control (no agent added), to which the ability of other concentration were normalised (100%).
b) Values represent means± SEM of at least three indipendent experiments. * inhibitory effect were significant at P-value≤0.05. ** enhancement effect were sinificant at P-value≤0.05.

Table3.11: Effect of *Moringa oleifera* extract on inhibition of AGEs formation, α-glucocidase activity, α-amylase activity and amelioration of acetylcholinesterase activity

MOE Treatment (μg/ml)[a]	% inhibition of AGEs formation at 72 hr (Mean ± SEM)	% Inhibition of α-Glucocidase activity (Mean ± SEM)	% inhibition of Amylase activity (Mean ± SEM)	%Enhance ACHE activity when added MOE (Mean ± SEM)	% Enhance ACHE activity when added MOE & paroxon 90 nM (Mean ± SEM)
0	0 ± 0.3	0.0 ± 0.06	0.0 ± 1.6	0 ± 0.7	0 ± 3.1
500	7.4 ± 0.6	13.6 ± 0.6*	-23.0 ± 1.7**	25.4 ± 3.8**	4.3 ± 2.5
1,000	-1.3 ± 1.1	13.6 ± 0.9*	-28.4 ± 4.1**	25.5 ± 5.1**	14.9 ± 0.04**
2,500	9.8 ± 1.2	17.0 ± 1.6*	-9.2 ± 2.9	17.4 ± 3.4**	11.9 ± 1.3**
5,000	21.7 ± 0.3	11.8 ± 1.3*	-6.7 + 0.9	8.5 ± 3.2	6.4 ± 1.7
7,500	32.7 ± 0.3*	18.1 ± 0.7*	36.2 ± 3.1*	1.3 ± 1.3	4.6 ± 1.1
10,000	45.4 ± 0.5*	14.3 ± 1.0*	22.6 ± 0.3*	2.8 ± 0.5	2.0 ± 1.2

a) Zero μg/ml represent the control (no agent added), to which the ability of other concentration were normalised (100%).
b) Values represent means± SEM of at least three indipendent experiments. * inhibitory effect were significant at P-value≤0.05. ** enhancement effect were sinificant at P-value≤0.05.

Table 3.12: Reference compounds were used for Inhibition of AGEs formation, α-glucocidase activity, amylase activity and amelioration of acetylcholinesterase activity

Inhibitor treatment	% inhibition of AGEs formation at 72 hr (Mean ± SEM)	% Inhibition of α-Glucocidase activity (Mean ± SEM)	% inhibition of Amylase activity (Mean + SEM)	% Inhinition ACHE activity (Mean + SEM)
Aminoguanidine	26.4 + 0.7(8.25mM)* 30.5 + 0.6*(16.5mM)* 50.2 + 0.5*(33 mM)*	-	-	-
Acarbose	-	30.5 + 0.8 (90 uM)* 34.0 + 0.3 (125 uM)* 42.1 + 0.3 (155 uM)*	44.4 ± 0.7 (40 uM)* 63.5 ± 1.2(75 uM)* 75.1 ± 0.3 (155 uM)*	-
Paroxon	-	-	-	27.8 ± 3.1 (90 nM)* 44.2 ± 1.8 (180 uM)*

Values represent means± SEM of at least three indipendent experiments. * inhibitory effect were significant at P-value≤0.05. ** enhancement effect were sinificant at P-value≤0.05.

3.7 The effect of herbal extracts on cellular functions, signaling networks and xenobiotic detoxifying system used HepG2 cell culture as the model.

3.7.1 Cytotoxic effect of herbal extracts on HepG2 cells using MTT assay.

In order to examine the effects of herbal extracts on hepatocellular cell, Firstly, an MTT assay was carried out to determine the optimum concentration of each herbal extracts to be used in the subsequent procedure. HepG2 cells were treated for 29 and 58 hr with herbal extracts at concentrations ranging between 100 to 3,000 μg/ml and polyphenol compounds at concentrations ranging between 5 and 100 μM. The cytotoxicity in the HepG2 cells was quantified with an MTT assay and expressed as the percentage of untreated cells. Figure 3.9 presented the % viability of cells following treatment with different agents. The results demonstrated that both *T. laurifolia* and *M. oleifera* had cytotoxic effect on

HepG2 cells in dose dependent manner. Loss of cell viability upon herbal treatment became much more pronounced with increasing time of culture. IC_{50} value of various extracts on HepG2 cells also exhibited distinctive patterns. At first cell-cycle, herbal extracts reach IC_{50} at 2,800 μg/ml and 1,300 μg/ml for *T. laurifolia* and *M. oleifera*, respectively. At 58 hr, second cell-cycle, IC_{50} was 540 μg/ml and 700 μg/ml for *T. laurifolia* and *M. oleifera*, respectively. The result indicated that *T. laurifolia* showed more cytotoxic effect than *M. oleifera*. Whereas phenolic compounds treatment indicated a slightly loss in viability and appeared only at high concentration range 75 to 100 μM. Among all the tested compounds, curcumin and apigenin was found to be the potent agents for induction of cell-death.Few to no toxic effect, however, was obtained with any other of the tested compounds.
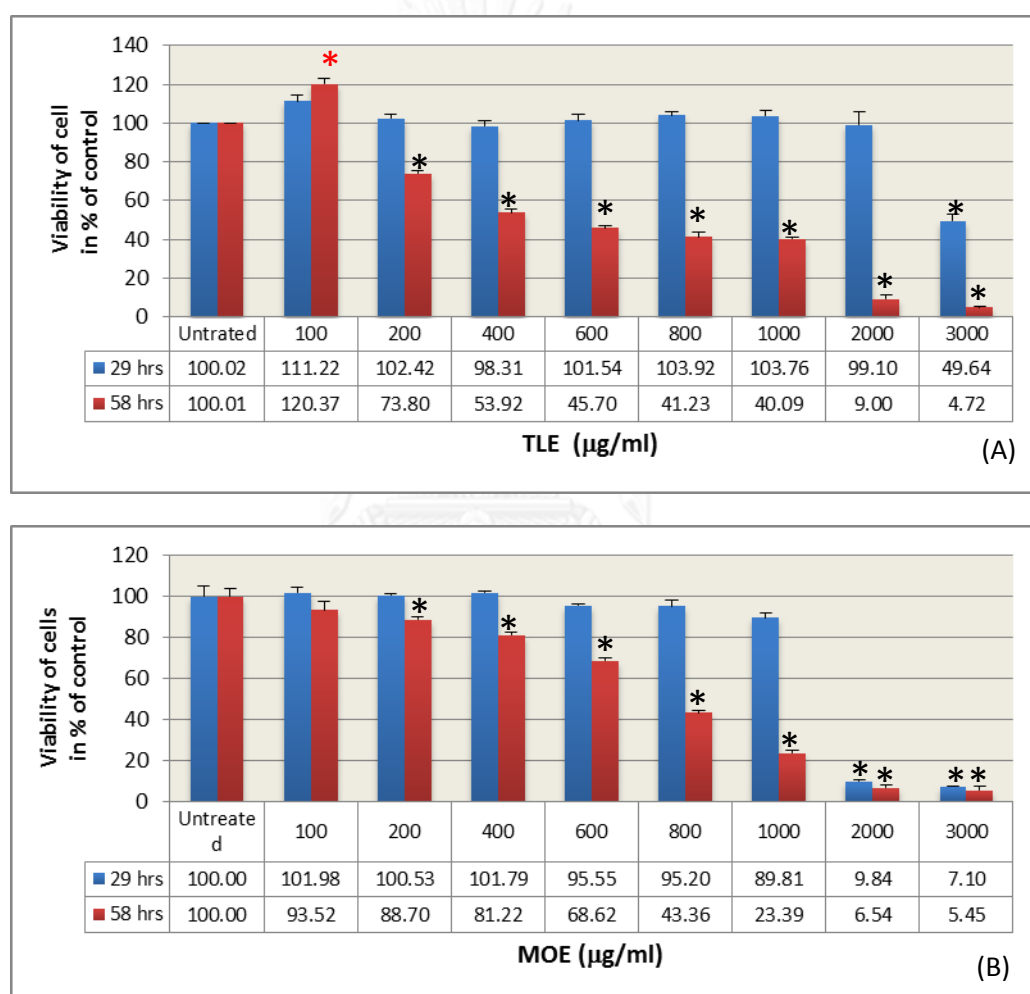
Figure 3.9: **Effect of herbal treatment on the viability of HepG2 cells using MTT assay.** This assay based on the formation of water-insoluble blue formasan dye from the yellow mitochonrial dye MTT^+. The cell viability was measured at 29 and 58 hr. The cells were exposed to different concentration of herbal extracts before MTT^+ dye was added. The results were expressed as a percentage of untreated control and represent as mean $\pm$ SEM of at least 4 independent experiments. *Significantly different from control was p-value $\leq$0.05.Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**

(A)

(B)

(C)
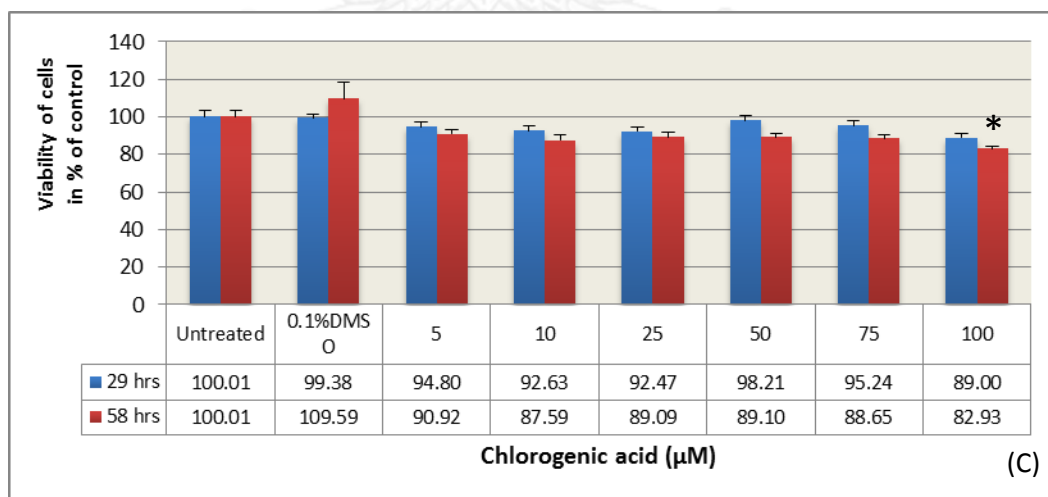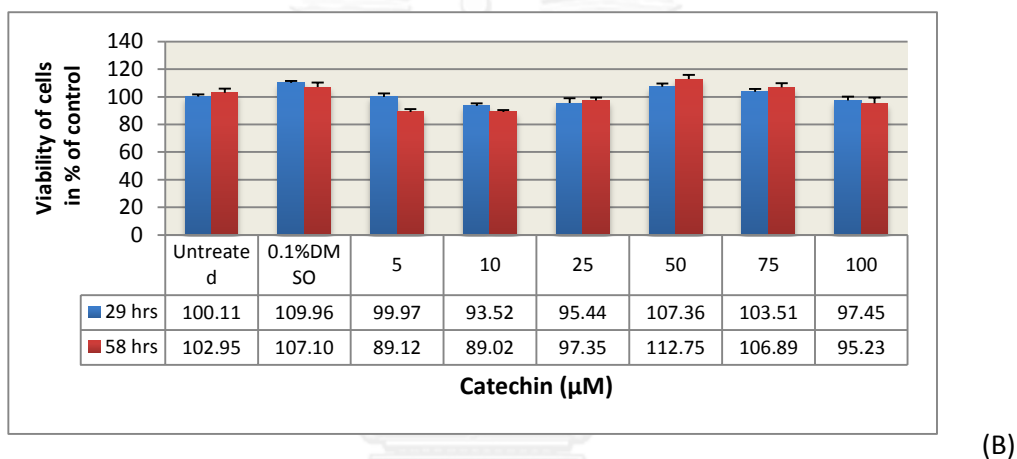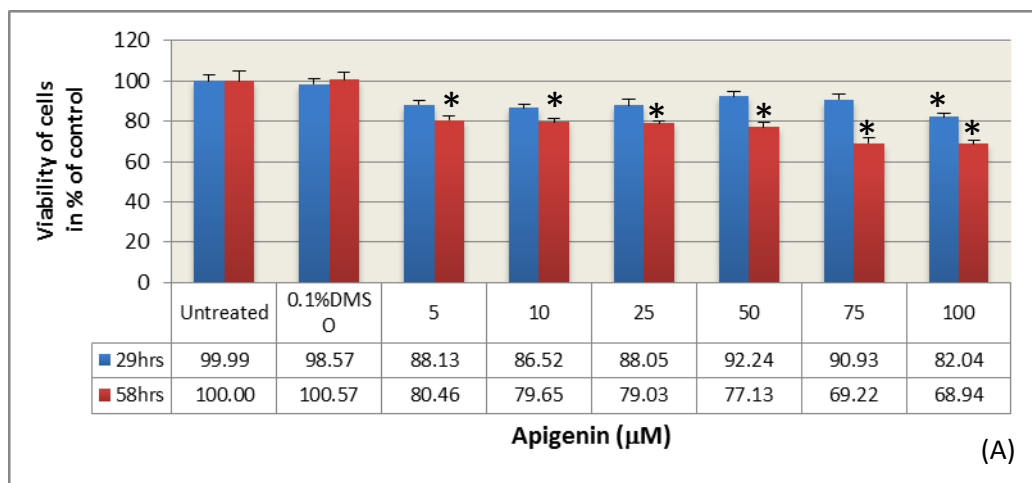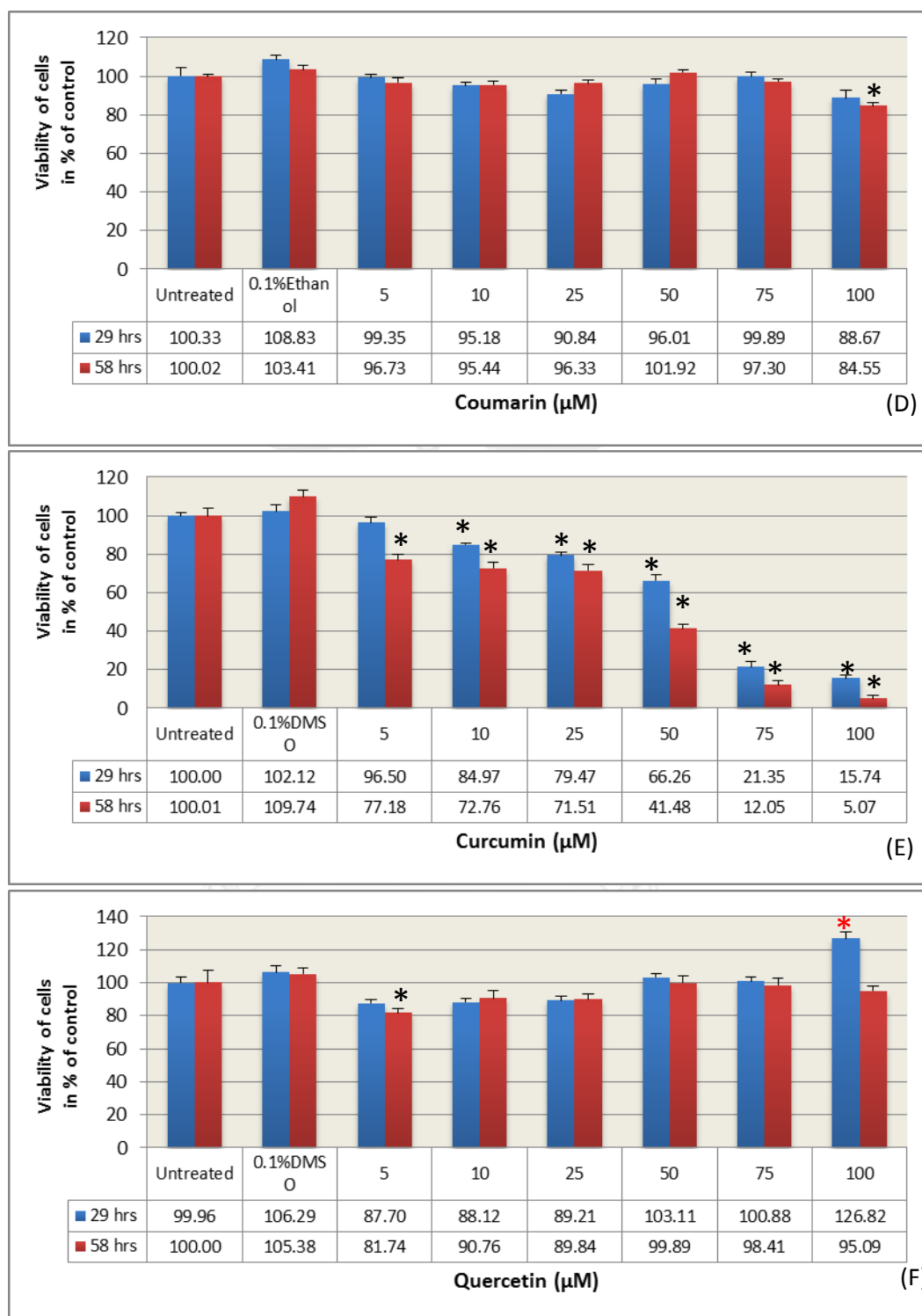
Figure 3.10: **Effect of polyphenols treatment on the viability of HepG2 cells using MTT assay.** This assay based on the formation of water-insoluble blue formasan dye from the yellow mitochonrial dye MTT[+]. The cell viability was measured at 29 and 58 hr. The cells were exposed to different concentration of pure polyphenols before MTT[+] dye was added. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with apigenin **(A),** catechin **(B),** chlorogenic acid **(C),** Coumarin **(D),** curcumin **(E)** and quercetin **(F).**

3.7.2. Determined the viability of HepG2 cells using neutral red (NR) assay

These cytotoxic effects of herbal extracts and pure polyphenols results from MTT data were confirmed by neutral red which were expressed as the percentage of untreated cells. A dose and time course for decreasing in cellular NR accumulation was shown in Figure 3.11. The results demonstrated that herbal treatment induced substantial cell death in a concentration-and time dependent manner. These NR data revealed that *M. oleifera* was relatively toxic in HepG2 cells. A 50% cell death occurred at 700 μg/ml for 29 hr and 300 μg/ml for 58 hr, respectively. *T. laurifolia* showed lower toxicity, and there was no IC_{50}-value determined for 29 hr and IC_{50} was 2,600 μg/ml for 58 hr, respectively. It was indicated that cytotoxic-mechanism of both herbs was different. In contrast to herbal treatment, the viability of HepG2 cells was not affected by any pure polyphenols treatment at level lower than 75 μM but 100 μM quercetin affected cell viability. As shown in Table 3.11 and Table 3.12, Good correlations between total protein level and the values of both MTT and NR assay were observed (r > 0.8) in 29 and 58 hr of incubation. Moreover, MTT assay and NR assay were shown significant correlation (r > 0.6) in 29 and 58 hr of incubation as shown in Table 3.13. Although the assays were based on different mechanism, both MTT and NR assays could be applied to screen the chemical toxicity.

TLE (μg/ml)	Untreated	100	200	400	600	800	1000	2000	3000
29 hrs	100.00	108.59	106.67	105.16	101.44	92.32	86.83	84.03	63.62
58 hrs	100.00	102.33	107.85	102.18	96.90	92.81	86.45	79.57	34.26

(A)

| MOE (μg/ml)	Untreated	100	200	400	600	800	1000	2000	3000
29 hrs	100.00	113.92	114.13	109.37	84.75	36.09	12.73	12.05	10.95
58 hrs	100.00	89.36	56.97	54.61	37.55	11.90	11.22	12.34	11.67

(B)
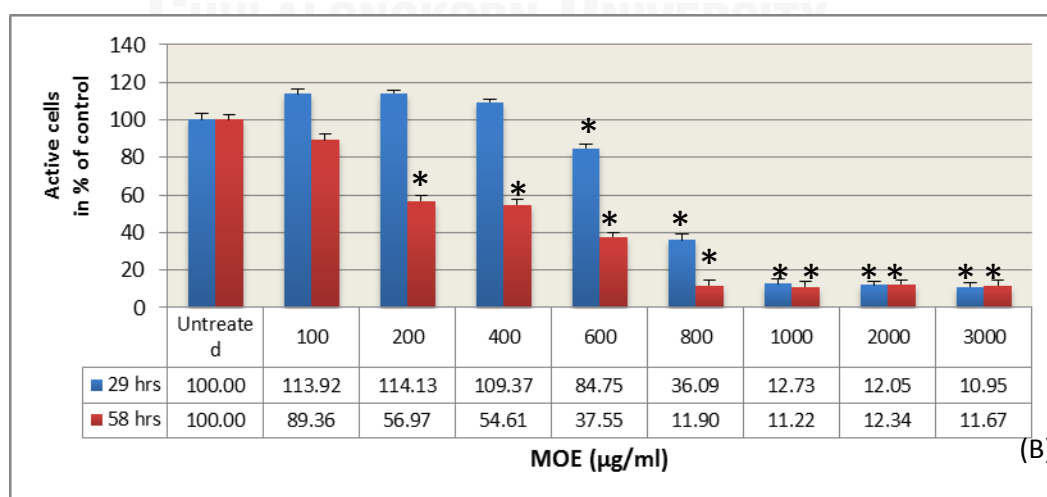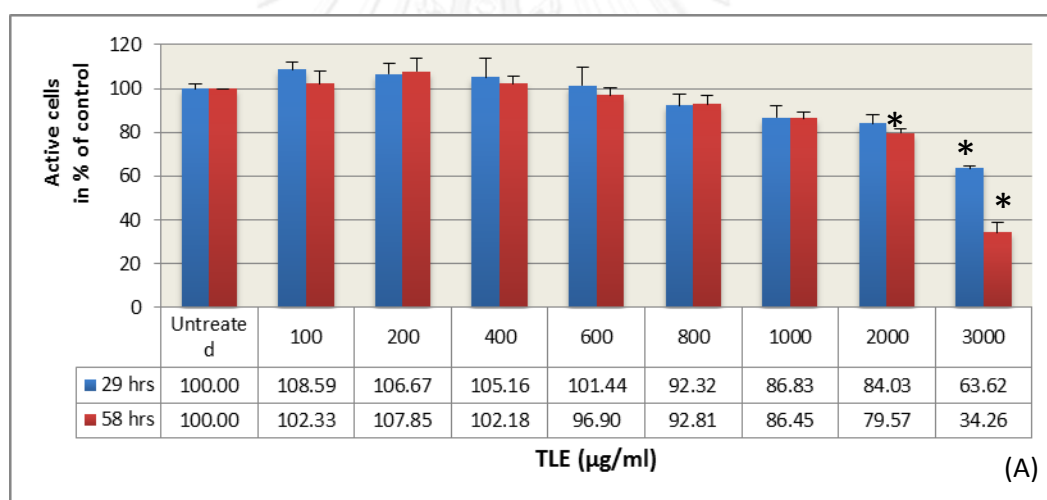
Figure 3.11: **Effect of herbal treatment on the viability of HepG2 cells**. The cell viability was measured at 29 and 58 hr of incubation using the NR assay and based on measurement of neutral red incorporation into the lysosomes of living cells. The cells were exposed to different concentration of herbal extracts before NR dye was added. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was P-value ≤ 0.05. Treated HepG2 cells with *T. laurifolia* (**A**) and *M. oleifera* (**B**)

Apigenin (µM)	Unteated	0.1%DMSO	5	10	25	50	75	100
29 hrs	99.80	100.08	100.52	99.45	100.07	100.30	99.83	98.44
58 hrs	99.86	101.65	99.94	98.82	95.32	92.96	95.05	97.06

(A)

Catechin (µM)	Untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	99.64	102.82	97.12	97.28	97.75	99.36	99.22	102.79
58 hrs	98.50	94.40	98.84	97.29	96.50	97.65	98.18	102.48

(B)

Chlorogenic acid (µM)	Untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	99.72	90.74	99.84	103.21	102.52	98.79	98.21	101.23
58 hrs	100.34	94.02	100.63	99.42	100.22	102.82	102.29	101.41

(C)

Figure 3.12: Effect of polyphenols treatment on the viability of HepG2 cells. The cell viability was measured at 29 and 58 hr of incubation using NR assay and based on measurement of Neutral red incorporation into the lysosomes of living cells. The cells were exposed to different concentration of polyphenols before NR dye was added. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤0.05. Treated HepG2 cells with apigenin **(A),** catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F).**

Table 3.13: Using Pearson's correlation coefficient r, to determine correlation with the findings of MTT, NR and the protein content.

	29 hr	protein	MTT	NR		58 hr	protein	MTT	NR
protein	Pearson Correlation	1	.840[**]	.861[**]	protein	Pearson Correlation	1	.918[**]	.747[**]
	Sig. (2-tailed)		.000	.000		Sig. (2-tailed)		.000	.000
MTT	Pearson Correlation	.840[**]	1	.612[**]	MTT	Pearson Correlation	.918[**]	1	.608[**]
	Sig. (2-tailed)	.000		.001		Sig. (2-tailed)	.000		.001
NR	Pearson Correlation	.861[**]	.612[**]	1	NR	Pearson Correlation	.747[**]	.608[**]	1
	Sig. (2-tailed)	.000	.001			Sig. (2-tailed)	.000	.001	

*. Correlation was significant at P-value ≤ 0.05 level (2-tailed).

**. Correlation was significant at P-value ≤ 0.01 level (2-tailed).

3.7.3. The effect of natural products against intracellular reactive oxygen species using DCFHDA assay

In order to evaluate the effect of herbal extracts on oxidative stress, levels of ROS production were examined using the DCF fluorescence method along with total GSH antioxidant levels and intracellular scavenging effects against oxidative stress in HepG2 cells. HepG2 cells were pretreated with herbs for 29 and 58 hr of incubation and then added DCFHDA. The changes of ROS in HepG2 cells were shown in Figures 3.14. Cells exposed to herbal extracts exhibited a rapid decreased of DCF fluorescence in a dose and time dependent manner. *M. oleifera* was more effective than *T. laurifolia*. A 50% inhibition of oxidative stress occurred at 1,053 μg/ml, and 313 μg/ml for 29 hr of incubation and 1,434 μg/ml, and 276 μg/ml for 58 hr of incubation for *T. laurifolia* and *M. oleifera,* respectively. While both herbs were moderately inhibited oxidative stress in HepG2 cells, polyphenols except quercetin and catechin showed no significant reduction of oxidative stress. Quercetin inhibited oxidative stress in dose-dependent manner. IC_{50} of quercetin was 58.5 μM for 29 hr and 42.2 μM for 58 hr of incubation, respectively. Catechin had a few inhibition of oxidative stress for 58 hr of incubation as shown in Figures 3.15.
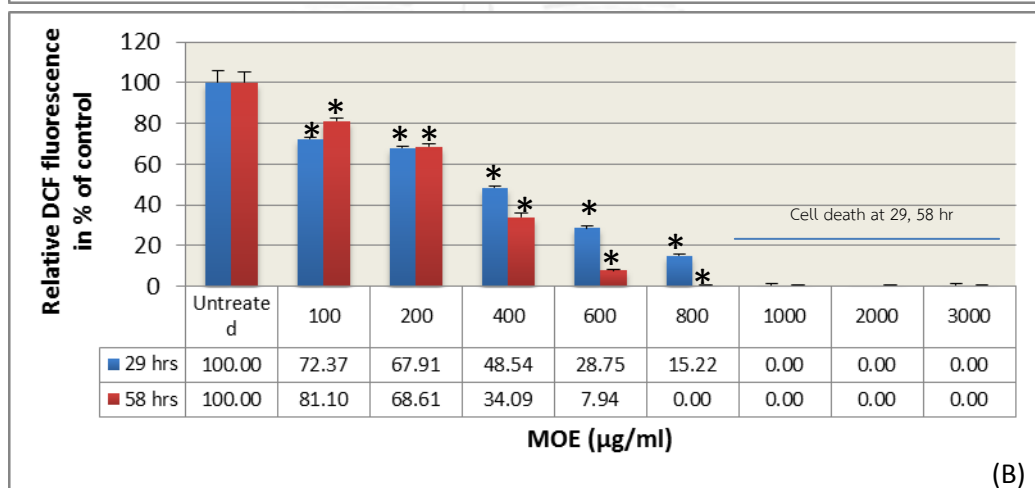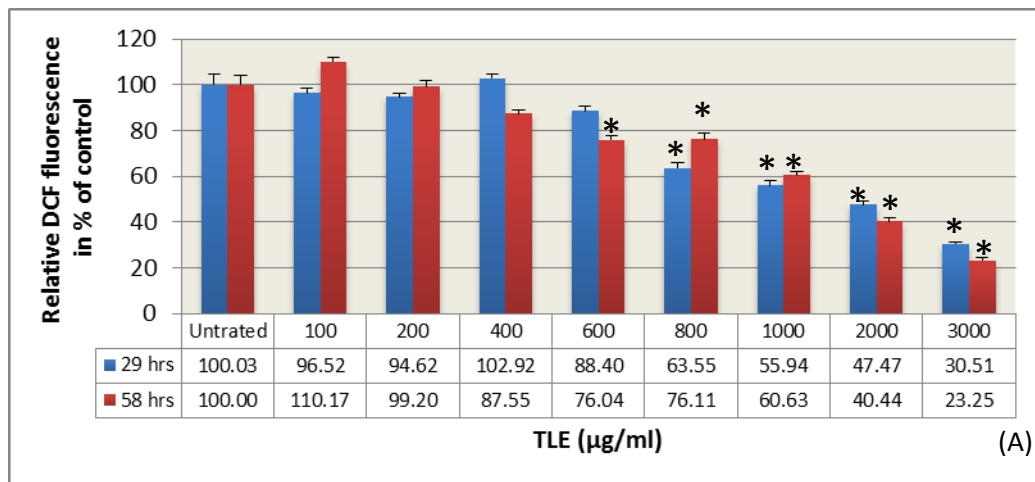
Fig 3.14: **Effect of herbal treatment on oxidative stress in HepG2 cells.** The oxidative stress levels were measured at 29 and 58 hr of incubation using the DCFHDA assay. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**

	Untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	100.00	103.93	101.78	99.58	96.81	98.98	97.47	94.62
58 hrs	100.00	89.06	96.54	96.13	89.07	84.14	83.05	84.00

Catechin (µM)

(B)

	Untreated	1%DMSO	5	10	25	50	75	100
29 hrs	100.00	88.16	96.31	95.35	92.34	91.90	97.02	99.99
58 hrs	100.00	88.76	106.72	108.95	105.37	103.51	103.09	103.18

Chrologenic acid (µM)

(C)

	Untreated	0.1%Ethanol	5	10	25	50	75	100
29 hrs	100.00	113.95	91.39	91.67	94.42	98.83	100.64	94.26
58 hrs	100.00	114.07	97.48	98.74	99.22	96.19	99.19	96.82

Coumarin (µM)

(D)
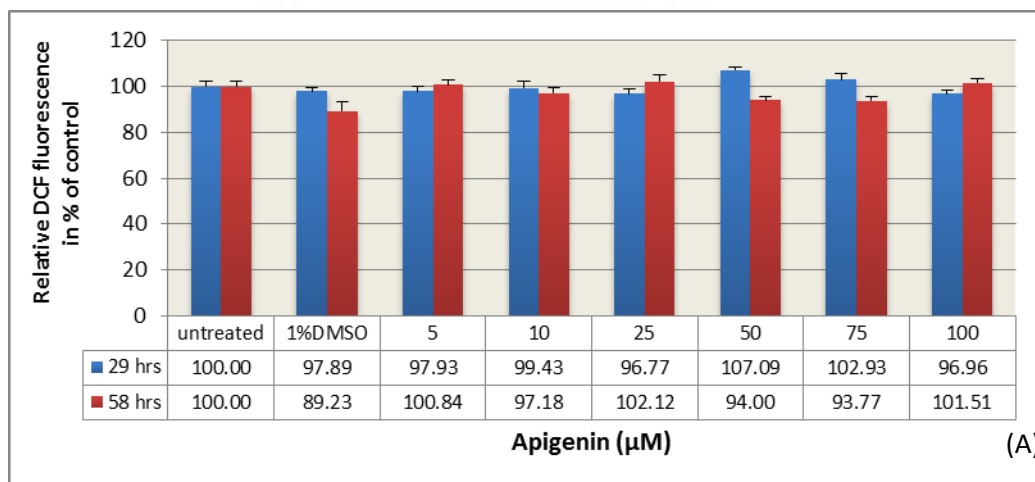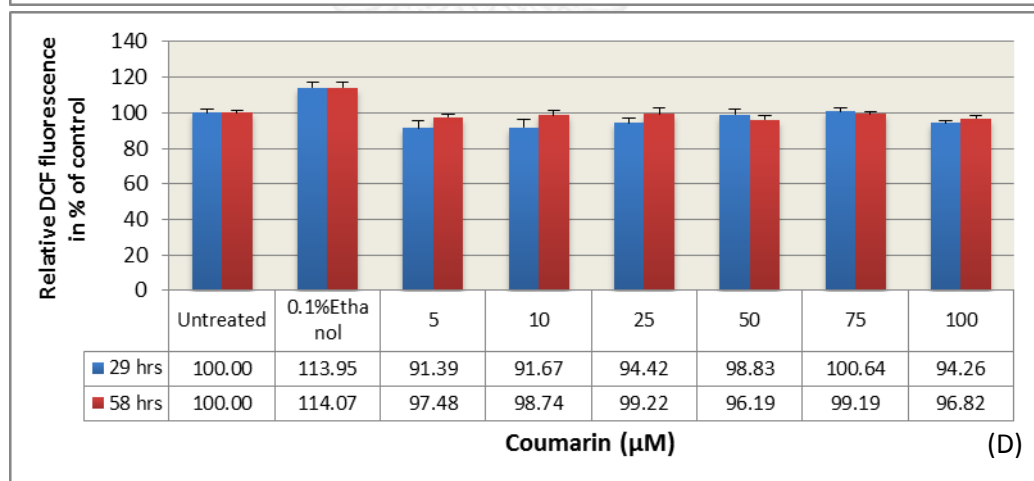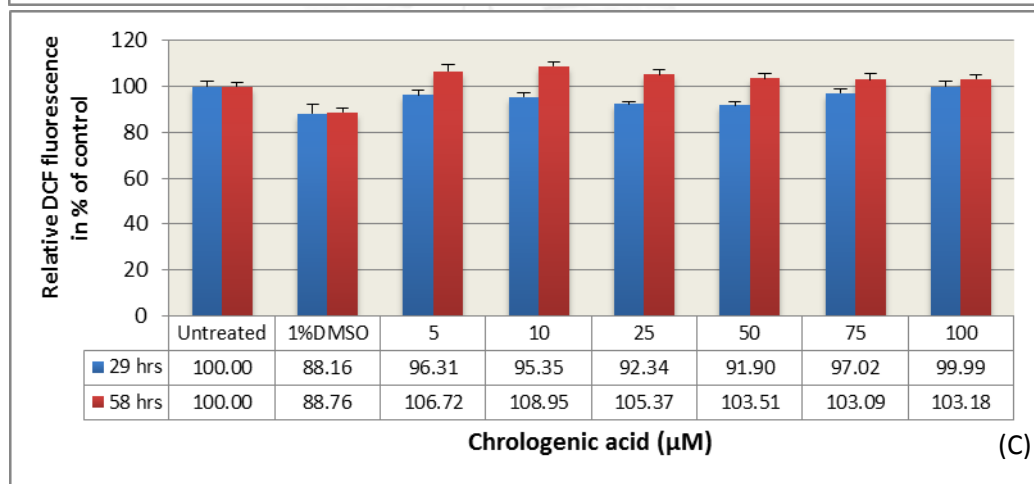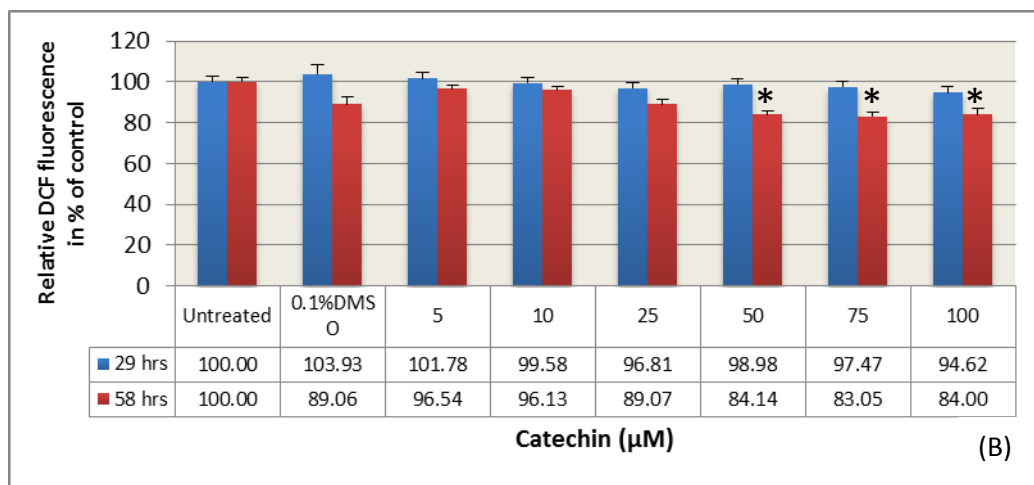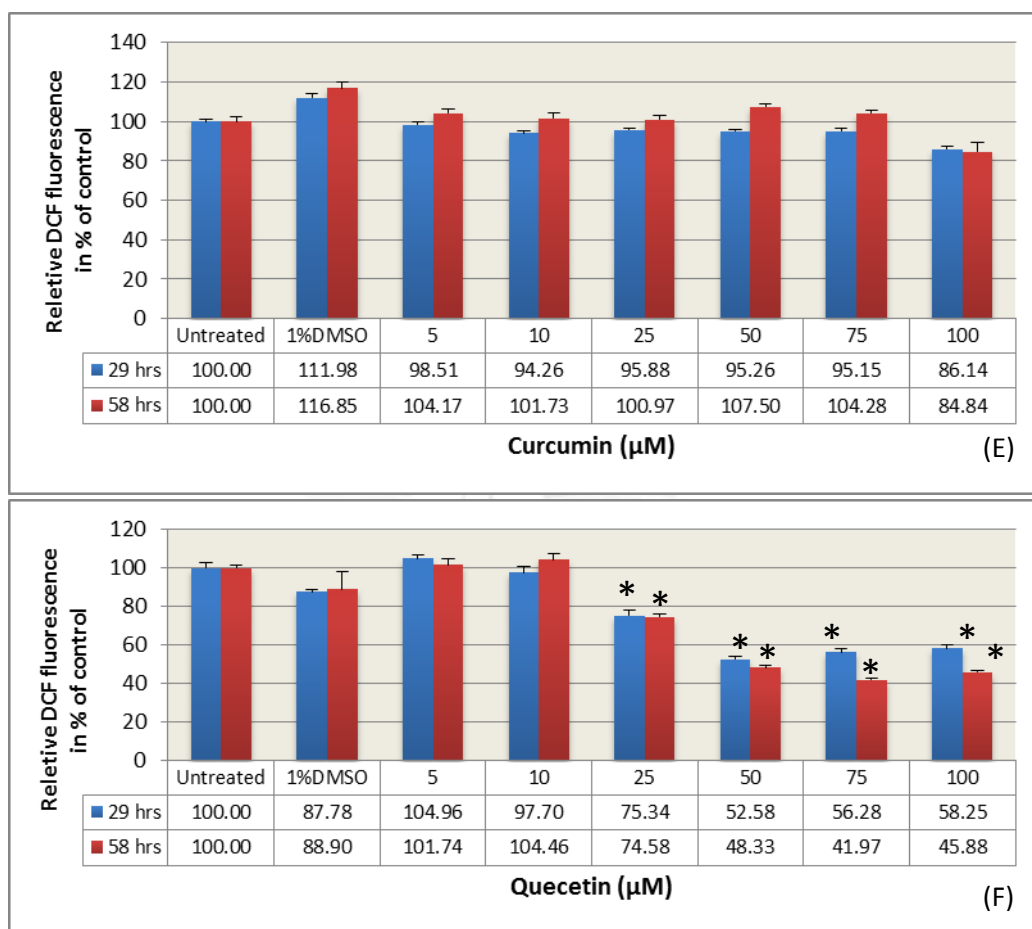
Figure 3.15: **Effect of polyphenols treatment on oxidative stress in HepG2 cells.** The oxidative stress levels were measured at 29 and 58 hr of incubation using the DCFHDA assay. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤0.05. Treated HepG2 with apigenin **(A)**, catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F)**.

3.7.4. Intracellular antioxidant status determined by Total GSH content within cells

Two approaches were used to explore the role of herbal extracts on intracellular antioxidant level. Firstly, effect on GSH levels were determined by treatment HepG2 cells alone with herbal extracts for 29 hr. Secondly, the protective effect of herbal extracts on ROS-induced cell death was estimated by pretreatment of HepG2 cells with herbal extracts then exposed to H_2O_2. As shown in Figure 3.10, cells were pretreated with *T. laurifolia* or *M. oleifera* these were shown biphasic response. The GSH level was significantly increased in dose-dependent manner but this effect was maintained at low concentrations. Maximal enhancing effect was observed at 100 µg/ml of *T. laurifolia* and 400 µg/ml of *M. oleifera*. After which, rising in *T. laurifolia* and *M. oleifera* concentration resulted in a dramatic decreased in the intracellular GSH level in a dose-dependent manner. Determination of glutathione level after treated HepG2 cells with polyphenols also showed biphasic effect in GSH level but difference in sensitivity and maximal response. Maximum GSH level was induced by 10 µM apigenin, catechin, quercetin, 25 µM chorlogenic acid, 50 µM coumarin, and 75 µM curcumin, respectively. The highest rising level of GSH, 170%, was induced by curcumin.
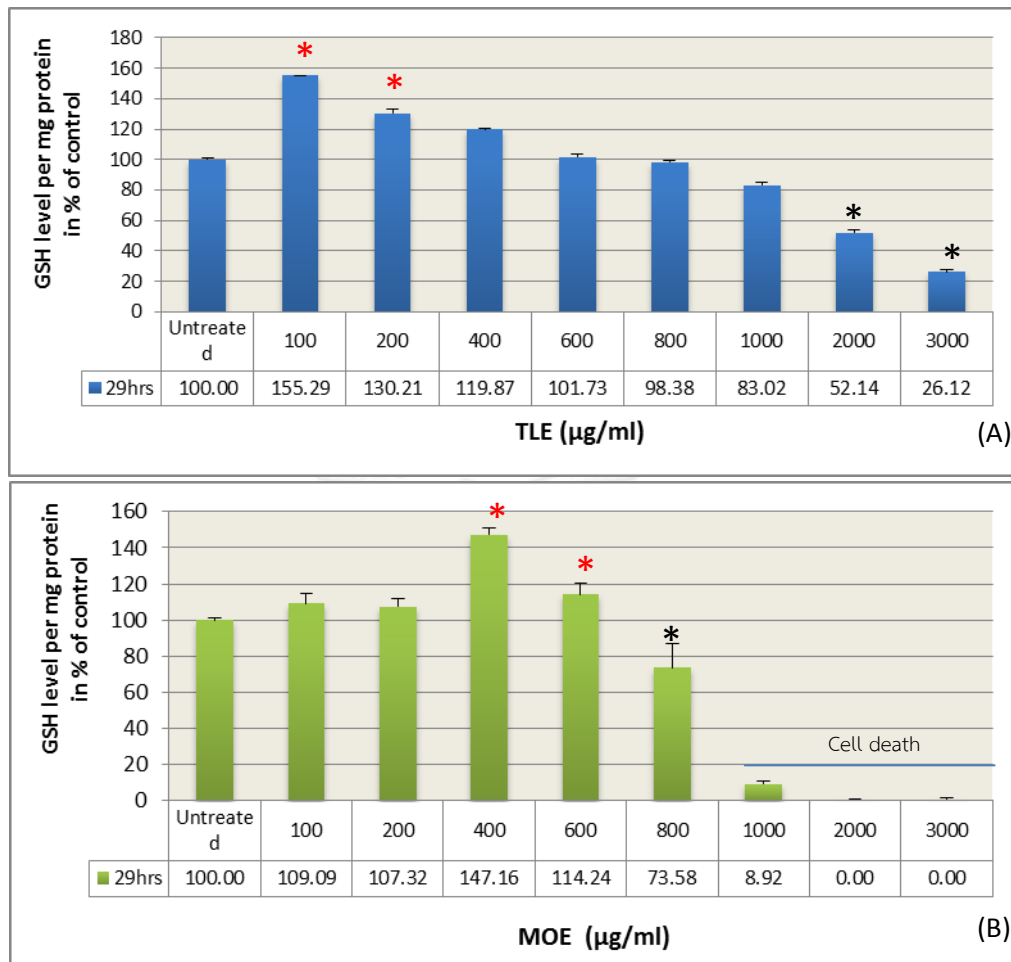
Figure 3.16: **Effect of herbal treatment on reduced glutathione (GSH) in HepG2 cells.** The GSH levels were measured using the NBT colorimetric assay. Total GSH level was calculated from GSH standard curve. GSH levels in control HepG2 cells were 7.4 nmol/mg proteins with a range of 3.0 – 9.3 μM/mg. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**

	untreated	0.1%DMSO	5	10	25	50	75	100
■ 29 hrs	100.00	106.81	116.42	134.67	129.62	112.13	108.38	84.45

Catechin (µM)

(B)

	untreated	0.1%DMSO	5	10	25	50	75	100
■ 29 hrs	100.00	106.81	115.73	121.49	124.57	112.74	100.66	102.30

Chrologenic acid (µM)

(C)

	untreated	0.1%DMSO	5	10	25	50	75	100
■ 29 hrs	100.00	106.81	145.30	147.13	145.57	155.95	116.91	106.90

Coumarin (µM)

(D)
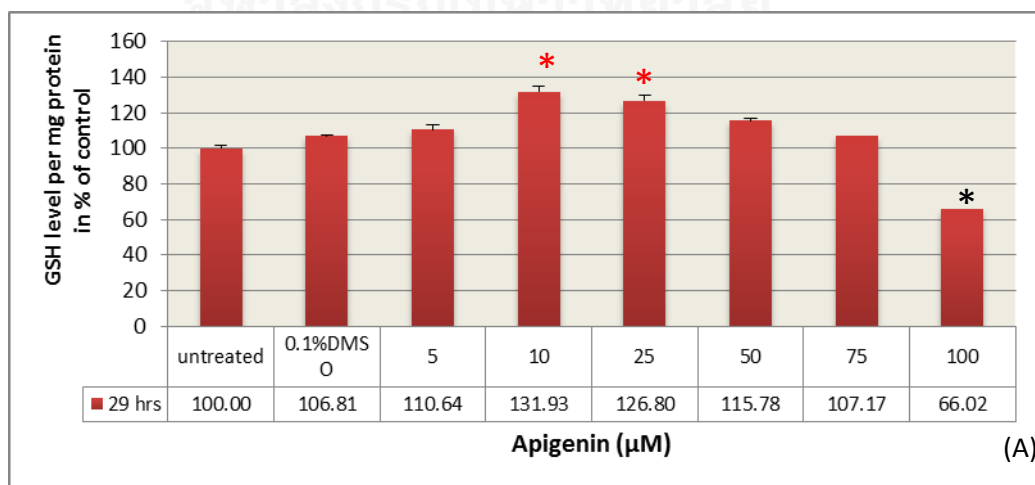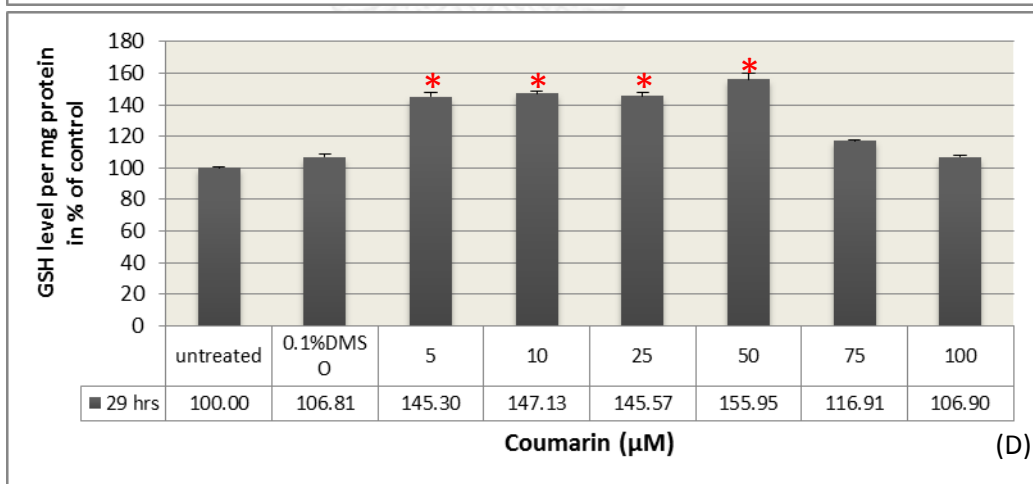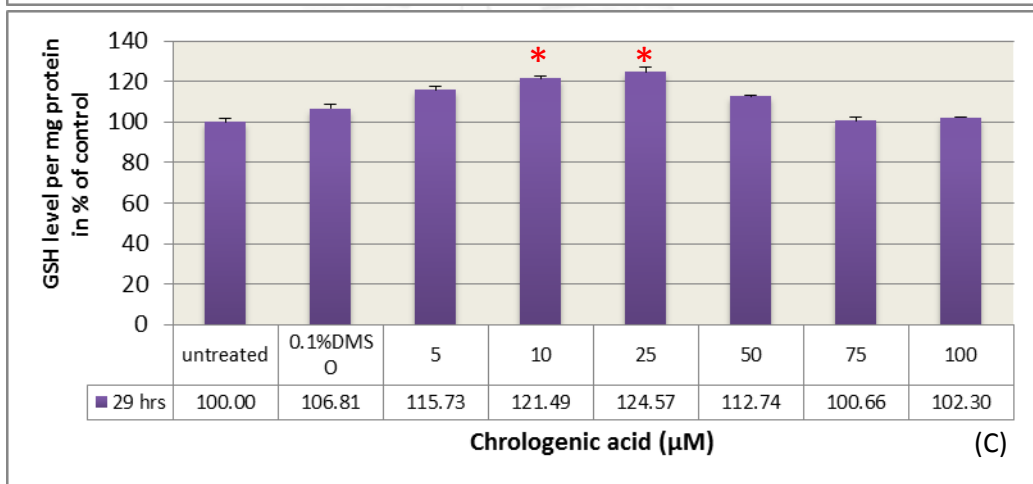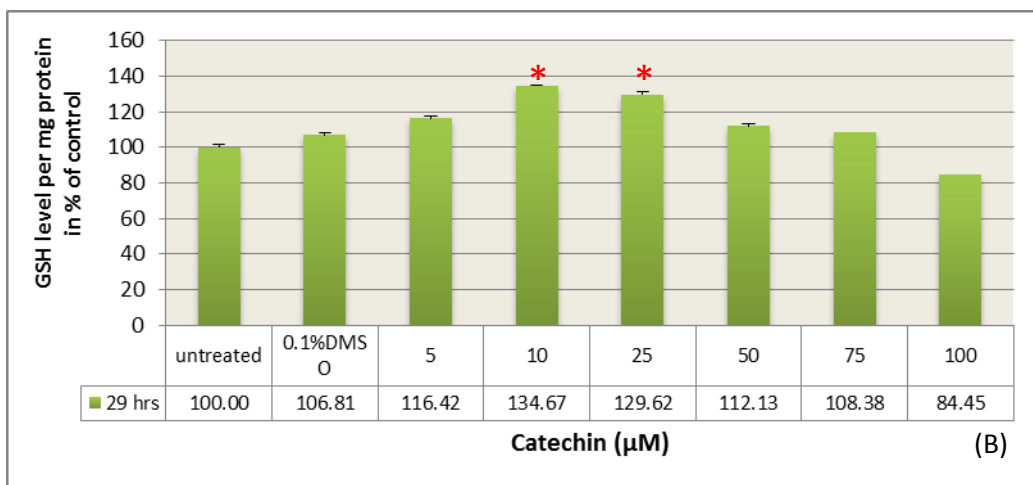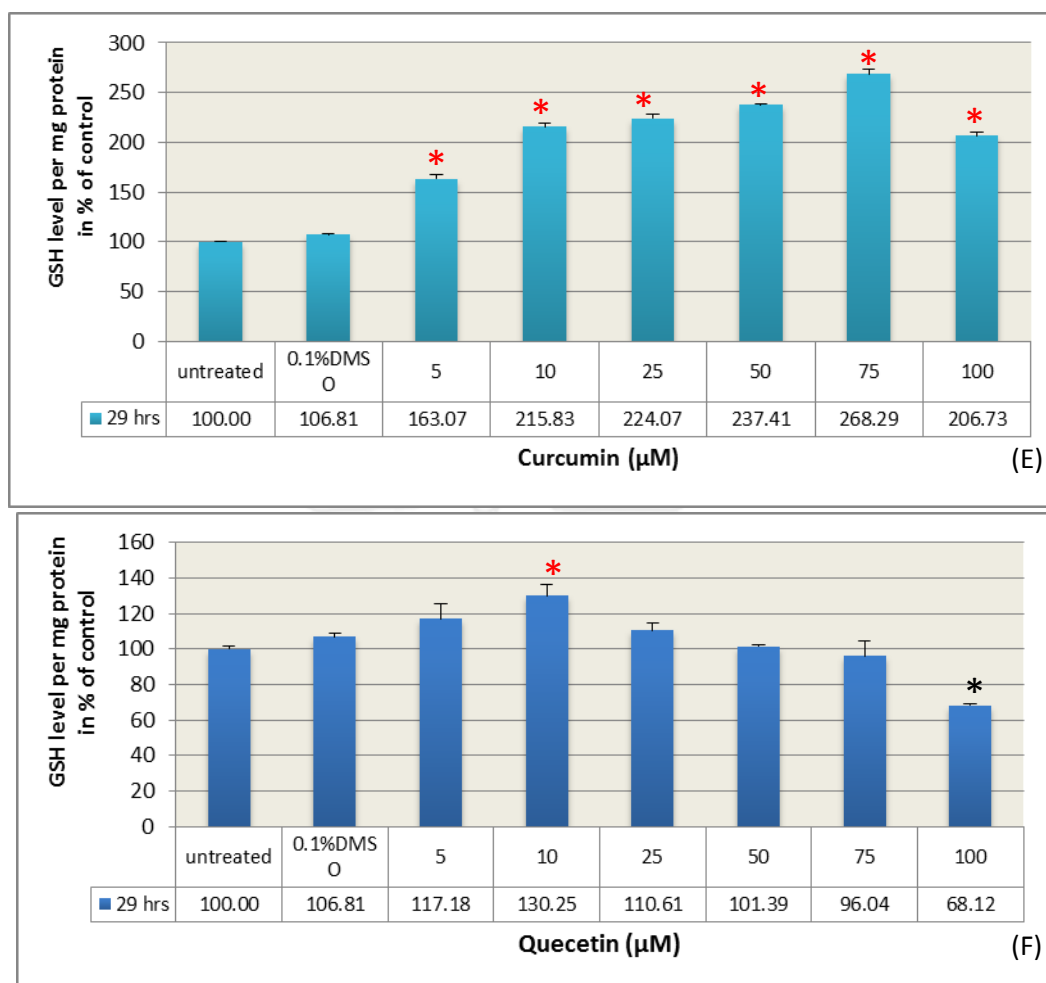
Figure 3.17: **Effect of polyphenols treatment on induced glutathione (GSH) in HepG2 cells.** The GSH levels were measured at indicated time points using the NBT colorimetric assay. Total GSH level was calculated from GSH standard curve. GSH levels in control HepG2 cells were 3.0-9.3 μmol/mg protein. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least 4 independent experiments. *Significantly different from control was p-value ≤0.05. Treated HepG2 with apigenin **(A),** catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F).**

We therefore tested the protective effect of herbal extracts on ROS-induced cell death in HepG2 cells. The result was shown in figure 3.18. Exposure of cells to 12.5 mM of H_2O_2 led to a significant reduction by 80% compared with control cells of intracellular GSH content after 2 hr. The pretreatment of HepG2 cells with *T. laurifolia* could block the decline of H_2O_2-induced GSH depletion which compared to the result of H_2O_2 treated cells alone. The protective effect of *T. laurifolia* against H_2O_2-induced GSH depletion was a dose-dependent manner. But GSH level did not change in *M. oleifera* pretreated cells. It was indicated that no protective effect of *M. oleifera* against H_2O_2-induced oxidative stress.
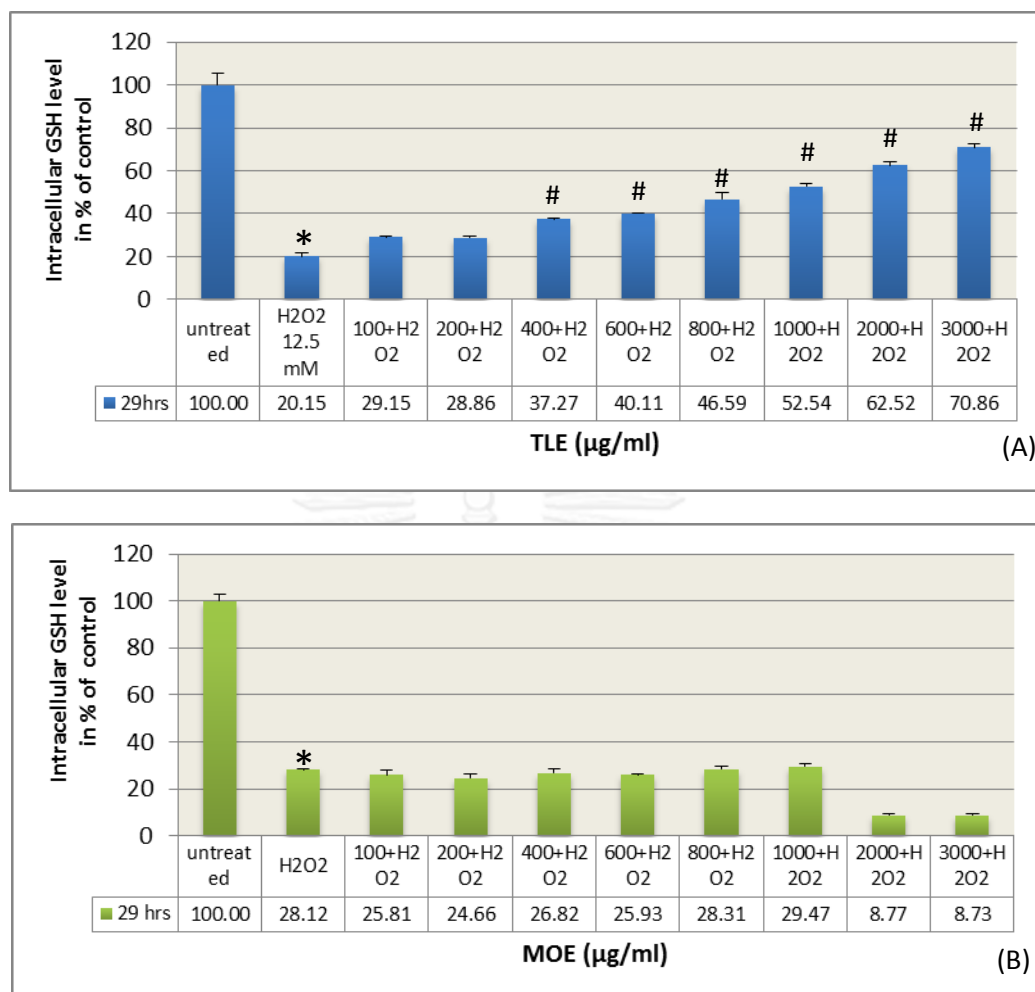
Figure 3.18: **Effect of herbal pretreatment on reduced glutathione (GSH) after 12.5 mM H_2O_2 treatment in HepG2 cells for 29 hr of incubation.** The GSH levels were measured using the NBT assay. Total GSH level was calculated from GSH standard curve. GSH levels in control cells were 3.0- 9.3 μmol/mg protein. The results were expressed as percentage of untreated control and represent mean ± SEM. * Significant different from control was p-value ≤ 0.05. [#] Significant different from positive H_2O_2-treated cells was p-value ≤ 0.05. Treated HepG2 with *T. laurifolia* (**A**) and *M. oleifera* (**B**)

3.7.5. The herbal effect on mitochondrial membrane potential

Mitochondria play pivotal roles in maintaining cell function. Mitochondrial dysfunction is central to theory of aging, with the production of high levels of reactive oxygen species (ROS) and plays a key role in the aging process and increases the incidence of age-related disorders such as neurodegenerative diseases. To study the herbal effect on mitochondrial function may provide better understanding in their protective effect in pathologies of aging. To investigate the effect of herbal extracts on mitochondrial membrane potential, HepG2 cells were exposed to herbal extracts or polyphenols 4, 29 and 58 hr. Treated cells were then stained with TMRM; cationic mitochondrial selective probe, and measured intracellular fluorescent intensity. Relative fluorescence intensity was calculated and compared with the untreated group. Results also were confirmed using confocal microscope images to detect the loss of red fluorescent component of TMRM upon depolarization state. In addition, a complete loss of fluorescence occurred following collapsed of $\Delta\psi_m$ with mitochondrial uncoupled carbonyl cyanide 3-

chlorphenyl hydrazone CCCP, a positive control in this study. Figure 3.19 illustrated the confocal image of the effects of 100 μM CCCP which depolarized mitochondria and decreased MMP. The results as shown in Figure 3.20, both herbal extracts significantly increased the mitochondrial membrane potential in biphasic fashion. The decline in mitochondrial $\Delta\psi_m$ was observed at higher concentration and correlated with the level of cells death. *M. oleifera* altered mitochondrial membrane potential even with the lowest incubation time (4 hr). On the other hand, the effect of *T. laurifolia* induced the alternation of mitochondrial membrane potential at 29 hr of incubation. Incubation HepG2 cells with polyphenols had an opposite effect; inhibition of mitochondrial membrane potential was observed. The fall in mitochondrial membrane potential was observed in apigenin, catechin and chlorogenic acid treatment. The effect of these 3 polyphenols on HepG2 cells was concentration- and time dependent. Apigenin was the most potent compounds caused substantial disruption of mitochondrial membrane potential, reaching IC_{50} in cells challenged with 100 μM for 29 hr and 89.4 μM for 58 hr which were relative to control cells. The effect of curcumin and quercetin on HepG2 cells was more complicated. Although there was a significant decreased in mitochondrial membrane potential at 29 hr, increased $\Delta\psi_m$ was observed at 58 hr of incubation. This effect was dose-dependent and more potent in quercetin over curcumin.
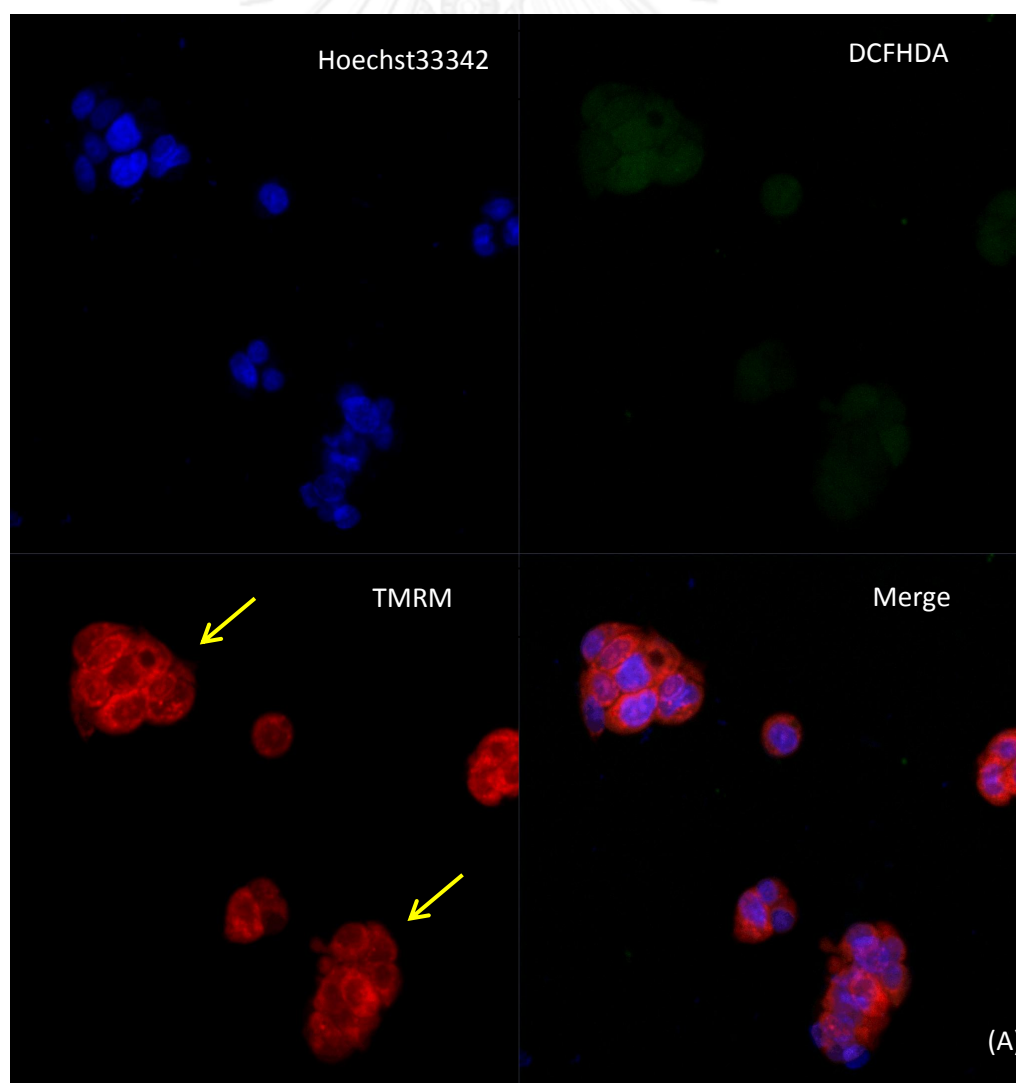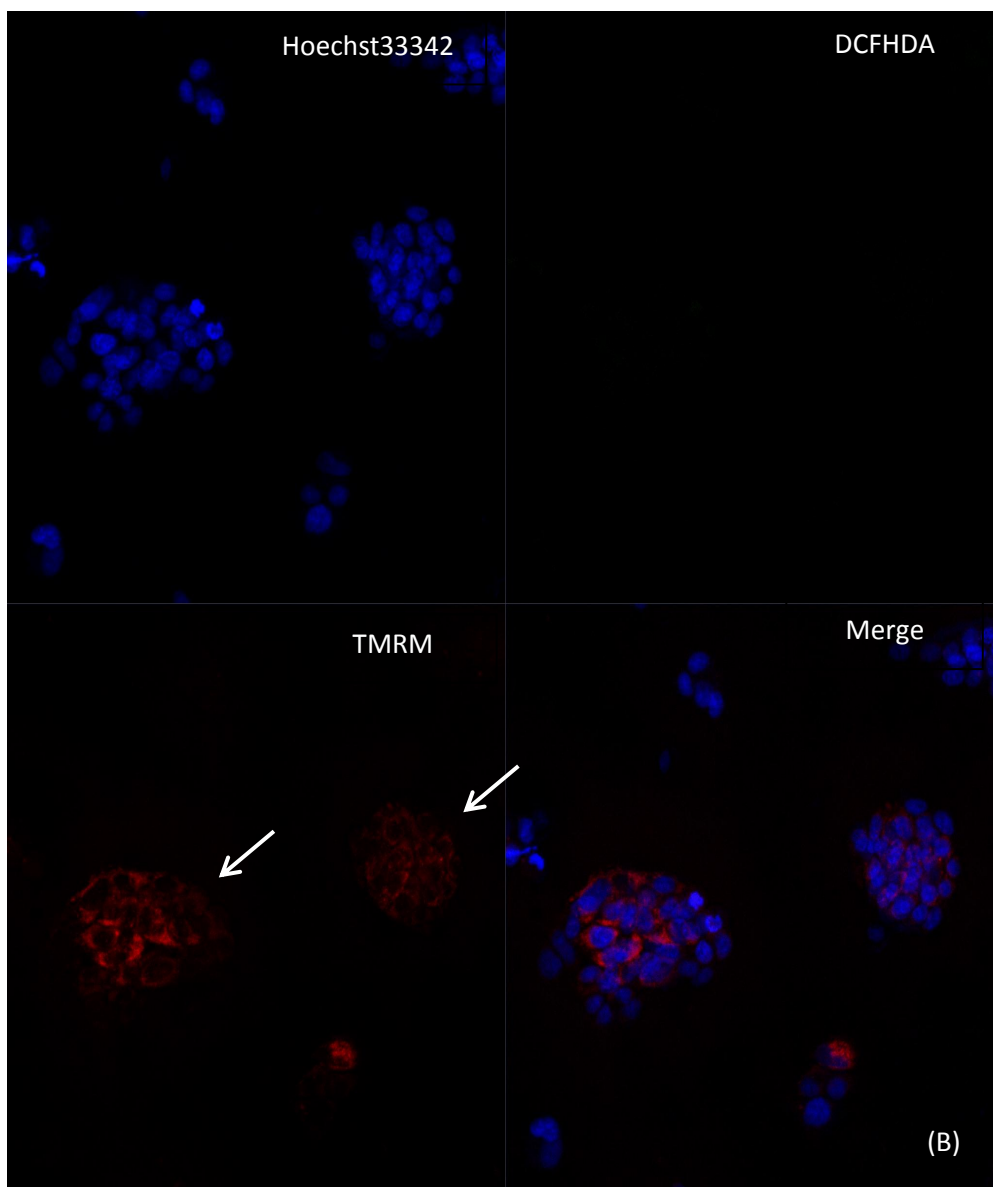
Figure 3.19: **Confocal analysis of mitochondrial membrance potential in HepG2 (100X).** Cells were added with DCFH-DA, Hoechst33342, and TMRM for 30 minutes then process under confocal microscopy in situ. Untreated HepG2 cells were noted as basal conditions (yellow arrow) **(A)**. Treated HepG2 cells with 100 μM CCCP for 30 min. CCCP caused mitochondrial depolarized led to low TMRM fluorescence intensity. Note the significant reduction of mitochondrial membrance potential compared to untreated cells (white arrow) **(B)**.
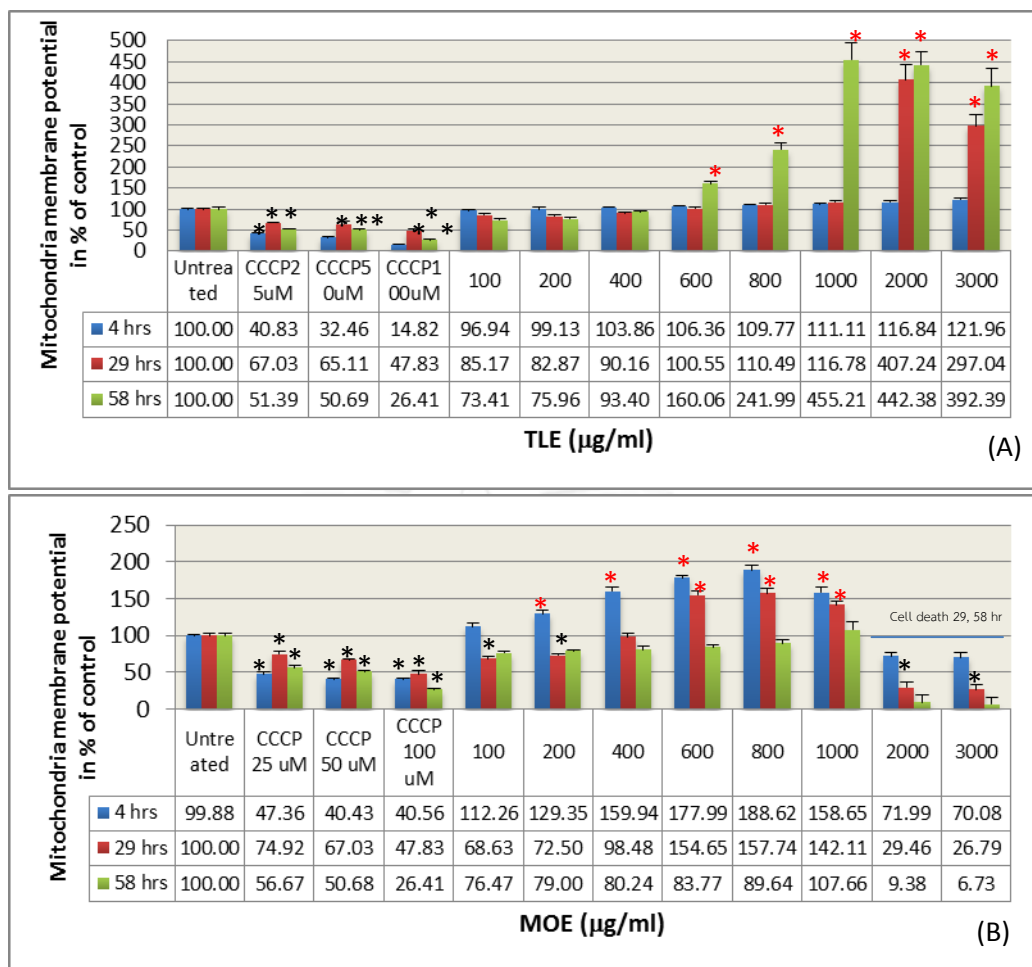
Figure 3.20: **Effect of herbal pretreatment on the mitochondrial membrane potential (△Ψ$_m$) in HepG2 cells.** The △Ψ$_m$ was measured at 4, 29 and 58 hr of incubation using TMRM staining assay. After pretreated cells with herbs, then added 20 μM TMRM for 30 min, followed by fluometric analysis. △Ψ$_m$ was calculated from fluorescence intensity of TMRM. CCCP, positive control, was used to confirm TMRM response. The results were expressed as a percentage of untreated control and represent mean ± SEM of at least three independent experiments. *Significant different from control was p-value ≤ 0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**

(B) Catechin (µM)

	Untreated	0.1%DMSO	CCCP 25 uM	CCCP 50 uM	CCCP 100 uM	5	10	25	50	75	100
■ 29hrs	100.00	103.41	62.92	58.28	44.89	102.25	96.34	90.80	90.05	90.64	90.20
■ 58 hrs	100.00	94.98	50.52	49.78	26.52	100.70	92.65	86.60	85.28	85.23	85.84

(C) Chlorogenic acid (µM)

	Untreated	0.1%DMSO	CCCP 25 uM	CCCP 50 uM	CCCP 100 uM	5	10	25	50	75	100
■ 29hrs	100.00	94.96	63.00	61.39	43.56	100.87	97.93	96.94	96.42	95.62	94.65
■ 58hrs	100.00	92.63	52.22	47.47	26.07	101.35	96.26	93.86	92.23	92.90	91.15

(D) Curcumin (µM)

	Untreated	0.1%DMSO	CCCP 25 uM	CCCP 50 uM	CCCP 100 uM	5	10	25	50	75	100
■ 29 hrs	100.00	96.25	65.73	60.04	44.11	93.89	92.24	89.72	82.42	77.11	77.15
■ 58 hrs	100.00	95.51	51.39	48.10	28.02	91.90	89.18	88.40	85.14	89.63	106.50

	untreated	0.1DMSO%	CCCP25 uM	CCCP50 uM	CCCP100uM	5	10	25	50	75	100
■ 29 hrs	100.00	106.47	94.66	75.38	62.49	101.05	105.36	103.10	102.99	106.06	103.31
■ 58 hrs	100.00	93.47	90.45	47.87	35.56	101.98	103.84	110.41	110.15	100.82	103.94

Coumarin (uM) (E)

	Untreated	0.1%DMSO	CCCP 25 uM	CCCP 50 uM	CCCP 100 uM	5	10	25	50	75	100
■ 29 hrs	100.00	97.74	73.67	65.56	52.57	105.00	108.94	112.37	87.74	78.33	72.96
■ 58 hrs	100.00	100.25	51.85	48.33	25.18	102.56	112.77	121.57	123.91	123.55	148.24

Quecetin (μM) (F)

Figure 3.21: **Effect of polyphenols treatment on mitochondrial membrane potential ($\Delta\Psi_m$) in HepG2 cells.** The $\Delta\Psi_m$ was measured at 29 and 58 hr of incubation using TMRM staining assay. After pretreated cells with polyphenols, then added 20 μM TMRM for 30 min, followed by fluometric analysis. $\Delta\Psi_m$ was calculated from fluorescence intensity of TMRM. CCCP, positive control, was used to confirm TMRM response. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with apigenin **(A)**, catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F)**.

3.7.6. The effect on mitochondrial function by measuring mitochondria ATP production.

Since herbal extracts affects the mitochondrial membrane potential, to determine the herbal extracts effect on mitochondria energy production. HepG2 cells were exposed to herbal extracts or polyphenols for 4, 29 and 58 hr of incubation. Treated cells were measured intracellular ATP level using luminancence assay and result was shown in Figure 3.22 and Figure 3.23. Intracellular ATP level of herbal treated HepG2 cells for 4 hr was slightly but significantly increased with raising herbal concentration to 2,000 or 3,000 μg/ml. In contrast, exposure to *T. laurifolia* and *M. oleifera* extracts for 29 hr significantly decreased ATP levels in all concentrations, and the effects were even pronounced in the 58 hr of incubation, in which almost complete depletion of ATP occurred with 2,000 to 3,000 μg/ml of herbal extracts. A 50% reducing in ATP level was obtained with 1,394 μg/ml, 668 μg/ml for *T. laurifolia*, and 485

μg/ml, 512 μg/ml *M. oleifera* for 29 and 58 hr of incubation, respectively. The alterations in intracellular ATP levels induced by polyphenols were observed, significantly increased ATP intracellular levels were found in catechin and chlorogenic acid-treated cells after 29 hr of incubation. However, no significant difference was found after that. After 58 hr of incubation, no significantly decreased ATP levels in cells that treated with apigenin, catechin, curcumin, and coumarin in all preparations, whereas there was significant increase in ATP levels with 5-10 μM of quercetin and 5-75 μM of Chlorogenic acid. These results had no correlation with the $\Delta\psi_m$ results indicated that it might be another factor affected these mitochondrial ATP production.

TLE (µg/ml)	untreated	100	200	400	600	800	1000	2000	3000
4hrs	100.00	102.52	94.71	94.39	97.50	99.22	98.46	112.34	122.54
29 hrs	100.00	94.54	92.08	84.53	81.05	79.77	70.46	28.83	15.71
58 hrs	100.00	88.32	86.00	64.50	65.54	37.98	31.70	0.12	0.22

(A)

MOE (µg/ml)	untreated	100	200	400	600	800	1000	2000	3000
4 hrs	100.00	79.74	76.07	75.48	77.38	82.98	91.07	93.42	35.69
29 hrs	100.00	86.73	74.78	48.63	45.52	33.95	33.75	0.30	0.26
58 hrs	100.00	116.47	89.83	67.88	44.97	16.40	5.17	0.00	0.00

(B)

Figure 3.22: **Effect of Herbal treatment on the intracellular ATP levels of HepG2 cells.** The ATP levels were measured at 29 and 58 hr of incubation using the luciferase ATPlite kit assay. Total ATP level was calculated from luminescence intensity of ATP standard curve. ATP level in untreated cells was 8.0 nmol/10^6 cells. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**
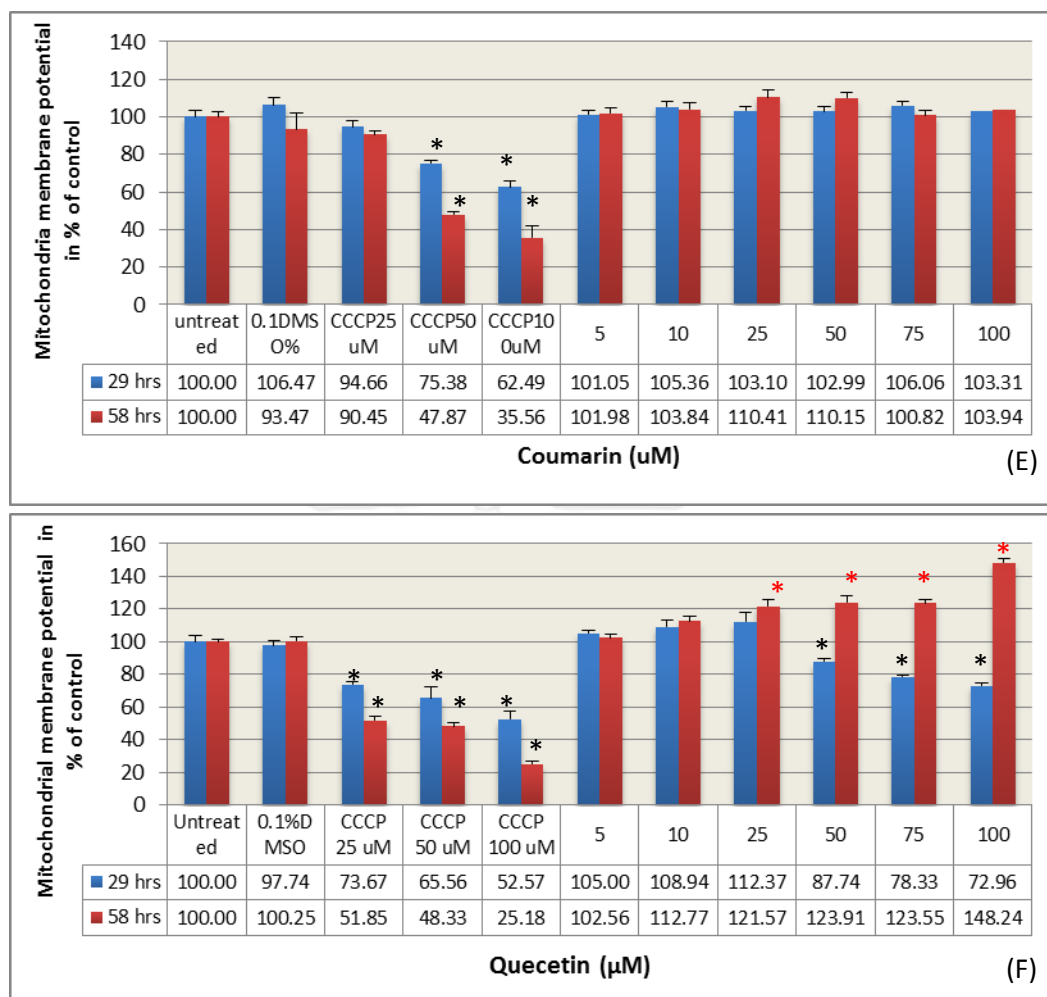
The figure consists of three bar charts (A, B, C) showing Intracellular ATP levels in % of control for different compounds at 29 hrs (blue) and 58 hrs (red).

(A) Apigenin (µM)

	Untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	100.00	97.21	91.96	97.88	94.90	85.14	87.76	92.99
58 hrs	100.00	101.20	109.53	109.20	101.98	92.07	89.35	84.84

(B) Catechin (µM)

	untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	100.00	107.14	142.78	167.59	167.92	165.56	144.01	137.79
58 hrs	100.00	97.22	85.06	85.24	96.65	89.11	95.93	93.52

(C) Chlorogenic acid (µM)

	untreated	0.1%DMSO	5	10	25	50	75	100
29 hrs	100.00	96.90	110.12	108.03	107.30	114.45	111.23	88.19
58 hrs	100.00	104.79	118.61	129.47	128.13	133.24	131.12	109.71
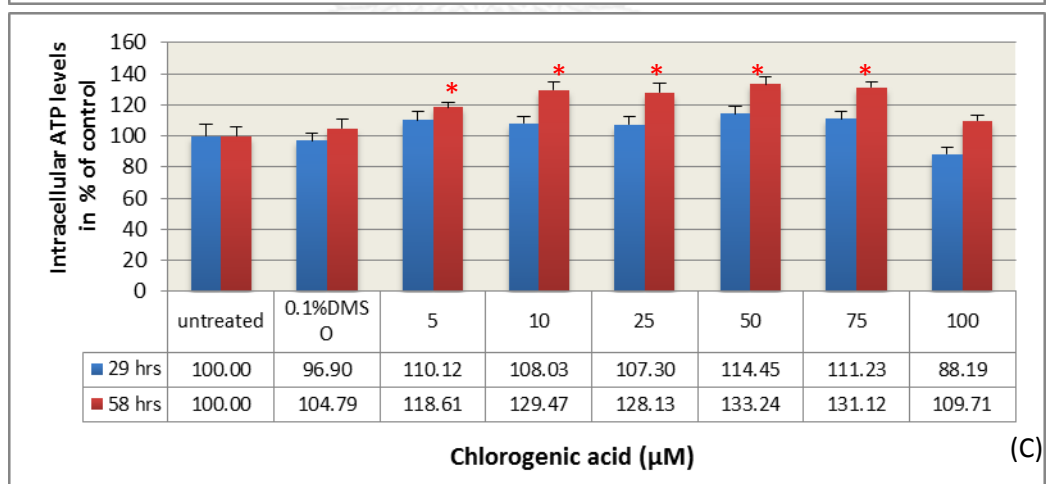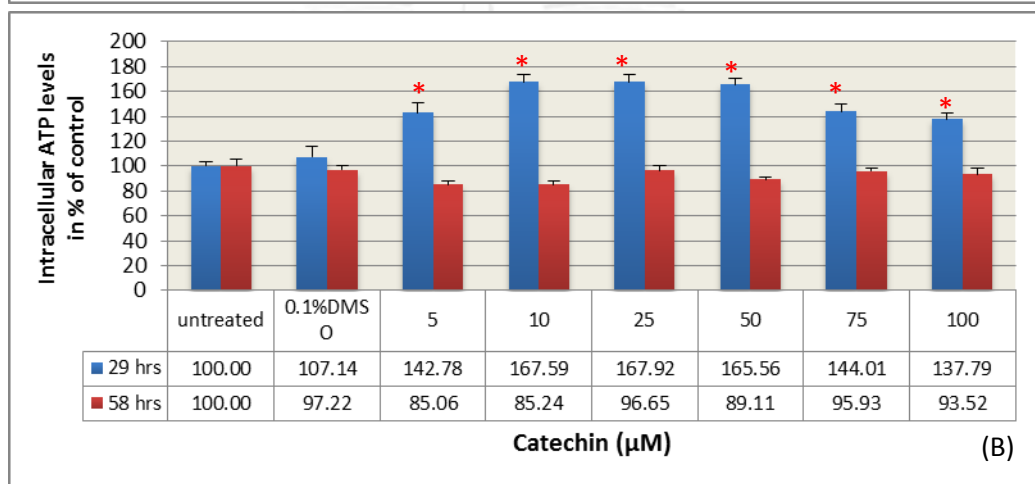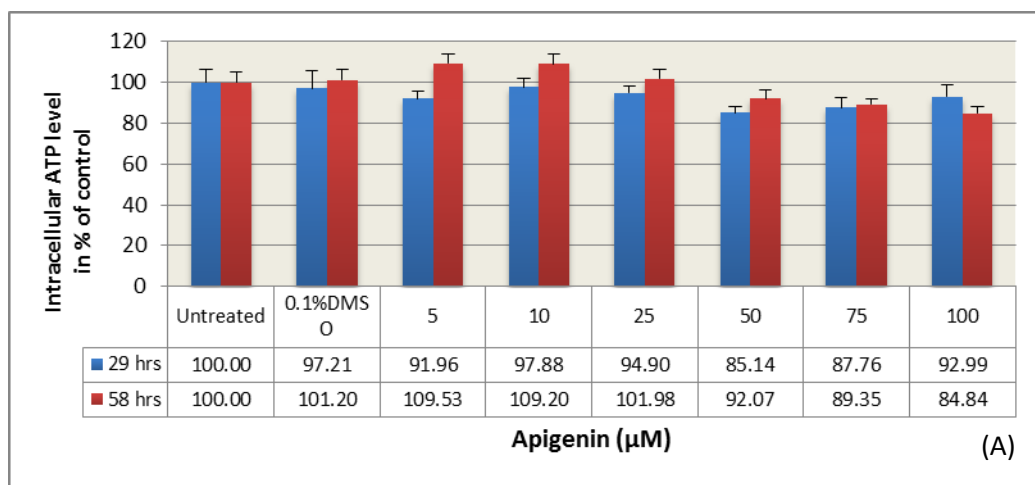
Figure 3.23: **Effect of polyphenols treatment on the intracellular ATP levels of HepG2 cells.** The ATP levels were measured at 29 and 58 hr of incubation using the luciferase ATPlite kit assay. Total ATP level was calculated from luminescence intensity of ATP standard curve. ATP level in control cells was 6.3 nmol/10^6 cells. The results were expressed as a percentage of untreated control levels and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with apigenin **(A),** catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F).**

3.7.7. The effect of herbal extracts on mitochondria calcium levels

There is a strong relationship between calcium signaling and mitochondrial bioenergetic parameters. To determine the effect of herbal extracts on intracellular calcium levels, HepG2 cells were incubated with herbal extracts; the mitochondria of the cells were loaded with calcium binding dye Rhod2-AM, and then monitored for increasing in intracellular Rhod2 fluorescence intensity. The changes in Rhod2AM-loaded cells were also recorded by confocal microscopy. Digitonin, the intracellular-free-calcium inducer, was used as the positive control. Treatment of HepG2 cells with 50 **μ**M of digitonin elevated intracellular calcium level. In the presence of 100 to 2,000 **μ**g/ml of herbal extracts, *T. laurifolia* did not change intracellular calcium level. Only 3,000 **μ**g/ml of *T. laurifolia* was enhancing the intracellular calcium level. In contrast, exposure of *M. oleifera* was more complicated, biphasic effect was observed at both 4 and 29 hr of incubation but differed in sensitivity. Low concentration, 100 to 400 **μ**g/ml, of *M. oleifera* would decrease the intracellular calcium, raising concentration of herbs 600 to 1,000 **μ**g/ml these would cause raising intracellular calcium levels by 50-65% compared with control. At very high concentrations which cytotoxic effect was seen, the elevation of intracellular calcium was attenuated. These data suggested that modulation of intracellular calcium levels were dose response.
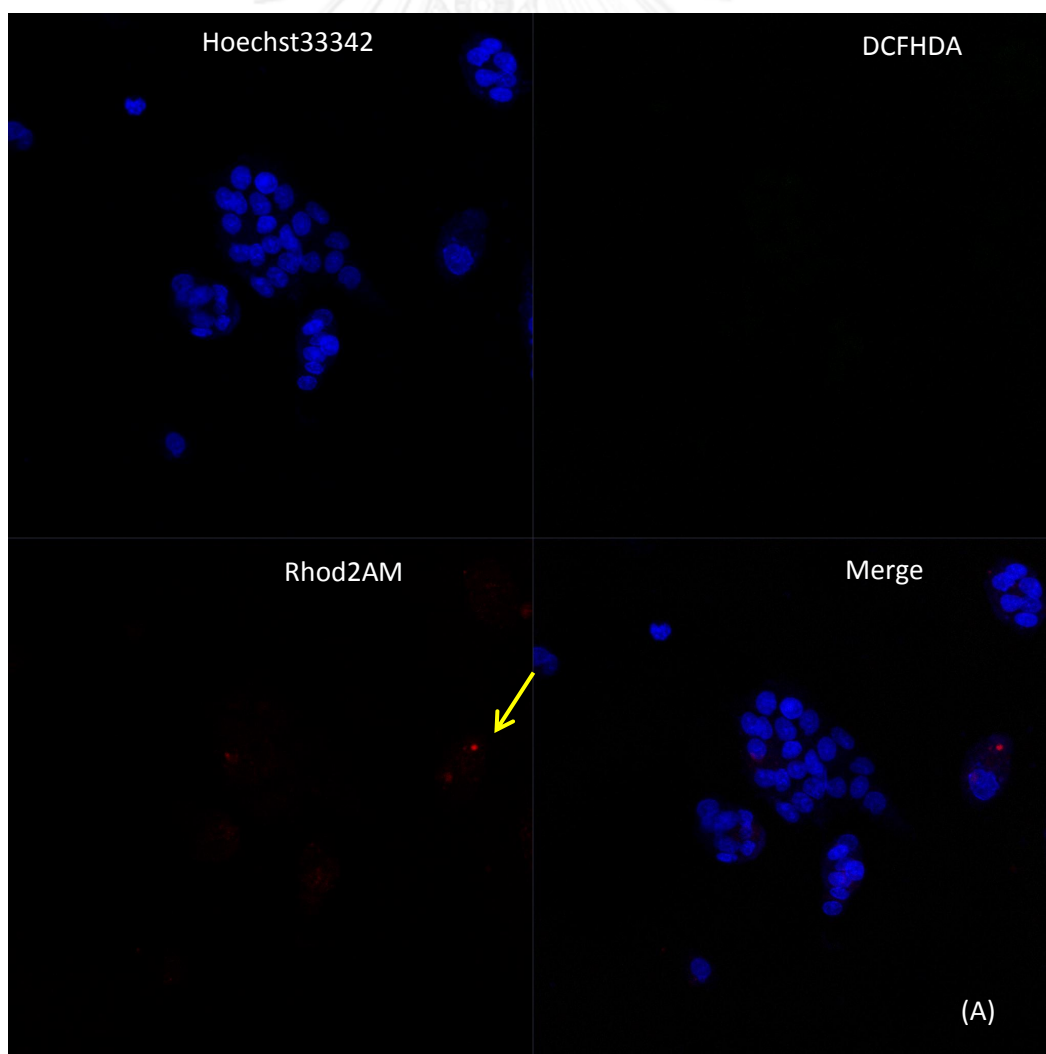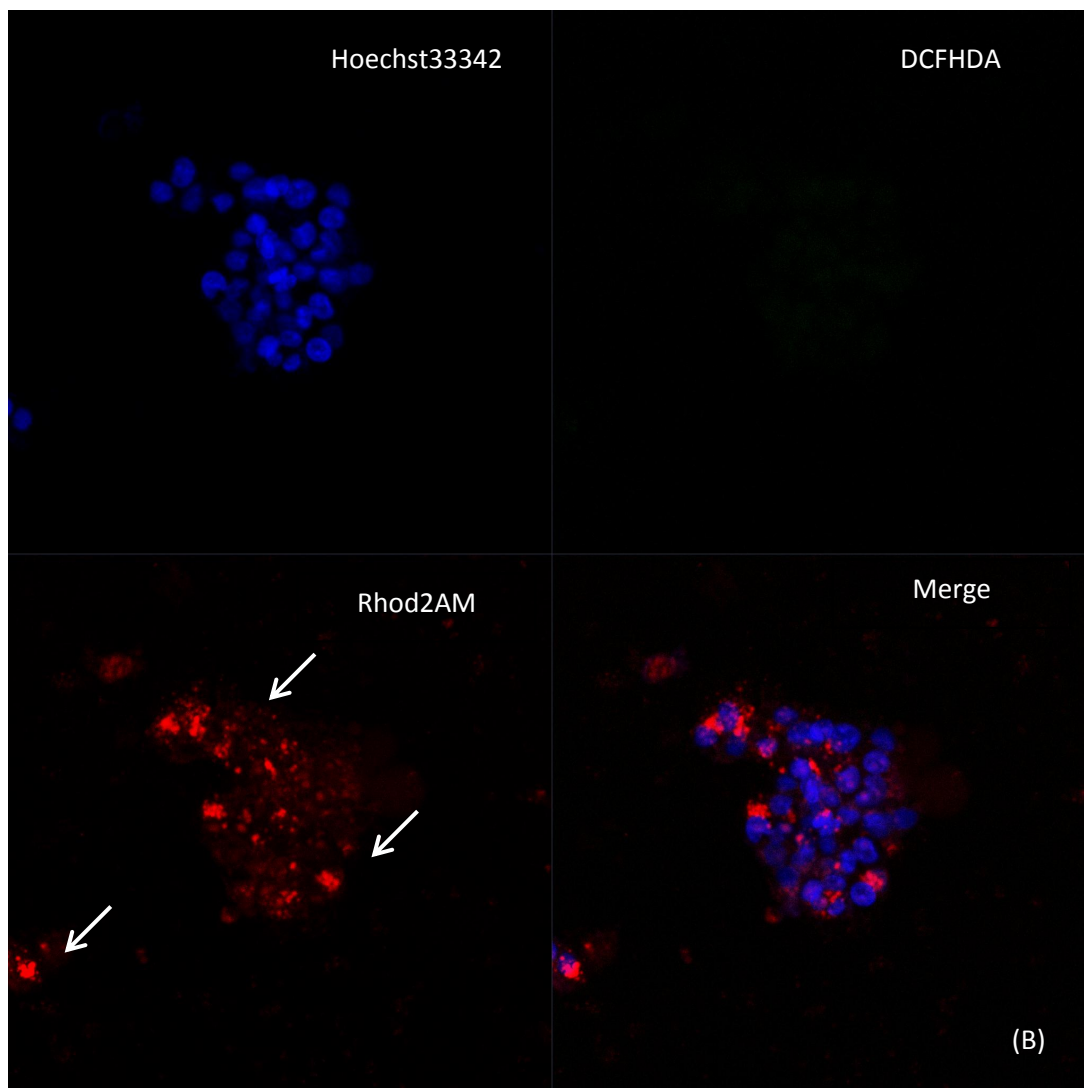
Figure 3.24: **Confocal analysis of the mitochondrial Ca^{2+} in HepG2 cells (100X).** Cells were added with DCFH-DA, Hoechst33342, and Rhod2AM for 30 min then process under confocal microscopy *in situ*. Untreated HepG2 cells were noted as basal condition (yellow arrow) **(A)**. Treatment HepG2 with 50 μM digitonin for 4 hr which is membrane permeabilized agant cause calcium storage in the ER releasing to cytoplasm. The releasing calcium is takeing up by mitochondria in order to control intracellular calcium concentration. As the result, increase Rhod2 fluorescence intensity was seen. Noted the significantly increase [Ca^{2+}]$_m$ compared to untreated cells(white arrow) **(B)**.
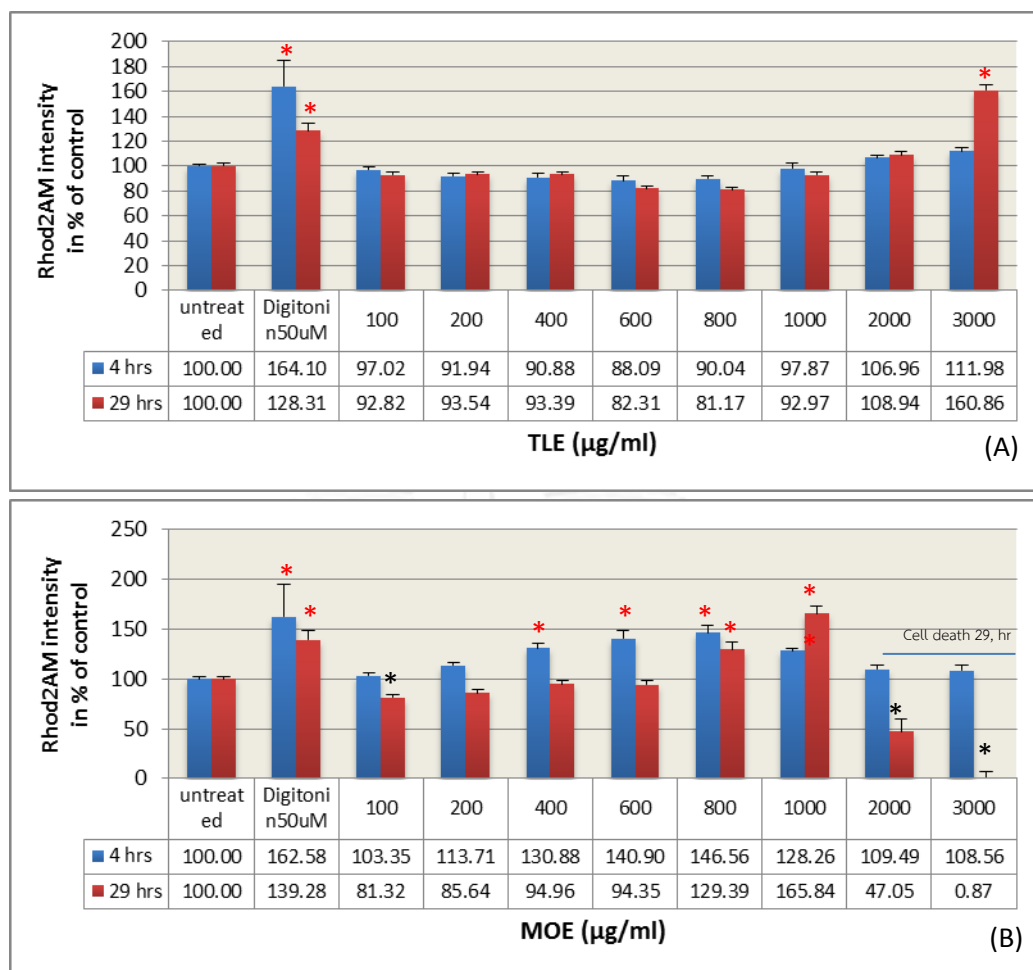
Figure 3.25: **Effect of herbal treatment on the mitochondrial calcium levels of HepG2 cells.** The $[Ca^{2+}]_m$ levels were measured at 4 and 29 hr of incubation using the specific mitochondrial Ca^{2+}, Rhod2AM dye. After treatment, cells were incubated with 5 µM Rhod2AM for 1 hr, followed by fluometric analysis. $[Ca^{2+}]_m$ was calculated from fluorescence intensity of Rhod2AM. Digitonin was used to confirm Rhod2AM response. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-valued ≤ 0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**
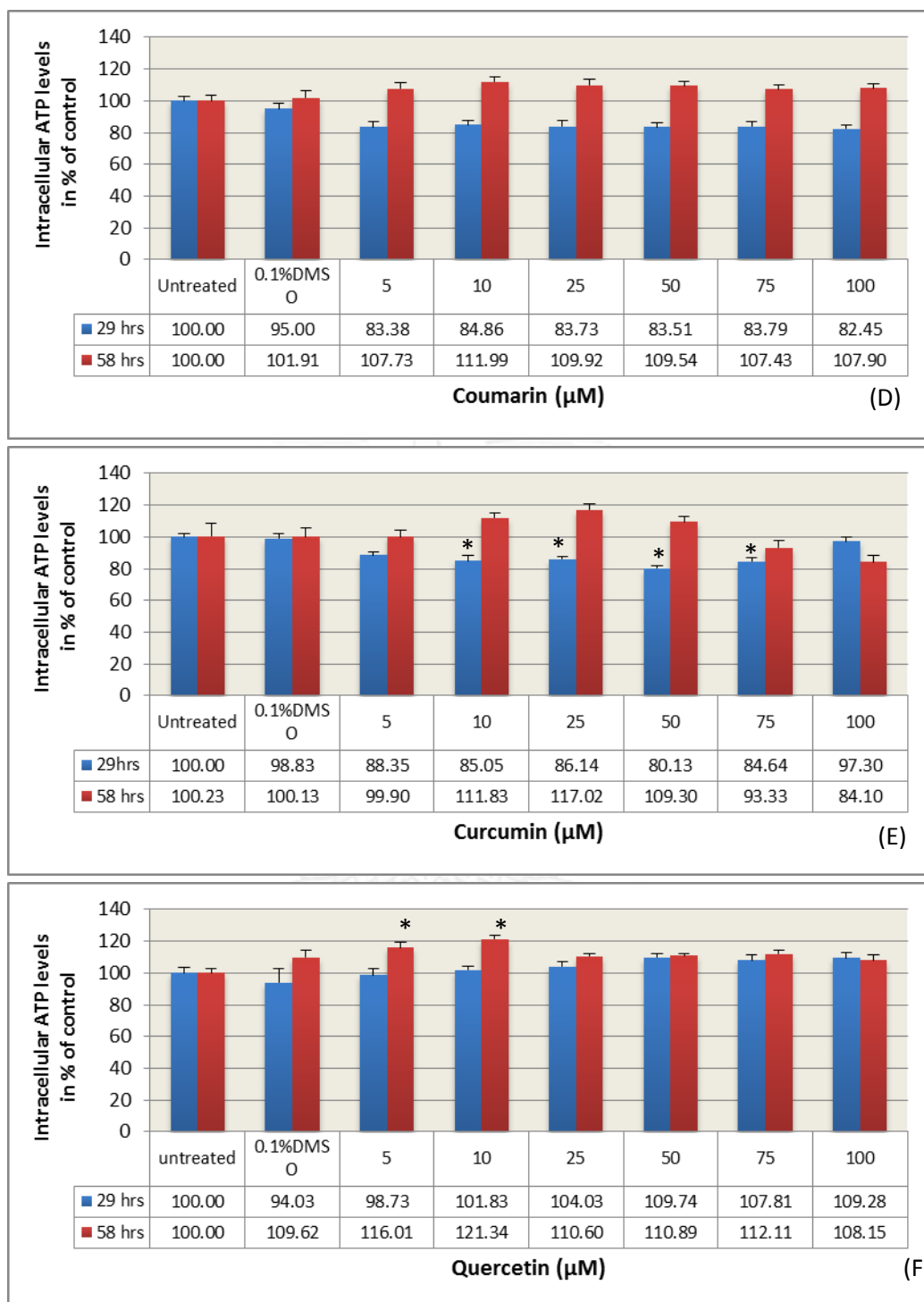
3.7.8. The ability of herbal extracts to modulate biotansformation phase III, P-gp-mediated transport.

Experiments were performed for determination the effect of herbal extracts on P-gp using rhodamine-123 (Rh-123) accumulation as an indicator. Rh-123 is the well-known fluorescent substrate of P-glycoprotein (P-gp). The increased accumulation of Rh-123 in the cells was usually resulting from inhibition of Rh-123 efflux. HepG2 cells were loaded with Rh-123 for 4 hr for allowing Rh-123 to accumulate within cells, and then measured intracellular Rh-123 fluorescence at 530 nm. Data were represented as the calculated percentage of Rho-123 that remaining in the herbal treated cells relatively to control cells. Verapamil, a known competitive P-gp inhibitor, was used as positive control. A rapid decreasing of intracellular Rh-123 was observed in verapamil treated cells after incubation in Rhodamine-123-free medium for 4 hr. The 50% inhibition was obtained with 50 µM verapamil. However, in the presence of herbal extracts, *T. laurifolia* treatment reduced marked accumulation of Rh-123 in HepG2

cells in a dose-dependent manner. Rh-123 efflux was drastically enhanced in treated cells. This observation suggested that *T. laurifolia* induced P-gp dependent efflux activity. Consistent with the results was shown in Figure 3.27; Rh-123 accumulation in HepG2 cells was slightly affected by *M. oleifera* treatment. Similarly as *T. laurifolia* result, after the cells were treated with curcumin, Rh-123 accumulation was increased in a dose-dependent way. It was suggested that curcumin enhanced P-gp dependent efflux. In contrast, no significant change in fluorescence after incubated with other polyphenols.

	untreated	verapamil 50uM	100	200	400	600	800	1000	2000	3000
4 hrs	100.00	50.87	109.12	115.02	120.71	123.67	135.05	133.01	137.01	130.43

TLE (µg/ml)   (A)

	untreated	verapamil 50uM	100	200	400	600	800	1000
4 hrs	100.76	44.75	112.06	119.23	103.35	93.25	93.64	90.19

MOE (µg/ml)   (B)
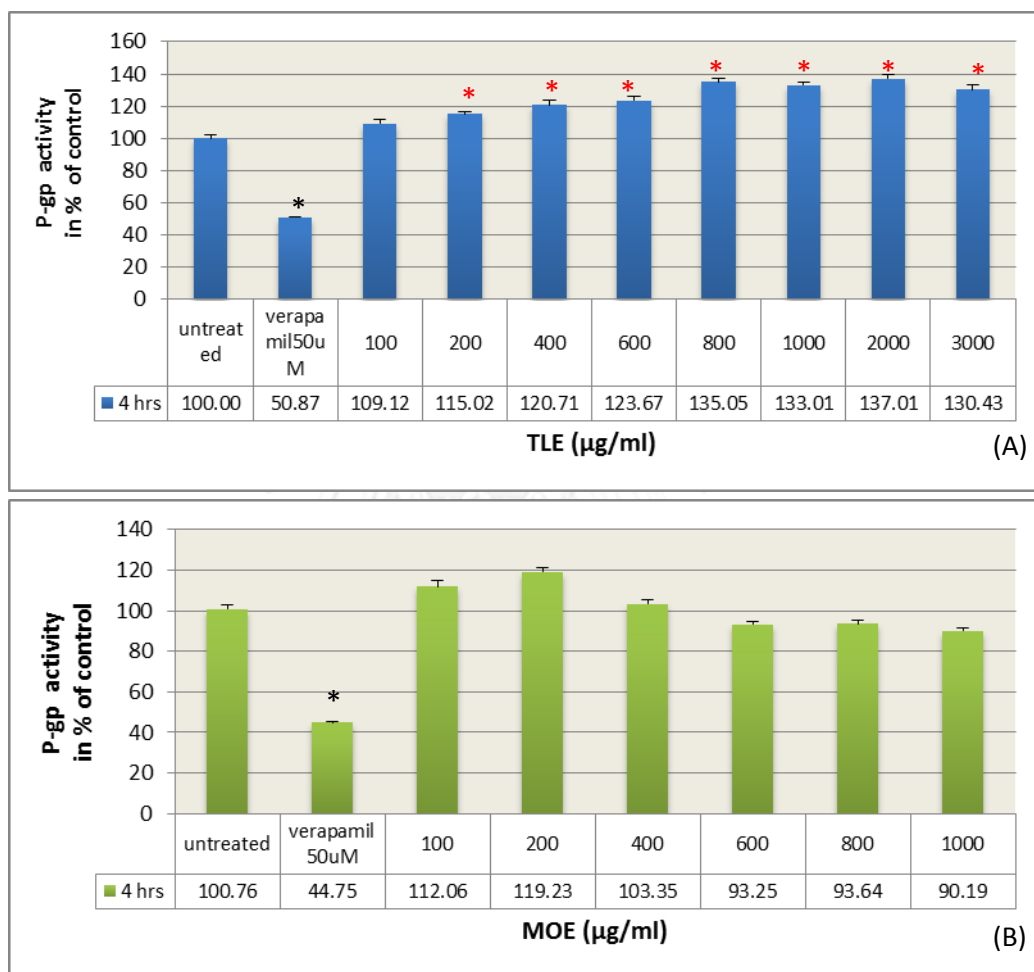
Figure 3.26: **Effect of herbal treatment on P-glycoprotein dependent rhodamine 123 efflux activity of HepG2 cells.** P-glyprotein activity levels were measured at 4 hr of incubation using the Rh-123 dye transport assay. Before treatment, cells were incubated with 20 µM Rh-123 for 1 hr, followed by treatment with herbal extracts for 4 hr and fluometric analysis was done. P-gp activity was inverted to fluorescence intensity of Rh-123. Verapamil was used to confirm Rh-123 response. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with *T. laurifolia* **(A)** and *M. oleifera* **(B)**

(A)

P-gp activity in % of control

4 hrs	Untreated	0.1%DMSO	verapamil 50 uM	verapamil 100 uM	verapamil 150 uM	5	10	25	50	75	100
	100.67	102.77	48.44	46.68	55.47	109.92	113.01	113.15	112.91	107.07	105.04

Apigenin (µM)

(B)

P-gp activity in % of control

4 hrs	Untreated	0.1%DMSO	verapamil 50 uM	verapamil 100 uM	verapamil 150 uM	5	10	25	50	75	100
	100.00	92.27	49.01	51.68	51.25	106.15	105.35	107.56	105.26	106.10	105.78

Catechin (µM)

(C)

P-gp activity in % of control

4 hrs	Untreated	0.1% Ethanol	verapamil 50 uM	verapamil 100 uM	verapamil 150 uM	5	10	25	50	75	100
	101.17	106.62	50.11	52.92	55.17	111.38	112.87	107.83	109.07	106.37	107.43

Chlorogenic acid (µM)
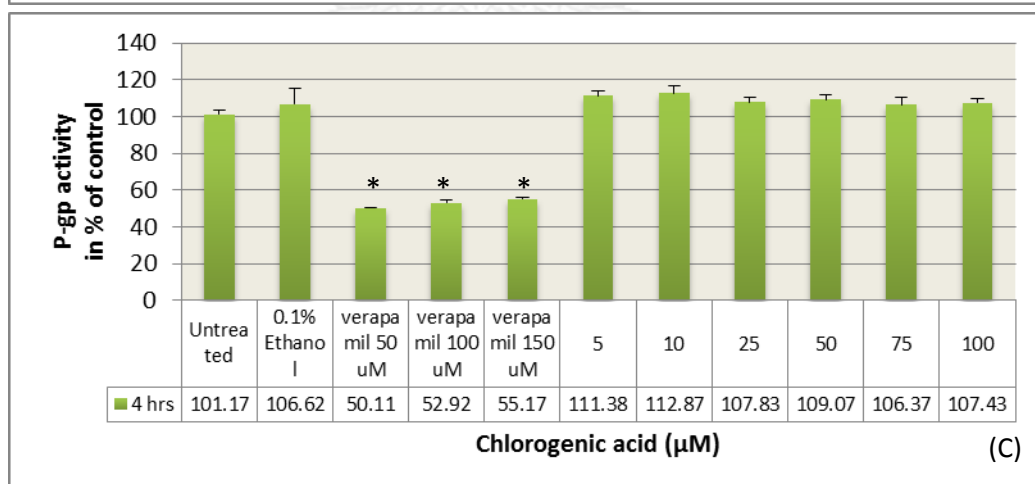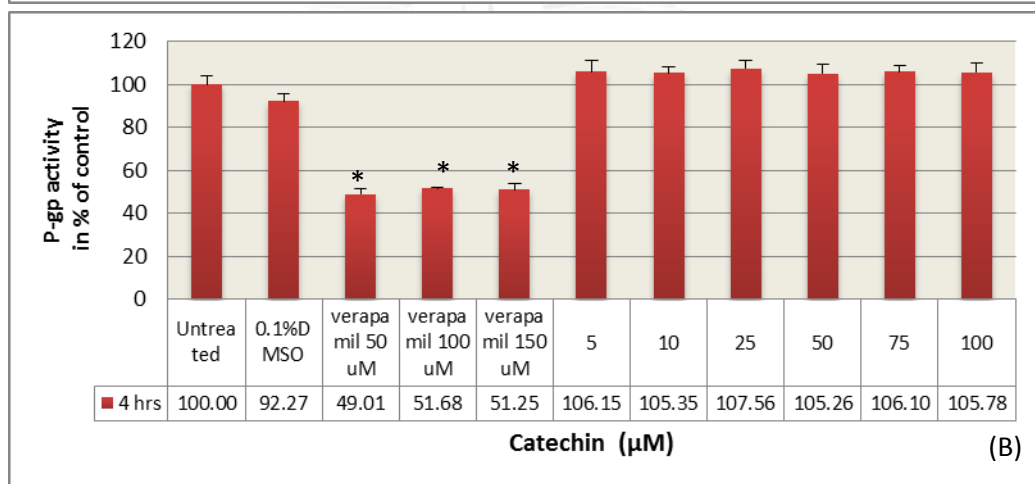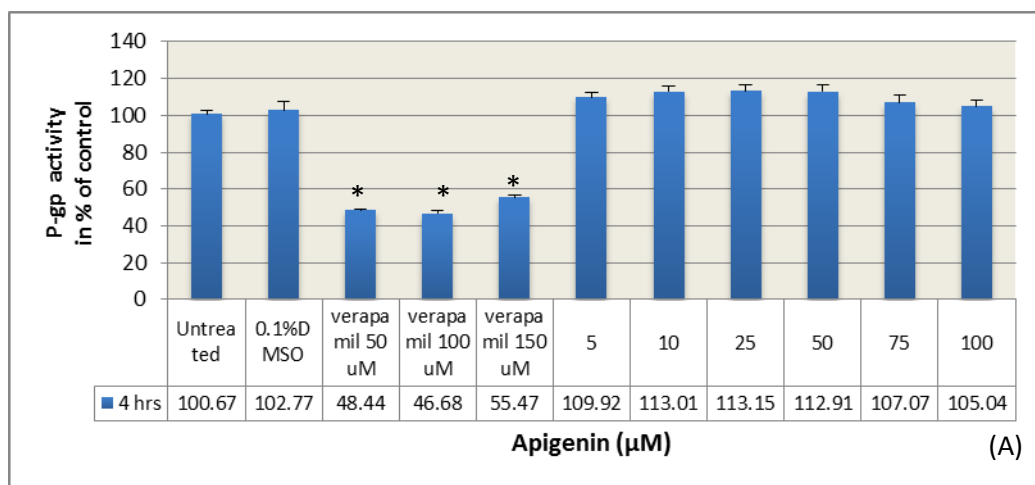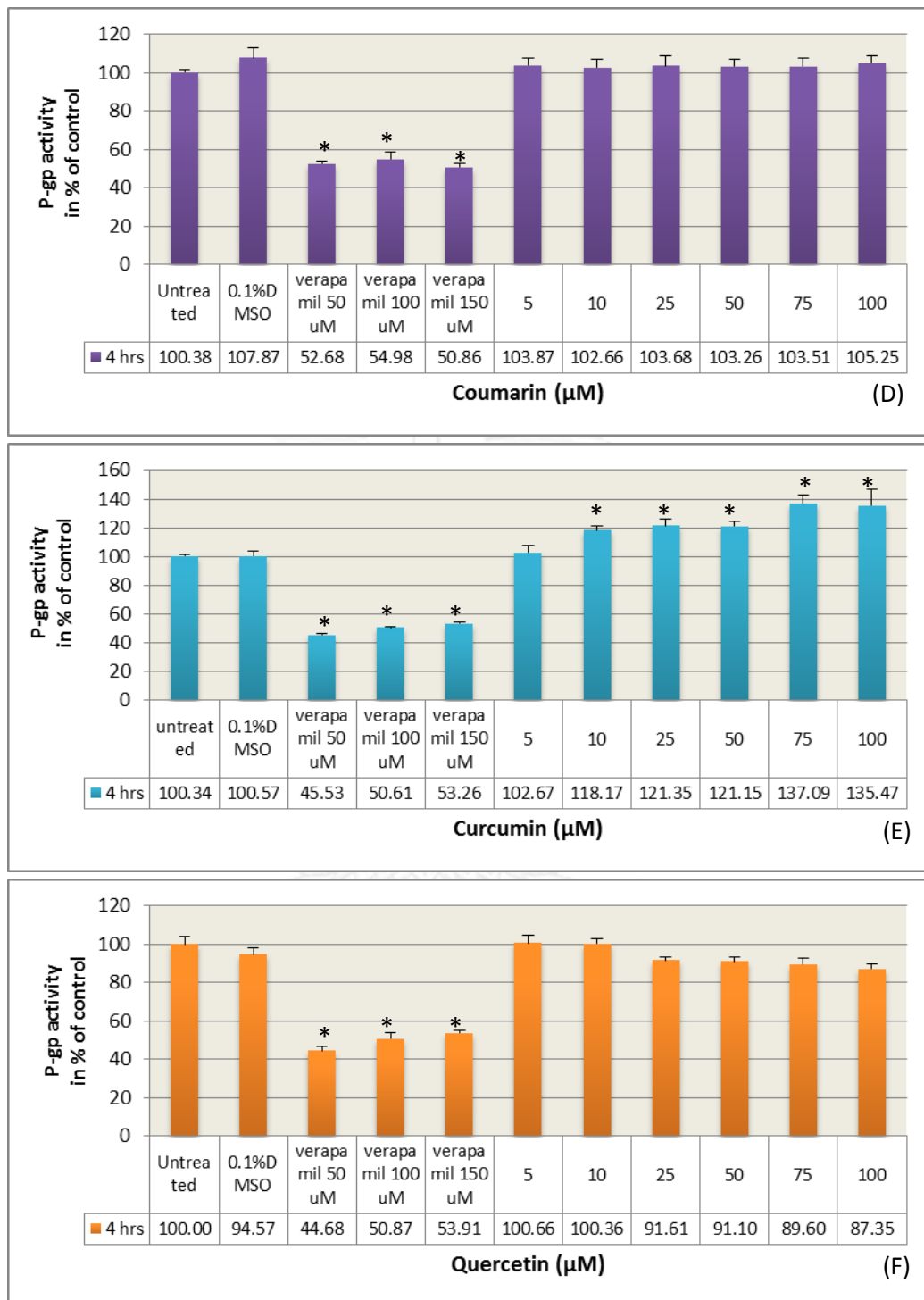
Figure 3.27: **Effect of polyphenols treatment on P-glycoprotein dependent rhodamine 123 efflux activity of HepG2 cells.** P-glyprotein levels were measured at 4 hr of incubation using the Rh-123 dye transport assay. Before treatment, cells were incubated with 20 μM Rho-123 for 1 hr, followed by treatment with polyphenols for 4 hr and fluometric analysis was done. P-gp activity was inverted to fluorescence intensity of Rho-123. Verapamil was used to confirm Rh-123 response. The results were expressed as a percentage of untreated control levels and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. Treated HepG2 with apigenin **(A),** catechin **(B)**, chlorogenic acid **(C)**, Coumarin **(D)**, curcumin **(E)** and quercetin **(F).**

3.7.9. *The ability of herbal extracts to modulate biotansformation phase I, CYP450s activity*

Since herb-drug interaction effects are based on the modulation effect of herbal extracts on the activity of CYP450s. Therefore, this study was futher investigated whether herbal extracts could affect CYP450s, the activity of CYP1A, CYP2A, 2B and 3A were measured. The known competitive inhibitors, ketoconazole (CYP3A), and quercetin (CYP1A) were used as the negative control and known inducer, rifampicin (CYP2A, 2B) was used as the positive control. The concentration of these compounds in this screening was set to 5-100 μM in consultation with several references. Although *T. laurifolia* exhibited weak induction on CYP1A (<50% induction) at 4 hr of incubation, *T. laurifolia* showed strong induction activity (>100% induction) on CYP1A enzymes at 29 hr in dose-dependent fashion. *M. oleifera* revealed less induction on CYP1A activity than *T. laurifolia* but *M. oleifera* showed inhibitory activity when raising level 2,000 to 3,000 μg/ml on CYP1A activity. Dose-dependent induction on CYP2A activity was observed for the *T. laurifolia* treatment for 4 hr while *M. oleifera* showed the inhibitory effect on CYP2A in dose and time dependent manner. When prolong incubation time to 29 hr, a substantial decreased was apparent in coumarin hydroxylation activity catalyzed by CYP2A in a concentration-dependent manner in both *T. laurifolia* and *M. oleifera*. Potency of *T. laurifolia* on CYP2A was less than *M. oleifera*. Nevertheless essentially no significantly induction of CYP2A activity by rifamcipin was seen within the range 5-10 μM. Herbal extracts did not significantly alter the activity of CYP2B at 4 hr of incubation. However, prolonged incubation time to 29 hr, it was demonstrated strongly induction in a dose-dependent manner. *T. laurifolia* showed remarkable induction effect over *M. oleifera*. Similar results were observed when assessed the effect on CYP3A activity at 4 hr, the herbal extracts showed no significantly change the CYP3A activity comparing to the known CYP3A inhibitors, ketoconazole. In addition, CPY3A4 activity was inhibited only 10% of 10 μM ketoconazole. However, the inhibitory potency of ketoconazole was increasing when prolonged incubation time to 29 hr. At this time, herbal extracts showed opposite effect. *T. laurifolia* revealed great enhancing enzyme activity (up to 100% induction) on CYP3A4-catalyzed BFC fluorescence substrate. It was summarized that both *T. laurifolia* and *M. oleifera* could interfere CYP450s enzymatic activities based on evidences showing the strong induction of *T. laurifolia* (>100% inducing) on CYP1A or CYP2B and 3A enzymes and an inhibitory activity against CYP2A at 29 hr of incubation as shown in Figure 3.28.

(A)

	Untreated	0.1% DMSO	TLE100	TLE200	TLE400	TLE600	TLE800	TLE1000	TLE2000	TLE3000	MOE100	MOE200	MOE400	MOE600	MOE800	MOE1000	MOE2000	MOE3000	Quercetin 50	Quercertin 75uM	Quercertin 100uM
4 hrs	100	103.9	105.1	120	132.5	142.5	128	121.8	106.2	99.32	112.9	138.5	159.3	176.2	150.6	142.7	85.52	76.45	87.15	71.77	69.16
29 hrs	100	101.7	118.4	126	154.4	178	208	231.2	298.3	459.7	94.71	118.7	144.1	244.4	187.9	117.3	0	0	78.09	77.71	74.93

CYP1A enzyme activity in % of control — Herbal extracts (µg/ml)

Cell death at 29 hr

(B)

	Untreated	0.1% DMSO	TLE100	TLE200	TLE400	TLE600	TLE800	TLE1000	TLE2000	TLE3000	MOE100	MOE200	MOE400	MOE600	MOE800	MOE1000	MOE2000	MOE3000	Rafimpicin5uM	Rafimpicin10uM
4 hrs	100	100	105.4	104.7	95.89	102.9	117.9	119.3	122.7	135.4	92.06	102.4	94.48	89.35	72.4	82.62	77.12	79.62	106.3	106.2
29 hrs	100	98.3	101.7	93.16	93.5	97.73	98.16	85.39	81.98	62.26	88.03	89.75	80.32	79.22	77.69	0	0	0	100.9	98.52

CYP2A enzyme activity in % of control — Herbal extracts (µg/ml)

Cell death at 29 hr

Chart (C): CYP2B enzyme activity in % of control

	untreated	0.1% DMSO	TLE100	TLE200	TLE400	TLE600	TLE800	TLE1000	TLE2000	TLE3000	MOE100	MOE200	MOE400	MOE600	MOE800	MOE1000	MOE2000	MOE3000	Rafimpicin5uM	Rafimpicin10uM
4 hrs	100	100	103	107.2	104.9	100.2	98.43	98.91	97.39	100.4	92.51	91.43	93.91	92.45	93.79	96.03	94.28	100	117	108.8
29 hrs	100	100	116.1	127.2	127.7	129.5	160.5	150.4	189.1	217.4	87.51	84.17	98.1	101.7	116	0	0	0	100.9	97.55

Herbal extracts (µg/ml)

Chart (D): CYP3A enzyme activity in % of control

	untreated	0.1% DMSO	TLE100	TLE200	TLE400	TLE600	TLE800	TLE1000	TLE2000	TLE3000	MOE100	MOE200	MOE400	MOE600	MOE800	MOE1000	MOE2000	MOE3000	ketoconazole10uM	ketoconazole20uM
4 hrs	100	100	92.44	93.16	102.6	95.96	93.04	95.48	84.98	91.69	81.72	103.7	98.8	98.57	113.6	93.65	98.51	109.5	96.15	90.91
29 hrs	100	96.72	94.97	90.17	101.8	94.4	111.3	159.1	167.1	242	76.55	86.6	105.3	124.2	109.5	0	0	0	70.03	68.92

Herbal extracts (µg/ml)

Figure 3.28: **The Effect of herbal extracts on CYP1A, CYP2A, CYP2B and CYP3A enzymatic activity in living HepG2 cells.** CYP450s activities were measured at 4 and 29 hr of incubation using the fluorescent metabolism assay. After treatment, cells were incubated with specific substates which were described under chapter 2, and fluometric analysis was done. Inhibitor; Ketoconazole, Quercetin or inducer; rafimpicin were used to confirm CYP450s response. The results were expressed as a percentage of untreated control and represent as mean ± SEM of at least three independent experiments. *Significantly different from control was p-value ≤ 0.05. CYP1A activity **(A)**, CYP2A activity **(B)**, CYP2B activity **(C)**, and CYP3A activity **(D)** after treated with herbal extracts.

3.8 Gene expression in HepG2 cells in response to herbal extracts exposure

To determine whether the herbal extract altered the cell physiology and function was correlated to mRNA levels in the hepatocyte cultures, the effects of herbal extracts on the relative expression of the biotransformation, lipid metabolism, inflammasome and their master transcriptional regulator genes were also examined in HepG2 cells. Non-toxic concentrations of 200, 400 and 600 µg/ml herbal extracts were incubated with cells and mRNA concentrations were analyzed after 29 hr. The expression profiles

of 30 genes were assessed following 29 hr of herbal exposure using RT-PCR. Exposure of HepG2 cells to herbal extracts resulted in altered gene expression over time as presented in Table 3.15 and Table 3.16. The relative fold change of each gene was listed. Although reports suggested that mRNA levels of most CYP enzymes were much lower in HepG2 cells, Transcripts of CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1, 3A4 and 3A7 but not 2C9 and 2C19 were present in our HepG2 cells. *T. laurifolia* and *M. oleifera* had *different gene expression* profiles, *T. laurifolia* showed modulate genes involved with biotransformation and cellular stress and toxicity while *M. oleifera* could modulate some of phase I-II biotransformation-related genes and lipid metabolism genes. The CYP450s-related genes were significantly up-regulated after *T. laurifolia* treatment for 29 hr, including CYP1A1, CYP1A2, and 2B6 genes. *T. laurifolia* also had a negative effect on CYP450s and their transcriptional regulator-related genes including CYP2D6, 2E1, 3A4, 3A7 and Nrf-1 expression at the mRNA levels. Moreover, the effect of *T. laurifolia* on induction of CYP1A1 and 1A2 mRNAs was similar to that already seen in enzymatic activity study. The expression of certain inflammatory regulated genes were significantly down-regulated following 29 hr *T. laurifolia* exposure, including HO-1, COX-1, and Bcl-2 while other inflammatory cytokines (e.g. COX-2, 5-LOX) were not found to be expressed in HepG2 cells under these condition. The expression profiles of other genes involved in oxidative or metabolic for lipid did not change significantly under the condition. Thus, HepG2 cells were modestly responsive to *M. oleifera* to some small extent, activated the expression of at least 6 biotransformation related genes. In contrast, almost lipid metabolism regulator genes were induced mRNA alterations. Only 600 µg/ml *M. oleifera* produced a significantly decreasing in lipid metabolism regulator genes expression including HMGCoAR, PPARα, PPARγ. The suppression was found in dose dependent manner. Other CYP450s genes with significantly altered expression include the down-regulation of 3A4, 3A7, GPX1A and SLUT1A, in addition the up-regulation of 2E1 and master transcriptional regulator of CYP family Nrf-2. No significantly change in inflamasome or oxidative sensor related mRNAs were detected in *M. oleifera*-treated cells.

Table 3.15: Effect of *T. laurifolia* on the mRNA expression of interesting genes at 29 hr of HepG2 cells treatment using RT-PCR analysis
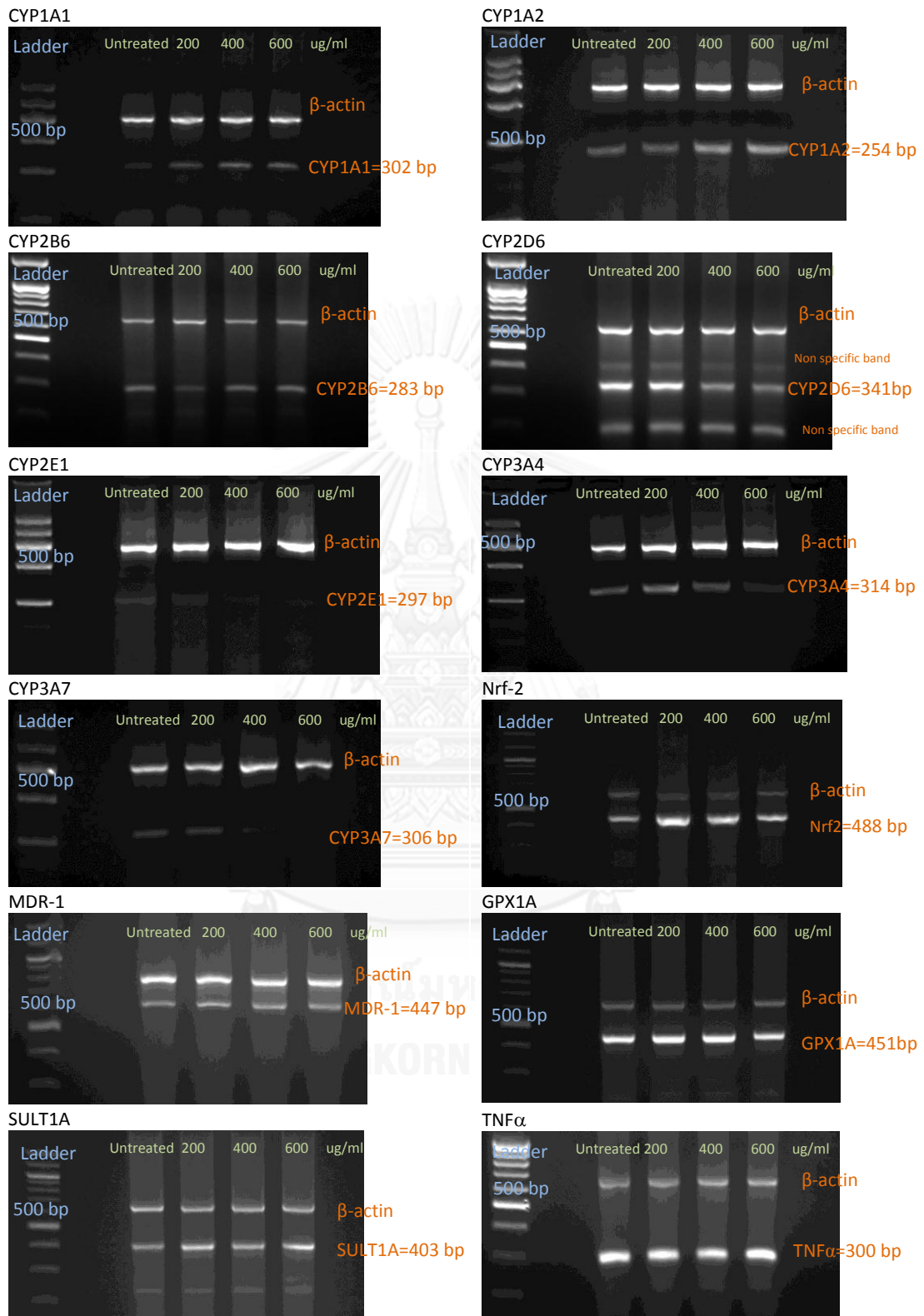
TLE Treatment (μg/ml)	Relative changing gene expression (fold)				
	CYP1A1	CYP1A2	CYP2B6	CYP2D6	CYP2E1
0	0.99 ± 0.05	1.00 ± 0.19	1.00 ± 0.09	1.00 ± 0.13	1.00 ± 0.05
200	1.45 ± 0.05	1.02 ± 0.14	0.92 ± 0.14	0.92 ± 0.05	0.70 ± 0.06*
400	1.80 ± 0.55*	1.29 ± 0.38	1.32 ± 0.20*	0.89 ± 0.01	0.59 ± 0.04*
600	1.71 ± 0.23*	1.34 ± 0.05*	1.30 ± 0.04*	0.80 ± 0.04*	0.56 ± 0.05*
concentration	CYP3A4	CYP3A7	Nrf-1	Nrf-2	MDR-1
0	1.00 ± 0.08	0.99 ± 0.32	1.00 ± 0.12	1.00 ± 0.05	1.00 ± 0.00
200	2.11 ± 0.23*	0.93 ± 0.15	1.03 ± 0.08	1.24 ± 0.06	0.95 ± 0.25
400	0.96 ± 0.03	0.72 ± 0.15	0.93 ± 0.08	1.07 ± 0.02	0.93 ± 0.12
600	0.57 ± 0.02**	0.58 ± 0.04*	0.72 ± 0.13*	0.79 ± 0.08	0.90 ± 0.10
concentration	GPX1A	SULT1	HO-1	TNFα	NF-κB
0	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.19	1.000±0.071	1.00 ± 0.12
200	1.09 ± 0.03	1.08 ± 0.02	1.07 ± 0.04	1.054±0.156	0.85 ± 0.28
400	0.95 ± 0.04	1.00 ± 0.01	0.82 ± 0.09	1.002±0.356	0.94 ± 0.21
600	0.88 ± 0.04	0.97 ± 0.07	0.57 ± 0.14*	0.954±0.034	0.75 ± 0.12
concentration	LDLR	HMGCoAR	PPARα	PPARγ	LXRα
0	1.00 ± 0.25	1.00 ± 0.07	1.00 ± 0.05	1.00± 0.16	1.00 ± 0.05
200	1.08 ± 0.20	1.02 ± 0.04	1.00 ± 0.04	0.98 ± 0.15	1.03 ± 0.07
400	1.06 ± 0.19	1.13 ± 0.07	1.04 ± 0.04	1.24 ± 0.06	1.06 ± 0.01
600	1.01 ± 0.09	1.08 ± 0.04	0.99 ± 0.02	1.12 ± 0.11	1.06 ± 0.01
concentration	COX-1	BAX	Bcl2	ALDH2	CYP2C9/2C19
0	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.05	N/A
200	1.08 ± 0.06	0.99 ± 0.21	1.01 ± 0.06	1.08 ± 0.07	N/A
400	1.11 ± 0.03	0.99 ± 0.22	0.95 ± 0.07	1.10 ± 0.16	N/A
600	0.68 ± 0.03*	1.00 ± 0.02	0.86 ± 0.02*	1.04 ± 0.01	N/A

The results were expressed as a relative fold of untreated control and represented as mean ± SEM of at least 3 independent experiments. *Significantly different from control was P-value ≤ 0.05.

Table 3.16: Effect of *M. oleifera* on the mRNA expression of interesting genes at 29 hr of HepG2 cells treatment using RT-PCR analysis

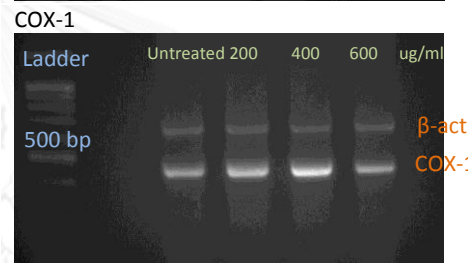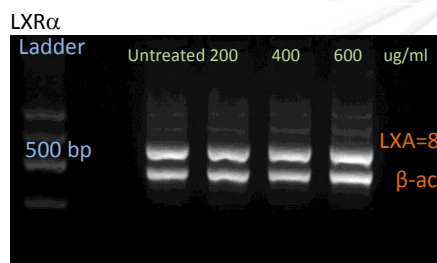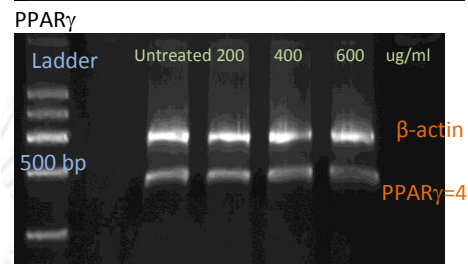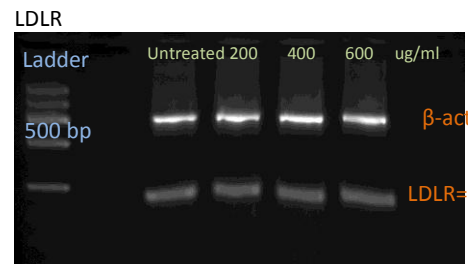| MOE Treatment (μg/ml)	Relative changing gene expression (fold)				
	CYP1A1	CYP1A2	CYP2B6	CYP2D6	CYP2E1
0	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.04
200	1.18 ± 0.08	1.21 ± 0.08	1.16 ± 0.03	0.95 ± 0.03	0.98 ± 0.04
400	1.28 ± 0.10	1.32 ± 0.10	1.27 ± 0.15	0.98 ± 0.03	1.32 ± 0.09*
600	0.99 ± 0.13	1.12 ± 0.11	0.92 ± 0.08	0.81 ± 0.02	1.14 ± 0.06
concentration	CYP3A4	CYP3A7	NRf-1	Nrf-2	MDR-1
0	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.10	1.00 ± 0.07	1.00 ± 0.03
200	0.92 ± 0.13	0.98 ± 0.03	0.99 ± 0.05	1.25 ± 0.07*	1.02 ± 0.01
400	0.66 ± 0.07*	0.79 ± 0.05	1.12 ± 0.04	1.27 ± 0.07*	1.02 ± 0.01
600	0.82 ± 0.06	0.73 ± 0.04*	0.95 ± 0.09	1.08 ± 0.08	0.95 ± 0.02
concentration	GPX1	SULT1A	HO-1	TNFα	NF-κB
0	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.05
200	1.04 ± 0.03	1.06 ± 0.03	0.95 ± 0.02	0.98 ± 0.04	0.99 ± 0.08
400	1.00 ± 0.02	1.03 ± 0.03	0.97 ± 0.07	0.93 ± 0.02	1.07 ± 0.03
600	0.87 ± 0.03*	0.83 ± 0.07*	0.81 ± 0.09	0.89 ± 0.07	1.08 ± 0.03
concentration	LDLR	HMGcoAR	PPARα	PPARγ	LXRα
0	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.04
200	0.91 ± 0.04	1.03 ± 0.01	0.89 ± 0.06	0.95 ± 0.09	1.06 ± 0.07
400	1.03 ± 0.03	0.97 ± 0.04	0.83 ± 0.06	0.93 ± 0.05	1.06 ± 0.06
600	1.06 ± 0.00	0.78 ± 0.07*	0.55 ± 0.06*	0.57 ± 0.01*	1.03 ± 0.08
concentration	COX-1	BAX	Bcl2	ALDH2	CYP2C9/2C19
0	1.00 ± 0.04	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.04	N/A
200	1.11 ± 0.07	0.99 ± 0.04	0.99 ± 0.03	0.92 ± 0.06	N/A
400	0.98 ± 0.03	1.16 ± 0.14	1.00 ± 0.02	0.91 ± 0.09	N/A
600	0.84 ± 0.08	0.85 ± 0.14	1.02 ± 0.07	0.72 ± 0.07*	N/A

The results were expressed as a relative fold of untreated control and represented as mean ± SEM of at least 3 independent experiments. *Significantly different from control was P-value ≤ 0.05.

CYP1A1

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP1A1=302 bp

CYP1A2

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP1A2=254 bp

CYP2B6

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP2B6=283 bp

CYP2D6

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
Non specific band
CYP2D6=341bp
Non specific band

CYP2E1

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP2E1=297 bp

CYP3A4

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP3A4=314 bp

CYP3A7

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
CYP3A7=306 bp

Nrf-2

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
Nrf2=488 bp

MDR-1

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
MDR-1=447 bp

GPX1A

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
GPX1A=451bp

SULT1A

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
SULT1A=403 bp

TNFα

Ladder Untreated 200 400 600 ug/ml
β-actin
500 bp
TNFα=300 bp

PPARA

PPARα=728 bp

Figure 3.29: **The effect of *T. laurifolia* on gene expression of HepG2 cells**. The mRNA levels were measured at 29 hr of incubation using RT-PCR. After treatment with 200, 400 or 600 μg/ml of *T. laurifolia*, total mRNA was isolated following by RT-PCR analysis. **β**-actin was used as an internal standard. Gene expression level was calculated from relative expression of interesting gene versus that of **β**-actin housekeeping gene. From left to right, untreated control, 200 μg/ml, 400 μg/ml and 600 μg/ml *T. laurifolia*. The result of pool PCR products from 3 experiments was shown.

CYP1A1

CYP1A2

CYP2B6

CYP2D6

CYP2E1

CYP3A4

CYP3A7

NRF2
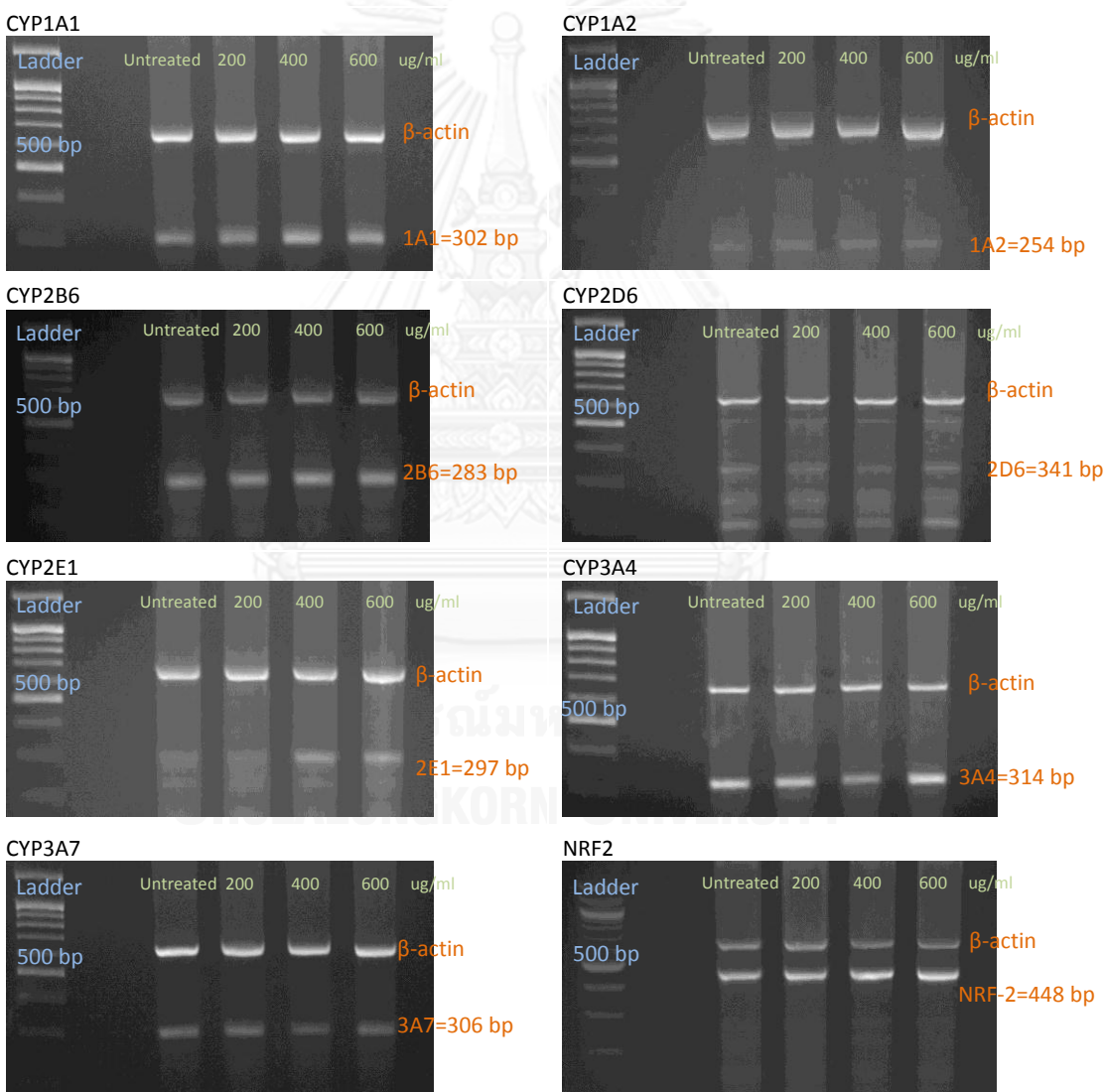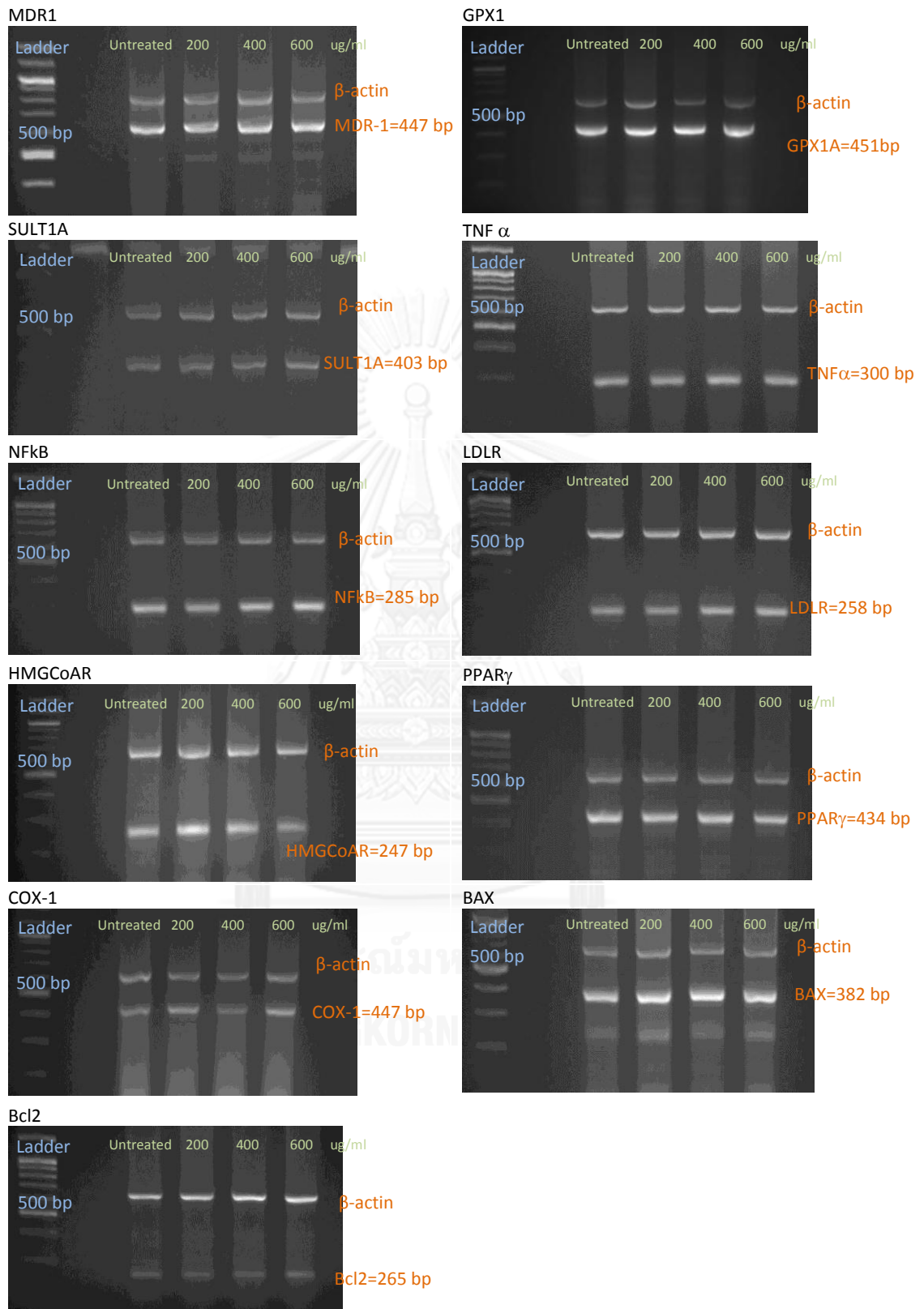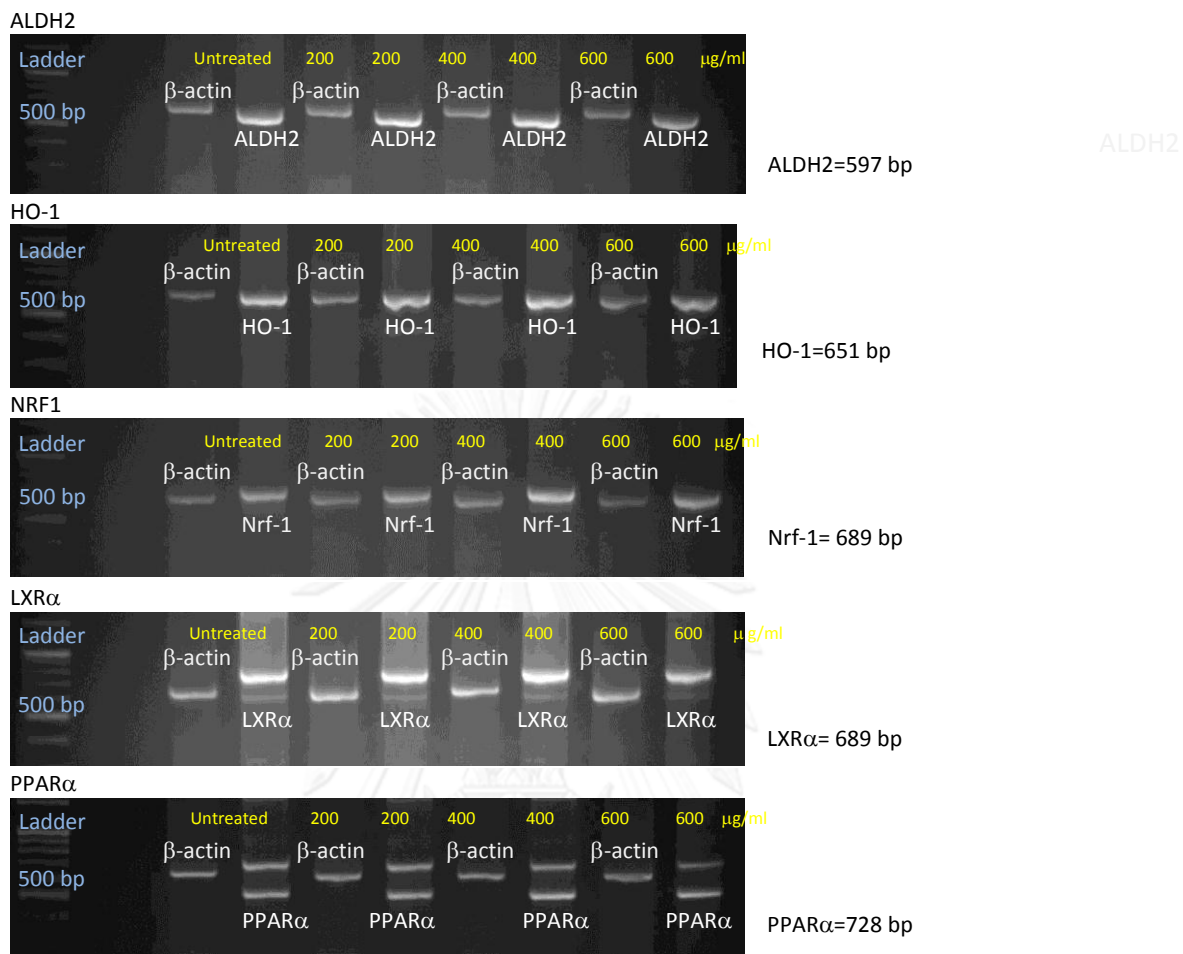
Figure 3.30: **The effect of *M. oleifera* on gene expression of HepG2 cells.** The mRNA levels were measured at 29 hr of incubation using RT-PCR. After treatment with 200, 400 or 600 μg/ml of *M. oleifera*, total mRNA was isolated following by RT-PCR analysis. β-actin was used as internal standard. Gene expression level was calculated from relative expression of interesting gene versus that of β-actin housekeeping gene. From left to right, untreated control, 200 μg/ml, 400 μg/ml and 600 μg/ml *M. oleifera*. The result of pool PCR products from 3 experiments was shown.

Chapter IV

Discussion

***Thunbergia laurifolia* Linn. and *Moringa oleifera* Lam. have antioxidant properties and could scavenging various kinds of free radicals.**

To determine active compounds and evaluate antioxidant properties of T. laurifolia Linn (TLE) and M. oleifera Lam (MOE) extracts. First, total polyphenols in the extracts were determined by Folin-Ciocalteu reagent which used to measure the total phenolics in plant materials for many years and accepted as a routine assay for rough estimation of the active compounds of herbal samples. However, it was obvious that the total phenolic content measured by the Folin–Ciocalteu procedure did not give a full picture of the quality or quantity of the phenolic constituents in the extracts. Hence, we had done other examination to confirm our results. Here, total phenolic content, total flavonoids content and total nitrate content of the herbal extracts were determined. The result showed that there were polyphenols, particularly be flavonoids and condensed tannins in the leaf of *T. laurifolia* and *M. oleifera*. Although *T. laurifolia* gave lower %yield of total crude extract but higher polyphenols than *M. oleifera*. Tannin/flavonol ratio examination suggested that polyphenol content in *T. laurifolia* might be monomeric rather than polymeric form. *M. oleifera*, in the other hand, *M. oleifera* contained high condense tannin suggested that polyphenols in *M. oleifera* was a complex structure form. Moreover, other chemicals such as nitrate were identified in the leaf of *T. laurifolia* and *M. oleifera*. Nitrate was mostly found in green-part of the plant especially leaf. Nitrate are considered to be the active constituents in many plants and found to have some beneficial effects such as enhance mitochondrial bioenergetics and biogenesis[292]. However, there were some reports on the determination and identification of polyphenols from the leaf of *T. laurifolia* and *M. oleifera* which was similar to our finding[37,47]. But the antioxidant activity of plant extracts was not limited to polyphenol[341]. The antioxidant activity may also contribute from the other antioxidant secondary metabolites such as volatile oils, carotenoids, indoles, alkaloids and some vitamins.

Evidences for the antioxidant ability of polyphenol isolated from natural products are documented in several publications based on both *in vitro* and *in vivo* studies[342,343]. Many studies have shown that phenolic compounds, particularly flavonoids, are potent free radicals scavengers[344]. In order to characterize antioxidant activity of a plant extract, we used different *in vitro* chemical-based assays to determine the antioxidant capacity of natural products including the popular ORAC, TEAC, and FRAP assay. These assays are based on different strategies, different advantage/disadvantage and provide different information about the ROS sample interaction. Hydrogen atom transferred (HAT) was the mechanism of the measurement about the classical ability of an antioxidant to quench free radicals by hydrogen donation while single electron transferred (SET) based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound. SET and HAT mechanisms almost always occur together in all samples, it is desirable to subject it for the tests that evaluate the

range of activities. The ORAC assay is based on HAT mechanism and provides excellent results about both hydrophilic and hydrophobic antioxidants against peroxyl radicals that model reactions of antioxidants with lipids in both food and physiological systems. But the ORAC reaction is temperature sensitive, close temperature control throughout the plate is essential. TEAC is based on SET mechanism and very useful to determine both hydrophilic and lipophilic antioxidant capacities of extracts because TEAC assay is simple, speedy, and inexpensive. But the ABTS radical used in TEAC assays is not found in mammalian biology and thus represents a "nonphysiological" radical source. FRAP assay, reducing potential determination is simple and robust. This assay measures the reducing capability based upon the ferric ion which could reflects the ability of compounds to modulate redox tone in plasma and tissues. But it does not relevant to antioxidant activity mechanistically and physiologically. Moreover, it does not measure activity of thiol antioxidants, such as glutathione. Results showed that *T. laurifolia* possessed antioxidant capacity greater than *M. oleifera* in all measurement methods. Both *T. laurifolia* and *M. oleifera* were shown strong antioxidant activity when compare with those of reported from other herbal extracts[345,346]. We also found the relationship between the antioxidant power and the total polyphenol, flavonoids, and condense tannins. There were high correlations among ORAC, TEAC, FRAP assay, the total polyphenol and the total flavonoids content.

The antioxidant property of *T. laurifolia* and *M. oleifera* was further confirmed by determination of free radical scavenging capacity and H_2O_2 elimination. It has been well established that antioxidant agents could scavenge free radicals, attenuated free radical formation, and consequently suppressed ed chain reaction in biological systems[19]. Among of free radical, most commonly reactive compounds found in biology system are superoxide radicals, hydroxyl radical, nitric oxide radical and hydrogen peroxide[347]. Superoxide radical is a precursor to other reactive oxygen species; plays an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances that thereby inducing tissue damage. In a biological system, superoxide is leaking from respiratory process and eliminated by superoxide dismutase. Results showed that $O_2^{\bullet-}$ scavenging activity of *T. laurifolia* was more pronounced than *M. oleifera*. Among the oxygen radicals, hydroxyl radical is the most reactive chemical species known. It can initiate chain reactions and induces oxidative damage to almost biomolecules such as all proteins, DNA, nucleic acid. Therefore, deoxyribose assay system was used to observe the ability of herbal extracts to scavenging OH•. Result shown that *M. oleifera* strongly inhibited OH• induced deoxyribose degradation than *T. laurifolia*. Hydrogen peroxide is an intermediate during endogenous oxidative metabolism. Hydrogen peroxide is not a free radical but it highly reactive oxidizing agent and has potential to produce the highly reactive hydroxyl radical through the Fenton reaction, particularly in pathological condition such as iron overload[348]. The decomposition of hydrogen peroxide into water may occur according to the antioxidant compounds act as a good electron donor which accelerates the conversion of H_2O_2 to H_2O. The result shows that H_2O_2 was abolished by *T. laurifolia*. Concerning of reactive species, nitric oxide radicals are the most implicated in inflammation related to pathological conditions[349]. It was shown that scavenging power of *T. laurifolia* was better than *M. oleifera*. When comparing the radical scavenging activity, it is found positive correlation between the high

polyphenol content and its radical scavenging activity. Polyphenol contents of herbal extracts have more correlation with its ability to scavenging $O_2^{\bullet}$ and H_2O_2 while it's NO• and OH• scavenging activities is further correlated with more specific, the flavonoids. In addition, radical scavenging activity of the extracts also shown high correlated with the presence of tannin and nitrate contents of the extract. Based on the results were obtained from both herb extracts exhibited strong antioxidant in different *in vitro* systems. They showed high content of polyphenols and prevented free radical formation more effectively than the standard, available synthetic antioxidants.

Thunbergia laurifolia Linn. and *Moringa oleifera* Lam. could protect biological molecules from oxidative damage and maintenance cellular redox stability

Considering the complexity involved in their *in vivo* mechanisms of action, a good antioxidant is not just a good radical scavenger and reducing compound but a molecule that can exert its antioxidant activity by protecting biology molecules from free radical damage. Therefore, only *in vitro* chemical method is not enough to evaluate and compare their antioxidant properties because these chemical assays do not consider relevant parameters involved in biological environments such as lipophilicity and bioavailability. Obtained *in vitro* antioxidant capacity indexes are not necessary reflect the antioxidant effects that would be associated with a particular sample *in vivo*. It is therefore not surprising that no correlation between the antioxidant potency of various polyphenols measured *in vitro* and their biological activity determined *in vivo* or at the cellular level has been observed in a large number of studies[343,350,351].

Oxidative stress plays an active role in alteration of normal physiological processes and development/progression of several diseases[19].These protective effects have been attributed to molecules found in fruits and vegetables that possess antioxidants properties. Most commonly targets of free radical attack in biological systems are the proteins, lipids, and nucleic acids[11,19].In this study, protective effect of herbal extracts was evaluated against oxidative stress that monitored molecules modification using free radicals as oxidative-stress markers including: Heinz body for protein protection, Hemolysis for lipid bilayer protection and comet assay for nucleic acids protection.

Cell membrane is a biological structure that is made of a phospholipid bilayer with embedded proteins. Polyunsaturated fatty acids molecules of cell membranes are particularly susceptible to oxidative alteration[11]. Free radical damage via lipid peroxidation can directly to breakdown of these membranes. In this study, erythrocytes cell membrane is used as the model. Human erythrocytes are constantly exposed to ROS and thus are highly susceptible to oxidative damage[352]. The results shown that free radical induced membrane lipid peroxidation that was strongly suppressed cooperatively by both *T. laurifolia* and *M. oleifera* suggested that herbal extracts were able to prevent hemolysis via attenuated the lipid peroxidation. Decreasing the lipid oxidation by natural antioxidants would contribute to the prevention of many diseases such as atherosclerosis[353]. However, high doses of herbal extracts

shown to increase hemolysis suggested that herbal exert pro-oxidant behavior when use inappropriate dosage.

The oxidation of proteins has been implicated in many pathological events[19]. Under oxidative conditions, free radical releases from chain reaction of polyunsaturated fatty acids peroxidation in cell membranes initiated intracellular Fenton reaction. Erythrocytes contain large amount of heme protein or hemoglobin which made it more susceptibility to ferrous ion catalyst. As a result of the denaturation of hemoglobins in erythrocytes, oxidized heme protein precipitated known as Heinz bodies. Assessment of Heinz bodies was a useful gauge in evaluating susceptibility of red blood cells to the oxidant stress[354]. Both *T. laurifolia* and *M. oleifera* were shown significant ability to suppress Heinz body formation. This data suggested that *T. laurifolia* and *M. oleifera* administration may protect proteins from oxidative damage.

In addition, other biological molecules are also susceptible to oxidative damage is nucleotide both RNA, DNA. In this study, the comet assay was used to demonstrate protection effect of herbal extracts against DNA damage in normal white blood cells (mononuclear cells) using hydrogen peroxide induced stress. The results suggested that the higher protective effects against DNA were achieved by *T. laurifolia*. *M. oleifera* was lower protective ability against oxidative damage in DNA. This might be explained that *M. oleifera was* low H_2O_2 scavenging property observed in chemical assay above. The most probable reason for the variation of DNA protection might be due to variation in the quantities of polyphenols. Polyphenols are potential protecting agents against the lethal effects of oxidative stress and offer protection of DNA by chelating redox-active transition metal ions. That was found the same in protein and lipid oxidation assay, although *T. laurifolia* showed the better result as a protective agent against oxidative stress in comet assay, high dosage pronouns excessive DNA damage by H_2O_2. It even induceed DNA damage in non-stressed lymphocytes, It was logical that *T. laurifolia*; (1.) acted as pro-oxidant, induced hydroxyl radicals generated by the Fenton reaction, (2.) had stimulated DNA repair processes and therefore additional DNA damage inflicted by hydrogen peroxide was more efficiently repaired. A further study is needed to identify underlying mechanism. This data suggest that *T. laurifolia* have antigenotoxic effects against oxidative stress.

It was suggested that polyphenols in the extracts were responsible to scavenge free radicals for protection of DNA against oxidative damage. Further, other herbal plants such as *Solanum nigrum* Linn[355], *Phyllanthus niruri*[356], and *Alpinia Oxyphylla* Miq.[357] have showed significant ability to protect against oxidative stress. Their prevention of oxidative DNA damage may help explain the cancer chemopreventive effects associated with a high consumption of fruit and vegetable diet. However, high doses of herbal extracts were shown to be cytotoxic- and genotoxic-agents. Other studies supportted this result since there was no benefit to be gained from the intake of high-dose antioxidant supplements[358,359]. Increasingly, it seems that the health hazards outnumber the beneficial effects of herbal medicine[360].

Eukaryotic cells display a plasma membrane redox system (PMRS) that transfers electrons from intracellular substrates to extracellular electron. An important role of PMRS is in the maintenance of extracellular concentrations of reduced ascorbic acid (ASC). This function of PMRS is especially important in erythrocytes because the presence of the PMRS in the erythrocyte provides the cell with an extra level of defense against extracellular oxidants and enables the cell to respond to changes in both intra- and extracellular redox environments. The electron acceptor used to investigate erythrocyte PMRS activity is the membrane impermeable oxidant ferricyanide. The reduction of ferricyanide to ferrocyanide occurs at the cell membrane and is measured spectrophotometrically. Recently, it has been shown that the activity of erythrocytes PMRS correlates with plasma antioxidant potential and that its activity increases as a function of human age[361]. Our observation of the activation of the erythrocyte PMRS by herbal extracts suggests that both *T. laurifolia* and *M. oleifera* can donate electrons to the PMRS for reduction of extracellular oxidants and for recycling of ascorbate. The electron donating ability of herbal extracts is less than that observed with quercetin. This evidence show that *T. laurifolia* and *M. oleifera* could enter human erythrocytes and played an important role in activating the erythrocyte PMRS, which results in transferring of reducing equivalents to the extracellular compartment. The ability of herbal extracts to activate the erythrocyte PMRS might consider as a protective mechanism operated against imbalance between increased oxidative stresses in all disease conditions and decreased in plasma antioxidant potential. It was concluded that herbal extracts exert their antioxidant ability at several levels that including radical scavenging, attenuated oxidative damage and modulation of cell antioxidant responses.

The beneficial of non-antioxidant-effect of *Thunbergia laurifolia* and *Moringa oleifera*

Medicinal plants have long been recognized to treat many diseases. These plants are potential benefits on hyperglycemia and might improve diabetes condition[362,363,364]. Several studies have suggested that intake of some plants such as sweet potato[365], green tea[366], berry[367] are associated with decreased level of plasma glucose by inhibiting the activities of digestive enzymes. In addition, medicinal plants appear to have a role in the regulation of hyperglycemia and metabolic dysfunctions. Most previous studies have focused on 2 digestive enzymes which play important role in hyperglycemia including α-glucosidase and α-amylase. Various *in vitro* assays are shown that many plants possess inhibitory effect on those carbohydrate hydrolyzing enzyme activities[368,369,370,371]. The present study demonstrated that *T. laurifolia* and *M. oleifera* possessed different capacity to inhibit α-glucosidase and α-amylase activities. In particular, *T. laurifolia* was more potent to inhibit α-glucosidase activity while *M. oleifera* was more potent to suppress α-amylase activity. The results suggest that herbal extracts contain various active compounds which exhibit different anti-hyperglycemic actions. These bioactive compounds could be flavonoids or phenolic acids previously showed a clear relationship with anti-diabetic activity[372].

Hyperglycemia can also lead to more serious conditions including accelerated the glycation of biomolecules. Increasing evidence indicates that long-term accumulation of glycated protein, advanced glycation end products (AGEs), is the initiating cause of the tissue damage occurring in diabetes[373]. Many herbal extracts and their polyphenol constituents show markedly suppressed the formations of AGEs[374]. It is likely that these make a contribution towards the well inhibition on the glycation of protein observed in this study. *T. laurifolia* and *M. oleifera* exhibited inhibition effect against the formations of AGEs *in vitro*. An inhibitory effect of *T. laurifolia* was as effective as that of aminoguanidine. Therefore, the effects of herbal extracts could be due to the different composition in phenolic compounds or other components. The present study indicated that *T. laurifolia* and *M. oleifera* could be useful in management of postprandial hyperglycemia.

Intoxication with the paraoxon (POX) organophosphorus compound still has frequently occurred in Thailand. Paraoxon is an inhibitor of critical enzyme acetylcholinesterase (EC 3.1.1.7)(AChE) with consequentially lead to excess acetylcholine accumulation and resulting in symptoms of cholinergic excess. Prevention the activity of AChE offers great potential for the treatment of insecticide poisoning. *T. laurifolia* has been used as a traditional medicine for treatment as an antipyretic as well as an antidote for several poisonous agents. So far, there is limited evidence for the protective role of these substances against organophosphate intoxication. Interestingly, *M. oleifera* was able to induce enzyme activity and effectively reverse AChE blocking effect of POX while *T. laurifolia* produced weak induction and failed to improve AChE activity against POX poisoning. This data suggest that herbal extracts were safe in terms of genotoxicity and general toxicity if consumers had taken the proper dose of these herbs.

The hepto-toxicity effect of *Thunbergia laurifolia* and *Moringa oleifera*

Antioxidants have been proclaimed as a "miracle drug" by many people to cure-all for many pathological conditions. This idea is based upon the notion that free radicals are highly reactive and could damage cells in an organism, causing aging. Unfortunately, the critical role of ROS is overlooked that ROS is also essential to our body in maintaining proper cell function and signaling network. The body knows how to maintain the redox balance and exogenous antioxidants such as vitamin C does not replace our endogenous system under a hormesis stress. In the other hand, excessive antioxidant disturbs the redox homeostasis[375,376], effectively repressing those cellular pathways responsible for recovery and adaptation mechanisms[377,378]. Many studies of antioxidant vitamin combinations have shown no benefit or even adverse effect of antioxidant combinations[379,380,381]. One secondary prevention trial showed an increase in all-cause mortality among women taking antioxidant supplements[382]. Some studies on type 2 diabetes mellitus[383] has been concerning about the negative (worsen) effect of fish oil administration on the glycemic control in diabetic patients. Several *in vitro* studies suggested that the antiproliferative effects of some polyphenol antioxidants on cancer cells are partially due to their prooxidant actions. It has been reported that many polyphenols including flavonoids such as quercetin, rutin, apigenin, delphinidin, resveratrol, curcumin, gallocatechin as well as EGCG and phenolics acids such as gallic acid, tannic acid, caffeic acid, are cytotoxicity[384,385,386,387] by

partially reduced endogenous antioxidant such as catalase and oxidative breakdown DNA strand[388]. The mechanism underlying this phenomenon is flavonoids which possess pyrogallol and/or catechol moieties in their structure found to be strong H_2O_2 generator through donating a hydrogen atom to an O_2^- and also possess inhibitory activities in liver microsomal lipid peroxidation. Indeed, flavonoids which generate H_2O_2 can scavenge free-radicals. The more H_2O_2 is generating, the more potent the radicals are trapping. Hydrogen peroxide has been pointed that might be the intermediated mediator behind many effects on cell function. It is reported to raise intracellular Ca^{2+}, activate transcription factors, repress expression of certain genes, promote or inhibit cell proliferation, be cytotoxic, activate or suppress certain signal transduction pathways, promote or suppress apoptosis[389].

Although herbal supplements have been used in humans due to the wide and easy availability, herbal toxicity has become an issue of concern. A safety assessment of the applied dose has been recommended due to the possibility of some adverse effect of this mode of consumption[390]. Herbs are quasi affected every organ or function in the body. Considering that liver is the principal site in the body for the degradation of these compounds and are responsible for the biotransformation of herbal medicine. It is possible to assume an adverse effect of this herbal medicine behavior is firstly takes placed on liver. Examples of herbal medicines suspected to cause liver injury are chaparral (*Larrea tridentata*), Dai-saiko-to (Sho-saiko-to, TJ-9), greater celandine, Mahuang (from Ephedra species), pennyroyal oil, saw palmetto (*Serenoa repens*), germander (*Teucrium chamaedris*), valerian, mistletoe[391,392,393]. Pyrrolizidine alkaloids, contained in plants like Heliotropium, Senecio, Symphytum (Compositae), are able to induce veno-occlusive disease[394]. However, so far not much is known about the hepatotoxic potential of herbal products and as it is a widely used phytomedicine it would be interesting to learn more about its toxicological profile. This study described the evaluation of safety of *T. laurifolia* and *M. oleifera* in relation to cause hepatotoxic potential. *M. oleifera* has been report about adverse effect on growth related and biochemical parameters. Treated animals had observable microscopical lesions in some organs[395] and had lower blood potassium levels[396] while there is only one report about *T. laurifolia* toxicity in animals[397].

A. Characterization of herbal extracts-mediated cytotoxicity

To evaluate cytotoxicity of herbal extracts on the proliferation of HepG2 cells, Time-course and concentration-dependent experiments were conducted to characterize the cytotoxic effect of *T. laurifolia* and *M. oleifera* in HepG2 cells which detected by MTT, NR and total protein determinations as well as investigated cell morphology by phase contrast microscopy. Time and dose dependent inducing cell death suggested that herbal extracts was solely attributed to its direct cytotoxicity. However, all of these effects were observed at concentrations much higher than what is normally ingested by humans. *M. oleifera* exhibited potent cytotoxic effect and proceeds much more rapidly than *T. laurifolia* did. In contrast, cell death induced by herbal extracts was not positively correlated with its polyphenol content. Moreover, parallel study on pure polyphenol compounds including flavonoids: apigenin, catechin, quercetin, coumarin, curcuminoid: curcumin and phenolic acid: chlorogenic acid were found

that mostly compounds were non-toxic or only slightly toxic on curcumin treatment. These data led us to hypothesize that herbal medicines often contain multi-active substances and multiple cellular molecules might be the targets of herbal medicine. The identification of these targets may provide molecular evidence for the herb's pharmacological activity and toxicity.

B. Role of herbal extracts on mitochondrial toxicity

Before declaring that specific dietary factors should be used in large or minimal amounts, it is necessary to have the best available scientific evidence about their mechanism. This will provide clues about their possible pharmaceutical exploration in the field of herbal medicine. Consequently, the present study was designed to evaluate the role of herbal extracts in modulating cellular activities during normal condition or induced oxidative stress condition. Since mitochondrial function as pivotal integrators of oxidize energy-linked substrates to powering energy needs of the cell. ROS is mainly produced within mitochondria, in particular by the respiratory chain[398]. It could provide that mitochondrial function as stress sensors and be capable of translating into survival/death signals that determined fate of the cell between life and death[399,400]. Damaged mitochondria were induced by oxidants usually directly led to predominant mitochondrial dysfunction in many diseases, may involve in varying degrees include mitochondrial depolarization, mitochondrial permeability transition, massive oxido-reductive stress, mtDNA mutations, low ATP production, dysfunction in calcium homeostasis, mitochondrial movement defect, and mitochondrial fusion and fission imbalance and/or the inhibition of glycolysis. In addition, mitochondrial pathologies are involved in the pathophysiological process of many neurodegenerative diseases[401]. All these disorders shared similar changed mitochondria, suggesting there were some connection of disease pathology and mitochondria. In order to gain a better understand of the molecular mechanism of the cytotoxic effect of herbal extracts, sign of mitochondrial dysfunction were determined including ATP, the mitochondrial membrane potential and calcium level in mitochondria.

Alteration in the mitochondrial membrane potential ($\Delta\Psi_m$) (MMP) was an important characteristic of mitochondrial dysfunction. A change in the mitochondrial membrane potential both hypo-polarization and hyper-polarization is a critical step in the induction of cell death. It has been demonstrated that the mitochondrial transmembrane potential is significantly correlated with oxidative stress[402] and the disruption of mitochondrial membrane potential is associated with many pathological conditions including hyperglycemia[403], hypoxia[404], and inflammation[405]. In the present study, mitochondrial membrane depolarization was studied by the observation the stability of the MMP using TMRM dye. *M. oleifera* treatment caused hyper-polarization occurred as rapidly as 4 hr while *T. laurifolia* induced mitochondria hyperpolarization after 29 hr of treatment and only at high dosages. Several studies have demonstrated a transient increase of MMP in cells by various kinds of stressor[406,407,408], suggested that herbal extracts were effectively induced cellular stress. Among of other pure polyphenols, loss of the mitochondrial membrane potential was only seen in apigenin treatment. The alteration in MMP was correlated with MTT results which based on measurement the mitochondrial metabolic enzyme activity

which suggested that *M. oleifera*, *T. laurifolia* and apigenin might interfere with cellular metabolic system which ultimately leads to cell death.

Further, this mechanism was confirmed by measurement ATP levels. Since modulation of $\Delta\Psi_m$ usually indicated about collapse in proton gradients which contributed to the alteration of cellular ATP formation that was ensuing cell death[409]. After prolonged culture with herbal extracts, we observed depletion in ATP production. The temporal pattern of cellular response to herbal extracts indicated that the depletion of cellular ATP level was associated with an elevation in MMP and declining in ATP production was also correlated with a corresponding increased cell death. These changes were followed by a reduction of MMP within 4 hr, a decline in ATP by 29 hr, and progression through cell death. The effect on ATP production could be perhaps involving in downregulation of the oxidative phosphorylation resulting in altering of MMP, further supporting the role of mitochondrial dysfunction because of herbal extract toxicity. It was possible that the compensatory mechanism of the cells used to protect itself from fuel overload by decreasing energy production and further contributed to the long-term deleterious effects of excess substrate availability. Herbal extracts were known to contain large amount of multi-nutrients which could increase fuel influx through the metabolic pathways overcomed the oxidative capacity of hepatic cells, large amounts of high-energy substrates might load the cells and impair their function. On the other side, pure polyphenolic compounds such as catechin, chlorogenic acid, coumarin and curcumin increased ATP formation in differenced pattern suggested that each polyphenols engage on interactions related to their chemical structures. These polyphenols acted on ATP formation were arisen without any effect on MMP, suggesting that maintenance of ATP is due to, some part, the preservation of the MMP. In addition to a primary role in ATP production, mitochondria is a key site for intracellular calcium storage and is in close proximity to the ER and function as Ca^{2+}-buffering system to help maintaining intracellular calcium homeostasis[400]. It was very important in continuing normal cellular functions in response to exogenous and endogenous stimuli. Dys-regulation of cellular calcium homeostasis ultimately ended up with enhancing cellular stress[410]. The alterations was observed in many pathological condition such as insulin resistance[411], allergic asthma[412], osteoporosis[413] or Alzheimer's disease[414].

Since we had observed an alternation in MMP and ATP production, further analyzed about role of $[Ca^{2+}]_m$ in mitochondria induced cellular stress was performed. Calcium levels in mitochondria were analyzed using the fluorescence Ca^{2+} indicators Rhod-2/AM using both confocal microscopy and spectrophotometer. Applying these herbal extracts to live HepG2 cells revealed the intricate relationship between increased calcium level and hyperpolarization of MMP, disturbance of ATP production and subsequent cell death. *T. laurifolia* increased calcium in HepG2 cells was significantly after prolonged incubation as at 29 hr, while *M. oleifera* was more effective to induced Ca^{2+} accumulation within the mitochondria. In addition, a dramatic induction of mitochondrial calcium was observed within first 4 hr of *M. oleifera* exposure. These results indicating that the herbal extracts affected the intracellular calcium homeostasis. One mechanism proposed to explain how *M. oleifera* might increase intracellular calcium levels possibly by inducing a calcium influx from the extracellular medium. Previously research showed

that *M. oleifera* also contain some inorganic minerals, particularly calcium and magnesium[415]. Prolonged and massive influx of Ca^{2+} into the mitochondria is reported to be one of the most prominent alterations in the disruption of mitochondrial calcium homeostasis[416]. Our data suggested that the exposure to either *T. laurifolia* or *M. oleifera* could impair mitochondria performance through at least two different mechanisms: energy production and signaling pathway.

The protective effect of *T. laurifolia* and *M. oleifera* on HepG2 cells.

Now, reactive oxygen species are thought to be involved in several cellular mechanisms. ROS are also known to perturb redox homeostasis[417] which considered to play a role in alteration of normal physiological processes and pathology of several diseases[418]. Indeed, it has been shown that natural polyphenols have an antioxidant capacity, free radical scavenging activity and can inhibit toxin-induced cellular oxidative damage. Several studies have demonstrated that the resveratrol found in wine[419], rutin or chlorogenic acid in apple juice[420], catechin in tea[421], and quercetin[422] have been shown to exert protective effects against cellular oxidative damage in different human cell lines. To study the influence of herbal extracts for modifying the endogenous oxidants, oxidative stress indicator was tested using DCFHDA staining. Herbal extracts showed significant intracellular ROS scavenging activity. Different pattern between intracellular ROS scavenging activity of *T. laurifolia* and *M. oleifera* were seen. *M. oleifera* was more potent suppressor oxidative indicator DCF than *T. laurifolia*, while quercetin was only pure polyphenol that had effect on intracellular oxidative status. These results were not correlated with antioxidant measurement by *in vitro* chemical method. This discrepancy could be also explained that it might be some other mechanism operated to inhibit/eliminate the activity of *T. laurifolia* in HepG2 cells. Other explanation was intracellular ROS measurement by DCFDA has some drawback because DCF detection was not specific for intracellular reactive oxygen species. Extracellular oxidant agents could also react with this indicator[423,424]; other experiment should be set-up to support this result. In addition, it should be noted that different polyphenols may also exert protective effects through other mechanisms, involving influences on signal transduction processes for example[425]. The reduction in ROS generation might not only induce immediate protection against oxidative damage but also provide delayed protection after the initial stimulating. Since studies have been confirmed that pro-inflammatory stimuli is associated with ROS generation and conditions of elevated oxidative stress can exist in cells after inflammation, natural antioxidants might exert protective effects by scavenging secondarily generated ROS resulting from inflammation-induced damage. For example, proanthocyanidins[426], catechin[427], quercetin[428] and pycnogenol[429] have been reported to inhibit pro-inflammatory transcription factors (e.g., NF-κB), TNFα production, and inflammatory enzymatic activity or blocking MAPK-mediated pathway. In many reports, polyphenols might exhibit anti-oxidative stress properties through enhancing antioxidant protection system such as inducting phase II detoxifying enzymes HO-1, GPX, and SULT and their nuclear receptors include Nrf-1/2, PPARγ and LXR[430,431,432]. Some of the nuclear receptors acted as sensors for dietary electrophilic molecules causing the transcription of specific genes codifying for proteins and protecting the cells from these electrophilic

molecules[433]. Several of the antioxidants have been shown to up-regulate these transcriptional activities with coordinated stimulation in the expression of antioxidants enzymes[434]. Interesting, we found several studies have been reported that Bcl-2 induction has been shown to be beneficial in prevention oxidative damage injury for example; prevented the generation of hydroxyl radical during ischemia/reperfusion injury[435]. Moreover, cells over-expressing Bcl-2 showed the elevation of cellular antioxidant levels especially SOD activity and GSH levels[436]. To study the secondary effects of a reduction in oxidative stress levels, several redox dependent genes that sensitive to an elevation of ROS such as Bcl-2, Bax, HO-1, and inflammation transcription genes such as COX-1, COX-2, 5-LOX, TNF-α and NF-κB were analyzed.

However, the gene response in HepG2 for *T. laurifolia* treatment for the 29 hr induction period didn't show any phase II genes modulation but only down-regulated HO-1. Likewise, *T. laurifolia* significantly suppress Bcl-2, Nrf-1, and COX-1 expression. Nrf-1 and COX-1 are constituently expressed throughout the body and function to maintain normal cellular performance. Study has been shown that inhibition of COX-1 could disturb the hemodynamic control of glomerular filtration[437]. Although this evidence showed *T. laurifolia* treatment lead to enhance stress, it should be noted that the possibility that other detoxifying genes might be expressed earlier or later and exert protective effects cannot be exclude. Different gene expression profile patterns were found with *M. oleifera* treatment. Phase II enzyme genes such as GPX and SULT were implicated. Whereas Nrf-2, the main nuclear receptor which responsible for these enzyme transcription, was up-regulated but at low dose than that cause phase II enzyme suppression. The result presumably that *M. oleifera* treatment may be actually perturbed the redox hoemostsis. Antioxidants nuclear sensor was provoked, in part, as defense system against *M. oleifera*. However, raising *M. oleifera* concentration caused potent toxic overwhelming the efficiency of cell defense system to cope with. Suppression of ROS effect on *T. laurifolia* and *M. oleifera* exhibited a reverse correlation with their cytotoxicity. One explanation was that HepG2 cells could metabolize herbal extracts to toxic reactive metabolites that interact with the target molecules to cause cell damage. Other mechanism that might explain was herbal extracts achieving their cytotoxicity by elimination of all ROS generated within cells which some ROS were crucial for maintenance normal cell physiology. Moreover, gene expression profile in slightly cytotoxicity herbal treatment might arise from effect from cytopathic secondary effect. Such changes were usually appeared to invoke quite a different gene expression compared to those provoked by lower concentration used. However, these factors are not enough to understand that result. Thus, further research on this discrepancy is required.

GSH is the most abundant non-protein thiol within cell. Evidence showed that cellular responses to drugs or toxins often involve changes in GSH content. It was suggested that GSH played a pivotal role in protection of cellular redox status against toxic agents[438]. Depletion of cellular GSH pool was associated with cellular susceptibility to oxidative stress[439]. Maintenance of GSH has a determinant role in the protection of the cell from damage[438]. Since the oxidative stress generally involves the GSH system, the effect on cellular redox status of herbal extracts treatment was further confirmed by two observations; the cellular GSH content was measured in the presence and absence of H_2O_2. GSH was

depleted by pre-treatment with H_2O_2[440]. This current study found that H_2O_2 induced ROS production, and subsequent reducing of GSH levels in HepG2 cells. Herbal treated cells gave biphasic effect. Exposure to non-toxic concentrations of *T. laurifolia* and *M. oleifera* extracts resulted in transient elevation of intracellular GSH content while the depletion of hepatic GSH after exposure with both toxic concentrations of *T. laurifolia* and *M. oleifera*. In addition, all pure polyphenols showed ability to induce an antioxidant response at the cellular level during prolonged exposure to 29 hr. This study confirmed that polyphenols offer significant protection that depending, in part, on the ability of the polyphenols to enhance endogenous antioxidant molecules. In order to further measured their ability to protect against H_2O_2 induced oxidative damage. Pre-treatment with *T. laurifolia* could protect the cells from H_2O_2-induced GSH depletion. In contrast, protection effect of *M. oleifera* against H_2O_2 was not found. Together with the results of our previous studies, these results indicated that *M. oleifera* could not scavenged H_2O_2 which paralleled that of *in vitro* chemical testing; H_2O_2 scavenging assay and comet assay. Our study clearly showed that when cell fate with oxidative damage, *T. laurifolia* exerted its protective effect by strengthening the intrinsic antioxidant defenses of HepG2 cells through modulation of intracellular GSH and thus protecting them by acting on the redox status. These results also demonstrated a strong correlation between the depletion in endogenous antioxidant GSH, mitochondrial dysfunction and cytotoxicity following herbal extracts treatment. Whereas, intracellular ROS level did not appear to correlate with other parameter suggested that DCFHDA might not be a good indicator to measure the intracellular ROS/RNS formation. Taken together, herbal extracts may minimize oxidative damage by reducing intracellular ROS formation, but also inducing mitochondria dysregulation and increasing cell death when inappropriate was used, thereby this effect had not been found in inflammation event. A novel pathological mechanism to explain the adverse effect of herbal extracts was herbal extracts might induce 'reductive stress' during excessive concentration treatment. Recently evidence showed that overexpression of anti-oxidative enzyme, heat shock protein 27, causes cardiomyopathy via reductive stress mechanism[441].

T. laurifolia and *M. oleifera* alter biotransformation system in HepG2 cells

Recently, a number of herb-drug interactions have been reported and received much attention. Herbal medicine is believed to be safety but many of studies documented about their ability to altered efficacy and/or adverse events[442]. Herbs are often administered in combination with therapeutic drugs, raising the possibility to modulate pharmacokinetic and/or pharmacodynamic of some drugs. Herb-drug interactions are generally due to enhanced or reduced drug bioavailability such as drug absorption, metabolism, distribution and excretion of drugs led to alter drug plasma concentration. The clinical outcome of pharmacokinetic herb-drug interactions is drug overdose or lack therapeutic effect for example which this change is clinically important for drugs with a steep concentration-response relationship or a narrow therapeutic index. The effect on drug pharmacodynamics is due to bio-activation of herbal constituents. Excessive concentration may act as pro-oxidant and generate reactive intermediates molecules. Firstly, these resultant reactive species could bind to DNA, lipid, and proteins,

leading to cellular damage. Secondly, herbs usually contain more than one active compound which appears to be critical for the herbal toxicity. Some substances in herb become active or even more toxic than the initial substance as a result of the inter-conversion reaction. Moreover, enzyme inhibition or induction is another potential mechanism leading to many of herb-drug interactions[442].

A. The effect T. laurifolia and M. oleifera on CYP450 enzymatic activities and genes expression.

As mentioned previously, herbs are often co-administered with drugs. The degradation (conversion) of drugs generally takes place in the liver which partly catalyzed by cytochrome P450 isoenzymes. These enzymes are metabolized numerous endogenous and exogenous compounds. In particular, herbal constituents have been reported to induce/inhibit of CYP450s. One of the underlying mechanisms of altered drug concentrations by concomitant herbal medicines is the induction or inhibition of hepatic CYP450s as herbal constituents and drugs are also broken down by the same enzyme system. The effects of combination of her-drugs can sometimes be important pharmacokinetic implications, even sometimes are the extent of becoming life-threatening[442]. As a result, it is important to recognize any modulation effects on CYPs from medicinal herbs. For this reason, this *in vitro* study was designed to study the ability of herbal extracts altered gene expression and enzymatic activities of CYP450s in order to evaluate the perpetrator of these herbs.

The present study revealed that *T. laurifolia* induced CYP1A1, CYP1A2, CYP2B6 and inhibited CYP2D6, CYP2E1, CYP3A7 gene expression in dose dependent manner. While a bimodal influence of *T. laurifolia* on CYPs mRNA expression, namely enhancement early on and suppression later was seen on CYP3A4 mRNA expressions. However, not all the CYP450s are inducible by *M. oleifera* treatment; the only relevant CYP450s appeared to be enhancement of CYP2E1 and suppression of CYP3A4 and CYP3A7. Since expression of CYP450 mRNA regulated by transcription factor Nrf-2, polyphenols were evidence to enhance its nuclear translocation and binding to antioxidant response element (ARE), thus leading to increased level and activity of detoxifying enzymes. This Nrf-2 was usually down-regulated by oxidative stress[443,444]. However, the modifications in Nrf-2 were not found in *T. laurifolia* treatment but weakly increasing in *M. oleifera* reflected that *M. oleifera* might induce the change in cellular oxidative/reductive balance. To change in CYP450 gene or protein expression did not necessarily lead to change in the activity of this enzyme. The ability of herbal extracts to alter CYP450s activities was evaluated *in vitro* in living HepG2 cells to confirm mRNA expression. These *in situ* enzymatic activities based on measurement of CYP450s specific substrates, coumarin derivative de-ethylation which had advantageous to avoid negative effect from microsomal isolation and focused only on assessing CYP1A, 2A, 2B and CYP3A activity. *T. laurifolia* and *M. oleifera* had ability to modulate all 4 subgroups of hepatic CYP450s activities. During the prolonged exposure the cells were more pronounce effect from herbal extract-induced CYP450s, indicated that herbal extracts could be oxidative metabolized by CYP450s enzymes. Exposure to *T. laurifolia* for 4 hr, we detected increasing CYP2A activity, which was shown to reduce in *M. oleifera* exposure while both herbal extracts induced CYP1A activity at this time. Later, when prolonged exposure

to herbal extracts up to 29 hr, the CYP1A, CYP2B and CYP3A activities elevated, but reduced in CYP2A activity were found.

In conclusion, both herbal extracts modulated some CYP450s gene expression and enzymatic activity. These results suggest that *T. laurifolia* might be involved in the regulatory mechanism of CYP450s induction at gene expression level, and their change in CYP450s gene expression might modulate protein level and their activities. The effects of herbal treatment were depending on treatment period and herbal concentration. It was suggested that herbal extracts might induced/suppressed these phase I biotransformation enzyme though direct and/or indirect stimulation/Inhibition pathway. Cells preferentially responded to herbal extracts by induced these biodegradation enzymes rather than suppressed them and *T. laurifolia* was more potent than *M. oleifera* for these phenomenon. *T. laurifolia* contained large amont of active compounds (more than *M. oleifera*) such as nitrate and flavonoids that might associated with modification of CYP450s. Nitrate could convert to NO via nitric oxide synthetase (NOS) pathway. CYP450s enzymes were significant targets for NO via S-nitrosylation and/or tyrosine nitration[445]. In addition, also, evidence had been shown that flavonoids could modulated CYP450-dependent hydroxylase activity and was found to correlate well with the oxidation potential for flavonoid aglycones[446]. This indicated the possibility of *T. laurifolia* / *M. oleifera* –drug interactions may occur when drugs that are metabolized by a certain CYP450s. Our result suggests that *T. laurifolia* / *M. oleifera*; (1) might increase some toxicants clearance through the elevation of CYP450-mediated metabolic activation such as those pesticides though CYP1A and CYP2B metabolism. (2) Some of these drug intermediate metabolites can have harmful effects on the body such as aldehyde from ethanol or methylecgonidine from cocaine. Thus, inhibit these CYP450s metabolism of these toxicant could be health benefit. (3) Enhance effects of some drugs. Recently study was showed that cocoa could increase CYP1A1 overexpression and enhance metabolism of estrogen which shown to be synergy between polyphenolic cocoa extract and non-cytotoxic tamoxifen concentrations[447]. But they might reduce active drug concentration may eventually occur; resulting in loss of therapeutic action such as bronchodilators and psychotropic drugs. Future studies should focus on study about potential of *T. laurifolia* / *M. oleifera* on CYP450 in herb–drug interactions *in vivo*.

B. *M. oleifera* could modulate Phase II biotransformation enzymes in gene expression level.

Beside of phase I reaction, performed mainly by CYP450 enzymes, another routes is responsible for the transformation of clinically medication is phase II reaction. After introduced or exposed reactive sites by phase I reaction (hydroxylation, reduction and oxidation), xenobiotic could be processed for conjugation reactions involving in glucuronidation, sulfation, acetylation or methylation reaction. This phase II reaction enhances water solubility of the resulting metabolites and facilitating their removal out of the body. This study has been shown to suppress phase II enzymes were only seen in *M. oleifera* treatment while exposure HepG2 cells to *T. laurifolia* for 29 hr did not alter these conjugation enzymes mRNA expression. From our result might lead to some suggestion that *T. laurifolia* did not affect phase II

biotransformation in part of glucuronidation, sulfation process whereas *M. oleifera* inhibited these enzyme expressions. This is considered to be due to the inhibition of conjugation capacity of phase II enzymes can lead to the manifestation of the adverse effects of xenobiotic. It is possible that following chronic exposure, the consumption of *M. oleifera* might reduce the protection of the liver against toxic agent.

C. *T. laurifolia* could modulate Phase III biotransformation enzymes activity.

The metabolites were generated by phase I and II reactions and they are excreted from the body with the aid of membrane efflux pumps system on the cell membrane. These drug efflux transporters systems were proposed as another detoxify mechanism cooperates with those intracellular transformation enzyme by accelerated the clearance potentially toxic products out of body. Phase III reactions involving in many trans-membrane proteins but mainly are responsible by P-glycoproteins. The result showed that *M. oleifera* exposure had no effect neither on rhodamine- 123 efflux results nor P-gp mRNA expression. In the other hand, *T. laurifolia* was the potent inducer of P-glycoprotein activity but did not change P-gp mRNA expression. All these results provided at least partially explanation about antidote effect of *T. laurifolia* against toxic agants. Nevertheless, some caution should be considered because increased P-gp activity consequently lead to rapidly decreased therapeutic level and increased clearance of clinical medication.

The effect of *T. laurifolia* and *M. oleifera* on lipid metabolism of HepG2 cells.

The liver is a major organ primary function for balance metabolic homeostasis[448]. Metabolic homeostasis processes include those that breakdown foreign substances or xenobiotics, as well as those of the endogenous chemicals or endobiotics such as lipids. Lipids are essential for life, necessary for maintenance normal aspects of cellular biology. They are tightly control by lipid homeostasis system in liver and adipose tissue that balanced production and elimination of these substances. The lipid metabolism in the liver is well-studied[449]. Disruption of lipid metabolism in the liver might trigger many other metabolic complications such as diabetes, obesity and atherosclerosis for example; Conversely, some herbal extracts can significantly alter lipid metabolism in the liver such as black sticky rice could lower hepatic LDLR mRNA expression[450]. Here, we designed experimental outlined to elucidate the effect of herbal extract in the regulation of lipid metabolism in the liver. Therefore, our result provided evidence that exposure of HepG2 cells to *M. oleifera* profoundly reduced HMGCoA reductase-, PPARγ-, PPARα-mRNA expressions while did not found significant gene expression modulation in *T. laurifolia* treatment except PPARγ-mRNA expression was induced. HMGCoA reductase was well known for its role in cholesterol biosynthesis. HMGCoA reductase inhibitor drug, statin, used for lowering lipid synthesis in the liver. In addition to the almost complete prevention of steatosis, there were large reductions in circulating cholesterol. It was indicated that *M. oleifera* could suppress lipid synthesis in the liver. HMGCoA reductase regulated by transcription factors PPAR superfamily[451,452]. These nuclear receptors actually represent the meeting point between the regulation of genetic transcription and cell physiology

response to dietary nutrients. The role of PPAR superfamily was literature reviewed by Daynes et al.[453] and Evans et al.[454] Generally, these transcription factor control the expression of numerous genes in the liver, including genes related to gluconeogenic, lipogenic and pro-inflammatory genes[455]. Transcriptional profiling of *M. oleifera* treatment reduced levels of PPARγ and PPARα, all of these evidence points toward a certain conclusion that *M. oleifera* might alter lipid metabolism in the liver. Agreement with some previously evidences that natural compound in tea down-regulated of PPARγ, C/EBP-β resulting in a decreased body fat mass[456]. In the other hand, PPARα/γ also was reported about its role in regulating mitochondrial function. Activation of PPAR result in protection against different stressors by induced mitochondrial biogenesis [in combination with the up-regulation of key mitochondrial and anti-apoptotic proteins (e.g. Bcl-2)] and ameliorates mitochondrial damage against oxidative stress. Thus, down-regulated PPAR seem to contribute to mitochondrial dysfunction and suggest that might be the underlying mechanism behind *M. oleifera* cyto-toxicity partly by impaired mitochondrial signaling regulators function. These results augment some factors should be considered in planning for adequate and appropriate *M. oleifera* used. *M. oleifera* could be beneficial for the treatment of metabolic or cardiovascular conditions but in the same time; it has been shown to have cytotoxicity properties that may be life-threatening in some instances.

Chapter V
Conclusion

Oxidative stress is a redox imbalance arises from the excess of free radicals, or malfunction of the antioxidant system which in favor of the former ones. It potentially leads to damage of cellular components such as lipids, proteins or DNA, subsequently impeding normal cell structure and functioning. Indeed, oxidative stress has been implicated in the pathogenesis of many human diseases, as well as to the aging process. Antioxidants are one way the body uses to defense and repair in order to minimize damage. Recent researches have focused on exogenous antioxidant administration to support the endogenous antioxidant system, thereby diminish oxidative damage, and arrest free radical-related functional decline processes. Herbal plants become increasing used because they are the excellent sources of antioxidants and easy availability. Herbal medicines promote to be safety but due to the fact that they often contain multiple active substances which target multiple cellular functions and , in addition, the patients tends to use in chronic conditions increase the possibility of interactions with concomitantly pharmacotherapy. However, quality, toxicological data and the cellular responses for these plants are mostly unknown and remained under-researched. To be clinically useful, more scientific data should be ensured to complete understanding of their pharmacological activity and toxicity. This present study was designed to investigate 2 most popular herbs widely used in Thailand: *Thunbergia laurifolia* (TL) and *Moringa oleifera* (MO). Both have been reputed to have many healthy beneficial and used since an ancient time in Thai traditional medicine. We studied their phytochemical profile, antioxidant efficacy, the impact on cellular redox balance, metabolism modulating affected both energy and xenobiotic detoxification potential in their extracts.

Screening by *in vitro* techniques, we found that lyophilized form of TL and MO in 80% ethanol extract possessed high antioxidant levels and have ability to scavenging some of the most common radicals in cellular system including superoxide radical, hydroxyl radical, nitric oxide and hydrogen peroxide. Their antioxidant activity are associated with the phenolic compounds contain in the extracts which believed to be major active phytochemical constituents. We next evaluated the protective effect of these herbal extracts on free radical-induced macromolecules degradation. The results showed that herbal extracts, particularly *T. laurifolia*, was significantly lowering the oxidative damage of lipid, protein, DNA determined by hemolysis inhibition, Heinz body inhibition, and Comet assay, respectively. In parallel, the herbal extracts possess ability to induced endogenous antioxidant molecules assayed by ePMRS test. We also test their beneficial effects such as anti-diabetic and antidote against pesticide. We found that both *T. laurifolia* significant inhibit α-amylase and α-glucosidase related to hyperglycemia and lowering AGEs formation while *M. oleifera* was significantly protective against organophosphate-induced acetylcholinesterase inhibition. Crude extracts were subsequently investigated for their cytotoxicity in HepG2 cell lines. The data was indicated that at high concentrations (over 1,000 μg/ml) for both extracts induced cytotoxicity using the neutral red assay and the MTT assay. The toxicity of these herbal extracts was evidently associated with mitochondrial dysfunction, partially, by interrupted mitochondrial

membrane potential, energy production and calcium homeostasis.The herbals extracts were subsequently examined about their role in maintaining cellular redox homeostasis determined by DCFH-DA staining and intracellular GSH levels. Both *T. laurifolia* and *M. oleifera* could reduce intracellular oxidative levels but only *T. laurifolia* could maintain redox balance when cell face oxidative injury. Next, we assessed the effect of herbal extracts on liver metabolism. The data showed that *T. laurifolia* significantly altered biotransformation system. *T. laurifolia* significantly change phase I, cytochrome P450 isoenzymes (CYP450s), gene expression profile and also enzymatic activity. In addition, *T. laurifolia* induced excretion process though up-regulated P-glycoprotein activity. These results supported the traditional medicinal use of *T. laurifolia* for detoxification. Unlike *T. laurifolia*, *M. oleifera* would rather to modulating lipid metabolism than effect on transformation of xenobiotic. *M. oleifera* was found significantly to modulated lipid biosysthesis by down-regulated mRNA expression of HMG-CoAR, and it regulatory transcription factor, PPARα1 and PPARγ.

The results indicate that these plants possesses antioxidant, free radical scavenging and anti - oxidative damage, maintaining cellular redox homeostasis, and has therapeutic potential to use as anti-diabetic, anti-hyperlipidemia or antidote. We suggest that the potential role of phenolic compounds constituents in modulating these beneficial for health promotion effects. While there is cause for optimism, one should keep in mind that some caution is in order with extra-explorations. Our results would be the index for further use by exploring molecular evidence about their toxicological information. Caution must be taken when mixing herbal supplements with drugs in order to assure the safety of these plants and avoid the complexity of the herb-drug-interaction.

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