ผลของแคปซูลสารสกัดขมิ้นชันต่อการเกิดภาวะเครียดออกซิเดชันในผู้ป่วยเบาหวานชนิดที่ 2

นางสาววรรณา บัวเผื่อน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา)
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ปีการศึกษา 2553

EFFECT OF CURCUMINOIDS EXTRACT CAPSULES ON OXIDATIVE STRESS IN TYPE II DIABETES MELLITUS

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmacology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2010

Thesis Title	EFFECT OF CURCUMINOIDS EXTRACT CAPSULES ON OXIDATIVE STRESS IN TYPE II DIABETES MELLITUS
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วรรณา บัวเผื่อน : ผลของแคปซูลสารสกัดขมิ้นชันต่อการเกิดภาวะเครียดออกซิเดชันในผู้ป่วยเบาหวานชนิดที่ 2. (EFFECT OF CURCUMINOIDS EXTRACT CAPSULES ON OXIDATIVE STRESS IN TYPE II DIABETES MELLITUS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. รัตยา ลือชาพุฒิพร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม ผศ.พญ.สมลักษณ์ จึงสมาน, นพ.ดร. สุธีร์ รัตนะมงคลกุล, 74 หน้า.

เบาหวานเป็นโรคเรื้อรังที่มีความผิดปกติทางเมแทบอลิกทำให้เกิดภาวะน้ำตาลในเลือดสูง ภาวะที่มีระดับน้ำตาลในเลือดสูงจะทำให้มีการสร้างรีแอคทีฟออกซิเจนสปีชีส์เพิ่มขึ้น ส่งผลทำให้เกิดการเปลี่ยนแปลงทางเคมีของโปรตีน ไขมัน ดีเอ็นเอ และกรดนิวคลีอิกในเนื้อเยื่อต่างๆ ก่อให้เกิดความเสียหายต่อเซลล์นำไปสู่ภาวะแทรกซ้อนของโรคเบาหวาน วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาผลของแคปซูลสารสกัดเคอร์คิวมินอยด์ต่อภาวะเครียดออกซิเดชันและเอนไซม์ต้านอนุมูลอิสระในผู้ป่วยเบาหวานชนิดที่สองที่ได้รับการรักษาด้วยยาลดระดับน้ำตาลในเลือดที่เหมาะสม โดยทำการศึกษาในผู้ป่วยโรคเบาหวาน 200 คน ให้รับประทานแคปซูลสารสกัดเคอร์คิวมินอยด์ (250 มิลลิกรัม) หรือแคปซูลยาหลอก ครั้งละ 2 แคปซูล วันละ 3 ครั้งเป็นเวลา 6 เดือนสำหรับเสริมการรักษา ทำการวัดพารามิเตอร์ของภาวะเครียดออกซิเดชันและเอนไซม์ต้านอนุมูลอิสระที่ 0, 3 และ 6 เดือน ผลการศึกษาพบว่าระดับมาลอนไดอัลดีไฮด์ (MDA) ที่ 3 และ 6 เดือนลดลงเมื่อเทียบกับค่าเริ่มต้นทั้งสองกลุ่ม อย่างไรก็ตามระดับมาลอนไดอัลดีไฮด์ (MDA) ความสามารถในการรีดิวส์เฟอริกในพลาสมา (FRAP) และ แอลฟา-โทโคฟีรอล ในกลุ่มเคอร์คิวมินอยด์ และยาหลอกไม่แตกต่างกัน ในขณะที่การทำงานของเอนไซม์ซุปเปอร์ออกไซด์ดิสมิวเตสในกลุ่มเคอร์คิวมินอยด์เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มยาหลอก ($p<0.05$) อย่างไรก็ตามระดับ เอนไซม์คะตะเลส กลูตาไธโอนทั้งหมด และ กลูตาไธโอนเปอร์ออกซิเดสไม่เปลี่ยนแปลง การศึกษานี้แสดงให้เห็นว่าภาวะเครียดออกซิเดชันที่เกิดขึ้นในพลาสมาของผู้ป่วยเบาหวานทั้งสองกลุ่ม สามารถควบคุมได้ซึ่งอาจเป็นผลมาจากการที่ผู้ป่วยได้รับยาในการรักษาโรคเบาหวานร่วมด้วยอยู่แล้ว ดังนั้น การศึกษานี้จึงสรุปได้ว่า การใช้แคปซูลสารสกัดเคอร์คิวมินอยด์เสริมการรักษาอาจจะมีส่วนช่วยเสริมสร้างเอนไซม์ในการต่อต้านอนุมูลอิสระในผู้ป่วยโรคเบาหวาน โดยส่งเสริมการทำงานของซุปเปอร์ออกไซด์ดิสมิวเตสซึ่งเป็นแอนติออกซิแดนท์ที่เกี่ยวข้องกับการป้องกันเซลล์โดยการทำลายสารอนุมูลอิสระ ดังนั้นการรักษาเสริมด้วยแคปซูลสารสกัดเคอร์คิวมินอยด์จึงอาจจะช่วยลดอนุมูลอิสระได้

สาขาวิชา.....เภสัชวิทยา..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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5187264220 : MAJOR PHARMACOLOGY

KEYWORDS : OXIDATIVE STRESS / CURCUMINOID / REACTIVE OXYGEN SPECIES / HYPERGLYCEMIA / ANTIOXIDANT ENZYME

WANNA BUAPHUAN : EFFECT OF CURCUMINOIDS EXTRACT CAPSULES ON OXIDATIVE STRESS IN TYPE II DIABETES MELLITUS. ADVISOR : ASST. PROF. RATAYA LUECHAPUDIPORN, Ph.D., CO-ADVISOR : ASST. PROF. SOMLAK CHUENGSAMARN, M.D., SUTHEE RATTANAMONGKOLGUL, M.D., Ph.D., 74 pp.

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia. Hyperglycemia accelerates generation of reactive oxygen species (ROS) contribute to increase the oxidative chemical modification of proteins, lipids, DNA and nucleic acids in various tissues, causing extensive cellular damage leading to complications of diabetes. The aim of this study was to investigate the effects of curcuminoids extract capsules on oxidative stress and antioxidant enzyme activities in type II diabetes mellitus. Two hundred diabetes mellitus patients treatment with appropriated hypoglycemic drugs were participated in this study. Patients received 2 capsules of either curcuminoids extract (250 mg) or placebo capsules 3 times a day for 6 months as a supplementation. The oxidative stress parameter and antioxidant enzymes activities were measured at 0, 3 and 6 months. The results showed that malondialdehyde (MDA) levels in plasma were decrease at 3 and 6 months when compared to baseline in both group. However, the MDA, ferric reducing ability of plasma (FRAP) and α-tocopherol levels in curcuminoids and placebo groups were not significantly difference between two groups. Interestingly, the activities of superoxide dismutase (SOD) were significantly increased in curcuminoids group when compare with placebo group ($p<0.05$). However, catalase, total glutathione, and glutathione peroxidase were not changed. This study demonstrated that oxidative stress occurred in the plasma of diabetic patients was controllable, might be due to patients receiving oral hypoglycemic drugs for treatment. In conclusion, this study demonstrated that the supplementation with curcuminoids extract capsules may enhance the effect on cellular antioxidant defense in diabetic patients by boosting superoxide dismutase activity which is antioxidant-related defenses to scavenge ROS. Additional therapy with curcuminoids extract capsules could possibly help reduce free radical activity.

Field of Study : .....Pharmacology..... Student's Signature.....

Academic Year : .....2010..... Advisor's Signature.....

Co-advisor's Signature.....

Co-advisor's Signature.....

ACKNOWLEDGEMENTS

The research studies described in this article would not have been possible without the collective pioneering vision from my advisor, Assistant Professor Dr. Rataya Luechapudiporn. I would like to express my profound gratitude and deep appreciation for her thoughtful advice, helpful guidance and continual support during the entire duration of the work.

I am sincerely grateful to my co-advisors, Assistant Professor Somlak Chuengsamarn and Dr. Suthee Rattanamongkolgul for their assistance and support, kindness, creative guidance, and constructive criticism throughout the course of this research.

I would like to thank HRH Princess Maha Chakri Sirindhorn Medical Center for this clinical trial study.

I would like to thank Dr. Chada Phisalaphong and the Government Pharmaceutical Organization for supporting curcuminoids extract capsules and placebo capsules.

I would like to thank to all lectures and staff of the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their facility, informative guidance and using instrument guidance.

Finally, I would like to express my infinite thanks and gratitude to all of my friends and my family for their endless love, kindness, understanding and encouragement that greatly support me in order to do this thesis possible.

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
α-tocopherol	Alpha-tocopherol
BHT	Butylated hydroxytoluene
CAT	Catalase
^{o}C	Degree Celcius
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
GPx	Glutathione peroxidase
Hb	Hemoglobin
H_2O_2	Hydrogen peroxide
IDDM	Insulin-dependent diabetes mellitus
MDA	Malondialdehyde
μg	Microgram
μl	Microliter
μM	Micromolar
mg	Milligram
ml	Milliliter
mM	Millimolar

min	Minute
M	Molar
nmol	Nanomole
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NIDDM	Non-insulin-dependent diabetes mellitus
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RBC	Red blood cell
GSH	Reduced glutathione
S.E.M.	Standard error of mean
STZ	Streptozotocin
$O_2^{\bullet-}$	Superoxide
SOD	Superoxide dismutase
TBARs	Thiobarbituric acid reactive substances
U	Unit

CHAPTER I

INTRODUCTION

Background and rationale

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin (Maritim et al., 2003). The number of adults with clinical diagnosis has been increasing dramatically worldwide. It has been estimated that the number of adults affected by diabetes in the world will grow from 135 million in 1995 to 300 million in the year 2025. There are more women than men with diabetes (King H et al., 1998; Rolo and Palmeira, 2006). In Thailand, the prevalence of type II diabetes among the population less than 35 years of age was 9.6% in 2001, with an increase of 20% over a period of 5 years (Aekplakorn et al., 2006).

Several studies have indicated that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential (Rahimi et al., 2005). Hyperglycemia accelerates generation of reactive oxygen species (ROS) results in abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms. Oxidative stress can lead to increase in oxidative chemical modification of proteins, lipids, DNA and nucleic acids in various tissues, potentially causing extensive cellular damage including the microvascular and macrovascular disorders leading to the development of cardiovascular complications in diabetes patients (Baynes, 1991, Osawa and Kato, 2005; Ramakrishna V and Jailkhani R, 2008). Many studies have been reported that antioxidants can protect the human body from free radicals and ROS effects (McCune and Johns, 2002; Chakraborty and Bhattacharyya, 2001). They may attenuate oxidative stress related tissue damage and pathophysiological complications. This has greatly stimulated to investigate the role of antioxidants including phenolic compounds in medicinal applications (Balasubramanyam et al., 2003).

Curcuminoids, a group of phenolic compounds of turmeric extract, has been claimed to be a potential antioxidant. Curcuminoids have been used widely for a long time in the treatment of inflammation and various diseases, such as in traditional Chinese medicine used to treat indigestion, hepatitis, jaundice, diabetes, atherosclerosis and bacterial infections. Curcuminoids in *C. longa* and other *curcuma* species are mainly curcumin, bis demethoxycurcumin and demethoxycurcumin (Itokawa et al., 2008). Curcumin has been shown to inhibit lipid peroxidation, and effectively scavenge superoxide and peroxyl radicals in membrane model (Priyadarsini, 1997). Curcumin protects islets against streptozotocin-induced oxidative stress by scavenging free radicals and retarded generation of reactive oxygen species in isolated islets from C57/BL6J mice. In this cell base model, curcumin prevents the reduction in levels of cellular free radical scavenging enzymes without causing overexpression of Cu/Zn superoxide dismutase (Meghana et al., 2007). In clinical study, the antioxidant activity of curcuminoids ameliorate oxidative damage resulted in a significant decrease in the antioxidant enzymes SOD and GPx concomitant with an increase GSH levels in RBC of β-thalassemia/Hb E patients (Kalpravidh et al., 2010). In Alzheimer's disease patient treated with curcuminoid enhanced intensity of Aβ uptake and induced intracellular phagocytosis, which leads to Aβ degradation (Zhang et al., 2006). Curcumin is reported as safely in clinical studies. A phase-one human trial with 25 subjects were premalignant conditions treated with tablets containing pure curcumin using up to 8000 mg per day for 3 months found no toxicity from curcumin (Cheng et al., 2001). Recently, much attention has been focused on suppression of oxidative stress by dietary antioxidant to assist in the prevention of diadetes mellitus (Osawa and Kato, 2005). So this study will focus on effect of curcuminoids extracts on the oxidative stress in diabetic patients.

Objective

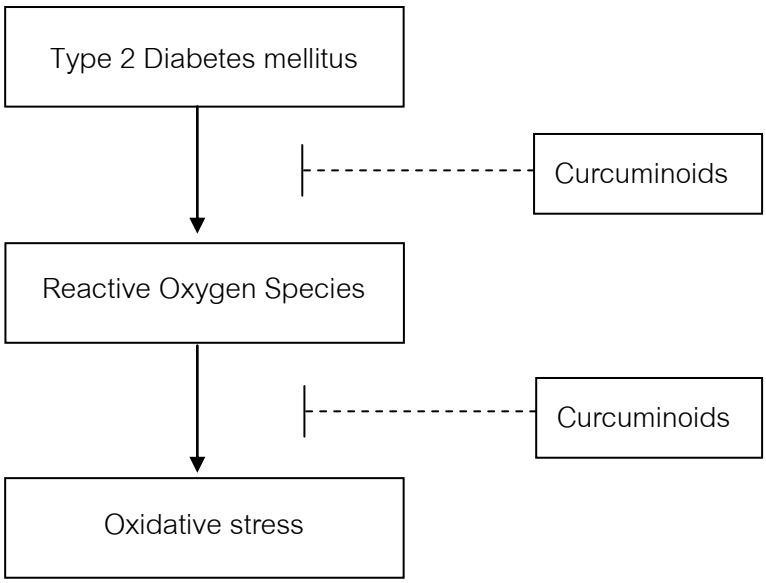
The objective of this present study was to determine

1. Effect of curcuminoids extract capsules on oxidative stress in type II diabetes mellitus
2. Effect of curcuminoids extract capsules on anti-oxidant enzymes in type II diabetes mellitus.

Hypothesis

Curcuminoids extract capsules are able to reduce oxidative stress and ameliorate oxidative damage in diabetic patient.

Conceptual Framework



Expected Benefit and Application

Knowledge from this study might be the suggestion to use curcuminoids supplementation to reduce oxidative damage in diabetic patients.

CHAPTER II

LITERATURE REVIEW

1. Diabetes mellitus

Diabetes mellitus is the most common endocrine metabolic disorder. It is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia causes the glucose autoxidation, proteins glycation, and the activation of polyol metabolism. These changes accelerated generation of reactive oxygen species (ROS) and increases in oxidative chemical modification of carbohydrate, lipids, DNA and proteins in various tissues causing cell injury and is implicated in the pathogenesis of vascular disease in type I and type II diabetes. The complications associated with diabetes are retinopathy, neuropathy, nephropathy, cardiovascular disease, erectile dysfumction and arteriosclerosis (Rahimi et al., 2005; Osawa and Kato, 2005).

1.1 Type I diabetes mellitus

Type I diabetes mellitus, which was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes have a small percentage (5 to 10%) of patients (Niedowicz and Daleke, 2005). Type I diabetes develops when the body's immune system destroys pancreatic beta cells, the only cells in the body that make the hormone insulin that regulates blood glucose. Risk factors for type I diabetes may be autoimmune, genetic, or environmental.

1.2 Type II diabetes mellitus

Type II diabetes mellitus, which was previously called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes are the majority of diabetes patients. It was usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce insulin. Type II diabetes is associated with older age, obesity, family history of

diabetes, history of gestational diabetes, impaired glucose metabolism, physical inactivity, and race/ethnicity.

In both diabetes, decreased uptake of glucose into muscle and adipose tissue leads to chronic extracellular hyperglycemia, which results in tissue damage and pathophysiological complications. These complications, including retinopathy and cataract formation, nephropathy, peripheral nerve damage, heart disease, and atherosclerosis (Niedowicz and Daleke, 2005).

2. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS)

ROS and RNS (Table 2.1) consist of free radicals and reactive species in these two groups. Breakdown products of lipids proteins, carbohydrates and nucleic acids are produced by ROS and RNS. Free radicals are molecules containing one or more unpaired electrons and could be positively or negatively charged or neutral in character. Superoxide anion, free hydroxyl radical and nitric oxide are important free radicals in human body and produce many other free radicals mainly from unsaturated fatty acids. They are unstable and destructive molecules which have affinity to give its unpaired electron to other cellular molecules or take another electron from other molecules to make stability. These free radicals are produced in cellular membrane mitochondria, lysosomes, nucleus, peroxisomes, endoplasmic reticulum and cytoplasm. The important non-radical species in human system are hydrogen peroxide, peroxynitrite, singlet oxygen peroxynitrous acid and peroxyl nitrite. Both ROS and RNS are reported to be involved in the etiopathogenesis of diabetes mellitus type II (Singh et al., 2009). Pathway of ROS formation have been shown in Figure 2.2

Table 2.1 Reactive oxygen and nitrogen species (Devasagayam et al., 2004).

Reactive species	Symbol	Half life (In sec)	Reactivity/Remarks
Reactive oxygen species:			
Superoxide	$O_2^{\bullet -}$	10^{-6} s	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	$^{\bullet}OH$	10^{-9} s	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	stable	Formed in our body by large number of reactions and yields potent species like $^{\cdot}OH$
Peroxyl radical	$ROO^{\bullet}$	s	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Organic hydroperoxide	ROOH	stable	Reacts with transient metal ion to yield reactive species
Singlet oxygen	$^1O_2$	10^{-6} s	Highly reactive, formed during photosensitization and chemical reaction
Ozone	O_3	s	Present as an atmospheric pollutant, can react with various molecules, yielding $^1O_2$

Reactive nitrogen species:

Nitric oxide	$NO^{\bullet}$	s	Neurotransmitter and blood pressure regulator, can yield potent oxidant during pathological states
Peroxynitrite	$ONOO^{-}$	10^{-3} s	Formed from NO and superoxide, highly reactive
Peroxynitrous acid	ONOOH	fairly stable	Protonated from of $ONOO^{-}$
Nitrogen dioxide	NO_2	s	Formed during atmospheric pollution

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3. Oxidative stress

In a normal healthy human, the generation of pro-oxidants in the form of ROS and RNS are effectively kept in check by the various levels of antioxidant defense. However, when it gets exposed to adverse physicochemical, environmental or pathological agents such as cigarette smoking, atmospheric pollutants, ultraviolet rays, radiation, over nutrition, toxic chemicals and advanced glycation end products (AGEs) in diabetes or in some disease states, result of imbalance between the formation of ROS and inactivation of these species by antioxidant defense system. The results are damage of DNA, proteins, carbohydrates and lipid constituents and compromise cell function leading to the development of type II diabetic complication, atherosclerosis and various human diseases (Devasagayam et al., 2004; Perez-Matute et al., 2009).

4. Oxidative stress in diabetes mellitus

Increased oxidative stress has been proposed to be one of the major causes of the hyperglycemia-induced diabetic complications. Continual hyperglycemia in the diabetic patients stimulates ROS formation from a variety of sources (Figure 2.1). Dominant among these sources is glucose autoxidation leading to the production of free radicals. Other factors include cellular oxidation/reduction imbalances and reduction in antioxidant defenses including decreased cellular antioxidant levels and a reduction in the activity of enzymes that dispose of free radicals . These sources include oxidative (Valko et al., 2007; Penckofer et al., 2002).

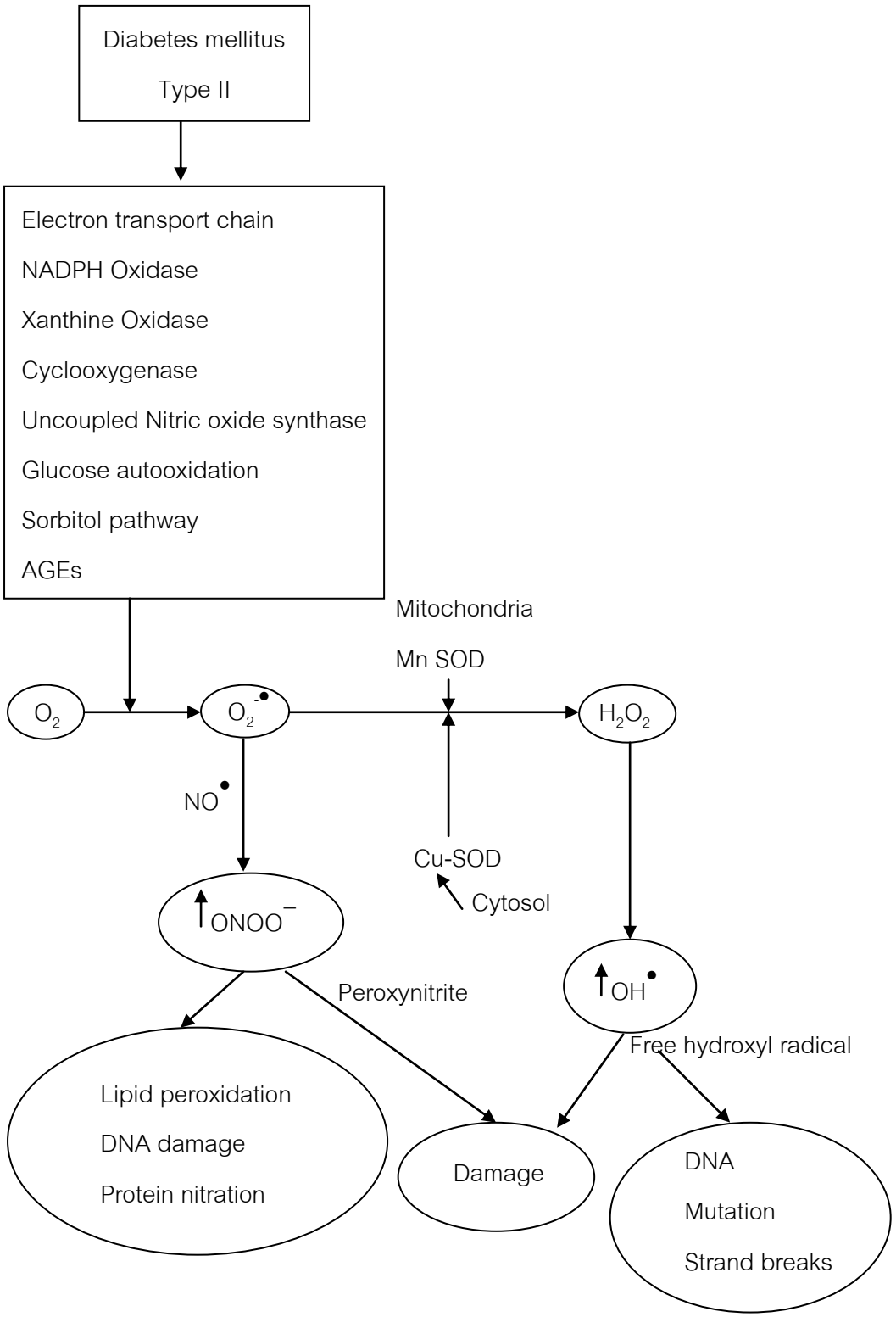


Figure 2.1: The different sources leading to enhanced generation of Reactive oxygen species (ROS) in diabetes (Sigh et al., 2009).

5. Mechanisms of hyperglycemia-induced damage

There are four main mechanisms that induce cell injury and cause diabetic complication

5.1 Increased polyol pathway flux: Hyperglycemia induces the polyol pathway, resulting in increased enzymatic conversion of glucose to sorbitol, with concomitant decrease in nicotinamide adenosine dinucleotide phosphate (NADPH) and glutathione (GSH), enhancing cellular sensitivity to oxidative stress. Hyperglycemia-induced excess production of superoxide significantly inhibits glucose-6-phosphate dehydrogenase, inhibiting the pentose phosphate pathway that is required for providing reducing equivalents to the antioxidant defense system (Branco et al., 2007).

5.2 Increased advanced glycation end-product (AGE) formation: There are three mechanisms of cells damage. The first mechanism shown at the top of the endothelial cell is the modification of intracellular proteins including, most importantly, proteins involved in the regulation of gene transcription. The second mechanism is that these AGE precursors can diffuse out of the cell and modify extracellular matrix molecules nearby, which changes signaling between the matrix and the cell and causes cellular dysfunction. The third mechanism is that these AGE precursors diffuse out of the cell and modify circulating proteins in the blood such as albumin. These modified circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology (Brownlee, 2005).

5.3 Activation of protein kinase C (PKC): Hyperglycemia results in increased enzymatic conversion of glucose to sorbitol, which is metabolized to fructose by sorbitol dehydrogenase, increasing the ratio NADH/NAD^+. This results in oxidized triose phosphates with de novo synthesis of diacylglycerol (DAG), which is a critical activating cofactor for the classic isoforms of protein kinase-C, -β, δ, and α. When PKC activated by intracellular hyperglycemia, it has a variety of effects on gene expression (Branco et al., 2007; Brownlee, 2005).

5.4 Increased hexosamine pathway flux: When glucose is high inside a cell, most of that glucose is metabolized through glycolysis, going first to glucose-6 phosphate, then fructose-6 phosphate, and then on through the rest of the glycolytic pathway. However, some of that fructose-6-phosphate gets diverted into a signaling pathway in which an enzyme called GFAT (glutamine: fructose-6 phosphate amidotransferase) converts the fructose-6 phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) N-acetyl glucosamine, is the substrate for the glycosylation of intracellular transcription factors, affecting the expression of many genes. The pathway has been associated with endothelial and microvascular dysfunction (Brownlee, 2005; Branco et al., 2007).

6. Antioxidant

Antioxidant is definite as any substance that neutralizes free radicals or their actions (Devasagayam et al., 2004). The antioxidant defenses against ROS and radicals have been widely studied (Perez-Matute et al., 2009). Defense mechanisms against free radical-induced oxidative stress involve: (I) preventative mechanisms, (II) repair mechanisms, (III) physical defenses, and (IV) antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko et al., 2007).

7. Biomarkers of oxidative stress

Methodologically, different strategies are available to determine alterations of the oxidative/anti-oxidative state, namely the direct detection of reactive species, the determination of antioxidants, and assessment of total anti-oxidative capacity and the quantification of molecules by the prevalence of ROS/RNS (Perez-Matute et al., 2009).

7.1 Lipid peroxidation: Many studies have been measured as markers of oxidative stress in diabetes (Niedowicz and Daleke, 2005). Lipids when reacted with free radicals to form a variety of products, including lipid hydroperoxides, isoprostanes, and malondialdehyde they can undergo the highly damaging chain reaction of lipid peroxidation leading to both direct and indirect effects. These molecules are generally reactive and some, such as isoprostanes, possess biological activity. Induction of diabetes in rats with streptozotocin (STZ) or alloxan uniformly results in an increase in thiobarbituric acid reactive substances (TBARS), an indirect evidence of intensified free-radical production (Maritim et al., 2003).

7.2 Ferric reducing ability of plasma (FRAP): FRAP assay is presented as a novel method for assessing ‘‘antioxidant power” (Benzie and Strain, 1996). FRAP summarizes the overall activity of antioxidant vitamin and enzymes. In diabetic with coronary heart disease patients found that significant decrease in FRAP levels when compared to the control levels (Pawar et al., 2011).

7.3 Glutathione (GSH): GSH is a small tripeptide that exists in the reduced (GSH) and the oxidized (GSSG) forms. GSH is synthesized in two steps, using the amino acids glycine, cysteine, and glutamate. GSH contains a free thiol group capable of undergoing disulfide bond formation with another molecule of GSH or another thiol-containing molecule. It classified as the primary intracellular free radical scavenger. It also plays a role in the maintenance of plasma antioxidant status and as the cofactor for many enzymes such as GPx, GSH reductase, and GSH-S-transferase. The relative amount of intracellular reduced and oxidized GSH is a measure of the cellular redox status. Levels of reduced GSH have been shown to decrease in many models of diabetes/ hyperglycemia and in diabetic patients (Niedowicz and Daleke, 2005). Type II diabetic patients had decreased erythrocyte GSH and increased GSSG levels (Atalay and Laaksonen, 2002). In STZ-treated diabetic rats, dietary GSH improved diabetic-induced oxidative stress in vivo (Osawa and Kato, 2005).

7.4 Glutathione peroxidase (GPx): GPx are found in the cytoplasm, mitochondria and nucleus. GPx convert hydrogen peroxide to water by using reduced glutathione as a hydrogen donor (Maritim et al., 2003). The effect of diabetes on GPx activity is highly variable with respect to the model of diabetes used and even the tissue type studied. GPx activity is increased in the renal cortex of STZ-induced diabetic rats and in erythrocytes from NIDDM patients. Conflicting results have been obtained, however. GPx activity was demonstrated to decrease in erythrocytes and reticulocytes from NIDDM and IDDM patients, though the decrease in IDDM patients was dependent on the presence of complications (Niedowicz and Daleke, 2005).

7.5 Catalase (CAT): CAT, located in peroxisomes, decomposes hydrogen peroxide to water and oxygen (Maritim et al., 2003). CAT activity is decreased in aortic endothelial cells and livers from alloxan-induced diabetic rabbits, in kidneys of STZ-induced diabetic rats, and in erythrocyte membranes from NIDDM patients (Niedowicz and Daleke, 2005).

7.6 Superoxide dismutases (SOD): The first enzyme involved in the antioxidant defense is the SOD. SOD converts $O2^{\bullet-}$ to hydrogen peroxide, a less reactive ROS. There are two types of SOD in the cell: the Cu,Zn-SOD found in the cytoplasm, nucleus, and lysosomes, and the Mn-SOD found in mitochondria, but can be released in to extracellular space. There is also an extracellular SOD found in plasma, lymph, ascites, and cerebrospinal fluid. The effect of diabetes on the activity of SOD is erratic, with no discernable pattern based on gender or species of animal, or duration of diabetes, or tissue studied (Niedowicz and Daleke, 2005; Maritim et al., 2003).

7.7 α-Tocopherol (Vitamin E): α-Tocopherol is a major lipophilic antioxidant, present in the cell membrane and plasma lipoproteins, functions as a chain-breaking antioxidant and serves to impede the propagation of free radical reaction in all cell membranes in the human (Bandyopadhyay et al., 1999; Devasagayam et al., 2004). Vitamin E levels are depleted in the plasma of NIDDM and IDDM patients, in normal

patients challenged with high concentrations of glucose and red blood cells treated with high concentrations of glucose (Niedowicz and Daleke, 2005).

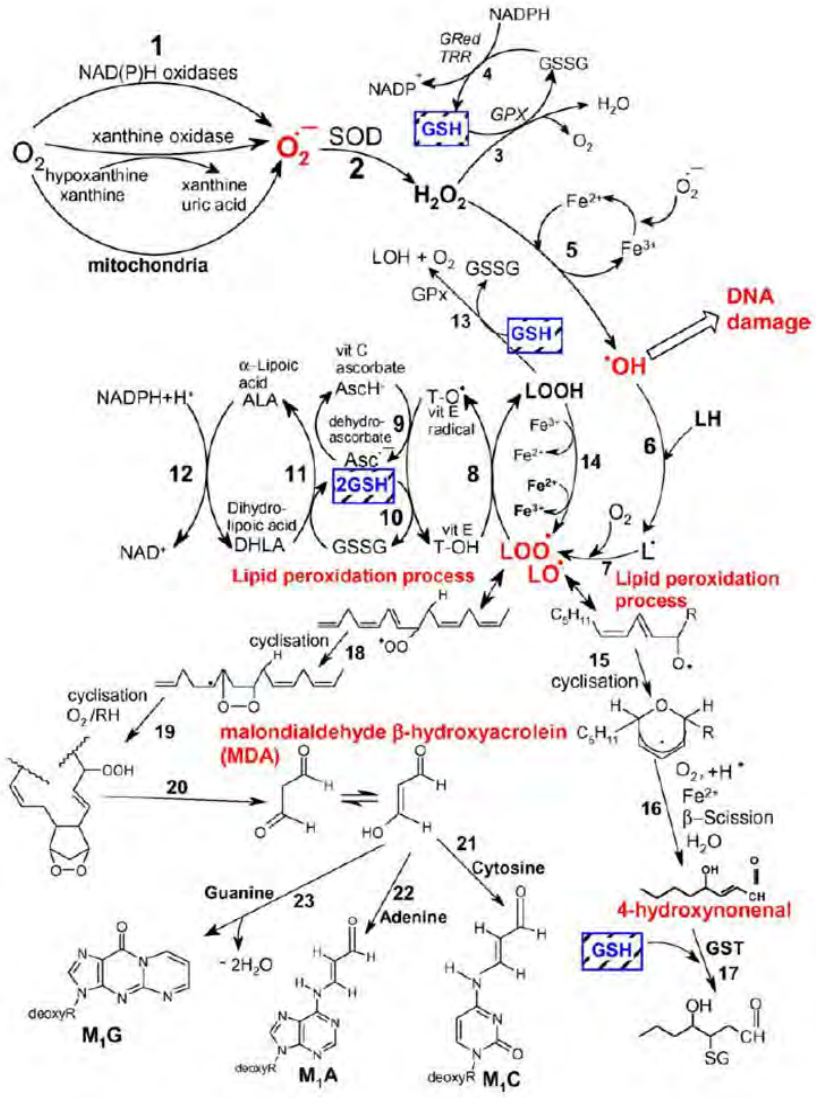


Figure 2.2: Pathway of ROS formation, the role of glutathione (GSH) and other antioxidants and lipid peroxidation process (Valko et al., 2007).

8. *Curcuma longa* Linn.

Class	:	Liliopsida
Subclass	:	Commelinids
Order	:	Zingiberales
Family	:	Zingiberaceae
Genus	:	Curcuma
Species	:	Curcuma longa

Curcuma longa or turmeric is a tropical plant native to southern and southeastern tropical Asia. Curcuminoids are mainly curcumin, bis-demethoxycurcumin and demethoxycurcumin (Figure 2.3). Curcumin is a diferuloylmethane presented in extract of the plant. Curcuminoids are responsible for the yellow color of turmeric and are found to be a rich source of phenolic compounds. They are derived from turmeric by ethanol extraction. Turmeric is widely consumed in the countries of its origin for a variety of uses, including as a dietary spice, a dietary pigment, and an Indian folk medicine for the treatment of various illnesses. Current traditional Indian medicine uses it for biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis. The old Hindu texts books have described it as an aromatic stimulant and carminative (Chattopadhyay et al., 2004; Aggarwal et al., 2005; Jayaprakasha et al., 2005).

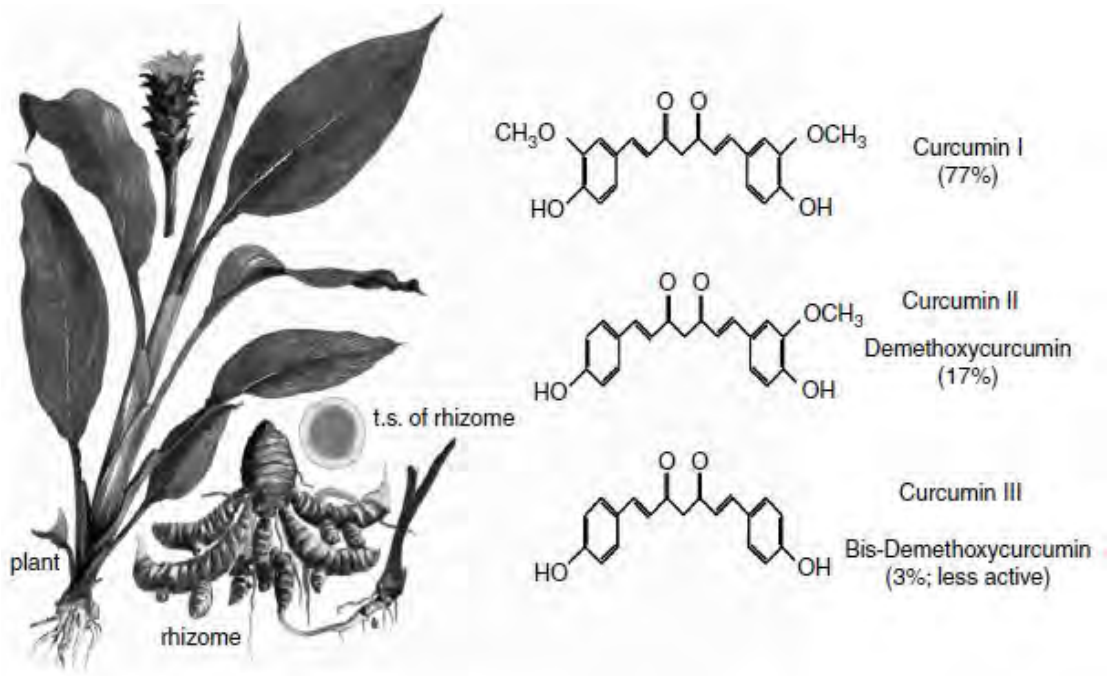


Figure 2.3: The plant *Curcuma longa* and chemical structure (Aggarwal et al., 2005)

8.1 Chemical properties

Curcumin is a bis-α,β-unsaturated β-diketone. The bis-keto form predominates in acidic and neutral aqueous solutions and in the cell membrane. At pH 3-7, curcumin acts as an extraordinarily potent H-atom donor. This is because, in the keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings contains a highly activated carbon atom, and the C-H carbon bonds on this carbon are very weak due to delocalisation of the unpaired electron on the adjacent oxygens. In contrast, above pH 8, the enolate form of the heptadienone chain predominates, and curcumin acts mainly as an electron donor, a mechanism more typical for the scavenging activity of phenolic antioxidants. Curcumin is relatively insoluble in water, but dissolves in acetone, dimethylsulphoxide and ethanol. Curcumin is unstable at basic pH, and degrades within 30 min to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2, 4-dioxo-5-hexanal, ferulic acid, feruloylmethane and vanillin. Curcumin has a structure of $C_{21}H_{20}O_6$ and molecular weight of 368.37 and a melting point of 183 ^{o}C. On ultraviolet-visible

spectrophotometric investigation, maximum light absorption of curcumin occurs at 420 nm (Sharma et al., 2005).

8.2 Pharmacokinetic properties

Oral consumption of curcumin in rats resultes in approximately 75% being excreted in the feces and only traces appeared in the urine whereas intra-peritoneal (i.p) administration accounted for similar levels of fecal excretion of curcumin, with only 11% found in bile suggesting poor absorption of curcumin from the intestine (Maheshwari et al., 2006). Curcumin exhibits low oral bioavailability in rodents and may undergo intestinal metabolism; absorbed curcumin undergoes rapid first-pass metabolism and excretion in the bile. In a study of high dose oral curcumin performed in Taiwan, Cheng and colleagues administered 0.5-8 g daily of curcumin for 3 months to patients with pre-invasive malignant or high risk pre-malignant conditions of the bladder, skin, cervix, stomach or oral mucosa. Serum curcumin concentrations were found to peak 1-2 h after oral intake and gradually decline within 12 h (Sharma et al., 2005).

8.3 Antioxidant activity and scavenging free radical of curcuminoid

Study in rats induced cataracts by galactosemic, the result show that both orally administrated curcumin and tetrahydrocurcumin prevented cataractgenesis in galactosemic rats effectively. Tetrahydrocurcumin also showed strong preventive activity xylose-induced cataract in cultured monkey lenses. The defense mechanism of tetrahydrocurcumin showed that scavenge ROS not only formed during hyperglycemia, but also induced antioxidative enzymes (GPx and SOD). Curcumin can be changed to tetrahydrocurcumin, a substance that has better antioxidant property than curcumin (Osawa and Kato, 2005). Used an erythrocyte cell model of healthy volunteers to the erythrocytes were treated with high levels of glucose (mimicking diabetes) in the presence or absence of curcumin (0-10 μM) in the medium at 37^{o}C for 24 h. The results show that curcumin prevents protein glycosylation and lipid peroxidation suggests that curcumin may inhibit oxygen radical production caused by high glucose concentrations in a cell-free system (Jain et al., 2006). Oral administration of tetrahydrocurcumin at 80

mg/kg body weight of diabetic rats for 45 days can reduced blood glucose and increase plasma insulin levels and increase the activities of SOD, CAT, GPx, glutathione-s-transferase, reduced glutathione, vitamin C and vitamin E in liver and kidney of diabetic rats with decrease thiobarbituric acid reactive substances (TBARS) and hydroperoxides formation in liver and kidney, indicated that tetrahydrocurcumin have antioxidant effect in type II diabetic rats (Murugan and Pari, 2006).

8.4 Clinical studies of curcumin

In patients with beta-thalassemia/HbE were received curcumin 500 mg daily for 6 months, MDA was reduced 30.73%, while the activities of enzyme SOD and GPx declined 15.30% and 18.91%, respectively. The antioxidant level of reduced glutathione (GSH) was improved 19.48% (Insain, 2004). Twenty-one β-thalassemia/Hb E patients were given 2 capsules of 250 mg each of curcuminoids (a total of 500 mg) daily for 12 months. The increased oxidative stress was shown by higher levels of MDA, SOD, GPx in RBC, serum NTBI, and lower level of RBC GSH. Curcuminoid administration resulted in improvement of all the measured parameters as long as they were administered. After 3 months withdrawal of treatment, all parameters returned close to baseline levels (Kalpravidh et al., 2010). In the influence of curcumin on the MDA level in 8 transfusion-independent and 8 transfusion-dependent beta-thalassemia patients who received curcumin 500 mg daily for 3 months. In transfusion-independent the erythrocyte MDA level was reduced about 41% and plasma MDA level was reduced about 25%, while the plasma MDA level in patients with transfusion-dependent beta-thalassemia was decreased approximately 12% (Mahathein, 2004). Ten cardiovascular patients administration of curcumin 500 mg/day for 7 days led to decreased serum lipid peroxidase 33%, increased HDL cholesterol 29%, and decreased total serum cholesterol 12%. In patients with atherosclerosis administration of curcumin 10 mg twice a day for 28 days lowered serum LDL and increased serum HDL levels (Goel et al., 2008). In tropical pancreatitis patients receive 500 mg of curcumin with 5 mg of piperine for 42 days reduction in the erythrocyte MDA level with increase in GSH level (Durgaprasad et al., 2005).

8.5 Safety

Acute toxicity study in mice indicated that oral administration of turmeric powder at the dose of 10 g/kg body weight produced no toxic effects. LD_{50} of 50% ethanolic extract administered orally, subcutaneously or intraperitoneally were more than 15 g/kg body (Sittisomwong et al., 1990). Administration of 1.2-2.1 g of oral curcumin daily to patients with rheumatoid arthritis in India for 2-6 weeks did not result in any reported adverse effects. In a study of high dose oral curcumin have suggested that, doses up to 8 g could be administered daily to patients with premalignant lesions for 3 months without overt toxicity (Cheng et al., 2001)

CHAPTERIII

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Curcuminoids treat capsules were supported by the government pharmaceutical organization. The following chemicals were purchased from Sigma Chemical Co., St Louis, U.S.A.: Sodium dodecyl sulfate (SDS), ethylene diamine tetraacetic acid-Na-salt (EDTA-Na salt), buthylated hydroxytoluene (BHT), thiobarbituric acid (TBA), tetraethoxypropane (TEP), sodium chloride (NaCl), sodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), α-tocopherol, sodium acetate trihydrate ($C_2H_3NaO_2.3(H_2O)$), glacial acetic acid, trizma hydrochloride (Tris-HCl), 2-4-6-tripyridyl-s-triazine (TPTZ), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), sulfosalicylic acid, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione reductase, standard glutathione, drabkin's solution, brij 35 solution, hemoglobin human, SOD assay kit, cupper zinc superoxide dismutase, potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), hydrogen peroxide (H_2O_2), *t*-buthyl hydroperoxide, ethylene diamine tetraacetic acid (EDTA)

Other chemicals were purchased from commercial sources as follows: trichloroacetic acid from Merck Germany, methanol, hexane, acetronitrile, isopropanol, ethanol, butanol (HPLC grade) from Lab Scan Co., Ltd Thailand.

1.2 Instruments

The following items were major equipments used in this study

1) Spectrofluorometer (Jasco model FP-777, Japan)

2) Spectrophotometer (Jasco model UVDEC 650, Japan)

3) Microplate reader (Wallac model 1420, U.S.A.)

4) HPLC system (Shimadzu, Japan) class LC 10

5) Refrigerated centrifuge (Becman coulter model Allegra X-12R, Germany)

2. Methods

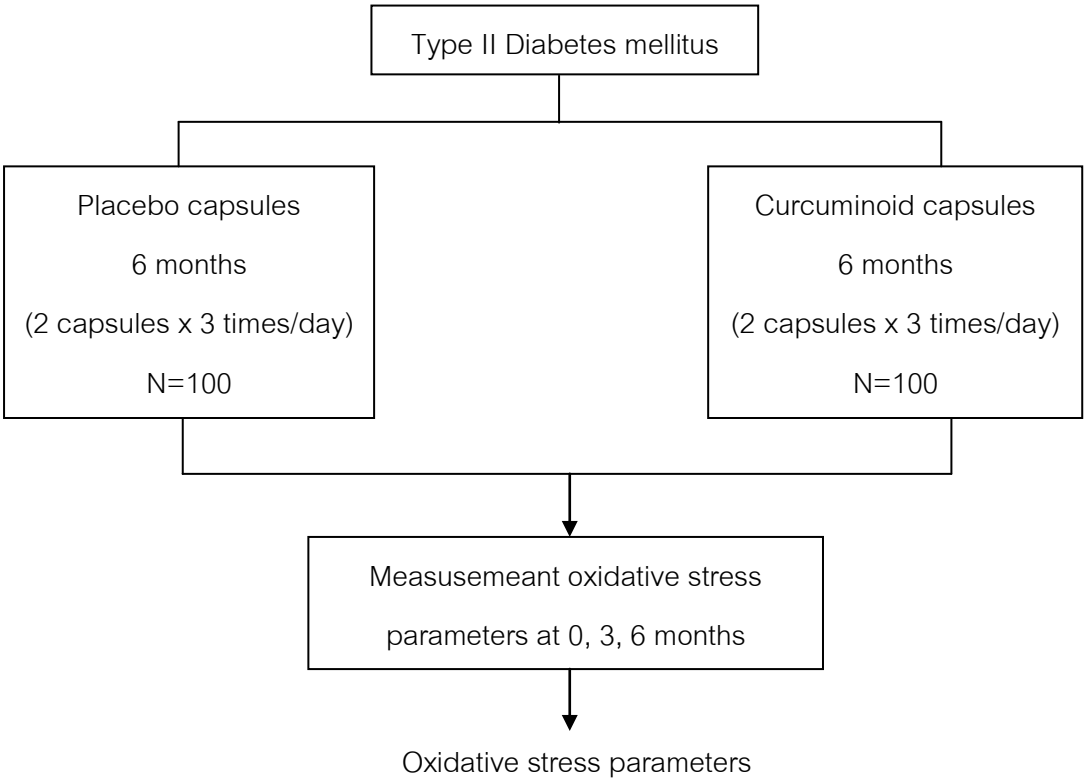
2.1 Subjects

Two hundred patients with type II diabetes mellitus more than 35 years of age were recruited from the HRH Princess Maha Chakri Sirindhorn Medical Center. All patients were participated in the research project heading “Antiaterogenic and antimetabolic effect of curcumin in type II diabetic patients”. All patients with inform consents were treated with the appropriate oral hypoglycemic drugs. In addition, subjects received 2 capsules of either curcuminoids or placebo 3 times a day for 6 months, according to which group they were randomized to. Both curcuminoids extract and placebo capsules were produced and standardized by the Government Pharmaceutical Organization. (See detail in appendix c).

2.2 Sample collection

Blood sample were collected at 0, 3 and 6 months for measurement of antioxidant enzyme and glutathione (GSH) levels in red blood cells and oxidative stress marker in plasma. Blood samples were obtained after a 12 hour overnight fasting into the EDTA tubes and then plasma was immediately separated by centrifugation at 3250

rpm at 4°C for 15 min. The buffy coat was discarded and the remaining erythrocytes were washed in saline solution three times. The erythrocytes were hemolysed by adding equal volume of ice-cold deionized water to yield a 50% hemolysate. The hemolysate was frozen at - 80 °C until used. Plasma was collected for detection of malondialdehyde level (MDA), total antioxidant activity and vitamin E. Hemolysate was collected for detection of superoxide dismutase (SOD), catalase (CAT), total glutathione (GSH) and glutathione peroxidase (GPx).



Plasma

1. Total antioxidant activity (FRAP assay)
2. Thiobarbituric acid reactive substances (TBARS)
3. α-tocopherol (Vitamin E)

Hemolysate

4. Superoxide dismutase (SOD)
5. Catalase (CAT)
6. Total glutathione (GSH)
7. Glutathione peroxidase (GPx)

Figure 3.1 Diagram of procedures

2.3 Determination of total antioxidant activity (Benzie and Strain, 1996)

In the ferric reducing ability of plasma (FRAP) assay, reductants ("antioxidants") in the sample reduce ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) at low pH, which present intense blue colored ferrous form and can be monitored at 593 nm (Figure 3.2). The change of absorbance is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample.

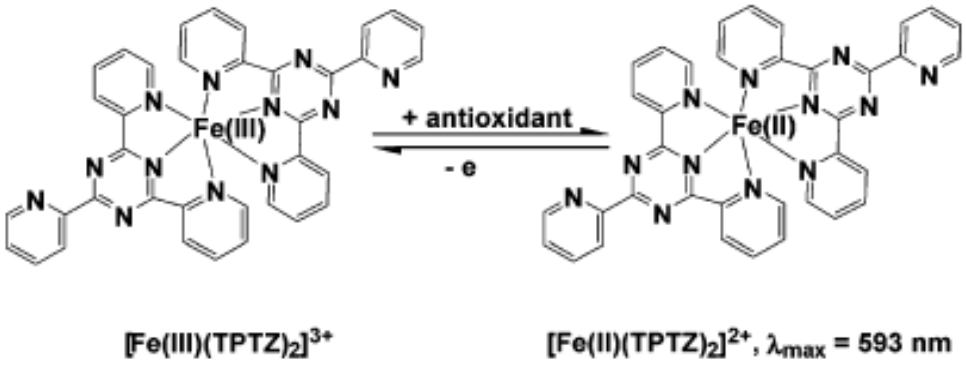


Figure 3.2 Principle of FRAP assay method

10 μl of plasma sample was pipette into 96 well microplate add deionized water 10 μl and add working FRAP reagent (300 nM sodium acetate buffer, pH 3.6, 10 nM TPTZ, 20 mM $FeCl_3.6H_2O$ ratio 10:1:1) 180 μl set left at room temperature for 5 minutes. The color development was recorded at 590 nm by microplate reader. Total antioxidant activity was expressed in μmol/l.

2.4 Determination of Thiobarbituric acid reactive substances (Asakawa and Matsushita, 1980)

Malondialdehyde, a secondary product of lipid peroxidation, reacts with thiobarbituric acid (TBA) in acidic medium forming an MDA-TBA_2 which adducts give's a pink colour (Figure 3.3).

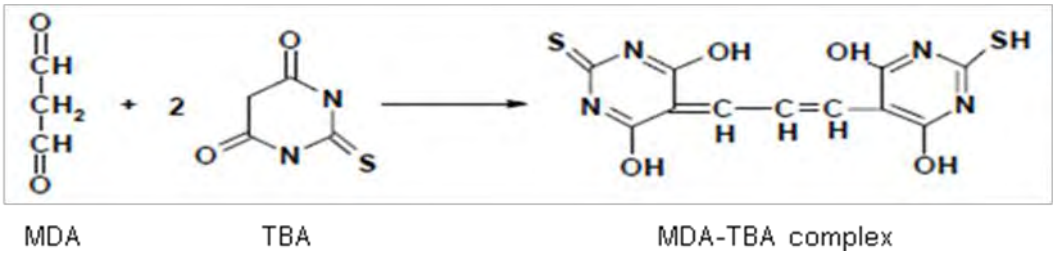


Figure 3.3 Principe of MDA assay method

25 μl of 100 mM butylated hydroxytoluene was added to inhibit lipid peroxidation occur during the process, and then 0.5 ml of 10% trichloroacetic acid was added and mixed for 1 min. A 0.25 ml of 5 mM EDTA was added into the mixture. After vortexing, 0.25 ml of 8% sodium dodecyl sulfate and 0.75 ml of 0.6% thiobarbituric acid were added and vortex, respectively. The reaction mixtures was then heating at 100 $^{\circ}$C for 1 hr, then samples were cooled to room temperature and added butanol 2 ml, vortex and then centrifuged at 3,000 rpm for 15 min. The pink color of supernatant was measured by spectrofluorometer, excitation and emission wavelength at 515 and 553 nm, respectively. TBARs levels of plasma were expressed as nmol/ml.

2.5 Determination of α-tocopherol (Vitamin E) (Seta et al., 1990)

α-Tocopherol can be determined by reverse phase HPLC method using UV monitor at 292 nm. 100 μl of plasma add with 100 μl of 10 mM PBS and vortex. Then 500 μl of ice-cold methanol were added and vortex for 30 sec. The 2.5 ml of hexane were added into the mixture and vortexed vigorously for 1 min and then centrifuged at 1,700 rpm at 4 $^{\circ}$C for 5 min. The 2 ml of hexane layer was transferred into test tube and dried under nitrogen and redissolved with 200 μl of mobile phase (75% acetronitrile: 25% isopropanol). The standard mixture of α-tocopherol (5, 10, 20, 40, 60 and 80 μl) were injected into the hypersil BDS C18 column (5 μm: 4.6 mm x 250 mm) by autosampler. The sample was injected 10 μl. The flow rate was 1.2 ml/min and the temperature of column was controlled at 50 $^{\circ}$C. α-tocopherol was expressed in nmol/ml.

2.6 Determination of superoxide dismutase (Fluka analytical assay kit)

The activity of SOD was measured by using SOD assay commercial kit (Sigma-Aldrich). Superoxide anions (O_2^-) are generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase. The superoxide anion then converts WST-1 to WST-1 formazan, a colored product that absorbance at 450 nm by microplate reader (Figure 3.4). However, in the presence of SOD these superoxide anion concentrations are reduced, yielding the lowers rate of WST-1-formazan formation.

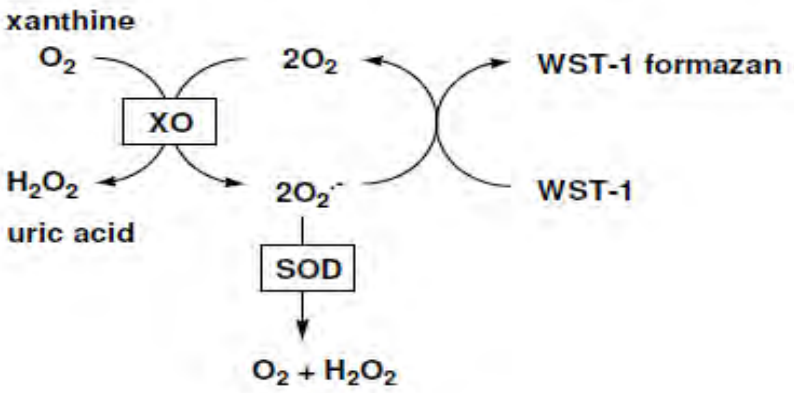


Figure 3.4 Principle of SOD assay method

Samples and standards were added to 96-well plate. WST working solution was added to each well. Enzyme working solution was added to the wells to initiate a reaction. The absorbance readings were taken at 450 nm every minute for 10 minutes by microplate reader. SOD activity were calculated from the percent inhibition of the reaction and expressed in U/gHb.

2.7 Determination of catalase activity (Pippenger et al., 1998)

The catalase activity was determined by catalyzed decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. The rate of disintegration of hydrogen peroxide into water and oxygen is proportional to the concentration of catalase.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

3 ml of 10 mM H_2O_2 in 50 mM potassium phosphate buffer was added to cuvet and pre-warm at 25°C for 5 min. 20 μl of hemolysate was added and record the change in absorbance at 240 nm between 30 and 210 sec by spectrophotometer. One unit of activity is defined arbitrarily as the amount of enzyme, which induces a change in A_{240} of 0.43 during the 3 min incubation. Catalase activity was expressed in KU/gHb.

2.8 Determination of total glutathione (Anderson, 1985)

Glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that has maximum absorbances at 405 nm, and the mixed disulfide, GSTNB, that is reduced by Glutathione reductase to recycle the Glutathione and produce more TNB (Figure 3.5). The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of glutathione in the sample.

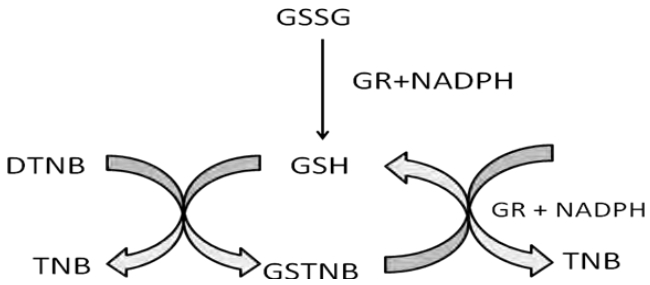


Figure 3.5 Principle of GSH recycling method

Preparation of sample by 0.5 ml of 4% sulfosalicylic acid adding into 0.5 ml of hemolysate, then centrifuge at 12,000 rpm for 15 min at 4°C. Supernatant was transferred to 96-well microplate. Then 80 μl of 0.01 sodium phosphate buffer with 1 mM EDTA pH 7.5 was added. Subsequently, 100 μl of reaction mixture (containing 1 mM of DTNB, 0.5 mM of NADPH, 1 iu of GSH reductase dissolved in 0.01 M of sodium phosphate buffer containing 1 mM of EDTA pH 7.5) was added immediately. After addition of the reagent, color development was recorded at 405 nm for 4 min by microplate reader. Total glutathione was expressed in μmol/gHb.

2.9 Determination of glutathione peroxidase activity (Paglia and Valentine, 1967)

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The hemolysate was added to the reaction mixture (consisted of 5 mM EDTA-Na salt, 0.1 M GSH, 10 unit/ml glutathione reductase, Tris-HCl buffer pH 8.0) and allowed to incubate for 5 min at 37°C. Then 7 mM cumene hydroperoxide was added as a starting reagent and the absorbance was monitored at 340 nm by spectrophotometer. The difference of absorbance per minute was used to calculate the enzyme activity by using an extinction coefficient of NADPH at $6.22 \times 10^3\ M^{-1}cm^{-1}$. Enzyme activities were reported in U/gHb.

2.10 Determination of Hemoglobin (Drabkin and Austin, 1935)

This procedure is based on the oxidation of hemoglobin and its derivatives to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin which has maximum absorbance at 540 nm.

$$Hb\text{-}Fe^{2+} + Fe^{3+}(CN)_6^{3-} \longrightarrow Hb\text{-}Fe^{3+} + Fe^{2+}(CN)_6^{4-}$$

$$Hb + Fe^{3}+ + CN^- \longrightarrow Hb\text{-}Fe^{3+}CN$$

The Drabkin's solution was prepared by reconstitute one vial of the Drabkin's reagent with 1,000 ml of water, then 0.5 ml of the 30% Brij 35 solution was added, mixed well. 20 μl of hemolysate was added into 5.0 ml of the Drabkin's solution. Mix well and allow standing for at least 15 min at room temperature. Then record absorbance at 540 nm by spectrophotometer. Determine the total hemoglobin concentration (mg/ml) from

the calibration curve of cyanmethemoglobin standard solution. Hemoglobin was expressed in mg/ml.

3. Statistical Analysis

Subject characteristics are given as mean $\pm$ standard deviation (SD). Data were presented as mean $\pm$ standard error of the mean (S.E.M). Data were analyzed by Student's t test to determine difference between curcuminoids and placebo group and repeated measures one-way analysis of variance (ANOVA) to determine overall differences between curcuminoids compared to placebo group. The parameters at 3 and 6 months of treatment in patients were analyzed the difference from baseline by Paired t-test. Values of $p<0.05$ were considered to be statistically significant.

CHAPTER IV

RESULTS

Part I: Subject characteristics

Clinical characteristics of type II diabetic patients presented in this study were summarized in Table 4.1. Sex, Age, Body mass index (BMI), Weight, Fasting plasma glucose (FPG), Cholesterol, Triglyceride, and HDL were comparable between curcuminoid and placebo groups. However LDL levels at 3th month were significantly increased in curcuminoid group compare with placebo group (Table 4.2).

Table 4.1 Characteristics of patients with diabetes mellitus at base line

Parameters	Placebo	Curcuminoid	P-value
Sex (male : female)	48 : 59	51: 56	0.68
Age (years)	59.63 $\pm$ 10.66	59.06 $\pm$ 10.96	0.70
Weight (kg)	68.34 $\pm$ 11.77	68.87 $\pm$ 15.24	0.78
BMI (kg/m^2)	26.38 $\pm$ 4.63	26.8 $\pm$ 5.42	0.55
Fasting plasma glucose (FPG) (mg/dl)	137.49 $\pm$ 38.36	137.5 $\pm$ 40.77	1.00
Cholesterol (mg/dl)	195.86 $\pm$ 44.89	196.92 $\pm$ 48.68	0.87
Triglyceride (mg/dl)	166.83 $\pm$ 101.97	152.27 $\pm$ 102	0.30
HDL (mg/dl)	49.3 $\pm$ 13.14	49.58 $\pm$ 10.74	0.87
LDL (mg/dl)	113.25 $\pm$ 33.6	117.4 $\pm$ 37.27	0.40

Value are given as mean $\pm$ SD

Table 4.2 Biochemical changes in placebo and curcuminoid group at 0, 3 and 6 months.

Parameters	Group	Duration of treatment		
		M0	M3	M6
Fasting plasma glucose (FPG) (mg/dl)	placebo	137.49 ± 38.36	143.26 ± 53.28	147.46 ± 50.16
	curcuminoid	137.5 ± 40.77	147.06 ± 52.16	137.77 ± 48.36
Cholesterol (mg/dl)	placebo	195.86 ± 44.89	195.41 ± 48.55	215.38 ± 160.48
	curcuminoid	196.92 ± 48.68	208.7 ± 44.64	204.70 ± 42.97
Triglyceride (mg/dl)	placebo	166.83 ± 101.97	157.6 ± 87.07	157.4 ± 85.2
	curcuminoid	152.27 ± 102	145.79 ± 86.27	138.18 ± 107.98
HDL (mg/dl)	placebo	49.3 ± 13.14	48.38 ± 12.66	48.74 ± 12.42
	curcuminoid	49.58 ± 10.74	49.52 ± 11.84	51.01 ± 13.39
LDL (mg/dl)	placebo	113.25 ± 33.6	119.04 ± 39.56	126.78 ± 40.64
	curcuminoid	117.4 ± 37.27	133.67 ± 39.61*	131.06 ± 38.44
BMI (kg/m^2)	placebo	26.38 ± 4.63	26.88 ± 4.44	27.22 ± 5.16
	curcuminoid	26.8 ± 5.42	29.17 ± 20.74	27.32 ± 5.58
Weight (kg)	placebo	68.34 ± 11.77	68.59 ± 11.62	68.65 ± 11.96
	curcuminoid	68.87 ± 15.24	69.28 ± 14.87	69.15 ± 14.44

Value are given as mean ± SD

*$P < 0.05$, different from placebo group

Table 4.3 Preliminary data of normal subjects compared with type II diabetic patients at baseline

Parameter	Normal subjects	DM at base line in placebo group	p-value	DM at base line in curcuminoid group	p-value
MDA (nmol/ml)	2.92 ± 0.19 (n=4)	3.56 ± 0.09 (n=88)	0.141	3.54 ± 0.09 (n=85)	0.130
α-Tocopherol (nmol/ml)	46.51 ± 5.08 (n=3)	27.62 ± 0.98** (n=88)	0.001	27.74 ± 1.14** (n=85)	0.003
FRAP (μmol/l)	614.70 ± 28.92 (n=3)	807.19 ± 18.25** (n=88)	0.006	791.94 ± 20.36 (n=85)	0.108
SOD (U/gHb)	1083.48 ± 135.03 (n=3)	1110.27 ± 34.62 (n=90)	0.889	1183.87 ± 42.07 (n=87)	0.662
CAT (KU/gHb)	6.99 ± 0.08 (n=3)	7.21 ± 0.10 (n=90)	0.700	7.30 ± 0.11 (n=87)	0.617
Total GSH (μmol/gHb)	6.87 ± 0.23 (n=4)	1.63 ± 0.14** (n=90)	0.000	1.97 ± 0.13** (n=87)	0.000
GPx (U/gHb)	24.20 ± 2.55 (n=3)	27.16 ± 0.63 (n=90)	0.400	27.95 ± 0.74 (n=87)	0.355

***P* < 0.01 significantly different from normal subjects. (Student's *t* test)

Data in table 4.3 showed the oxidative parameters of normal subjects compared to those of diabetic patients participated in this study. Parameters that indicated the occurring of the oxidative stress status in diabetic patients were the depletion of α-tocopherol and total GSH. FRAP which is the parameter summarized the overall activity of antioxidants in plasma, was increased to protect patients against free radicals. However, the MDA level and the activity of antioxidant enzymes in red blood cell including SOD, CAT and GPx were not significantly different from those of normal subjects.

Part II: Oxidative stress status

4.2.1 The Malondialdehyde (MDA) level

MDA is the most often measured index of lipid peroxidation. The MDA levels of diabetic patients at baseline, 3 and 6 months were not significantly different between the curcuminoids group and placebo group. In addition, data analysis by repeated measures ANOVA on baseline, 3 and 6 months also showed no significantly difference between curcuminoids and placebo group (p = 0.22). When calculated by paired t-test, data showed a significant decreased in placebo group at 3 and 6 month when compared to baseline (2.87 $\pm$ 0.09, 2.44 $\pm$ 0.10 *vs*. 3.56 $\pm$ 0.09 nmol/ml; p = 0.00, respectively) and those at 6 month decreased significantly (p = 0.00) in placebo group when compared with 3 month. While, MDA in curcuminoids group decrease significantly at 3 and 6 month when compared to base line (2.70 $\pm$ 0.07, 2.27 $\pm$ 0.09 *vs*. 3.54 $\pm$ 0.09 nmol/ml; p = 0.00) and those at 6 month significant decreased in placebo group when compared with 3 month (p = 0.00) (Figure 4.1).

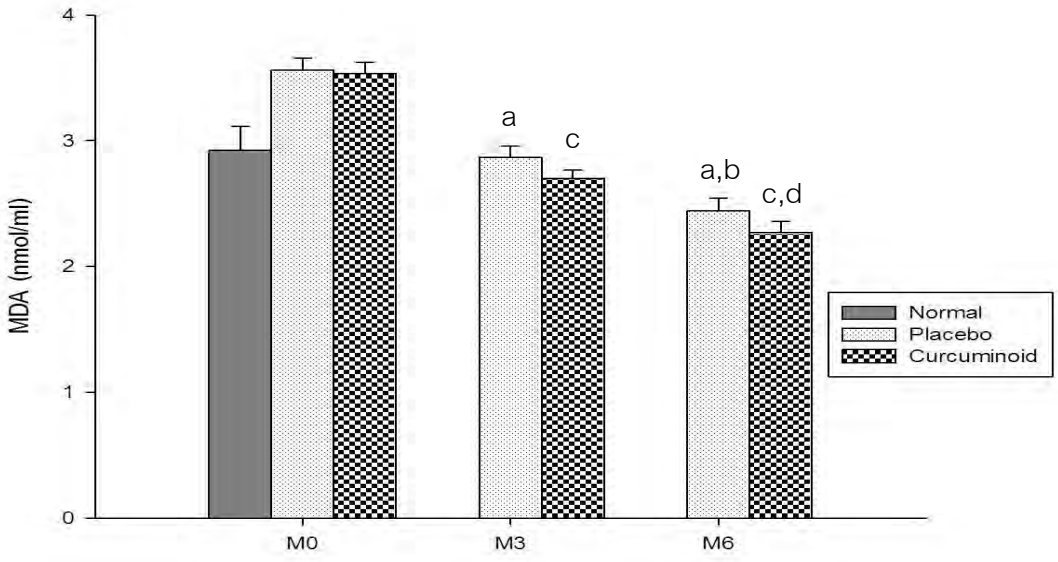


Figure 4.1 Levels of MDA (mean $\pm$ S.E.M) in plasma of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[a] $p < 0.05$ significantly difference from base line in placebo group

[b] $p < 0.05$ significantly difference from 3 month in placebo group

[c] $p < 0.05$ significantly difference from base line in curcuminoid group

[d] $p < 0.05$ significantly difference from 3 month in curcuminoids group

4.2.2 α-Tocopherol (Vitamin E) level

α-Tocopherol is usually measured to assess the antioxidant status in the blood. The α-tocopherol levels at base line in curcuminoid and placebo group were significantly lower than normal subjects (27.74 ± 1.14 and 27.62 ± 0.98 *vs*. 46.51 ± 5.08 nmol/ml; p= 0.003 and p = 0.001, respectively) When treatment for 3 months, α-tocopherol levels were increased in both group and did not show a significant different from normal subjects (32.58 ± 1.36 and 33.13 ± 1.56 *vs*. 46.51 ± 5.08 nmol/ml; p = 0.06 and p= 0.120, respectively). However, the levels were lower than normal again when treatment for 6 months (26.53 ± 1.53 and 28.37 + 1.65 *vs*. 46.51 ± 5.08 nmol/ml; p = 0.018 and p= 0.048, respectively). When calculated by paired t-test, data shown a significant increased in placebo group at 3 month when compared to baseline (33.13 ± 1.56 *vs*. 27.62 ± 0.98 nmol/ml; p = 0.000) and those at 6 month significant decreased in placebo group when compared with 3 month (p = 0.031). While, α-tocopherol levels in curcuminoids group increase significantly at 3 month when compared to base line (p = 0.001) and those at 6 month significant decreased in curcuminoids group when compared to those at 3 month (26.53 ± 1.53 *vs*. 32.58 ± 1.36 nmol/ml; p = 0.003, respectively).

When compare between curcuminoid and placebo group, the α-tocopherol levels at baseline, 3 and 6 months were not significantly different In addition, data analysis by repeated measures ANOVA on baseline, 3 and 6 months also showed no significantly difference between the curcuminoid and placebo treated group (p = 0.59) (Figure 4.2).

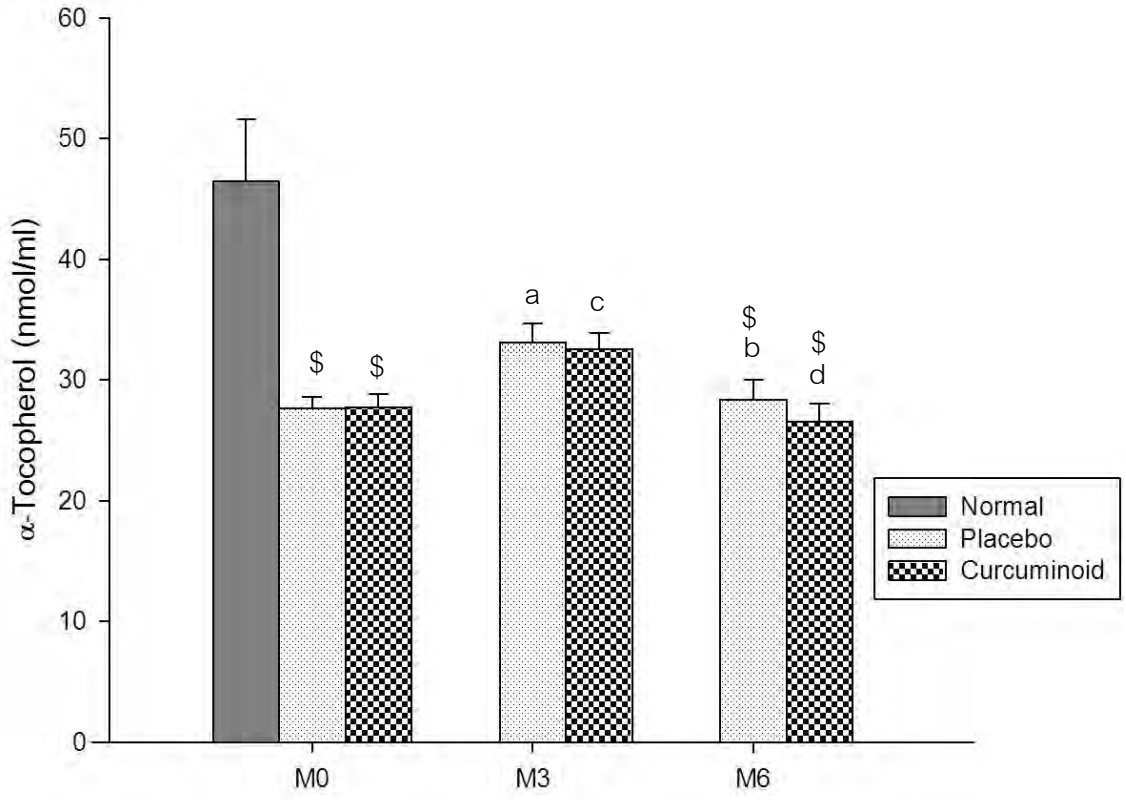


Figure 4.2 Levels of α-tocopherol (mean ± S.E.M) in plasma of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[$] $p < 0.05$ significantly difference from normal subjects (Student's *t* test)

[a] $p < 0.05$ significantly difference from base line in placebo group (Paired t-test)

[b] $p < 0.05$ significantly difference from 3 month in placebo group

[c] $p < 0.05$ significantly difference from base line in curcuminoid group

[d] $p < 0.05$ significantly difference from 3 month in curcuminoid group

4.2.3 Ferric reducing ability of plasma (FRAP) assay

FRAP assay determine the total antioxidant capacity as an indicator of oxidative stress. FRAP summarizes the overall activity of antioxidant vitamins and enzymes. The FRAP levels at baseline, 3 and 6 months were not significantly different between the curcuminoids group and placebo group. In addition, data analysis by repeated measures ANOVA among baseline, 3 and 6 months also showed no significantly difference between curcuminoid and placebo group ($p = 0.31$).

When calculated by paired t-test, data shown a significant decreased in placebo group at 6 month when compared to baseline (731.69 ± 23.13 *vs*. 804.09 ± 18.25 μmol/l; $p = 0.003$) and those at 6 month significant decreased in placebo group when compared with 3 month (731.69 ± 23.13 *vs*.785.60 ± 19.51 μmol/l; $p = 0.010$). While, FRAP levels in curcuminoids group decreased significantly at 6 month when compared to base line (689.46 ± 21.41 *vs*. 791.94 ± 20.36 μmol/l; $p = 0.000$) and those at 6 month significant decreased in curcuminoids group when compared with 3 month (689.46 ± 21.41 *vs*.767.43 ± 20.22 μmol/l; $p = 0.000$).

When compare between placebo group and normal subjects, FRAP levels at baseline significantly increased (804.09 ± 18.25 *vs*. 614.70 ± 28.92 μmol/l; $p = 0.006$) (Figure 4.3). Although FRAP levels at baseline in curcuminoids group did not show a significant difference from normal levels, when the treatment continue to 6 months, FRAP levels in both group could decrease to normal levels.

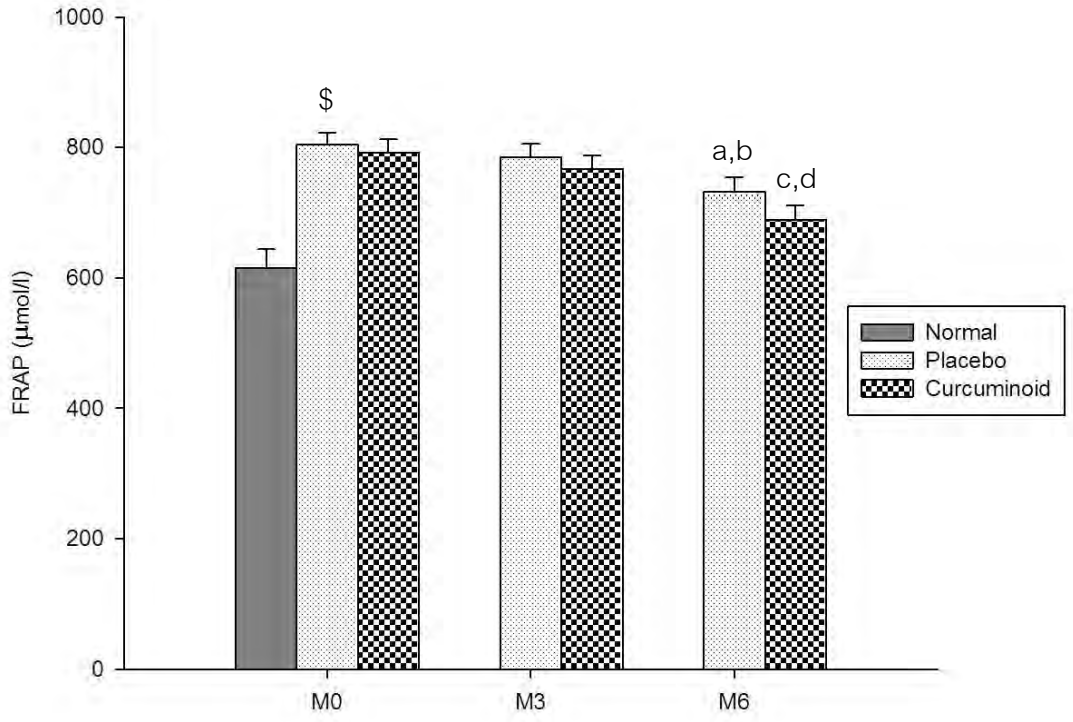


Figure 4.3 Levels of FRAP (mean ± S.E.M) in plasma of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[$] $p < 0.05$ significantly difference from normal subjects (Student's *t* test)

[a] $p < 0.05$ significantly difference from base line in placebo group (Paired t-test)

[b] $p < 0.05$ significantly difference from 3 month in placebo group

[c] $p < 0.05$ significantly difference from base line in curcuminoid group

[d] $p < 0.05$ significantly difference from 3 month in curcuminoid group

Part III: Antioxidant enzyme activity

4.3.1 Superoxide dismutase activity

The SOD activities at baseline and 3 month were not significantly different between the curcuminoids group and placebo group. Interestingly, the significantly increase of SOD activities in curcuminoids group compare to placebo at 6 month were found (2166.92 $\pm$ 67.19 *vs*. 1935.94 $\pm$ 50.06 U/gHb; p = 0.007). In addition, data analysis by repeated measures ANOVA among baseline, 3 and 6 months also showed significantly increase of SOD activities in curcuminoids group compare to placebo groups (p = 0.033).

When calculated by paired t-test, data showed a significant increase of SOD activities in placebo group at 3 and 6 month when compared to baseline (1460.87 $\pm$ 48.86, 1935.94 $\pm$ 50.06 *vs*. 1110.27 $\pm$ 34.62 U/gHb; p = 0.000, respectively) and those at 6 month significant increased in placebo group when compared to those at 3 month (1935.94 $\pm$ 50.06 *vs*. 1460.87 $\pm$ 48.86 U/gHb; p = 0.000). Same as placebo, SOD activities in curcuminoids group significantly increased at 3 and 6 month when compared to base line (1572.47 $\pm$ 66.29, 2166.92 $\pm$ 67.19 *vs*.1183.87 $\pm$ 42.07 U/gHb; p = 0.000) and those at 3 and 6 month were significantly difference (1572.47 $\pm$ 66.29 *vs*. 2166.92 $\pm$ 67.19 U/gHb; p = 0.000).

When compare placebo and curcuminoids group with normal subjects, SOD activities significantly increased at 6 months (1935.94 $\pm$ 50.06 and 2166.92 $\pm$ 67.19 *vs*. 1083.48 $\pm$ 135.03 U/gHb; p = 0.003, p = 0.004, respectively) (figure 4.4).

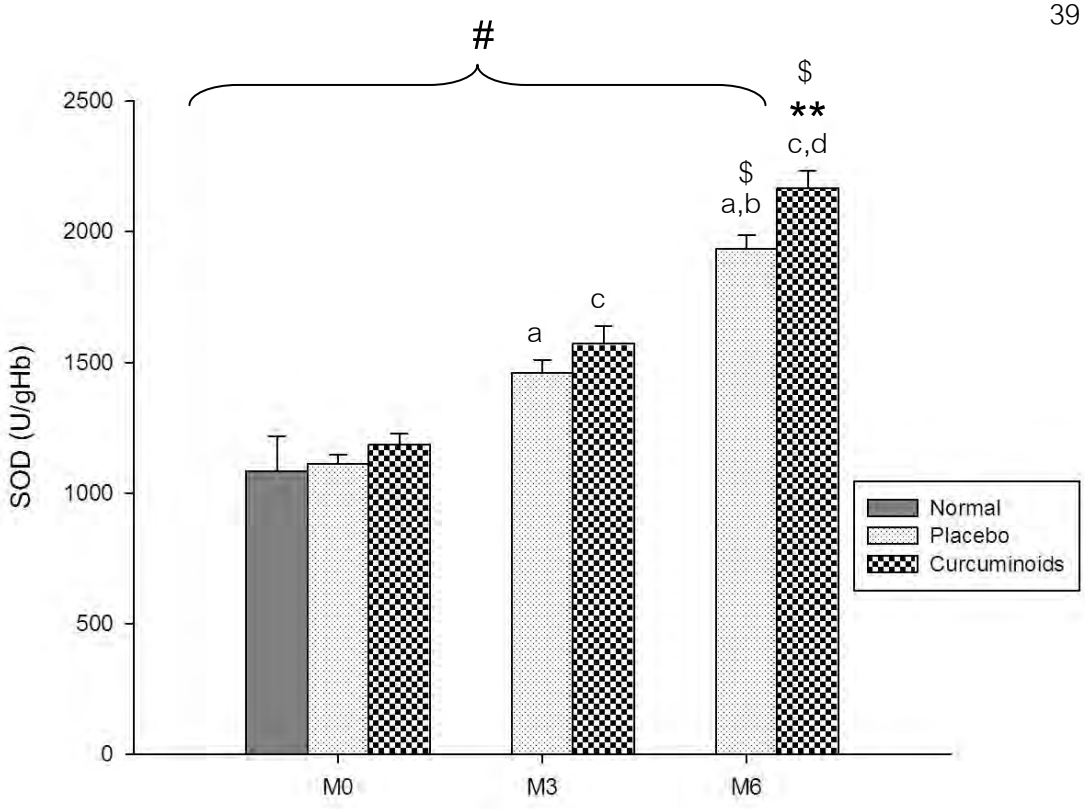


Figure 4.4 SOD activity (mean ± S.E.M) in red blood cells of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

***P* < 0.01 significantly difference from placebo group in the same month. (Student's *t* test)

[$] *p* < 0.05 significantly difference from normal subjects (Student's *t* test)

[#] *p* < 0.05 significantly difference from placebo group. (Repeated measures ANOVA)

[a] *p*< 0.05 significantly difference from base line in placebo group (Paired t-test)

[b] *p* < 0.05 significantly difference from 3 month in placebo group

[c] *p* < 0.05 significantly difference from base line in curcuminoid group

[d] *p* < 0.05 significantly difference from 3 month in curcuminoid group

4.3.2 Catalase activity

The CAT activities at baseline, 3 and 6 months were not significantly different between the curcuminoids group and placebo group. In addition, data analysis by repeated measures ANOVA on baseline, 3 and 6 months also showed no significantly difference between curcuminoid and placebo group (p = 0.32). When calculated by paired t-test, data shown a significant decreased in placebo group at 3 and 6 month when compared to baseline (6.34 $\pm$ 0.13, 6.55 $\pm$ 0.09 *vs*.7.21 $\pm$ 0.10 KU/gHb; p = 0.000). Same as placebo, CAT activities in curcuminoids group significant decrease at 3 and 6 months when compared to base line (6.47 $\pm$ 0.11, 6.67 $\pm$ 0.09 *vs*. 7.30 $\pm$ 0.11 KU/gHb; p = 0.000). (Figure 4.5)

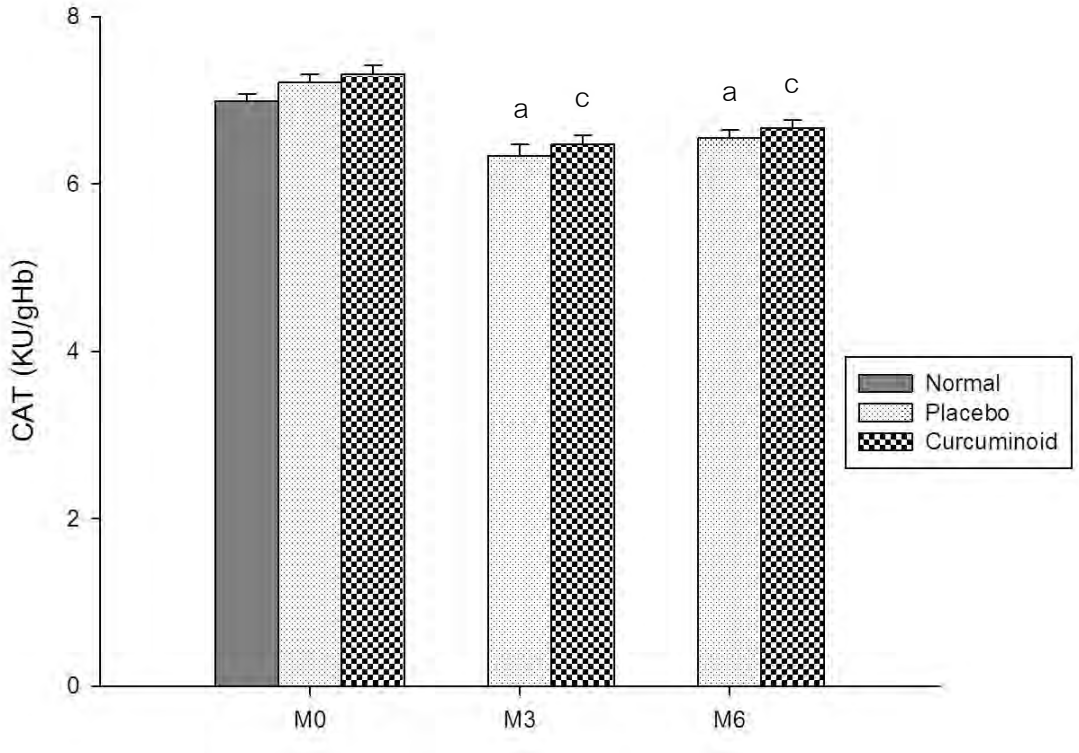


Figure 4.5 CAT activity (mean $\pm$ S.E.M) in red blood cells of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[a] $p < 0.05$ significantly difference from base line in placebo group (Paired t-test)

[c] $p < 0.05$ significantly difference from base line in curcuminoid group

4.3.3 Total glutathione (GSH) level

Glutathione is an antioxidant that has been widely used as an index of oxidative stress. The total glutathione levels in curcuminoids group at baseline, 3 and 6 months were not significantly different compare to placebo groups. In addition, data analysis by repeated measures ANOVA on baseline, 3 and 6 months showed significantly increase between curcuminoids group compare to placebo groups (p = 0.01). When calculated by Student t-test, data showed that total glutathione levels at baseline both in placebo and curcuminoids group were dramatically decreased when compared with normal subjects (1.63 ± 0.14 and 1.97 ± 0.13 *vs*. 6.87 ± 0.23 μmol/gHb; p = 0.000, respectively). However, when treatment continues to 3 and 6 months, these total glutathione levels were still much lower than those in normal subjects (1.99 ± 0.21 and 1.78 ± 0.12 in placebo group; 2.32 ± 0.21and 2.07 ± 0.13 in curcuminoids) (figure 4.6).

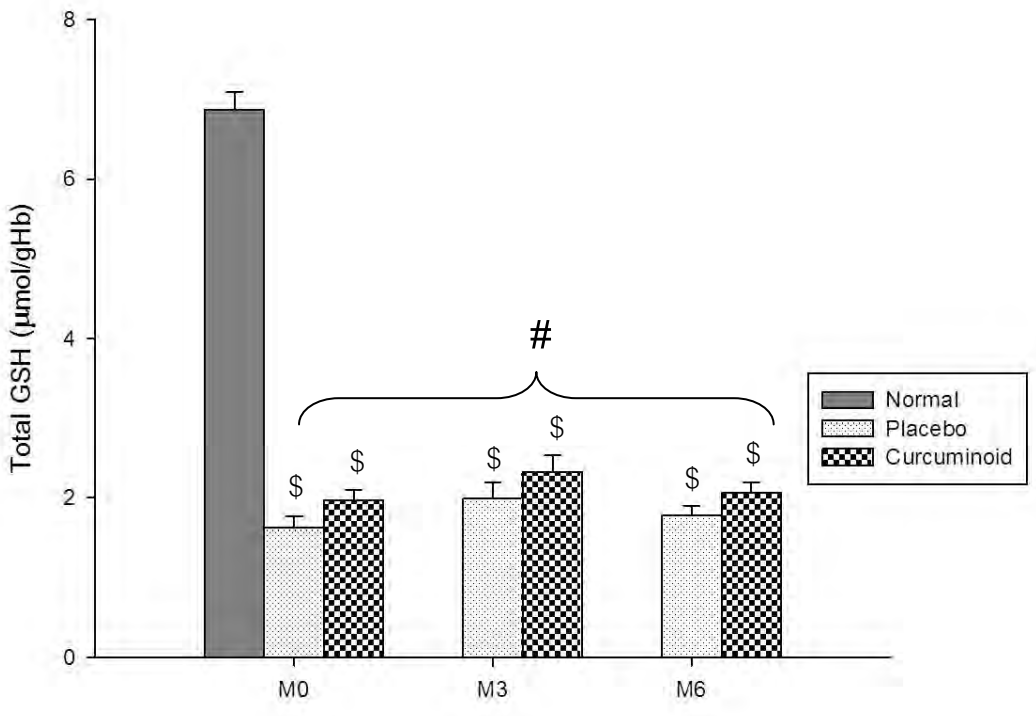


Figure 4.6 Levels of total GSH (mean ± S.E.M) in red blood cells of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[#] $p < 0.05$ significantly difference from placebo group. (Repeated measures ANOVA)

[$] $p < 0.05$ significantly difference from normal subjects (Student's *t* test)

4.3.4 Glutathione peroxidase activity

The activities of GPx at baseline, 3 and 6 months were not significantly different between the curcuminoids and placebo group. In addition, data analysis by repeated measures ANOVA on baseline, 3 and 6 months also showed no significantly difference between curcuminoids and placebo group (p = 0.29). When calculated by paired t-test, data showed a significant decrease in placebo group at 3 and 6 months when compared to baseline (25.65 $\pm$ 0.68 and 25.82 $\pm$ 0.56 *vs*. 27.16 $\pm$ 0.63 U/gHb; p = 0.001, p = 0.000, respetively). While, GPx acivities in curcuminoids group were significant decreased at 3 and 6 months when compared to base line (26.74 $\pm$ 0.74 and 26.78 $\pm$ 0.70 *vs*.27.95 $\pm$ 0.74 U/gHb; p = 0.006, p = 0.001, respectively). (Figure 4.7)

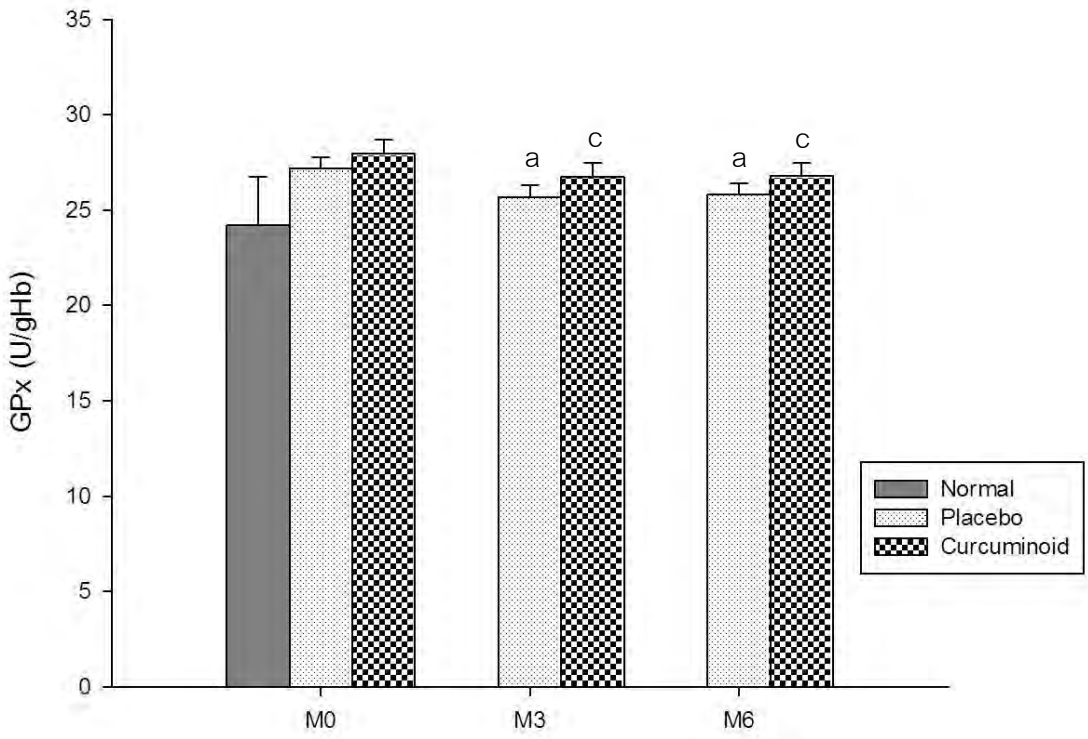


Figure 4.7 GPx activity (mean $\pm$ S.E.M) in red blood cells of type II diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 months.

[a] $p < 0.05$ significantly difference from base line in placebo group (Paired t-test)

[c] $p < 0.05$ significantly difference from base line in curcuminoid group

CHAPTER V

DISCUSSION AND CONCLUSION

oxidative stress play an important role in the pathogenesis of diabetic complications since hyperglycemia cause increased production of free radicals leading to increase oxidative stress (Willems et al., 1998). The present study was evaluated the effect of curcuminoids extract capsules on oxidative stress and anti-oxidant enzyme in the patients suffering from NIDDM. Clinical characteristics of diabetic patients at baseline such as sex, age, BMI, weight, fasting plasma glucose (FPG), cholesterol, triglyceride, and HDL were not significantly different between curcuminoids and placebo groups. In the present investigation, we measured oxidative stress markers including MDA, FRAP and α-tocopherol in plasma and antioxidant enzyme in red blood cells such as superoxide dismutase, catalase, glutathione peroxidase, and total glutathione of diabetic patients.

Oxidative stress in plasma

Oxidative stress could be evaluated by various indices such as MDA, an end product of lipid peroxidation (Firoozrai et al., 2007) and FRAP. Lipid peroxidation is an important biological consequence of oxidative cellular damage in patients with DM (Lapolla et al., 2005). FRAP levels, the parameter of total plasma anti-oxidant capacity, revealed a good achievement of oxidative stress in several pathologies and in particular in the diabetes mellitus (Bonnefont-Rousselot et al., 2000).

In the present study, oxidative stress in type II diabetic patients was existed before participating, by occurring the increase of FRAP level, the depletion of α-tocopherol, and total GSH at baseline in both diabetic patient groups compare to normal subjects (see in Table 4.3). Oxidative stress might be generated by free radicals that being continuously produced in the body by different mechanisms which can oxidize multiple fatty acid side chains to lipid peroxides (Gutteridge, 1995). These peroxidation reactions were countered by antioxidants present in plasma and red blood cells. Among the endogeneous antioxidants, α-tocopherol was the most important

vitamin and nonenzymatic chain-breaking lipid soluble antioxidant with a particular function of scavenging peroxyl radicals to prevent lipid peroxidation (Byer and Bowman, 1993; MacDonald-Wicks and Garg, 2003). Consistent to this result, the decrease of α-tocopherol in NIDDM patients compared to normal subjects (Ramakrishna and Jailkhani, 2008; Nourooz-Zadeh et al., 1997) were also reported.

This study did not show a significant difference in plasma level of MDA between type II diabetic and normal subjects, due to the small number of normal subjects in this study. Nakhjavani et al have reported the higher level of MDA in diabetic patient than those in normal control (3.45 ± 0.87 *vs*. 2.91 ± 0.59, P<0.001), these MDA level was similar to the present study both in diabetic patient and normal (Table 4.3). So regarding to the previous report of normal level, the MDA level in this type II diabetes were higher than normal subjects in general. Several studies have showed the elevated levels of MDA in plasma of type II diabetic subjects (Noberasco et al., 1991; Nacitarhan et al., 1995; Nakhjavani et. al., 2010). Since type II diabetes patients recruited in this study have not seen any cardiovascular complications, and MDA levels was not so high, suggesting the mildly oxidative stress status was occurred in plasma.

The increasing in FRAP level in type II diabetic was revealed. The level of FRAP in diabetic patients were higher than normal subjects, indicating that a compensatory increased of the antioxidant status is induced as a response to free radical over production in type II diabetes. However, one study reported the existence of increased total antioxidant power in the presence of normal lipid peroxidation in plasma and saliva of type I diabetic patients indicated the existence of oxidative stress (Astaneie et al., 2005). In alloxan-induced diabetic rats, the FRAP value showed an elevated in comparison with normal group (Houcher et al., 2007). So this study recommended MDA level, FRAP value and α-tocopherol level used as appropriated oxidative stress indicators in plasma of diabetic patients

When supplementation with curcuminoids extracts capsules, the MDA, FRAP and α-tocopherol at 3 and 6 months of treatment were measured. MDA showed

significantly decreased in both diabetic groups compared with baseline and those at 6 month decreased significantly compared with 3 month. While the increasing of α-tocopherol levels in both group were not showed a significant different from normal subjects at 3 months of treatment. However, the levels of α-tocopherol were lower than normal subjects again when treatment for 6 months. When the treatment continue to 6 months, FRAP levels in both group could decrease to normal levels when compared with normal subjects, indicating a reduction of oxidative status at 3 and 6 months after treatment with curcuminoids or placebo capsule in plasma of type II diabetic patient. In addition MDA, FRAP and α-tocopherol at baseline 3 and 6 months were not significant difference in curcuminoids group compare to placebo group. These results indicated that a reduction of oxidative status in diabetic type II patients was not affected by curcumnoids extract, because alterations of oxidative stress status were not found between curcuminoids and placebo group. The result was inconsistent with previous studies, in patients with tropical pancreatitis after treatment with 500 mg of curcumin plus 5 mg of piperine for 6 week, there was a significant reduction in the erythrocyte MDA level in the curcumin group compared to the placebo group (Durgaprasad et al., 2005). In preclinical study, curcuminoids was capable to protect against lead-induced neurotoxicity by reduced the lipid peroxidation when co-incubation of the neurons with the curcuminoids (Dairam et al., 2007).

The curcuminoids extract has no significant effect on oxidative stress status in plasma of this type II diabetes. The reason to explain this result may be due to the low bioavailability of curcumin. Shoba et al. reported that piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects.

However, in type II diabetic patients that participating in this study was treated with oral hypoglycemic drugs resulting in improvements oxidative stress status. Because hypoglycemic drug seems to have antioxidant properties such as glibenclamide, a member of the second generation sulfonylureas have restored liver CAT and SOD in STZ-induced diabetic rat. Glipizide seem to play a prominent role in

scavenging free radicals and restoring antioxidant activities in the tissues of diabetic animals. (Rahim et al., 2005). In gliclazide-treated type II diabetic patients were significant improvements in the oxidative status by increase SOD in red blood cell (Jennings and Belch, 2001).

This study demonstrated that oxidative stress occurred in the plasma of diabetic patients was controllable, might be due to patients receiving hypoglycemic drugs for treatment, therefore a decrease in MDA level or altered in FRAP and α-tocopherol between curcuminoid and placebo group at 3 and 6 months were not found.

Antioxidant enzyme in red blood cell

The most important defense mechanism against free radicals in the body is mediated by the actions of antioxidants. The main system of defense against damage from free radicals is an enzymatic system that opposes oxidation. Antioxidant enzymes primarily account for intracellular defense. The three major antioxidant enzymes are SOD, GPx and CAT. The SOD catalyzes the destruction of the O_2^- free radical. It protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals, a less reactive ROS. The GPx to convert hydrogen peroxide to water by using reduced glutathione as a hydrogen donor (Maritim et al., 2003). The enzyme CAT has a predominant role in defending against the oxidative damage caused by high concentration of hydrogen peroxide. Hydrogen peroxide is a byproduct of normal cellular respiration and it also formed from superoxide anion, which is also a free radical. The GSH functions as a direct free-radical scavenger. It is an important scavenger of free radicals and a potent endogenous antioxidant which helps to protect cells from oxidative injury. Besides its role in the maintenance of the redox potential within the cell, it is also a key component of the enzymatic antioxidant system. The enhance free radical generation in hyperglycemic condition, can also directly interact with intracellular reduced GSH and result in its depletion. Measurement of these antioxidants can be used for evaluating oxidative stress in red blood cells of diabetic patients.

In this study, the antioxidant enzymes activity in red blood cell including CAT, GPx and SOD were not significantly differenced from normal subjects (see in Table 4.3). While, total GSH levels at baseline both in placebo and curcuminoids group were dramatically decreased when compared with normal subjects, indicating that GSH was used to ameliorative biological system from oxidative stress by directly interact with free radical generation in hyperglycemic condition. The antioxidant enzyme activities in diabetes have been reported in several studies which may be decreased or unchanged depended on various conditions such as the glycemic control, duration and complication of DM. The erythrocyte SOD and GPx activities in fairly controlled group of type II diabetic patients were not significant difference with normal control group (Likidlilid et al., 2010). The erythrocyte GPx activity was significantly lower in diabetic patients compared with normal control (Martin-Gallan et al., 2003). In alloxan-induced diabetic rats, the SOD, CAT, and GPx activities in the liver and pancreas in diabetic rats significantly decreased as compared to normal rats (Hamden et al., 2009). In the other hand GPx activity was not significantly different in IDDM compared to normal subjects (Firoozrai et al., 2007). Hyperglycemia induces over production of oxygen free radicals in diabetes to cause the decrease levels of enzymatic (GPx, SOD, catalase in RBC) and non enzymatic antioxidants (β-carotene, retinol, vitamin C & E and uric acid) of RBC in NIDDM patients (Ramakrishna and Jailkhani, 2008).

When supplementation with curcuminoids extracts capsules, the data showed a significant increase of SOD activities both curcuminoids and placebo group at 3 and 6 month compared to baseline. While, the CAT and GPx activities showed significantly decrease in both curcuminoids and placebo group at 3 month and 6 month of treatment compare to baseline. However, the total glutathione levels in both curcuminoids and placebo group were not booster but still much lower than those in normal subjects. The alteration of these antioxidative enzymes at 3 and 6 months in red blood cells of type II diabetic patients were found after treatment with either curcuminoids or placebo capsules. In addition, there were no significantly difference in CAT, GPx, and total GSH between curcuminoids and placebo group. These results indicated that alterations of CAT, GPx and total GSH in diabetic type II patients were not affected by

supplementation with curcumnoids extract capsules. While, the SOD activity in curcuminoids group were significantly increased after treatment up to 6 month compare with placebo group. So this study suggested that curcuminoids can ameliorated the oxidative status by increase SOD activity in type II diabetic patients. The finding of some study also demonstrated that the diabetes-associated reductions in antioxidant enzymes can be ameliorated by insulin and/or antioxidant therapy (Sindhu et al., 2004). In addition, these results were consistent with previous studies in the cell models that curcumin could increase the level of SOD on 6-hydroxydopamine-induced cytotoxicity in MES23.5 cell, which inhibited the oxidative stress (Wang et al., 2009). In animal models activities of SOD were significantly decrease in streptozotocin (STZ)-induced oxidative stress in albino wistar rats which also restored to normal after curcumin treatment (Hussein and Abu-Zinadah, 2010). In cyclosporine induced renal dysfunction and oxidative stress in rat kidneys which was effectively reversed by curcumin treatment (Tirkey et al., 2005). In streptozotocin-induced diabetic rat the results showed that SOD activity was significantly lower in red blood cells. Interestingly, feeding curcumin and turmeric to the diabetic animals prevented decrease of SOD activities in red blood cells (Suryanarayana et al., 2007). Curcumin can significantly reduce the elevated MDA levels and increase SOD activities in rat induced oxidative renal damage by acetaminophen (Cekmen et al., 2009).

This study demonstrated that curcuminoids may be assisting to boosting SOD activities in scavenging free radical. However, the mechanisms of curcuminoids on SOD for scavenging free radical were remains unclear. The increased SOD activity may be due to the increased expression of SOD gene product by curcumin treatment (Thiyagarajan and Sharma, 2004). Shahed and coworkers (2001) have been reported that curcumin up-regulates the SOD gene expression in rat kidney after ischemia/reperfusion injury. In myocardial ischemia in rat, the elevated level of percent xanthine oxidase activity in the diseased group was shown to be effectively counteracted by administration of curcumin (Manikandan et al., 2004).

Limitations and suggestion of this study

Limitations of this study were the treatment with various hypoglycemic drugs, which can affect the oxidative stress conditions. Further study should design to be matching hypoglycemic drugs, age, gender both curcuminoids and placebo groups. Another suggestion was doses of curcuminoids, in this trial doses may be insufficient due to poor absorption, rapid metabolism and rapid systemic elimination of curcuminoids (Anand et al., 2007). Increase doses of curcuminoids or use of adjuvant like piperine that interferes with glucuronidation to improve the bioavailability was suggested. There were likely to see changes of other parameter. In β-thalassemia/Hb E patients, treatment with curcuminoids 500 mg daily for 12 months (Kalpravidh et al., 2010) could ameliorate oxidative damage. Unlike in diabetes patients, no medications treated for thalassemia except folic acid, so the confounding factor was minimized and the effects of curcuminoids on antioxidant enzymes were obvious. Moreover, thalassemia patients were excess iron overload leading to fenton reaction; the chelating properties of curcuminoids were act as free-radical scavengers. Then excess iron overload could be inhibiting result improvement of oxidative stress status (Arezzini et al., 2004).

Conclusion

In conclusion, this study demonstrated that supplementation with curcuminoids extract capsules have a potential role in enhancing effect on cellular antioxidant defense by boosting superoxide dismutase activity which are antioxidant-related defenses and scavenging ROS in diabetic patients. Additional therapy with curcuminoids could possibly help reduce free radical activity, though, CAT, total GSH and GPx were not affected by curcuminoids extract capsules. So this study suggested that curcuminoids extract capsules, may be benefit as a supplementary antioxidant approach to the management of type II diabetes mellitus patients.

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APPENDICES

APPENDIX A

1. Validation of FRAP assay

Inter-run precision % CV of the assay for measure of FRAP level of $FeSO_4.7H_2O$ and normal subject control

Std. FRAP conc. (μmol/l)	Abs.	Abs.	Abs.	Abs.	Abs.	Mean	SD	%CV
0	0.050	0.058	0.056	0.058	0.055			
0	0.048	0.057	0.059	0.056	0.054	0.055	0.004	6.472
300	0.246	0.256	0.261	0.256	0.256			
300	0.243	0.257	0.258	0.256	0.261	0.255	0.006	2.328
600	0.453	0.461	0.465	0.458	0.462			
600	0.447	0.453	0.464	0.457	0.457	0.458	0.005	1.179
900	0.653	0.668	0.672	0.670	0.684			
900	0.638	0.650	0.669	0.652	0.652	0.661	0.014	2.061
1200	0.859	0.847	0.864	0.856	0.859			
1200	0.832	0.847	0.857	0.863	0.859	0.854	0.010	1.129
1500	1.035	1.056	1.053	1.073	1.024			
1500	1.049	1.044	1.053	1.061	1.028	1.048	0.015	1.445
1800	1.258	1.252	1.261	1.244	1.291			
1800	1.225	1.248	1.243	1.243	1.266	1.253	0.018	1.406
2100	1.446	1.452	1.465	1.457	1.457			
2100	1.429	1.429	1.438	1.445	1.453	1.447	0.012	0.838

FRAP normal subject control (μmol/l)

Control	1	2	3	4	5	Mean	SD	%CV
N1	667.69	662.93	670.10	677.53	660.04	667.66	6.78	1.02
N2	563.16	566.26	571.12	576.96	562.95	568.09	5.96	1.05
N3	609.95	601.82	612.52	602.47	614.95	608.34	5.93	0.97

2. Validation of TBARs assay

Inter-run precision % CV of the assay for measure of MDA level in standard TEP and normal subject control

Std. MDA conc. (nmol/ml)	Intensity	Intensity	Intensity	Intensity	Intensity	Mean	SD	%CV
0.1	22.77	19.42	22.46	22.89	24.95			
0.1	22.26	22.72	24.99	23.43	24.02	22.99	1.59	6.92
0.2	45.18	41.61	50.90	49.38	47.93			
0.2	44.39	53.68	48.15	48.32	48.94	47.85	3.42	7.14
0.4	84.34	83.41	102.80	93.28	91.43			
0.4	88.43	91.03	87.88	98.31	95.02	91.59	6.05	6.60
0.8	170.60	164.00	195.90	188.00	193.00			
0.8	170.00	172.90	163.40	180.50	182.80	178.11	11.68	6.56
1.2	235.30	255.90	270.70	276.50	271.20			
1.2	271.70	255.50	262.20	256.20	260.00	261.52	11.98	4.58
2.4	511.40	502.00	508.30	522.20	539.40			
2.4	474.70	504.00	543.60	508.90	510.90	512.54	19.52	3.81
3.6	741.20	736.40	729.30	770.40	775.80			
3.6	722.40	723.00	774.50	737.50	768.20	747.87	21.87	2.92

MDA normal subject control (nmol/ml)

Control	1	2	3	4	5	Mean	SD	%CV
N1	3.00	3.04	3.26	3.36	2.95	3.12	0.18	5.69
N2	2.54	2.64	2.41	2.34	2.80	2.55	0.18	7.16
N3	3.51	3.12	3.65	3.43	3.08	3.36	0.25	7.35
N4	2.67	2.57	2.72	2.33	3.00	2.66	0.24	9.03

3. Validation of α-tocopherol

Inter-run precision % CV of the assay for measure of α-tocopherol level of standard α-tocopherol and normal subject control

Std.α-tocopherol conc. (nmol/ml)	AUC	AUC	AUC	AUC	AUC	Mean	SD	%CV
12.5	1953	2218	2246	2027	2100	2108.8	124.29	5.89
25	4254	5130	4242	4557	4302	4497.0	376.29	8.37
50	8950	9636	8084	9336	9211	9043.4	590.35	6.53
100	19359	20315	18461	18764	19595	19298.8	726.63	3.77
150	29200	31147	26481	29740	27483	28810.2	1848.22	6.42
200	36969	39996	36535	37643	35229	37274.4	1758.31	4.72

α-tocopherol normal subject control (nmol/ml)

Control	1	2	3	Mean	SD	%CV
N1	34.09	36.03	40.98	37.04	3.55	9.59
N2	51.82	43.40	48.88	48.03	4.27	8.89
N3	56.86	55.29	51.19	54.45	2.93	5.38

4. Validation of SOD assay

Inter-run precision % CV of the assay for measure of SOD activity of standard SOD and normal subject control

Std.SOD conc. (U/ml)	Rate	Rate	Rate	Rate	Rate	Mean	SD	%CV
10	0.003	0.002	0.002	0.002	0.002			
10	0.002	0.003	0.002	0.003	0.002	0.002	0.000	16.87
5	0.009	0.012	0.009	0.007	0.007			
5	0.010	0.012	0.009	0.007	0.007	0.009	0.002	19.02
2	0.028	0.030	0.028	0.027	0.026			
2	0.030	0.030	0.028	0.025	0.027	0.028	0.002	6.18
1	0.043	0.047	0.044	0.039	0.041			
1	0.043	0.046	0.046	0.039	0.037	0.043	0.003	7.72
0.5	0.064	0.062	0.057	0.055	0.052			
0.5	0.069	0.061	0.054	0.054	0.053	0.058	0.006	9.67
0.2	0.074	0.081	0.074	0.068	0.071			
0.2	0.081	0.084	0.081	0.073	0.070	0.076	0.006	7.31
0.1	0.081	0.096	0.090	0.086	0.082			
0.1	0.082	0.093	0.092	0.088	0.088	0.088	0.005	5.98

SOD normal subject control (U/gHb)

Control	1	2	3	4	5	Mean	SD	%CV
N1	992.48	1003.18	963.33	1120.84	1161.68	1048.30	87.30	8.33
N2	842.38	850.63	888.07	818.96	945.89	869.19	49.56	5.70
N3	1306.49	1242.30	1449.32	1190.80	1475.83	1332.95	125.58	9.42

5. Validation of CAT assay

Inter-run precision % CV of the assay for measure of CAT (KU/gHb) activity of normal subject control

Control	1	2	3	4	5	Mean	SD	%CV
N1	6.82	6.85	6.79	6.90	6.96	6.86	0.07	0.96
N2	7.19	7.14	7.22	7.11	7.03	7.14	0.08	1.06
N3	7.00	6.95	6.98	7.06	6.89	6.98	0.06	0.87

6. Validation of total GSH

Inter-run precision % CV of the assay for measure of standard GSH and normal subject control

Std.GSH conc. (μmol/l)	Rate	Rate	Rate	Rate	Rate	Mean	SD	%CV
0	0.004	0.004	0.004	0.003	0.003			
0	0.004	0.004	0.003	0.003	0.003	0.004	0.0003	9.12
0.1	0.005	0.005	0.005	0.005	0.005			
0.1	0.005	0.005	0.005	0.004	0.004	0.005	0.0004	7.61
0.25	0.008	0.007	0.007	0.007	0.007			
0.25	0.008	0.007	0.007	0.006	0.007	0.007	0.0004	6.09
0.5	0.011	0.010	0.010	0.009	0.009			
0.5	0.010	0.010	0.010	0.009	0.009	0.010	0.0006	6.26
1.5	0.021	0.021	0.020	0.018	0.018			
1.5	0.022	0.021	0.020	0.019	0.019	0.020	0.0013	6.73
3	0.038	0.038	0.036	0.034	0.036			
3	0.040	0.041	0.038	0.038	0.039	0.038	0.0020	5.34
6	0.068	0.070	0.065	0.062	0.068			
6	0.065	0.068	0.059	0.057	0.063	0.064	0.0041	6.44
9	0.092	0.095	0.082	0.079	0.088			
9	0.088	0.093	0.082	0.080	0.090	0.087	0.0058	6.69
12	0.114	0.115	0.103	0.101	0.111			
12	0.115	0.119	0.104	0.101	0.112	0.109	0.0067	6.11
16	0.139	0.146	0.128	0.127	0.139			
16	0.141	0.144	0.126	0.127	0.138	0.135	0.0078	5.75
20	0.158	0.163	0.151	0.149	0.163			
20	0.159	0.161	0.154	0.152	0.165	0.157	0.0056	3.53

Total GSH normal subject control (μmol/gHb)

Control	1	2	3	Mean	SD	%CV
N1	7.14	7.42	7.41	7.32	0.16	2.19
N2	6.50	6.45	7.00	6.65	0.30	4.56
N3	6.51	6.76	5.77	6.35	0.51	8.07
N4	7.41	6.56	7.53	7.17	0.53	7.40

7. Validation of GPx assay

Inter-run precision % CV of the assay for measure of GPx (U/gHb) activity of normal subject control

Control	1	2	3	4	5	Mean	SD	%CV
N1	19.60	18.08	19.90	20.28	22.87	20.15	1.74	8.61
N2	28.77	29.85	26.09	29.54	30.23	28.90	1.66	5.74
N3	22.95	23.70	21.08	25.58	24.38	23.54	1.68	7.14

8. Validation of Hemoglobin

Inter-run precision % CV of the assay for measure of hemoglobin of standard hemoglobin and normal subject control

Std.Hb conc. (mg/ml)	Abs	Abs	Abs	Abs	Mean	SD	%CV
0	0.000	0.000	0.000	0.000			
0	0.000	0.000	0.000	0.000	0.00	0.00	0.00
60	0.085	0.099	0.097	0.088			
60	0.086	0.100	0.098	0.089	0.09	0.01	6.81
120	0.173	0.200	0.195	0.177			
120	0.174	0.201	0.195	0.177	0.19	0.01	6.59
180	0.261	0.301	0.291	0.265			
180	0.261	0.301	0.292	0.265	0.28	0.02	6.51

Hb normal subjects control (mg/ml)

Control	1	2	3	4	5	Mean	SD	%CV
N1	424.63	422.53	426.74	418.32	430.95	424.63	4.71	1.11
N2	420.42	424.63	418.32	422.53	416.21	420.42	3.33	0.79
N3	430.95	435.16	424.63	422.53	437.26	430.11	6.42	1.49

APPENDIX B

1. FRAP level in plasma of type II diabetes mellitus

Group	FRAP (μmol/l)		
	M0	M3	M6
Placebo	804.09 ± 18.25	785.60 ± 19.51	731.69 ± 23.13
Curcuminoid	791.94 ± 20.36	767.43 ± 20.22	689.46 ± 21.41
P-value	0.657	0.518	0.183

2. MDA level in plasma of type II diabetes mellitus

Group	MDA (nmol/ml)		
	M0	M3	M6
Placebo	3.56 ± 0.09	2.87 ± 0.09	2.44 ± 0.10
Curcuminoid	3.54 ± 0.09	2.70 ± 0.07	2.27 ± 0.09
P-value	0.826	0.145	0.212

3. α-Tocopherol level in plasma of type II diabetes mellitus

Group	Vitamin E (nmol/ml)		
	M0	M3	M6
Placebo	27.62 ± 0.98	33.13 ± 1.56	28.37 ± 1.65
Curcuminoid	27.74 ± 1.14	32.58 ± 1.36	26.53 ± 1.53
P-value	0.935	0.790	0.415

4. SOD activity in red blood cells of type II diabetes mellitus

Group	SOD (U/gHb)		
	M0	M3	M6
Placebo	1110.27 ± 34.62	1460.87 ± 48.86	1935.94 ± 50.06
Curcuminoid	1183.87± 42.07	1572.47± 66.29	2166.92 ± 67.19
P-value	0.177	0.175	0.007

5. Catalase activity in red blood cells of type II diabetes mellitus

Group	Catalase (KU/gHb)		
	M0	M3	M6
Placebo	7.21 ± 0.10	6.34 ± 0.13	6.55 ± 0.09
Curcuminoid	7.30 ± 0.11	6.47 ± 0.11	6.67 ± 0.09
P-value	0.533	0.430	0.355

6. Total glutathione in red blood cells of type II diabetes mellitus

Group	Total Glutathione (μmol/gHb)		
	M0	M3	M6
Placebo	1.63 ± 0.14	1.99 ± 0.21	1.78 ± 0.12
Curcuminoid	1.97 ± 0.13	2.32 ± 0.21	2.07 ± 0.13
P-value	0.077	0.257	0.114

7. Glutathione peroxidase activity in red blood cells of type II diabetes mellitus

Group	GPx (U/gHb)		
	M0	M3	M6
Placebo	27.16 ± 0.63	25.65 ± 0.68	25.82 ± 0.56
Curcuminoid	27.95 ± 0.74	26.74 ± 0.74	26.78 ± 0.70
P-value	0.418	0.277	0.280

8. Hemoglobin level in red blood cells of type II diabetes mellitus

Group	Hb (mg/ml)		
	M0	M3	M6
Placebo	370.96 ± 4.09	405.18 ± 6.73	420.84 ± 4.76
Curcuminoid	371.60 ± 5.06	400.29 ± 4.82	415.62 ± 4.31
P-value	0.921	0.558	0.418

APPENDIX C

Subjects

Subjects were participated from two hundred patients with type II diabetes mellitus more than 35 years of age were recruited from the HRH Princess Maha Chakri Sirindhorn Medical Center. All patients were participated in the research project heading "Antiatherogenic and antimetabolic effect of curcumin in type 2 diabetic patients". All patients with inform consents were treated with the appropriate oral hypoglycemic drugs.

Intervention

Both curcuminoids extract and placebo capsules were produced and standardized by the Government Pharmaceutical Organization of Thailand, packing 250 mg per capsule. In addition, subjects received 2 capsules of either curcuminoids or placebo 3 times a day for 6 months, according to which group they were randomized to.

Inclusion criteria

Inclusion criteria were combined research project heading "Antiatherogenic and antimetabolic effect of curcumin in type 2 diabetic patient".

1. Diabetic patients 35 years of age or above, the level of blood sugar after fasting for 12 hours $\geq$ 126 mg/dl and less than 210 mg/dl and not using insulin, or metformin in treatment during the first 5 years after diagnosis of diabetes.
2. Cholesterol $\geq$ 200 mg/dl, TG $\geq$ 150 mg/dl, LDL $\geq$ 100 mg/dl, HDL $\leq$ 35 mg/dl
3. BP $\geq$ 130/85 mm Hg or take anti-hypertension drug.
4. BMI $\geq$ 25

Exclusion criteria

Exclusion criteria were combined research project heading "Anti artherogenic and anti metabolic effect of curcumin in type 2 diabetic patients".

1. Secondary peripheral arterial disease (PAD)

2. Cardiovascular diseases such as coronary arterial disease and cerebrovascular disease.
3. Renal failure and serum creatinine > 2.0 mg/dl or the dialysis.
4. Hepatitis with the ALT$\geq$ 3 times.
5. The patients received metformin hypoglycemic herbs or turmeric.
6. Type I diabetic patient and younger than 35 years of age.
7. High glucose condition from other causes or treatment steroids drugs, cancer, tumors of the pancreas, etc.
8. The patient had an underlying infection / inflammation that affect on the level of CRP.
9. Pregnant woman
10. Gallbladder disease or history of gallbladder surgery.

Ethical approval

This study was approved by the Srinakharinwirot University Ethics Committee for Human Research and written informed consent was obtained from all subjects.

The sample size calculation

$$n = \frac{2\delta^2[Z, - \alpha/2 + Z1 - \beta]^2}{(\mu 1 - \mu 2)^2}$$

α type I error = 0.05

β type II error = 0.20

δ = 0.019

$\mu 1 - \mu 2$ = 0.045

n = 93

n estimated = 100

n is the number of sample size in each experimental group.

n calculated using a formula from the Paired t-test.

62 หมู่ 7 อำเภอองครักษ์
จังหวัดนครนายก 26120
โทร.0-3739-5085 ต่อ 10513

เอกสารรับรองโครงการวิจัย

โดย

คณะกรรมการจริยธรรมสำหรับการพิจารณาโครงการวิจัยที่ทำในมนุษย์

SWUEC เลขที่หนังสือรับรอง 30/2550

ชื่อโครงการ	ประสิทธิผลของขมิ้นชันต่อการลดภาวะโรคหลอดเลือดแข็งตัวและการควบคุมภาวะเมตะบอลิกในผู้ป่วยเบาหวานชนิดที่ 2
	Antiatherogenic and Antimetabolic Effect of Curcumin in Type 2 Diabetic Patients
ชื่อหัวหน้าโครงการ / หน่วยงานที่สังกัด	พญ.สมลักษณ์ จึงสมาน / ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
SWUEC **รหัสโครงการ**	30/2550
สถานที่ทำการวิจัย	ศูนย์การแพทย์ฯ คณะแพทยศาสตร์ มศว
เอกสารรับรอง	- แบบเสนอโครงการวิจัยเพื่อขอรับการพิจารณา - หนังสือให้ความยินยอมเข้าร่วมโครงการ - คำชี้แจงอาสาสมัคร
รับรองโดย	คณะกรรมการจริยธรรมสำหรับการพิจารณาโครงการวิจัยที่ทำในมนุษย์ (6/2550) A
วันที่รับรอง	21 สิงหาคม 2550
วันหมดอายุ	20 สิงหาคม 2551

หนังสือรับรองฉบับนี้ออกโดยความเห็นชอบในการพิจารณาจากคณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ตามประกาศเฮลซิงกิ

ลงนาม.....
(รองศาสตราจารย์ นายแพทย์วันชัย บุพพันเหรัญ)
ประธานคณะกรรมการจริยธรรม ฯ

ลงนาม.....
(ศาสตราจารย์ นายแพทย์สมเกียรติ วัฒนศิริชัยกุล)
คณบดีคณะแพทยศาสตร์

62 หมู่ 7 อำเภอองครักษ์
จังหวัดนครนายก 26120
โทร.0-3739-5085 ต่อ 81516

เอกสารรับรองโครงการวิจัย

โดย

คณะกรรมการจริยธรรมสำหรับการพิจารณาโครงการวิจัยที่ทำในมนุษย์

SWUEC เลขที่หนังสือรับรอง 9/2552

ชื่อโครงการ	ประสิทธิผลของขมิ้นชัน ต่อการลดภาวะโรคหลอดเลือดแข็งตัวและการควบคุมภาวะเมตะบอลิก รวมทั้งผลต่อการป้องกันชะลอการเกิดโรคเบาหวานชนิดที่ 2ในกลุ่มผู้ป่วยที่เริ่มมีความผิดปกติของระดับน้ำตาลก่อนเป็นโรคเบาหวานและมีภาวะดื้อต่ออินซูลิน
	Antiatherogenic and Antimetabolic Effect of Curcumin therapy in the Prevention and Delay of Type 2 Diabetic in Patients with Impaired Glucose Tolerance and Insulin Resistance.
ชื่อหัวหน้าโครงการ / หน่วยงานที่สังกัด	ผู้ช่วยศาสตราจารย์ แพทย์หญิงสมลักษณ์ จึงสมาน / ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
SWUEC รหัสโครงการ	SWUEC 9/2552
สถานที่ทำการวิจัย	ศูนย์การแพทย์ฯ คณะแพทยศาสตร์ มศว
เอกสารรับรอง	- แบบเสนอโครงการวิจัยเพื่อขอรับการพิจารณา - หนังสือให้ความยินยอมเข้าร่วมโครงการ - เอกสารให้คำแนะนำผู้เข้าร่วมโครงการ - แบบบันทึกข้อมูลผู้เข้าร่วมโครงการวิจัย
รับรองโดย	คณะกรรมการจริยธรรมสำหรับการพิจารณาโครงการวิจัยที่ทำในมนุษย์ 1/2552
วันที่รับรอง	10 มีนาคม 2552
วันหมดอายุ	9 มีนาคม 2553

หนังสือรับรองฉบับนี้ออกโดยความเห็นชอบในการพิจารณาจากคณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ตามประกาศเฮลซิงกิ

ลงนาม.....
(รองศาสตราจารย์ นายแพทย์วันชัย บุพพันเหรัญ)
ประธานคณะกรรมการจริยธรรมฯ

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