PHARMACODYNAMIC STUDY OF CURCUMINOIDS IN NORMAL VOLUNTEERS

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Thesis
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PHARMACODYNAMIC STUDY OF CURCUMINOIDS IN NORMAL VOLUNTEERS.

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ABSTRACT

Free radicals or reactive oxygen species (ROS) now play an important role in many biochemical processes involving various diseases in humans such as cancer, neurological disease, and some genetic diseases, thalassemia etc. These reactive species oxidize all biomolecules, protein, lipids, and DNA which lead to damage of cell structures and functions. Normally, our body is protected against free radicals by enzymatic and non-enzymatic anti-oxidant defense systems. The anti-oxidant system also includes low molecular weight substances, plant polyphenols, vitamin C and E etc. Among the potential therapeutic anti-oxidants, curcuminoids are the herb of choice because of their various biological activities such as anti-oxidant, anti-inflammation and anti-cancer. We, therefore, evaluated their anti-oxidant effects *in vitro* and the pharmacodynamics of curcuminoids in 20 normal volunteers taking a single oral dose of 6 gram curcuminoids capsules. Each 250 mg curcumonoids capsule consisted of curcumin: demethoxycurcumin : bis-demethoxycurcumin in the ratio of 1 : 0.3 : 0.1

In vitro results showed that curcuminoids had better anti-oxidant properties than vitamin C and vitamin E. Curcuminoids can protect red blood cell lysis induced by 2,2´ azobis (2-methylpropioarnidine) dihydrochloride (AAPH).

Curcuminoids are quickly metabolized and bound to adipose tissue. The normal volunteers received folic acid for 14 days followed by another pharmacodynamic study of single oral 6 gram curcuminoids. The results did not differ from the previous experiment implying that folic acid did not have any effect.

Reactive oxygen species determined by flow cytometer revealed that ROS decreased with increasing time for the first hour after taking curcuminoids and then increased to maximum level at 24 h. The volunteers showed a slight decrease in malondialdehyde level after curcuminoids administration for 1 h and then returned to baseline level while the level of reduced glutathione (GSH) and malondialdehyde (MDA) were the same at every time point. All parameters evaluated before and after receiving folic acid did not show significant difference.

KEY WORDS : CURCUMIN / CURCUMINOIDS / PHARMACODYNAMICS / FREE RADICALS / ANTIOXIDANT

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บทคัดย่อ

ปัจจุบันอนุมูลอิสระ มีส่วนเกี่ยวข้องกับโรคต่างๆ ของคนเป็นจำนวนมาก เช่น โรคมะเร็ง, โรคทางสมอง, และโรคทางพันธุกรรมบางชนิด เช่น โรคธาลัสซีเมีย ทั้งนี้เพราะอนุมูลอิสระเหล่านี้ไปทำปฏิกิริยาออกซิเดชั่น กับสารชีวโมเลกุล เช่น โปรตีน, ไขมัน, และ ดี เอ็น เอ ในเซลล์ โดยปกติร่างกายของคนเรามีวิธีกำจัดอนุมูลอิสระด้วยสารต้านอนุมูลอิสระที่เป็นเอนไซม์ หรือสารที่มีน้ำหนักโมเลกุลต่ำ หรือได้จากอาหาร เช่น วิตามินซี และวิตามินอี เป็นต้น นอกจากนี้ยังมีสมุนไพรที่มีมากในประเทศที่มีฤทธิ์เป็นสารต้านอนุมูลอิสระที่ดีมาก ได้แก่ ขมิ้นชัน ผู้วิจัยจึงนำมาศึกษาฤทธิ์ของขมิ้นชันในหลอดทดลอง และ PHARMACODYNAMIC ในอาสาสมัครปกติจำนวน 20 คน ที่ได้รับขมิ้นชันแคปซูลครั้งเดียว 6 กรัม โดยขมิ้นชันแคปซูลที่นำมาใช้มีปริมาณเคอร์คิวมินอยส์อยู่แคปซูลละ 250 มก. โดยประกอบด้วย เคอร์คิวมิน : ดีเม็ทธอกซีเคอร์คิวมิน : บิส-ดีเม็ทธอกซีเคอร์คิวมิน ในอัตราส่วน 1:0.3:0.1

จากการทดลองในหลอดทดลอง พบว่า เคอร์คิวมินอยส์ มีคุณสมบัติเป็นสารต้านอนุมูลอิสระ ที่ดีกว่าวิตามินซี และวิตามินอี และสามารถป้องกันการแตกตัวของเม็ดเลือดแดง จากการวิเคราะห์ระดับของเคอร์คิวมินอยส์ในพลาสม่าของอาสาสมัครชาย และหญิง ไม่มีความแตกต่างกัน เคอร์คิวมินอยส์จะถูก เมตะบอไลซ์อย่างรวดเร็ว และส่วนใหญ่น่าจะจับเนื้อเยื่อไขมัน

หลังจากที่ได้ศึกษา PHARMACODYNAMIC ของอาสาสมัครปกติครั้งที่ 1 แล้ว อาสาสมัครจะได้รับกรดโฟลิคเป็นเวลา 14 วัน แล้วศึกษา PHARMACODYNAMIC ของขมิ้นชันอีกครั้ง พบว่ากรดโฟลิคไม่มีผลกระทบใดๆ ส่วนการวิเคราะห์อนุมูลอิสระ (ROS) โดยโฟลไซโตมิเตอร์ พบว่า ค่าอนุมูลอิสระจะลดลงไปจากเดิมที่เวลา 0 นาที แล้วลงไปต่ำสุดในเวลา 1 ชม. หลังจากได้รับยา และกลับสู่ระดับเดิมที่ 24 ชม. ส่วนปริมาณรีดิวซ์กลูตาไธโอน (GSH) และ มาลอนไดอัลดีไฮด์ (MDA) จะมีค่าใกล้เคียงกันที่เวลาต่างๆ กัน และนอกจากนี้พบว่าระดับของสารต่างๆ ในร่างกายก่อนและหลังได้รับกรดโฟลิคไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
AC	acetylcurcumin
BDMC	bisdemethoxycurcumin
CAT	catalase
CBC	complete blood count
DNA	deoxyribonucleic acid
DMC	demethoxycurcumin
DTNB	5,5′-dithiobis-(2-nitrobezoic acid)
EDTA	ethylenediamine tetrachloroacetic acid
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
GR	glutathione reductase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione
Hb	hemoglobin
HCl	hydrochloric acid
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide
KCl	potassium chloride
LOOH	lipid hydroperoxide
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean cell volume

LIST OF ABBREVIATIONS (cont.)

MDA	malondialdehyde
MPO	myeloperoxidase
NADPH	nicotinamide adeninedinucleotide phosphate
NBT	nitroblue tetrazolium
$NO^{\bullet}$	nitric oxide
NS	non-splenectomized
NTBI	non transferrin bound iron
$OH^{\bullet}$	hydroxyl radical
PUFA	polyunsaturated fatty acid
RBC	red blood cells
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
THC	tetrahydrocurcumin
WBC	white blood cells

CHAPTER I
INTRODUCTION

Nowadays, free radicals and reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, peroxyl radicals, and hydrogen peroxide are involved in different human diseases. They have been implicated in many disorders, ranging from cancer, neurodegenerative diseases, atherosclerosis, ageing and some hereditary disorder such as thalassemia. Because ROS can injure the tissue by oxidizing and leads to oxidative stress biomolecules such as lipid, protein, amino acids, and DNA.

Normally, the antioxidants are substances capable of counteracting the damaging effects of oxidation in body tissue. Antioxidants are divided into two classes based on mechanism of action: (i) chain-breaking antioxidants, such as vitamin E and beta-carotene, “break the chain” of free radical formation by donating an electron to stabilize an existing free radical; and (ii) preventive antioxidants are enzymes that scavenge initiating radicals before they start an oxidation chain such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).

Moreover, animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities.

Curcuminoids are active polyphenolic compounds found in the rhizome of turmeric (*Curcuma longa* L.), is most widely known for its use as a spice and coloring agent for food. The interest in this herb has grown in recent years based on its putative beneficial pharmacological effects including antioxidant, anti-inflammatory, and cancer chemopreventive actions (1-8). Curcuminoids contain three major yellow pigments; curcumin(diferuloylmethane), demethoxycurcumin(p-hydroxycinnamoyl, feruloylmethane) and bis-demethoxycurcumin (di-p-hydroxycinnamoylmethane) (9).

Numerous studies have indicated that curcuminoids especially curcumin have potentially antioxidant, anticancer and anti-inflammatory properties. An *in vitro* study, curcuminoids provide a protection of hemoglobin from oxidation at a concentration as low as 0.08 mM (10). They also can inhibit lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates. Curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals which an important to the initiation of lipid peroxidation and maintaining the activities of antioxidant enzyme like superoxide dismutase, catalase and glutathione peroxidase at higher levels (11, 12).

The antioxidant activity of curcumin increases when the phenolic group with a methoxy is at the ortho position. Apparently, the presence of both β-diketone moiety and phenol is necessary for optimal antioxidant function of curcumin. If generated by one-electron oxidant, the phenoxyl radicals from demethoxycurcumin and bisdemethoxycurcumin were more reactive and damaging than that of curcumin, because their higher reduction potentials. Therefore, demethoxycurcumin and bisdemethoxycurcumin are considerably inferior physiological antioxidants to curcumin (13). The β-diketone moiety of tetrahydrocurcumin (THC) must exhibit antioxidative activity by cleavage of the C-C bond at the active methylene carbon between two carbonyl in the β-diketone moiety (14).

In vivo experiment in rats, when administered curcumin orally in dose of 1 to 5 g/kg found that 75% of curcumin was excreted in feces while only traces appeared in the urine (15). Curcumin (up to 5 μg/ml) added to microsomes suspensions disappeared within 30 min and it was similar in hepatocyte suspensions.

Several pharmacokinetic studies have addressed the absorption, metabolism, and tissue distribution of curcumin both in animal (rat) and human study (5). Oral and intraperitoneal administration of [^{3}H] curcumin led to the fecal excretion of most of radioactivity but intravenous administration were well excreted in the bile of cannulated rats (16), whereas measurement of plasma levels with radioactivity have shown that curcumin is poorly absorbed from the gut (17-19). After oral administration of 400 mg curcumin to rats, about 60% of the dose was absorbed and could not detect curcumin colorimetrically in heart blood and only traces (<5μg/ml) in portal blood and negligible quantities in liver and kidney (<20 μg/tissue) were

obserbed . These studies indicated that curcumin is transformed during absorption in intestine, and the transformed product(s), which is more polar and colorless than curcumin, enters the serosal side.

Pharmacodynamic studies was carried out to determine anti-inflammatory activity; oral administration of curcumin in acute inflammation was found to be as effectve as cortisone or phenylbutazone, and one-half as effective in cases of chronic inflammation (20). In monkeys, curcumin was shown to inhibit neutrophil aggregation associated with inflammation (21). Curcumin analogue's anti-inflammatory properties may be attributed to its ability to inhibit pro-inflammatory arachidoic acid, as well as neutrophil function during in flammatory states. Curcumin may also be applied topically to animal skin to counteract inflammation and irritation associated with inflammatory skin conditions and allergies (20).

Animal studies in rats and mice as well as *in vitro* studies utilizing human cell lines have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion (22), angiogenesis (23), and tumor growth (24). Curcumin was also found to inhibit cell proliferation and tumor growth (25, 26). Turmeric and curcumin are also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both *in vitro* and in vivo studied (27-30). The anticarcinogenic effects of turmeric and curcumin are due in part to direct antioxidant and free-radical scavenging effect; but they also enhance the body's natural antioxidant system, increasing glutathione levels, thereby aiding in hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation. When comparing effect of curcumin, demethoxycurcumin, bis demethoxycurcumin and tetrahydrocurcumin on tumor promotion, the results indicate that pure curcumin and demethoxycurcumin have the same potent inhibitory effects, but bis demethoxycurcumin and tetrahydrocurcumin are less active (31).

A number clinical studies were reported; double blind crossover clinical trial in 18 patients with "definite" rheumatoid arthritis, found significant improvement of morning stiffness, walking time, joint swelling following two weeks of therapy with dose of 1200 mg/day administered orally in all patients (32).

The effect of curcumin on serum levels of cholesterol and lipid peroxides in 10 healthy human volunteers, a dose of 500 mg of curcumin per day for 7 days

significantly decrease total serum lipid peroxide (33%), increase HDL cholesterol (29%) and decrease total serum cholesterol (11.63%). The results suggest curcumin as a chemopreventive substance against arterial disease (33).

Adverse reaction and toxicity; in rat, the long term administration of curcuminoids at therapeutic dose (10 mg/kg BW/day) did not produce any toxicity. However, at higher dose, it may affect the function and morphology of liver in a reversible manner. The oral curcumin is not toxic to human even at the very high dose of 8000 mg/day taken for 3 months (34).

Turmeric is listed by the U.S. FDA as an herb generally regnized as safe for its intended use as a spice, seasoning and flavoring. Curcuma extract can be administered safety to patients (colorectal cancer) at dose up to 2.2 g daily, equivalent to 180 mg of curcumin (35).

Although, there have been considerable interest in beneficial effects of curcuminoids in human health, the pharmacodynamics of orally delivered curcuminoids have not been fully studied. This study will investigate *in vitro* effect of curcuminoids as an antioxidant and the pharmacodynamics properties of orally delivered curcuminoids in normal volunteer after a single six grams administration.

Since in Thailand, there are many thalassemaia patients who will be benefit from the antioxidant effect of curcuminoids and those patients receive folic acid regularly, therefore it will be interesting to analyze the effect of folic acid on pharmacodynamic of curcuminoids.

Objectives

1. To determine antioxidant of curcuminoids *in vitro* and after ingestion of a single six grams oral administration in normal volunteers at various times at 15, 30, 45, 60, 75 min, 1.5, 2, 4 ,8 and 24 hours after drug administration.
2. To determine *in vitro* inhibitory effect of curcuminoids on human erythrocyte hemolysis.
3. To determine ROS at various times at 15, 30, 45, 60, 75 min, 1.5, 2, 4 ,8 and 24 hours after drug administration in whole blood by flow cytometric method.
4. To determine the level of lipid peroxide as malondialdehyde (MDA) at various times at 15, 30, 45, 60, 75 min, 1.5, 2, 4 ,8 and 24 hours after drug administration in RBCs.
5. To determine the level of glutathione (GSH) at various times at 15, 30, 45, 60, 75 min, 1.5, 2, 4 ,8 and 24 hours after drug administration in RBCs.
6. To determine the correlation between the level of curcuminoids in plasma and the antioxidant activity.
7. To elucidate the effect of folic acid on curcuminoid pharmacodynamics

CHAPTER II
LITERATURE REVIEW

1. Curcumin

Phytochemicals are naturally occurring substances found in plants. There has been considerable public and scientific interest in the use of phytochemicals derived from dietary components to combat human diseases, especially the two commonest killers in the developed world, cardiovascular disease and cancer. The dried ground rhizome of the perennial herb *Curcuma longa* Linn., called turmeric in English, haldi in Hindi and ukon in Japanese, has been used in Asian medicine since the second millenium BC (36). Its utility is referred to in the ancient Hindu scripture, the Ayurveda. In addition to its aromatic, stimulant and colouring properties in the diet, turmeric is mixed with other natural compounds such as slaked lime and has been used topically as a treatment for wounds, inflammation and tumors. In contrast to the maximum dietary consumption of 1.5g per person per day in certain South East Asian communities, smaller quantities of turmeric tend to be used for medicinal purposes (37). The appeal of turmeric as a colouring, food preservative and flavouring is global according to the Food and Agriculture Organization of the United Nations, over 2400 metric tons of turmeric are imported annually into the USA for consumer use.

1.1 Major chemical constituents

The compounds extracted from the rhizome of *Curcuma longa* contain volatile oil (6%) that are composed of a number of monoterpenes and sesquiterpenes, including zingiberene, curcumene, α and β turmerone among others. The coloring principles (5%) are curcuminoids, 50-60% of which are a mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 1). Curcumin has a unique

CURCUMIN **Diferuloylmethane**

DEMETHOXYCURCUMIN **p-Hydroxy-cinnamoyl-feruloyl-methane**

BISDEMETHOXYCUCUMIN **pp'-Dihydroxy-dicinnamoyl-methane**

Figure 1 Structure of curcuminoid and its derivertives (38).

conjugated structure including two methoxylated phenols and the enol form of a β-diketone. Each curcuminoid differs in the side chain in the chemical structure. Other constituents include sugar, protein and resins. Curcumin, which gives yellow color to tumeric rhizomes, is one of the active ingredients responsible for the biological activity. It melts at 176-177 °C and form red-brown salts with alkalis. Curcumin is unstable at neutral and basic pH values and is degraded to ferulic acid (4-hydroxy-3-methoxycinnamic-acid) and feruloyl-methane (4-hydroxyl-3-methoxycinnamoyl-methane. Therefore curcumin should be stable in the stomach and small intestines between the pH 1 and 6 and the degradation of curcumin is extremely slow in these conditions (38). Curcumin is soluble in ethanol, alkali, ketone, acetic acid, and chloroform but it is insoluble in water.

1.2 Pharmacokinetic properties

1.2.1 Preclinical pharmacokinetics

The absorption, metabolism and tissue distribution of curcumin has been studied in at least 10 studies performed in rodents over the past three decades. In an early study, a dose of 1 g/kg was administered to rats in the diet (15). About 75% of the dose was excreted in the faeces and negligible amounts appeared in the urine. A few years later, a study of oral curcumin administered to rats demonstrated 60% absorption of curcumin and presented evidence for the presence of glucuronide and sulphate conjugates in urine (39). The same investigators proceeded to study the bioavailability of curcumin using ^{3}H-radiolabelling; oral administration resulted in the vast majority of the oral dose being excreted in faeces, and only one-third was excreted unchanged (17). Intravenous and intraperitoneal administration of curcumin in rodents resulted in large quantities of curcumin and metabolites in bile, which were characterised as mainly tetrahydrocurcumin and hexahydrocurcumin glucuronides (16, 18). After intravenous dosing, more than 50% of the dose was excreted in the bile within 5 h; these data were presented as evidence that curcumin undergoes transformation during absorption *via* the intestine and is possibly subject to entero-hepatic recirculation (18). Such an hypothesis was originally presented by Holder, Plummer and Ryan (16) based on their studies of the fate of curcumin in rats.

A more recent study of intraperitoneal curcumin (0.1g/kg) in the mouse has suggested that curcumin was first biotransformed to dihydrocurcumin and tetrahydrocurcumin, and that these compounds were subsequently converted to monoglucuronide conjugates (40). Preclinical studies of oral dosing of curcumin in rats using modern high pressure liquid chromatography (HPLC) techniques demonstrate small amounts of curcumin in plasma with higher levels of curcumin glucuronide and curcumin sulphate in plasma, and small quantities of hexahydrocurcumin, hexahydrocurcuminol and hexahydrocurcumin glucuronide (41), as summarised in Figure 2. This preclinical work was extended using suspensions of isolated human hepatocytes or liver or gut microsomes (42). The data suggested that metabolic reduction occurs very rapidly, in a matter of minutes. A study of high dose curcumin (2% in the diet, equating to approximately 1.2 g curcumin per kg body weight) for 14 days has shown that low nanomolar levels are detectable in plasma, with concentrations in liver and colon mucosal tissue ranging from 0.1 to 1.8 nmol/g tissue (43). In a study of oral curcumin (2 g/kg) in rats performed in Bangalore, India, the investigators suggested that co-administration of piperine may increase systemic bioavailability following oral dosing by as much as 154%, potentially by inhibition of xenobiotic glucuronidation (44). Piperine is primarily found in the fruit of the pepper vine, piper nigrum, and can also be found in other vegetables and spices such as hot jalapeno peppers. It is also said to give peppercorns their hot, biting and pungent taste.

1.2.2 Clinical pharmacokinetics

In comparison to the preclinical work presented above, comprehensive pharmacokinetic data in humans is lacking. In a clinical study to parallel their work in rats, Shoba and colleagues administered 2 g of pure curcumin powder to fasting volunteers resulting in low curcumin concentrations detected in plasma (less than 10 ng/ml) 1 h post-dose (44). In the same study, co-ingestion of curcumin with 20 mg of the pepper constituent l-piperoylpiperidine appeared to increase curcumin's bioavailability by 2000%. In a study of high dose oral curcumin performed in Taiwan, Cheng and colleagues (34) 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

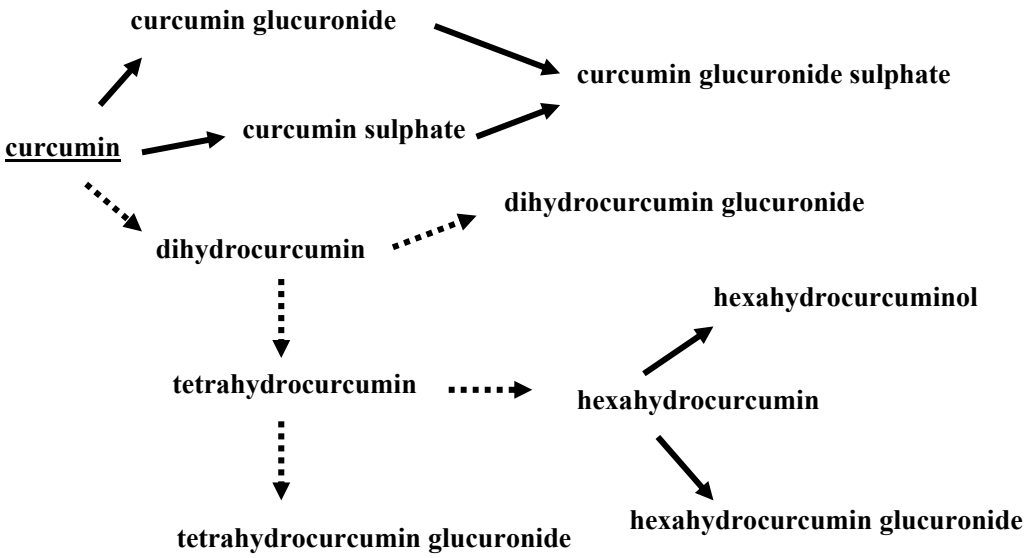

Figure 2. Metabolic pathways of curcumin in rodents and ex vivo culture of rat and human hepatocytes (5).

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. The 8 g/day dose resulted in a peak serum concentration of 1.75 ± 0.80 μM. In a study performed in Michigan, USA, 50–200 mg of micronised curcumin was administered to 18 healthy volunteers as an oral dose with orange juice, resulting in no evidence for the presence of curcumin in the serum at the limit of quantitation, which was approximately 0.63 ng/ml (45).

Two clinical phase I dose escalation studies have been performed in Leicester, England, over the past 5 years. In the first study using a standardised oral Curcuma extract, doses up to 180 mg of curcumin per day were administered to patients with advanced colorectal cancer for up to 4 months without overt toxicity or detectable systemic bioavailability (35). In a subsequent phase I study in 15 patients with advanced colorectal cancer refractory to standard chemotherapies, a curcuminoid formulation was consumed orally for up to 4 months, equating to curcumin doses between 0.45 and 3.6 g daily (46). Levels of curcumin and its metabolites in plasma, urine and faeces were analysed by HPLC and mass spectrometry. Oral consumption of 3.6 g of curcumin daily resulted in levels of drug and conjugates in plasma near the limit of detection of the assays used. Surprisingly, analysis of urine suggested the presence of curcumin and its conjugates in all samples from patients consuming this dose. Such chromatographic peaks were not seen in any extracts of urine samples from patients on the lower doses. In the six patients consuming 3.6 g of curcumin daily, urinary levels varied between 0.1 and 1.3 μM (curcumin), 19 and 45 nM (curcumin sulphate) and 210 and 510 nM (curcumin glucuronide). Since the measurement of compliance is increasingly perceived to be an important component of intervention trials, the consistent presence of curcumin and its conjugates in urine observed in patients consuming 3.6 g of curcumin daily is of potential relevance to the clinical advancement of curcumin as a chemopreventive agent. Since urinary analysis of drug-derived species constitutes an easily accessible and a reproducible test for ensuring general compliance, larger studies of this dose level are merited to confirm the consistent presence of curcumin and its conjugates and to define inter- and intra-individual variability.

In order to study the levels of curcumin in hepatic tissue following oral dosing, 12 patients with liver metastases from colorectal cancer received 0.45–3.6 g of oral curcumin daily for 7 days prior to hepatic surgery (47). Levels of curcumin and its metabolites were measured by HPLC in portal and peripheral blood, bile and liver tissue. Low nM levels of the parent compound and its glucuronide and sulphate conjugates were found in peripheral blood samples taken 1 h after the seventh dose of curcumin and in portal blood samples taken 6–7 h after the seventh dose of curcumin. Whilst curcumin was not found in liver tissue resected and preserved 6–7 h after the seventh dose of curcumin, trace levels of products of its metabolic reduction were detected. It was concluded from this pilot study that doses of oral curcumin required to produce hepatic levels sufficient to exert pharmacological activity are probably not feasible in humans using this pharmaceutical formulation.

Summarizing the data from pilot and Phase I clinical studies performed with curcumin, it appears that low systemic bioavailability following oral dosing is consistent with the findings in preclinical models presented above. Efficient first-pass and some degree of intestinal metabolism of curcumin, particularly glucuronidation and sulphation, may explain its poor systemic availability when administered *via* the oral route, as suggested by the detection of metabolites in the plasma from patients consuming high doses of curcumin daily. A daily oral dose of 3.6 g of curcumin results in pharmacologically efficacious levels in colorectal tissue, with negligible distribution of the parent drug in hepatic tissue or other tissues out with the gastrointestinal tract. Urinary analysis of drug-derived species may constitute an easily accessible and a reproducible test for ensuring general compliance with high doses of oral curcumin.

1.3 Safety

Recent concerns regarding the safety of selective enzyme inhibitors in large-scale chemoprevention trials emphasize the importance of carefully evaluating any potential toxicity of agents at the preclinical and early clinical trial levels. It cannot be assumed that diet-derived agents will be innocuous when administered as pharmaceutical formulations at doses likely to exceed those consumed in the dietary

matrix. Anecdotal reports suggest that dietary consumption of turmeric up to 1.5 g per person per day, equating to a maximum of 150 mg/day of curcumin, are not associated with adverse effects in humans (37).

Studies of curcumin in animals have confirmed a lack of significant toxicity since an early report in which doses up to 5 g/kg were administered orally to Sprague–Dawley rats (15). Systematic preclinical studies funded by the Prevention Division of the US National Cancer Institute did not discover adverse effects in rats, dogs or monkeys of doses up to 3.5 g/kg body weight (BW) administered for up to 3 months (48). One report of dietary curcumin suggested ulcerogenic activity in the stomach of the albino rat (49), but this finding has not been confirmed in subsequent studies. In more recent preclinical studies of curcumin, no toxicity has been observed from 2% dietary curcumin (approximately 1.2 g/kg BW) administered to rats for 14 days(43) or from 0.2% dietary curcumin (approximately 300 mg/kg BW) administered to mice for 14 weeks (19).

Although very few clinical studies of oral curcuminoids have reported any discernible toxicity, it has not been clearly stated by the investigators of most of these studies which methods or which scales have been used to assess potential toxicity. 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 (32). In a study of high dose oral curcumin in Taiwan, Cheng and colleagues administered up to 8 g daily of curcumin for 3 months to patients with pre-invasive malignant or high risk pre-malignant conditions, stating that no toxicity was observed (34). In patients with advanced colorectal cancer treated in the UK, curcumin was well tolerated at all dose levels up to 3.6 g daily for up to 4 months (46). Two types of gastrointestinal adverse events were reported by patients, which were probably related to curcumin consumption: one patient consuming 0.45 g daily and one patient consuming 3.6 g daily developed diarrhoea (US National Cancer Institute (NCI) grades 1 or 2) one month and four months into treatment, respectively. One patient consuming 0.9 g curcumin daily experienced nausea (NCI toxicity grade 2), which resolved spontaneously despite continuation of treatment. Two abnormalities were detected in blood tests, both possibly related to treatment: a rise in serum alkaline phosphatase

level was observed in four patients, consistent with NCI grade 1 toxicity in two patients and grade 2 toxicity in two patients; serum lactate dehydrogenase rose to more than 150% of pre-treatment values in three patients. These abnormal blood test results may have been related to disease progression rather than treatment toxicity.

1.4 Antioxidant actions

Pulla Reddy and Lokesh (1992) (11) observed that curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals, which are the initiators of lipid peroxidation. The effect of curcumin on lipid peroxidation was also studied in various models by several authors. The lipid peroxidation has a main role in the inflammation, in heart diseases, and in cancer. Curcumin is a good antioxidant and inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (12). Unnikrishnan and Rao (1992) (50) studied the antioxidative properties of curcumin. It was demonstrated that, curcumin protects (52%) hemoglobin from nitrate-induced oxidation to methemoglobin at 400 μM concentration.

Sreejayan and Rao (1994) (51) have reported that three curcuminoids were inhibitors of lipid peroxidation in rat brain homogenates and rat liver microsomes. All of these compounds were more active than tocopherol as reference and curcumin showed the better results. In the case of curcumin, the methoxy group seems to play a major role. The phenolic hydroxyl and the methoxyl groups on the phenyl ring and the 1,3-diketone system seem to be important structural features that can contribute to these effects. The diketone system is a potent ligand for metals such as iron, used in these experiments. Another fact proposed is that the antioxidant activity increases when the phenolic hydroxyl group is at the ortho position with respect to methoxy group. The photodynamic action of some drugs and pigments is also mediated through $^1O_2$. Light-induced diseases including erythropoietic protoporphyria, pellagra and cataractogenesis have been attributed in part to the toxicity of $^1O_2$. Thus, curcumin may be used in singlet oxygen-mediated diseases diseases as a pharmacologic agent. Curcumin would be augmented also through induction of Glutathione peroxidase (GPx) and the 4-hydroxynonenal (4-HNE) metabolizing glutathione S-transferase

(GST) isozymes. Such a postulate is consistent with the view that the regulation of the expression of GST is linked to the pro-antioxidant environment in the cell (52). Ahsan et al., (1999) (53) studied structure activity relationship between curcumin, bisdemethoxycurcumin and demethoxycurcumin. Curcumin was found to be most effective in the DNA cleavage reaction, rate of formation of hydroxyl radicals and a reducer of Cu (II) followed by bisdemethoxycurcumin and demethoxycurcumin.

Curcumin is reported to be a powerful antioxidant to repair both oxidative and reductive damage caused to proteins by radiation (54). Antioxidant mechanism of curcumin, in the presence of ethyl linoleate as one of the polyunsaturated lipids was reported (55). During the antioxidation process, curcumin reacted with four types of linoleate peroxyl radicals. Six reaction products were observed in the reaction and these have novel tricyclic structures, including a peroxyl linkage. On the basis of the formation pathway for their chemical structures, an antioxidant mechanism of curcumin in polyunsaturated lipids was proposed, which consisted of an oxidative oupling reaction at the 30-position of the curcumin with the lipid and a subsequent intramolecular Diels–Alder reaction. Further, a relatively high concentration of curcumin gave three dimers as radical termination products in addition to the coupling products with curcumin and the lipid hydroperoxide. The structural analysis of these dimers and quantitative analysis of their production rates revealed that radical termination mainly occurred at the 2-position of curcumin. The contribution of the pathway for production of these dimers to the antioxidant mechanism of curcumin was estimated from the concentration-dependent data of the antioxidant activity and formation rates of these termination products. The A–A termination (dimer formation) was estimated to contribute at least about 40% of the entire antioxidant process against ethyl linoleate oxidation (56). Das and Das (2002) (57) demonstrated that curcumin is a potent singlet oxygen quencher at physiological or pharmacological concentration. Additionally, singlet oxygen quenching by low concentration of curcumin in aqueous solutions is a physiologically relevant property of this compound, which can explain its effect inprotecting skin against UV light. Singlet molecular oxygen is an electronically excited species of oxygen is known to produce in mammalian cells under normal and pathophysiological conditions.

Jayaprakasha, Jena, Negi and Sakariah (58-60) reported antioxidant and antimutagenic activity of turmeric oil, which was isolated from spent turmeric oleoresin. Turmeric oil has been fractionated to get three fractions using silica gel column chromatography. Turmeric oil and its fractions were tested for antioxidant activity using the b-carotene–linoleate model system and the phospomolybdenum method. Fraction eluted with benzene showed maximum antioxidant capacity. Turmeric oil contained aromatic turmerone (31.32%), turmerone (15.08%) and curlone (9.7%), whereas most active fraction has aromatic turmerone (44.5%), curlone (19.22%) and turmerone (10.88%) as major compounds. These fractions were also used to determine their protective effect against the mutagenicity of sodium azide by means of the Ames test. All the fractions and turmeric oil exhibited antimutagenicity markedly. The antioxidant effects of turmeric oil and its fractions may provide an explanation for their antimutagenic action. Bond dissociation enthalpies (BDEs) for the curcumin-related compounds have been calculated using density functional theory (DFT) methods (61, 62)). It was reported that the antioxidant mechanism of curcumin was a H-atom abstraction from the phenolic group, not from the central CH_2 group in the heptadienone link. Curcumin, methylcurcumin, and half-curcumin had similar O-H BDEs, indicating that the two phenolic groups in curcumin were independent of each other.

Recently, Gayathri, Kalpana, Jamuna, and Srinivasan (2004) (63) reported that the loss (27–71%) of β-carotene in vegetables was observed during the two domestic methods of cooking commonly used, namely, pressurecooking and open pan boiling. However, presence of antioxidant spice turmeric generally improved the retention of b-carotene. Recent report (64) Daniel, Limson, Dairam, Watkins, and Daya (2004) showed that curcumin could chelate toxic metals (cadmium and lead), and potentially reduces their neurotoxicity and tissue damage in rat brain homogenate.

1.5 Pharmacodynamics in humans

1.5.1 Dose–effect relationships

Although any substantial data in favour of a dose–response relationship for any biomarker of curcumin's activity is currently lacking, several observations in human volunteers and patients suggest that curcumin may possess systemic biological activity at low oral doses. In a small study performed in Taiwan, a single oral dose of 20 mg curcumin appeared to induce contraction of the gall bladder assessed by ultrasound scanning in human volunteers, compared to amylum placebo (65).

In a pilot study performed in Leicester, England, doses up to 180 mg of curcumin per day were administered to patients with advanced colorectal cancer for up to 4 months (35). Two potential biomarkers of curcumin's systemic efficacy were evaluated. In three patients taking 36 mg of curcumin daily, lymphocytic activity of the detoxification enzymes, GST, decreased gradually with time from a pre-treatment GST value of 64 ± 19 nmol/min/mg protein to 26 ± 13 nmol/min/mg protein on day 29 of treatment. This decline was not observed at the higher dose levels and was not reproduced in a subsequent study of higher doses in the patients with the same disease (46). Similarly, consumption of curcumin did not affect blood leukocyte levels of the oxidative DNA adduct, pyrimido-[1,2α]purin-10(3H)-one-2′-deoxyguanosine (M_1G), described for the first time in patients with colorectal cancer, although interesting observations were made regarding *GST* isoenzyme genotypes and baseline leukocytic M_1G adduct levels.

In contrast to leukocyte M_1G and GST, the inducibility of prostaglandin (PG) E_2 production in whole blood *ex vivo* may represent a surrogate biomarker for assessing the pharmacological activity of curcumin at a systemic level. As discussed above, COX-2 is an important target for chemoprevention and its pharmacological modulation holds implications for cancer treatment. At least part of curcumin's effect on inducible PGE_2 production in human blood can be attributed to inhibition of *COX2* transcription, which may be due to the inhibition of the NFκB-activating enzymes IKK-α/β (66). The effect of curcumin described in an *ex vivo* assay developed using

blood from healthy volunteers (67) was associated with plasma levels detected in the 10^{-8} M range in patients with advanced colorectal cancer (46), less than a hundredth of the concentration of curcumin shown *in vitro* to elicit an effect in blood or colon cells(41, 68, 69). Blood was taken immediately pre-dose or 1 h post-dose on days 1, 2, 8 and 29 of treatment with 3.6 g of curcumin daily (46). Following addition of acetylsalicylic acid (200 μM) to eliminate COX-1 activity, whole blood was incubated for 24 h in the presence of lipopolysaccharide (LPS, 10 μg/ml) (67). In the trial described above, oral administration of curcumin did not impact on basal PGE_2 levels in leukocytes, nor did doses of 0.45–1.8 g daily alter LPS-induced PGE_2. In contrast, consumption of 3.6 g of curcumin daily affected LPS-induced PGE_2 levels (46). When values obtained immediately pre- or 1 h post-dose on days 1, 2, 8, and 29 were pooled for the six patients consuming this dose, PGE_2 levels observed post-dose were 46% lower ($P = 0.028$) than those measured immediately pre-dosing. The difference reached significance on the first day and 29th day of treatment, but not on day 2 or day 8 (46). Although these results suggested that consumption of 3.6 g of curcumin daily was linked with inhibition of PGE_2 induction in blood taken post-dose compared to blood taken pre-dose, overall time-dependent trends were not identified and no dose-response has been demonstrated for this biomarker. Although the *ex vivo* assay described using human blood is limited in its clinical application by the high inter-individual and high intra-individual variability (67), the results suggest the feasibility and potential utility of measurement of PGE_2 levels in target tissue as a biomarker reflecting potential anticancer activity of curcumin. It should also be noted that curcumin sulphate and products of metabolic reduction of curcumin also inhibited PGE_2 production in colon cells grown *in vitro*, although their inhibitory potency appeared lower than that of parent curcumin in these cells(41).

1.5.2 Anti-inflammatory effects

Curcumin's suppression of the inflammatory response may involve inhibition of the induction of COX-2, iNOS and production of cytokines such as interferon-γ, at least in part due to its suppression of the Janus kinase (JAK)-STAT signalling cascade *via* its effect on the Src homology 2 domain-containing protein tyrosine phosphatases (SHP)-2 (70). In myeloma cells, curcumin has also been shown to inhibit STAT3

phosphorylation and thus suppress interleukin-6 production (71). Compatible with these immunological effects, data from a chemical model of inflammatory bowel disease suggest that curcumin may be of value in the treatment of this disease (72).

A number of teams have studied the effect of oral curcumin on inflammatory diseases in humans. Satoskar and colleagues (73) found a significant anti-inflammatory effect objectively and subjectively from 400 mg thrice daily for 5 days in post-operative patients. In a double-blind study, Deodhar and colleagues (32) administered 1200 mg curcumin four times daily to 18 patients with rheumatoid arthritis for 2 weeks; they reported a significant improvement in the patients' inflammatory symptomology without apparent toxicity. Two teams have studied the effects of oral curcumin on ophthalmological conditions(74). In one study, 375 mg of curcumin was administered thrice daily to patients with chronic anterior uveitis for 12 weeks, resulting in a suggestion of improvement in the condition. In a subsequent study, the same dose of curcumin was administered to eight patients with idiopathic inflammatory orbital pseudotumours for 6–22 months (75). Complete response was observed in half the patients up to 2 years of follow-up. Although histopathological details were not presented in this report, inflammatory orbital pseudotumour is now generally attributed to low-grade non-Hodgkin's lymphoma; hence this result suggests potential anti-cancer activity.

1.6 Anti-cancer effects

Curcumin's induction of apoptosis in cancer cells by a variety of mechanisms described above, as well as its inhibition of DNA topoisomerase II at micromolar concentrations (76), hints at its potential for chemotherapeutic activity in the treatment of cancer. Published anecdotes of curcumin's activity as a topical treatment for cancer can be found, most notably Kuttan's (77) report of turmeric as a topical treatment for oral cancers and leukoplakia. This research group reported a reduction in the size of the lesions in 10% of the 62 patients treated, but there was no control group, no assessment of anti-inflammatory activity and no chemical analysis of the preparation applied.

In one of the pilot studies performed in Leicester, UK, low doses (36–180 mg) of curcumin were administered daily to patients with progressive advanced colorectal cancer, refractory to standard chemotherapies, for up to 4 months (35). Five out of fifteen patients treated in this study experienced radiologically stable disease for three months or longer, and a significant decrease in venous levels of a tumour marker, carcino-embryonic antigen, was observed in one patient. In a subsequent study performed in patients with progressive advanced colorectal cancer, doses of 0.45–3.6 g of curcumin were administered daily: radiologically stable disease was observed in 2 out of 15 patients for up to 4 months of treatment (46). The variable natural history of colorectal cancer makes these results from pilot studies difficult to interpret, but there is perhaps a hint of cytostatic activity using macroscopic measures in this patient group. Cheng and colleagues (34) in Taiwan investigated curcumin's potential anticancer activity in patients with pre-invasive malignant or high-risk pre-malignant conditions of the bladder, skin, cervix, stomach or oral mucosa. They administered doses of 1–8 g of curcumin (500 mg of curcumin per capsule, 99% pure) daily for 3 months; they noted that doses above 8 g per day were not tolerated by patients on account of the bulky volume of the number of capsules that had to be consumed daily. Histological improvement was noted in one of two patients with presumed bladder carcinoma *in situ*, two of seven patients with oral leukoplakia, one of six patients with stomach metaplasia, one of four patients with cervical intra-epithelial neoplasia (CIN) and two of six patients with Bowen's disease of the skin. Conversely, in one of four patients with CIN and one of seven patients with oral leukoplakia, the treatment failed to prevent the development of invasive malignancy during the 3-month study period. The small numbers of patients with each condition studied and the lack of blinding of the interpreting pathologists make definite conclusions impossible, but the results, particularly the photographic representations presented, re-emphasise the biological activity that curcumin might possess in a range of human tissues.

2. Free Radical

A free radical is defined as any species that contains one or more unpaired electrons(78). Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals, such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$) radicals, and certain nonradical oxidizing agents, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and ozone (O_3), that can be converted easily to into radicals (78). ROS are produced during normal metabolism and are involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of neutrophils and macrophages (2).

The chemical characteristics of molecular oxygen, or dioxygen (O_2) are based on the presence in the external orbital of two unpaired electrons with the same spin, therefore, O_2 is by definition a "free radical" (Figure 3). This conformation implies that O_2 attempts to oxidize other molecules, i.e. to capture a pair of electrons to form two electron pairs (this reduced form of O_2 is the peroxide anion, usually reported in its protonated form, i.e. hydrogen peroxide, H_2O_2). The one electron reduction of O_2 forms the superoxide radical ($O_2^{\bullet -}$) while the tetravalent reduction produces intramolecular rearrangements with rupture of the covalent bond of O_2 and the formation of two molecules of O^{2-} that once protonated result into two molecules of water.

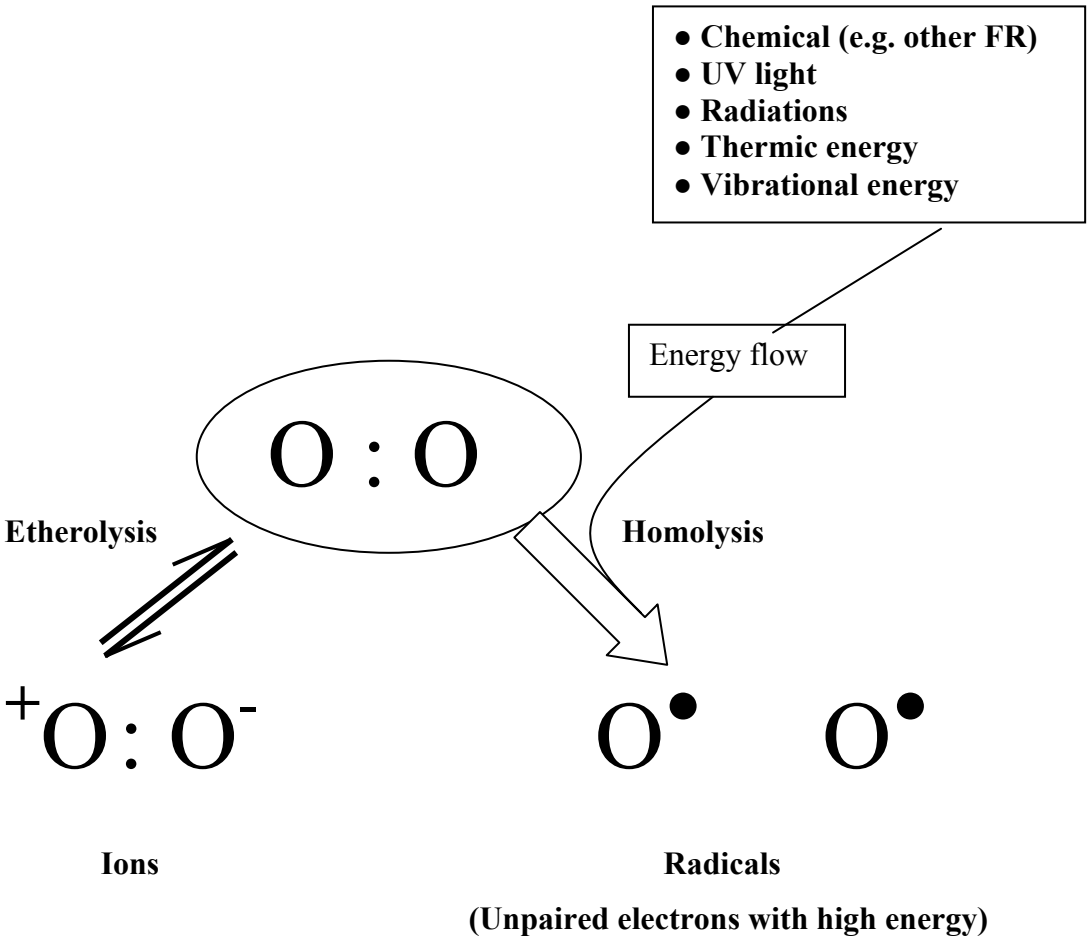

Figure 3 Mechanism of formation of free radical (FR) species. (78, 79).

Free radicals can be formed in three ways. One is formed by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining on the paired electrons. Second is by the loss of single electron from a normal molecule. The last, it may be formed by the addition of a single electron to a normal molecule. The electron transfer is far more common process in biological systems than homolytic fission which requires high energy input from either high temperatures, UV light or ionizing radiation. Heterolytic fission, in which the electron of covalent bond are retained by only one of fragment of molecule does not result in free radical but in charged ions. The process by which free radical and ions are formed are illustrated below (80).

Radical formation by electron transfer : $A + e^- \rightarrow A^{-\bullet}$

Radical formation by homolytic fission : $X : Y \rightarrow X^{\bullet} + Y^{\bullet}$

Ion formation by heterolytic fission : $X : Y \rightarrow X:^- + Y:^+$

Several reactive oxygen species (ROS) are shown in Table 1. Among them, the most frequently studied are given for examples.

2.1 Superoxide ($O_2^{\bullet -}$)

The superoxide anion creates from molecular oxygen by the addition of an electron, in spite of being a free radical, not highly reactive. It lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it is produced. The formation of superoxide takes place spontaneously, especially in electron-rich aerobic environment in vicinity of the inner mitochondrial membrane with the respiratory chain (Figure 4). Superoxide is also produced endogenously by flavoenzymes, e.g., xanthine oxidase activated in ischemic-reperfusion (81). Two molecules of superoxide rapidly dismutate to hydrogen peroxide and molecular oxygen and this reaction is further accelerated by superoxide dismutase (SOD).

The fundamental reactions of superoxide chemistry are as follows :

$$X + O_2 \longrightarrow X^+ + O_2^-$$

Table 1 Some of the most relevant reactive oxygen species (79).

Radicals	**Nonradicals**
Superoxide, $O_2^{\bullet -}$ Hydroxyl, $OH^{\bullet}$ Peroxyl, $RO_2^{\bullet}$ Alkoxyl, $RO^{\bullet}$ Hydroperoxyl, $HO_2^{\bullet}$	Hydrogen peroxide, H_2O_2 Hypochlorous acid, HOCl Ozone, O_3 Singlet oxygen, $^1O_2$ Peroxynitrite, ONOO

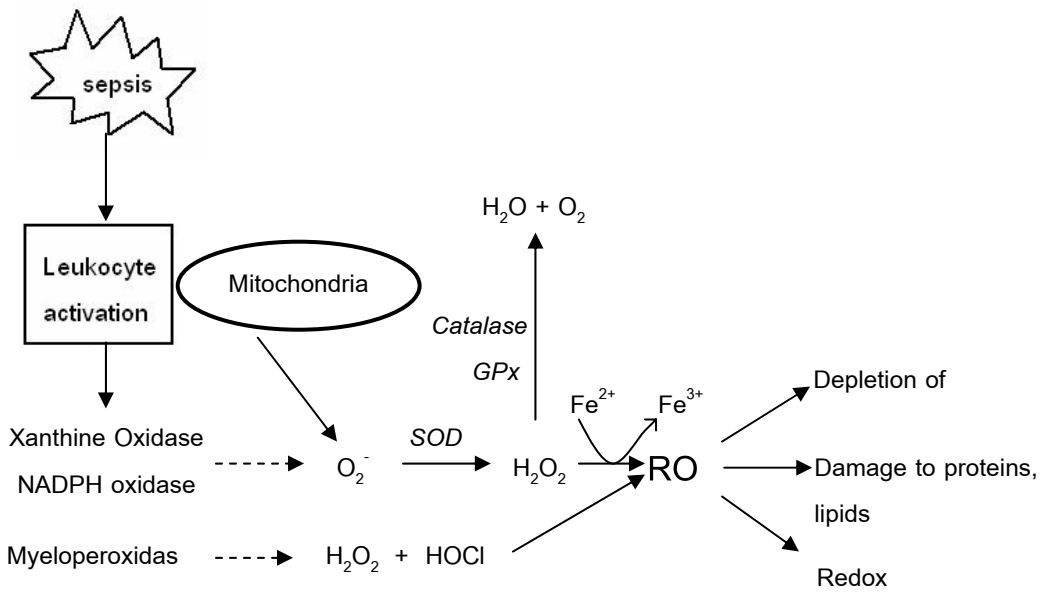

Figure 4 A variety of enzymatic and nonenzymatic processes can generate reactive oxygen species (ROS) in mammalian cells (82).

The single electron transported from a donor, X (such as hemoglobin, cytochrome, quinone, thiol, or redox metal) to ground state oxygen forms superoxide and the oxidized donor, X^+, the dismutation of superoxide requires proton to form hydrogen peroxide (34):

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

2.2. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is not a free radical but highly important because it is also able to diffuse easily through cellular membranes. Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxysomes, and mitochondria. Even in normoxia conditions, the hydrogen peroxide production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M (33). In plant and animal cells, superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\cdot-}$, thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity (32). Besides that, it plays a radical forming role as an intermediate in the production of more reactive ROS molecules including hypochlorous acid (HOCl) by the action of myeloperoxidase, an enzyme present in phagosomes of neutrophils (35) and, more importantly, formation of $OH^\bullet$ via oxidation of transition metals. Another important function of H_2O_2 is carried out in its role as an intracellular signaling molecule.

$$H^+ + Cl^- + H_2O_2 \longrightarrow HOCl + H_2O$$

H_2O_2 once produced by the above mentioned mechanism is removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases, and peroxiredoxins (35).

2.3. Hydroxyl radical ($OH^\bullet$)

Due to its strong reactivity with biomolecules, $OH^\bullet$ is probably capable of doing more damage to biological systems than any other ROS (36, 37). The radical is formed hydrogen peroxide in a reaction catalyzed by metal ions (Fe^{2+}or Cu^+), often bound in complex with different proteins or other molecules because of hydrogen

peroxide can easily break down in the presence of transition metals (32). This is known the Fenton reaction:

$$H_2O_2 + Fe^{2+}/Cu^{+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}/Cu2^{+} \quad \text{(reaction 1)}$$

Superoxide also plays an important role in connection with reaction 1 by recycling the metal ions:

$$Fe^{3+}/Cu^{2+} + O_2^{\bullet -} \longrightarrow Fe^{2+}/Cu^{+} + O_2 \quad \text{(reaction 2)}$$

The sum of reaction 1 and 2 is the Haber-Weiss reaction; transition metals thus play a role in the formation of hydroxyl radicals, which may release from proteins such as ferritin.

2.4. Singlet oxygen ($^1O_2$)

This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA, 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen. Several natural sensitizers are known to catalyze oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADPH). Some of these sensitizers are also found in foods and cosmetics. Others are used for therapeutic purposes (anticancer treatment) and are sensitive to visible light. The presence of metals contributes to increase the production of singlet oxygen, as well as superoxide anion, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet oxygen may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet O_2 formation may account for the chemiluminescence observed during lipid peroxidation.

2.5. Hypochlorous acid (HOCl)

Hypochlorous acid (HOCl) is powerful oxidizing agent. It is produced by the enzyme myeloperoxidase (MPO) in activated neutrophil. Hypochlorous acid can oxidize thiols, ascorbic acid, and NAD(P)H leading to chlorination of DNA bases, especially pyrimidines, and tyrosine residues in proteins. In addition, it is able to cross

membranes, causing damage to mebrane proteins on its passage, especially to sulfhydryl group (-SH group) and methionine residues (78).

3. Sources of free radicals

A variety of enzymatic and non-enzymatic processes can generate ROS in mammalian cells (Figure 2). Among the most important sources are the reactions catalyzed by the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidoreductase (XOR), and myeloperoxidase (MPO). NADPH oxidase is a multiple-subunit complex that generates $O_2^{\bullet -}$ in one-electron reduction of oxygen using electrons supplied by NADPH. The enzyme consists of two membrane-bound subunits (gp91 and p22) and at least three cytoplasmic subunits (p47, p67, G-protein Rac2). The leukocyte NADPH oxidase is inactive under resting conditions. Several different stimuli, such as lipo-polysaccharide or various proinflammatory mediators (tumor necrosis factor-α, interleukin-lβ, and interleukin-6), activate the oxidase in leukocytes. Stimulation of leukocytes leads to translocation of cytoplasmic subunits of NADPH oxidase and a small G protein (known as Rac2 in humans) to the plasma membrane subunits resulting in a massive generation of $O_2^{\bullet -}$ (83). NADPH oxidase is critical for the killing of microorganisms by formation of ROS in leukocytes. Genetic defects of gp91, p22, p47, or p67 are the basis for chronic granulomatous disease. NADPH oxidase is also present in nonphagocytic cells, including vascular endothelial cells, smooth muscle cells, fibroblasts, and the carotid body (84).

Xanthine oxidoreductase (XOR) is widely distributed in mammalian tissues but is largely concentrated in endothelial and epithelial cells. XOR has two identical 150-kDa subunits, and its cofactors include molybdopterin, two iron-sulfur centers, and flavin adenine dinucleotide. XOR has two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (7). Both can reduce oxygen to $O_2^{\bullet -}$, but XDH has a greater affinity for NAD. XOR conversion occurs via two different routes. Treatment of XDH with proteases transforms it to XO irreversibly. Reversible conversion of XDH to XO occurs due to conditions that oxidize critical cysteines, and it can be prevented by cysteine donors. Both forms of XOR catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid, the terminal two reactions in the purine degradation

pathway. Although the basal expression of human XOR is low, a variety of factors, such as hypoxia, lipopolysaccharide, proinflammatory mediators (tumor necrosis factor-α, interleukin-lβ, and interleukin-6), steroids, and prolactin, up-regulate its transcription. Hyperoxia, on the other hand, inhibits XOR transcription and decreases XOR activity by blocking its phosphorylation (85).

Myeloperoxidase (MPO) catalyzes the oxidation of chloride to HOCl in the presence of H_2O_2 in neutrophils and macrophages and thereby generates an array of highly reactive oxidants. Macrophages have one third of the MPO found in neutrophils. Reactions of HOCI with $O_2^{\bullet -}$ and iron (reaction. 3 and 4) lead to further formation of ROS. Hypochlorous acid also reacts with nitrite to yield the nitrating and chlorinating compound, nitrylchloride (NO_2C1) (reaction. 5) (86, 87).

$$HOCl + O_2^{\bullet -} \longrightarrow OH^- + O_2 + Cl^- \qquad \text{(reaction 3)}$$

$$HOCl + Fe(II) \longrightarrow OH^- + Fe(III) + Cl^- \qquad \text{(reaction 4)}$$

$$HOCl + NO_2^- \longrightarrow NO_2Cl + OH^- \qquad \text{(reaction 5)}$$

In addition to the pathways mentioned above, mitochondria have been shown to be another important source of ROS generation. More than 90% of total cellular oxygen is reduced to water via electron carriers of the mitochondrial respiratory chain. Isolated mitochondria have been shown to generate $O_2^{\bullet -}$ in the presence of inhibitors of electron transfer (88, 89). Therefore, under basal conditions they have been considered as the main $O_2^{\bullet -}$ radical source in the cell.

4. Oxidative stress

The chemistry of O_2 and ROS has both physiological and toxicological implications that have been extensively investigated in the last century as documented by an impressive number of publications in literature, and now it represents a wide and well-defined area of research in biology and medicine (78). Toxicological aspects of ROS in

aerobes are dependent on a complex series of factors and are commonly described within the unifying concept of 'oxidative stress'. The heterogeneous nature and redundancy of endogenous and exogenous sources of ROS (Figure 5) have represented a major danger for the survival of primordial as well present-time living organisms.

Oxygen is the primary oxidants in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. Oxidative stress results from the metabolic reactions that use oxygen and it has defined as a disturbance in equilibrium status of pro-oxidant/antioxidant systems in intact cells (90). When oxidative events occur, the pro-oxidant systems outbalance the antioxidant, potentially producing oxidative damage to lipid, proteins, carbohydrates, and nucleic acids, ultimately leading to cell death in severe oxidative stress (91, 92). Mild, chronic oxidative stress may alter the antioxidant system by inducing or repressing proteins that participate in these systems, and by depleting cellular antioxidant such as glutathione and vitamin E.

Oxidative stress is the result of oxygen free radicals which are generated in excess in the human body and can happen by a number of mechanisms. Cells can tolerate mild oxidative stress and often respond to it by increasing production of antioxidant defense enzymes. Severe oxidative stress, however, can lead to cell injury or cell death. Depending on the cell-type used and the means by which oxidative stress is imposed, injury, and death can result from damage to DNA, proteins, and/or lipid. Oxidative stress occurs in most, if not all, human disease, for example, cancer, Alzheimer's disease, cataracts, arthritis, diabetes, Parkinson's disease, ischemic-reperfusion injury, cardiovascular disease and thalassemia.

Oxidative stress is a mechanism with a central role in the pathogenesis of cancers. In cancer, oxidative stress is known to cause the mutation of DNA including frameshift mutation, base-pair substitution that lead to the mutation of tumor suppressor genes and make the critical initial events in carcinogenesis (93).

The oxidative stress can produce the mitochondrial aberrations, including both mitochondrial MDA (mtDNA) mutation and alterations in mitochondrial genomic functions have been identified in cancer of the bladder, colon, head, neck, kidney, liver, lung, and stomach (94).

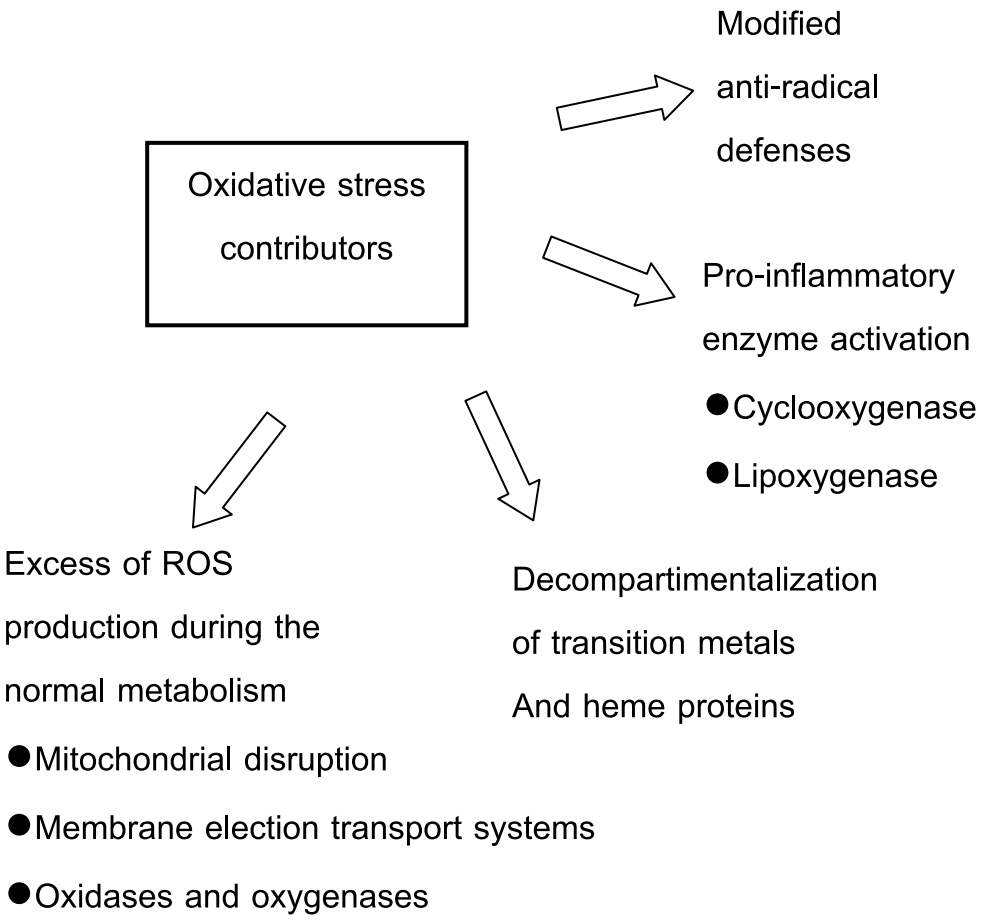

Figure 5 Some of the most relevant contributors to oxidative stress (79).

Endothelium dysfunction, the earliest expression of the atherosclerotic process is associated with biochemical changes that are transformed into structural changes of arterial wall. Overproduction of free radicals accounts for oxidative stress that is causing substantial damage to low density lipoprotein (LDL), endothelial cells, other tissue cells and finally leads to endothelial dysfunction. Oxidative stress has positive role of various mediations such as statins, angiotensin converting enzyme inhibitors, estrogens that consequently to the endothelial dysfunction (95).

The oxidative stress in thalassemic red blood cell (RBCs) results from the presence of excess unpaired globin chains, high intracellular content of nonhemoglobin iron and low concentration of normal hemoglobin. The unpaired globin chains in thalassemic cells are not stable. These unstable hemoglobin subunits can generate the free oxygen radical species such as superoxide (O_2^-) and hydroxyl radical (OH^-). The oxygen radical can start the oxidative damage to thalassemia red blood cells (RBCs). In condition that present of trace metals, especially copper and iron, have been implicated as causative agents in excessive generation of endogenous free oxygen radicals. These metals can catalyst the reaction of oxidative stress by Fenton reaction and initiate chain reaction that promote the membrane peroxidation including protein peroxidation and lipid peroxidation in thalassemic red blood cells (RBCs) (96).

5. Antioxidant defence systems

Because radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, an extensive range of antioxidant defences, both endogenous and exogenous, are present to protect cellular components from free radical induced damage. These can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins.(78) (Figure 6)

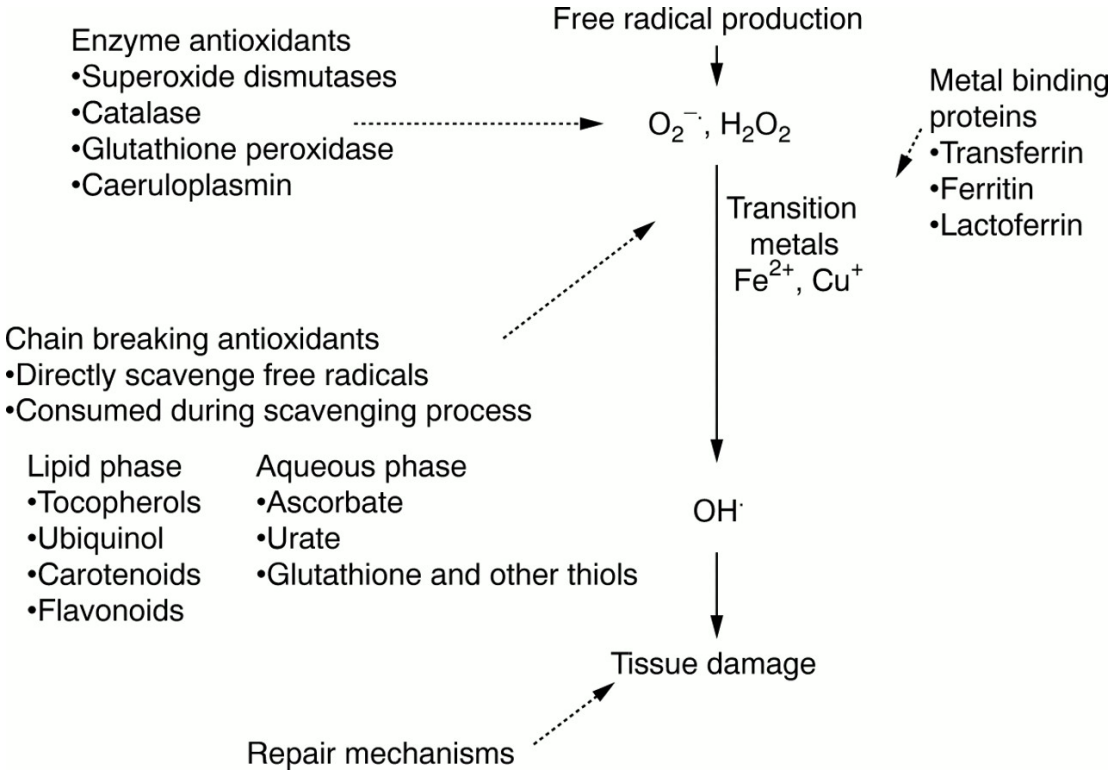

Figure 6 Antioxidant defenses against free radical attack (97).

THE ANTIOXIDANT ENZYMES

Catalase

Catalase is a heme-containing protein that decomposes H_2O_2 to H_2O and O_2. It is present in the peroxisomes of mammalian cells (98) and there have been reported that it could be present in other cell compartments. At high concentration of H_2O_2, the enzyme itself alone can detoxify H_2O_2 into H_2O and O_2.

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

At lower concentration of H_2O_2 in the presence of a suitable hydrogen donor (AH_2) such as methanol, ethanol, the peroxidase activity becomes predominant (81).

Glutathione peroxidase

Glutathione peroxidase (GSH-Px) is a selenoprotein, which is capable of reducing hydrogen peroxide as well as lipid hydroperoxide (ROOH) in the presence of reduced glutathione (GSH), H_2O and the reduction product of hydroperoxides, as in the following equation.

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} ROH + GSSG + H_2O$$

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} H_2O + GSSG$$

Glutathione peroxidase is located in both the cytolic and mitochondrial compartment. It is the most important H_2O_2-removing enzymes in mammalian cells, which contain the element selenium at their active site. This enzyme has high activity in liver and red cells, moderate activity in heart and lung, and low activity in muscle (99).

The catalytic mechanism proposed for reduction of hydroperoxides by glutathione peroxidase involving oxidation active site selenolate (Se^-) to selenenic acid (SeOH). Upon addition of one molecule of GSH, the selenenic acid is transformed to a selenenylsulfide adduct with glutathione (Se-SG), which can regenerate the active

selenolate and glutathione disulfide (GSSG) by the addition of a second molecule of GSH. Thus, in the reaction, two molecules of GSH are oxidized to GSSG that subsequently can be reduced by glutathione reductase (GR), the major mammalian GSSG-reducing enzyme.

Superoxide dismutase

One of the body's primary defense mechanisms against free-radical damage is the superoxide dismutase (SOD) family of enzymes. The discovery of superoxide dismutase (SOD, EC 1.15.11) was first isolated in 1938 as a copper green protein by McCord and Fridovich (100), while examing the reduction of cytochrome *c* by xanthine oxidase. The enzyme is ubiquitous. All SODs are metalloproteins which catalyze the conversion of superoxide radical to hydrogen peroxide and ground state oxygen, as shown in the following reaction.

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

There are three distinct types of SOD based on the metal ion in their active sites that have been observed from a wide range of organisms. They are Cu/Zn containing SOD (CuZnSOD), iron containing SOD (FeSOD) and manganese containing SOD (MnSOD). The FeSOD and MnSOD are characteristics of prokaryotes and are related to extensive sequence homologies. While the CuZnSOD, which is the characteristic of eukaryotic cytosols, show no sequence homology with Mn/FeSOD. The mitochondria containing a MnSOD has a high degree of sequence homology with the prokaryotic enzyme.

THE CHAIN BREAKING ANTIOXIDANTS

Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts. In general, the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing

the further propagation of the chain reaction. Such antioxidants can be conveniently divided into aqueous phase and lipid phase antioxidants (97).

Lipid phase chain breaking antioxidants

These antioxidants scavenge radicals in membranes and lipoprotein particles are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E. Vitamin E occurs in nature in eight different forms, which differ greatly in their degree of biological activity. The tocopherols (α, β, γ, and δ) have a chromanol ring and a phytyl tail, and differ in the number and position of the methyl groups on the ring. The tocotrienols (α, β, γ, and δ) are structurally similar but have unsaturated tails. Both classes of compounds are lipid soluble and have pronounced antioxidant properties. They react more rapidly than polyunsaturated fatty acids with peroxyl radicals and hence act to break the chain reaction of lipid peroxidation. In addition to its antioxidant role, vitamin E might also have a structural role in stabilizing membranes (101). The absorption, transport, and regulation of plasma concentrations of vitamin E in humans has been reviewed by Kayden and Traber (102), although in general the metabolism of vitamin E is not well described. Vitamin E will not prevent the initial formation of carbon center radicals in a lipid rich environment, but does minimize the formation of secondary radicals. An α-tocopherol is the most potent antioxidant of the tocopherols and is also the most abundant in humans. It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. This resonance stabilized radical might subsequently react in one of several ways. The α-tocopherol might be regenerated by reaction at the aqueous interface with ascorbate (103) or another aqueous phase chain breaking antioxidant, such as reduced glutathione or urate (104). Alternatively, two α-tocopheroxyl radicals might combine to form a stable dimer, or the radical may be completely oxidized to form tocopherol quinone. The carotenoids are a group of lipid soluble antioxidants based around an isoprenoid carbon skeleton (105). The most important of these is β-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen (106), but can also trap peroxyl radicals at low oxygen pressure with an

efficiency at least as great as that of α-tocopherol. Because these conditions prevail in many biological tissues, the carotenoids might play a role in preventing in vivo lipid peroxidation (107) . The other important role of certain carotenoids is as precursors of vitamin A (retinol). Vitamin A also has antioxidant properties , which do not, however, show any dependency on oxygen concentration. Flavonoids are a large group of polyphenolic antioxidants found in many fruits, vegetables, and beverages such as tea and wine (108). Over 4000 flavonoids have been identified and they are divided into several groups according to their chemical structure, including flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). Epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases such as coronary heart disease (CHD)(109, 110). However, little is currently known about the absorption and metabolism of flavonoids and epidemiological associations might be a consequence of confounding by other factors. Available evidence suggests that the bioavailability of many flavonoids is poor (111), and plasma values very low, although there is some evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage (112). Apart from flavonoids, other dietary phenolic compounds might also make a small contribution to total antioxidant capacity (113). Ubiquinol-10, the reduced form of coenzyme Q10, is also an effective lipid soluble chain breaking antioxidant (114). Although present in lower concentrations than α-tocopherol, it can scavenge lipid peroxyl radicals with higher efficiency than either α-tocopherol or the carotenoids, and can also regenerate membrane bound α-tocopherol from the tocopheryl radical (115). Indeed, whenever plasma or isolated low density lipoprotein (LDL) cholesterol is exposed to radicals generated in the lipid phase, ubiquinol-10 is the first antioxidant to be consumed, suggesting that it might be of particular importance in preventing the propagation of lipid peroxidation (116). However, work to clarify further its role has been hampered by the ease with which ubinquinol-10 becomes oxidized during sample handling or analysis.

Aqueous phase chain breaking antioxidants

These antioxidants will directly scavenge radicals present in the aqueous compartment. Qualitatively the most important antioxidant of this type is vitamin C (ascorbate) (117). In humans, ascorbate acts as an essential cofactor for several enzymes catalyzing hydroxylation reactions. In most cases, it provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity. Its best known role is as a cofactor for prolyl and lysyl oxidases in the synthesis of collagen. However, in addition to these well defined actions, several other biochemical pathways depend upon the presence of ascorbate (118). In addition to its role as an enzyme cofactor, the other major function of ascorbate is as a key chain breaking antioxidant in the aqueous phase (119). Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals, and singlet oxygen. During its antioxidant action, ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate. The semidehydroascorbyl radical is relatively stable owing to dispersion of the charge associated with the presence of a single electron over the three oxygen atoms, and it can be readily detected by electron spin resonance in body fluids in the presence of increased free radical production (120). Dehydroascorbate is relatively unstable and hydrolyses readily to diketogulonic acid, which is subsequently broken down to oxalic acid. Two mechanisms have been described by which dehydroascorbate can be reduced back to ascorbate; one is mediated by the selenoenzyme thioredoxin reductase (121) and the other is a non-enzyme mediated reaction that uses reduced glutathione (122). Dehydroascorbate in plasma is probably rapidly taken up by red blood cells before recycling, so that very little, if any, dehydroascorbate is present in plasma (123). Apart from ascorbate, other antioxidants are present in plasma in high concentrations. Uric acid efficiently scavenges radicals, being converted in the process to allantoin (124). Urate might be particularly important in providing protection against certain oxidizing agents, such as ozone (125). Indeed, it has been suggested that the increase in life span that has occurred during human evolution might be partly explained by the protective action provided by uric acid in human plasma (126) . Part of the antioxidant effect of urate

might be attributable to the formation of stable non-reactive complexes with iron, but it is also a direct free radical scavenger. Albumin bound bilirubin is also an efficient radical scavenger (127), and it has been suggested that it might play a particularly crucial role in protecting the neonate from oxidative damage , because deficiency of other chain breaking antioxidants is common in the newborn. The other major chain breaking antioxidants in plasma are the protein bound thiol groups. The sulphydryl groups present on plasma proteins can function as chain breaking antioxidants by donating an electron to neutralize a free radical, with the resultant formation of a protein thiyl radical. Albumin is the predominant plasma protein and makes the major contribution to plasma sulphydryl groups, although it also has several other antioxidant properties (128). Albumin contains 17 disulphide bridges and has a single remaining cysteine residue, and it is this residue that is responsible for the capacity of albumin to react with and neutralize peroxyl radicals. This property is important in view of the role albumin plays in transporting free fatty acids in the blood. In addition, albumin has the capacity to bind copper ions and will inhibit copper dependent lipid peroxidation and hydroxyl radical formation. It is also a powerful scavenger of the phagocytic product hypochlorous acid, and provides the main plasma defense against this oxidant (129). Because albumin itself is damaged when it acts as an antioxidant, it has been viewed as a sacrificial molecule that prevents damage occurring to more vital species. The high plasma concentration of albumin and a relatively short half life mean that any damage suffered is unlikely to be of biological importance. However, in vitro work has shown that protein thiyl radicals can themselves act as a potential source of reactive oxidants. The thiyl radical can abstract an electron from a polyunsaturated fatty acid to initiate the process of lipid peroxidation, a reaction that can be inhibited by ascorbate and retinol. The antioxidant effects of albumin and other proteins have been shown to decrease at high concentrations and it has been suggested that this is because thiyl radicals can oxidatively damage other molecules. The importance of these findings to the antioxidant role of albumin in vivo remains unclear. Reduced glutathione (GSH) is a major source of thiol groups in the intracellular compartment but is of little importance in the extracellular space (90). GSH might function directly as an antioxidant, scavenging a variety of radical species, as well as acting as an essential factor for glutathione peroxidase (discussed above). Thioredoxin

might also function as a key intracellular antioxidant, particularly in redox induced activation of transcription factors (130) .

INTERACTION BETWEEN CHAIN EREAKING ANTIOXIDANTS

Although the actions of chain breaking antioxidants have been considered separately above, it is vital to remember that in vivo complex interactions between antioxidants are likely to occur. For instance, it is likely that ascorbate will recycle the tocopheryl radical at the aqueous–lipid interface, so regenerating tocopherol (103). This might be crucial in ensuring that tocopherol concentrations are maintained in lipoproteins and membranes. In a similar manner, glutathione can regenerate ascorbate from dehydroascorbate. A complex interplay is therefore likely to exist between antioxidants, making it difficult to predict how antioxidants will function in vivo. It therefore becomes meaningless to ask which antioxidant is most important: the answer will depend on the circumstances existing in a particular microenvironment at a specific time, and on the nature of the oxidant injury taking place. A second important property of chain breaking antioxidants is their ability to act as pro-oxidants. In certain circumstances, the presence of an antioxidant might paradoxically lead to increased oxidative damage. For instance, it has been reported that the administration of vitamin C can sometimes lead to an increase in oxidative damage, particularly if iron is also administered. Similarly, it has been clearly shown in vitro that tocopherol might promote LDL oxidation in the absence of an aqueous phase antioxidant such as ascorbate (131) . Whether these reactions are important in vivo is as yet unclear. However, the possibility that antioxidants may have pro-oxidant effects in vivo must be considered when designing and interpreting the results of clinical trials of antioxidant supplementation.

THE TRANSITION METAL BINDING PROTEINS

As discussed above, transition metal binding proteins (ferritin, transferrin, lactoferrin, and caeruloplasmin) act as a crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein, caeruloplasmin,

might also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron (132) .

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

Fe^{2+} is the form of iron that drives the Fenton reaction and the rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect.

6. Folic acid

Folic acid, also known generically as folate or folacin, is a member of the B-complex family of vitamins, and works in concert with vitamin B 12. Folic acid functions primarily as a methyl-group donor involved in many important body processes, including DNA synthesis. Therapeutically, folic acid is instrumental in reducing homocysteine levels and the occurrence of neural tube defects. It may play a key role in preventing cervical dysplasia and protecting against neoplasia in ulcerative colitis. Folic acid also shows promise as part of a nutritional protocol to treat vitiligo, and may reduce inflammation of the gingiva. Furthermore, certain neurological, cognitive, and psychiatric presentations may be secondary to folate deficiency. Such presentations include peripheral neuropathy, myelopathy, restless legs syndrome, insomnia, dementia, forgetfulness, irritability, endogenous depression, organic psychosis, and schizophrenia-like syndromes.

Folic acid is a water-soluble member of the B-complex family of vitamins. Folic acid is composed of three primary structures, a hetero-bicyclic pteridine ring, para-aminobenzoic acid (PABA), and glutamic acid. Because humans cannot synthesize this compound, it is a dietary requirement. Although folic acid is the primary form of folate used in dietary supplements or fortified foods, it comprises only 10 percent or less of folates in the diet. Dietary folic acid, or the form naturally found in foods, is actually a complex and variable mixture of folate compounds, such as polyglutamate (multiple glutamate molecules attached) conjugate compounds, reduced folates, and tetrahydrofolates. Although folates are abundant in the diet, cooking or processing destroys these compounds. The best folate sources in foods are green, leafy vegetables; sprouts, fruits, brewer's yeast, liver, and kidney also contain high

amounts of folates. Human pharmacokinetic studies indicate folic acid has very high bioavailability, with large oral doses of folic acid substantially raising plasma levels in healthy subjects in a time- and dose-dependent manner. Subsequent to high-dose oral administration of folic acid (ranging from 25-1,000 mg/day), red blood cell (RBC) folate levels remain elevated for periods in excess of 40 days following discontinuation of the supplement. Folic acid is poorly transported to the brain and rapidly cleared from the central nervous system. The primary methods of elimination of absorbed folic acid are fecal (through bile) and urinary. (133-136) After ingestion, the process of conversion of folic acid to the metabolically active coenzyme forms is relatively complex. Synthesis of the active forms of folic acid requires several enzymes, adequate liver and intestinal function, and adequate supplies of riboflavin (B2), niacin (B3), pyridoxine (B6), zinc, vitamin C, and serine. After the formation of the coenzyme forms of the vitamin in the liver, these metabolically active compounds are secreted into the small intestine with bile (the folate enterohepatic cycle), where they are reabsorbed and distributed to tissues throughout the body. Despite the biochemical complexity of this process, evidence suggests oral supplementation with folic acid is able to increase the body's pool of the active reduced folate metabolites (such as methyltetrahydrofolate) in healthy individuals (137). Enzyme defects, malabsorption or digestive system pathology, and liver disease can result in impaired ability to activate folic acid to the required coenzyme forms in the body. Evidence indicates some individuals have a severe congenital deficiency of the enzyme methyltetrahydrofolate reductase, which is needed to convert folic acid to the 5-methyltetrahydrofolate coenzyme form of the vitamin. The existence of milder forms of this enzyme defect is strongly suspected and likely interacts with dietary folate status to determine risk for some disease conditions (138-142). In individuals with a genetic defect of this enzyme (whether mild or severe), greater dietary exposure to foods rich in folates and supplemental folates in the form of folinic acid or 5-methyltetrahydrofolate might be preferable to folic acid supplementation.

Mechanisms of Action

Folic acid's primary mechanisms of action are through its role as a methyl donor in a range of metabolic and nervous system biochemical processes, as well as being necessary for DNA synthesis. Serine reacts with tetrahydrofolate, forming 5,10-methylenetetrahydrofolate, the folate derivative involved in DNA synthesis. A methyl group is donated to cobalamin (B12) by 5-methyltetrahydrofolate, forming methylcobalamin. With the help of the enzyme methionine synthase, methylcobalamin donates a methyl group to the amino acid metabolite homocysteine, converting it to the amino acid methionine. Methionine subsequently is converted to S-adenosylmethionine (SAMe), a methyl donor involved in numerous biochemical processes.

Deficiency States and Symptoms

Folic acid deficiency is considered to be one of the most common nutritional deficiencies. The following may contribute to a deficiency of folic acid: deficient food supply; defects in utilization, as in alcoholics or individuals with liver disease; malabsorption; increased needs in pregnant women, nursing mothers, and cancer patients; metabolic interference by drugs; folate losses in hemodialysis; and deficiencies in enzymes or cofactors needed for the generation of active folic acid (143). Absorption of folic acid appears to be significantly impaired in HIV disease, irrespective of the stage of the disease (144). Signs and symptoms of folate deficiency include macrocytic anemia, fatigue, irritability, peripheral neuropathy, tendon hyper-reflexivity, restless legs syndrome, diarrhea, weight loss, insomnia, depression, dementia, cognitive disturbances, and psychiatric disorders (145). Elevated plasma homocysteine can also indicate a dietary or functional deficiency of folic acid.

Clinical Indications

Anemia

Folic acid has a long history of use in conjunction with vitamin B 12 for the treatment of macrocytic anemia. Depending on the clinical status of the patient, the dose of folic acid required to reverse macrocytic anemia varies, but the therapeutic dose is usually 1 mg daily. Duration of therapy to reverse macrocytic anemia can be as short as 15 days after initiation of supplementation, or it may require prolonged supplementation.

Cervical dysplasia

Research points to an association between folate status in adults and cervical dysplasia; (146) however, its role as an efficacious therapeutic intervention is unclear. One report suggests folic acid supplementation (10 mg folic acid for three months) reverses cervical dysplasia in women taking oral contraceptives (147). In another study, 154 individuals with grade 1 or 2 cervical intraepithelial neoplasia were randomly assigned either 10 mg folic acid or placebo daily for six months. No significant differences were observed between supplemented and unsupplemented subjects regarding dysplasia status, biopsy results, or prevalence of human papilloma virus type-16 infection (148). It is possible certain subsets of women (perhaps those with an oral contraceptive-induced deficiency) might be more amenable to treatment; however, additional research is required to clarify the therapeutic role of folic acid in cervical dysplasia.

Gout

There is no evidence demonstrating efficacy of folic acid supplementation in gout. Although some in vitro evidence suggests folate compounds are potent inhibitors of xanthine oxidase activity, (149) it appears pterin aldehyde, a photolytic breakdown product of folic acid, and not folic acid itself, is responsible for the observed inactivation of xanthine oxidase(150).

Available evidence has shown no ability of supplemental folic acid in oral daily doses up to 1,000 mg to significantly lower serum urate concentration, or to decrease urinary urate or total oxypurine excretion in hyperuricemic subjects (151).

Homocysteinemia

An abnormally high plasma level of homocysteine, the de-methylated derivative of the amino acid methionine, is an independent risk factor for cardiovascular disease. Elevated plasma homocysteine has been connected to increased risk of neural tube defects and other birth defects, as well as to schizophrenia, Alzheimer's disease, cognitive decline, osteoporosis, rheumatoid arthritis, kidney failure, and cancer (152). The activated coenzyme form of folic acid (5-methyltetrahydrofolate) is needed for optimal homocysteine metabolism, since it acts as a methyl donor, providing a methyl group to vitamin B12. The methylated form of vitamin B12 (methylcobalamin) subsequently transfers this methyl group to homocysteine. The result is a recycling of homocysteine to methionine, resulting in reduction in elevated plasma homocysteine. In healthy subjects even low doses of folic acid can lower homocysteine levels. A dose of 250 mcg daily for four weeks reduced homocysteine an average of 11.4 percent in healthy 18- to 40-year-old women. A dose of 500 mcg daily for the same duration reduced levels an average of 22 percent (153). In a separate study, 650 mcg daily for six weeks resulted in an average plasma homocysteine reduction of 41.7 percent (154). In subjects with cardiovascular disease, 800 mcg folic acid daily resulted in an average decrease in homocysteine levels of 23 percent, (152) while 2.5 mg daily resulted in an average decrease of 27 percent. (155) In subjects receiving the higher dose, 94 percent experienced some degree of reduction in homocysteine (156). Evidence suggests individuals with higher initial homocysteine levels are likely to experience a greater reduction following folic acid supplementation (155). In addition to helping reduce blood levels of homocysteine, folic acid may also aid peripheral blood flow by increasing nitric oxide (NO) in vascular endothelial cells. Impaired endothelial NO activity is an early marker for cardiovascular disease, particularly atherosclerosis. In fact, most of the risk factors for atherosclerosis are associated with poor vasodilation due to insufficient NO production. Chronic, unopposed exposure of the vascular

endothelium to homocysteine compromises the production of adequate amounts of NO, which leads to injury of the endothelial lining and the initiation/exacerbation of atherosclerosis and/or thrombus formation. Folic acid appears to improve NO synthesis by reducing plasma homocysteine levels, enhancing the availability of key endothelial NO cofactors, and reducing the production of superoxide anions, the net effect of which is improvement of peripheral blood flow (157). In a recent doubled-blind, placebo-controlled, crossover study of individuals with coronary heart disease, researchers found supplementation with high-dose folic acid (30 mg per day) improved blood flow to the heart muscle via the coronary arteries. Using positron emission tomography (PET scanning), researchers at Massachusetts General Hospital noted significant improvement in coronary blood flow with folic acid supplementation compared to placebo. The improvement was especially enhanced in areas of the heart that had shown reduced blood flow prior to supplementation. Folic acid supplementation also significantly lowered the study participants' blood pressure. The findings from this high-dose folate study demonstrate another significant way this nutrient benefits the cardiovascular system (158). Although excellent results have been achieved with folic acid monotherapy, available evidence suggests an additive effect exists between folic acid and vitamins B6, B12, and betaine with respect to lowering homocysteine levels. Combinations of these nutrients typically produce greater reductions in homocysteine than does folic acid alone (159). Furthermore, the addition of vitamin C, L-arginine, tetrahydrobiopterin, and polyunsaturated fatty acids (PUFAs) has been suggested as a means of enhancing the effect of folic acid on endothelial NO production (157).

Pregnancy

Low dietary intake of folic acid increases the risk for delivery of a child with a neural tube defect (NTD). Periconceptional folic acid supplementation significantly reduces the occurrence of NTD (160). Supplemental folic acid intake during pregnancy results in increased infant birth weight and improved Apgar scores, along with a concomitant decreased incidence of fetal growth retardation and maternal infections(161).

Drug-Nutrient Interactions

A number of drugs can interfere with the pharmacokinetics of folic acid. Cimetidine and antacids appear to reduce folate absorption (162). Sulfasalazine interferes with folic acid absorption and conversion to the active form (163). Supplementation with folic acid (15 mg/day for one month) prevents folate deficiency in patients with inflammatory bowel disease treated with sulfasalazine (164). Continuous long-term use of acetaminophen and aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs appears to increase the body's need for folic acid (163). Although the mechanism is unclear, anticonvulsants, antituberculosis drugs, alcohol, and oral contraceptives produce low serum and tissue concentrations of folate (165). Folic acid reduces elevated liver enzymes induced by methotrexate therapy in rheumatoid arthritis; however, it had no effect on the incidence, severity, and duration of other adverse events (166). Folic acid supplementation prevents nitric oxide synthase dysfunction induced by continuous nitroglycerin use (167). Anti-seizure medications, including carbamazepine and phenobarbital, appear to utilize folic acid during hepatic metabolism. Folic acid supplementation can increase metabolism of these drugs, thus lowering blood levels of the drugs and possibly resulting in breakthrough seizures. Initiating folic acid therapy after starting these drugs in individuals should be done with caution (168). The anticonvulsant drugs phenytoin and valproic acid appear to interfere with folate absorption (169). Folic acid supplementation, at a time of day other than when taking an anticonvulsant, may be helpful to prevent deficiency. There is conflicting information regarding the effects of folate supplementation in individuals treated with antifolate medications such as methotrexate (MTX) and 5-fluorouracil (5-FU). There is evidence folic acid might inhibit the activity of these drugs, although in some cases it may increase activity. In fact, the folic acid metabolite, folinic acid (also known as 5-formyltetrahydrofolate and leucovorin), is often used to "rescue" normal tissue after MTX or 5-FU therapy. Folic acid supplementation does not appear to interfere with methotrexate's anti-arthritic or anti-inflammatory activity. Since these medications are used to treat a wide range of malignant and nonmalignant disorders, indiscriminate use of folates should be avoided until further investigation is conducted.

Nutrient-Nutrient Interactions

Some concern exists that supplementation with high doses of folic acid could mask a vitamin B12 deficiency, resulting in neurological injury secondary to undiagnosed pernicious anemia. If there is any possibility of B12-induced anemia in an individual needing folate therapy, dual therapy with B12 and folate should be administered. Some authors have suggested folic acid supplements might interfere with intestinal zinc absorption: however, doses as high as 15 mg folic acid daily do not appear to have any significant effect on zinc status in healthy, non-pregnant subjects (168).

Side Effects and Toxicity

In doses typically administered for therapeutic purposes, folic acid is considered non-toxic. At doses of 15 mg daily and above, gastrointestinal complaints, insomnia, irritability, and fatigue have been mentioned as occasional side effects. Folic acid is considered safe during pregnancy, with an established recommended intake of 800 mcg daily.

Dosage

The dose of folic acid required varies depending on the clinical condition. For lowering homocysteine, a minimum dose of 800 mg daily is generally used. The most common therapeutic dose is in the range of 1-3 mg daily. Doses greater than 10 mg daily have been used in conditions such as cervical dysplasia. Dosages of over-the-counter folic acid supplements are restricted to no more than 800 mg of folic acid per serving, although prescription forms of folic acid are available in higher doses.

CHAPTER III
MATERIALS AND METHODS

1. Subjects

Twenty normal volunteers, 13 males and 7 females, ages ranging from 18 to 50 years were participated in this study. All subjects were healthy on clinical assessment and were given written informed consents. This trial was approved by The Ethic Committee on Research Involving Human Subject, Faculty of Medicine Siriraj Hospital, Mahidol University.

2. Intervention

Curcuminoid capsules (250mg) were donated from The Government Pharmaceutical Organization. The pharmacodynamics properties of curcuminoids after a single oral dose of 6,000 mg were studied in normal volunteers. Subjects were abstained from food for 10 hours and blood samples of 20 ml each were collected before dose administration and at 15, 30, 45, 60, 75 minutes, 1.5, 2, 4 ,8 and 24 hours after drug administration. After washing out for one week volunteers took 5mg folic acid for 14 days, in day 14 concomitant curcuminoids and folic acid were taken and blood was collected.

3. **Inclusion criteria** of subjects

1. No history of all type of allergy
2. Not use of any drug during the past month.
3. No smoke and drink alcohol
4. No pregnant and lactation
5. Sign in informed consents

4. Blood collection

Twenty milliliters of whole blood in ethylenediamine tetrachloroacetic acid (EDTA) as anticoagulant from normal subjects were collected before and after receiving folic acids. Blood samples were collected to check complete blood count (CBC). Whole blood in EDTA tube was centrifuged at 800 x g, 4°C for 10 minutes to remove buffy coat and plasma. Then, red cells were washed three times with 5 mM phosphate bufferred saline (PBS), pH 7.4 at 800 x g, 4°C for 10 minutes. Red cells were resuspended with the same buffer to make up 50% red cell suspension. This suspension and blood plasma were aliquoted to analyse various hematological and biochemical parameters.

5. Biochemical parameters

Parameters of oxidative stress and antioxidant status in this research were malondialdehyde, reactive oxygen species, and reduced glutathione.

6. Instruments

6.1 Autopipette pipetman, Gilson, France
6.2 Analytical balance, Balance Scaltec, model BC52, Scaltec, Germany
6.3 Analytical balance, Model R160P, Sartorius research
6.4 Centrifuge, Sorvall MC12V, rotor F-12/M.18, Dupont, USA
6.5 Magnetic stirrer, Magnestir, Lab-Line Instrument Inc., USA
6.6 pH meter, Model RL150, Russell, USA
6.7 Refrigerated centrifuge, Sorvall RC 26 plus, rotor SS-34, Dupont, USA
6.8 Spectrophotometer, UV-160, Shimadzu, Japan
6.9 Spectrophotometer, UV-1601, Shimadzu, Japan
6.10 Vacuum pump, Model DOA-V122-FD, MFG. Corp., Benton Harbor, USA
6.11 Vortex, Genie 2, USA
6.12 Waterbath, Imperial III, Lab-Line Instrument Inc., USA
6.13 Reciprocal shaking waterbath, Model 50, Precision Scientific, USA
6.14 FACSort flow cytometer, Becton-Dickenson, USA
6.15 CO_2 incubator, Shel Lab, USA

6.16 CellQuest software, USA

6.17 Analytic balance, Mettler AJ 150, Switzerland

6.18 Spectrophotometer, Jasco ModelUVIDEC-650, Japan

7. Glasswares and supplies

7.1 Aluminium foil, Reynolds metals company, USA

7.2 Beakers

7.3 Medicine Dropper

7.4 Duran 500, 1000 ml

7.5 Eppendorf 1.5 mlband 2 ml

7.6 Filter paper, diameter 70 mm and 90 mm circle, Whatman no.1, USA

7.7 Parafilm, 4 inch x 125 ft roll, size M, American nation, USA

7.8 Pipette tips 200 and 1000 µl

7.9 Plasteur pipettes

7.10 Suction volumetric flask

7.11 Tube 10x100 mm, 16x100 mm, 16x150 mm Pyrex, USA, and glass micritubes 3 ml

7.12 Volumetric flask 10, 25, 50, 100, 200, 500, 1000 and 2000 ml

8. Chemicals

Table 2 Chemicals used in this study

	Chemical name	Formula	Molecular weight	Trade mark
1	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	$C_{18}H_{18}N_4O_6S_4 \cdot (NH_3)_2$	548.7	Calbiochem
2	6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)	$C_{14}H_{18}O_4$,	250.29	Sigma

	Chemical name	Formula	Molecular weight	Trade mark
3	Potassium persulfate	$K_2S_2O_8$	270.33	Sigma
4	2,2'-azobis(2-methylpropionamidine) dihydrochoride (AAPH)	$C_8H_{18}N_6 \cdot 2HCl$	271.2	Cayman Chemical
5	Ascorbic acid	$C_6H_8O_6$	176.10	Sigma
6	Curcumin	$[HOC_6H_3(OCH_3)CH=CHCO]_2CH_2$	368.38	Sigma
7	Dichlorofluorescin diacetate	$C_{24}H_{16}O_7Cl_{20}$	487.30	Sigma
8	2,2-diphenyl-1-picylhydrazyl(DPPH)			Fluka
9	Disodium ethylenediamine tetrachloroacetic acid (EDTA)	$C_{10}H_{14}N_2O_8$ $Na_2H.2H_2O$	372.20	Sigma
10	Disodium hydrogenphophate	$Na_2HPO_4.7H_2O$	268.07	Amersham
11	Dimethyl sulfoxide (DMSO)	$(CH_3)_2SO$	78.13	Sigma
12	5, 5-Dithiobis-(2-nitrobenzoic acid)	$C_{14}H_8N_2O_8S_2$	396.30	Sigma
13	Glacial metaphosphoric acid	$(HPO_3)n$	HPO_3 (40-50%) $NaPO_3$ (50-60%)	Merck
14	Glutathione (reduced form)	$C_{10}H_{17}N_3O_6S$	307.30	Sigma
15	Glutathione reductase			Sigma
16	Hydrogen peroxide	H_2O_2	34.00	Sigma

	Chemical name	Formula	Molecular weight	Trade mark
17	Methanol	CH_3OH	32.04	Merck
18	Sodium azide	NaN_3	65.01	Sigma
19	Sodium arsenite	$NaAsO_2$	129.9	Sigma
20	Sodium chloride	NaCl	58.44	Sigma
21	Sodium citrate dihydrate	$C_6H_5Na_3O_7.2H_2O$	294.10	Merck
22	Sodium dihydrogenphophate	$NaH_2PO_4.H2O$	137.99	Amersham
23	Sodium hydroxide	NaOH	40.00	Merck
24	Thiobarbituric acid	$C_4H_4N_2\ O_2\ S$	144.1	Sigma
25	α -Tocopherol	$C_{29}H_{50}O2$	430.7	Sigma
26	Trichloroacetic acid	$C_2HCl_3\ O_2$	163.39	Merck

9. Reagent preparation

9.1 Reagents for preparation of red blood cells

9.1.1 5 mM phosphate bufferred saline, pH 7.4

Na_2HPO_4 .$7H_2O$ 1.0616 g, NaH_2PO_4 .H_2O 0.14352 g and NaCl 8.5 g were mixed with 800 ml of distilled water. The mixture was adjusted pH to 7.4 and adjusted volume to 1000 ml with distilled water. This solution was kept at 4°C and used within 3 months.

9.2 Reagents for analyzing antioxidant capacity of curcuminoids

9.2.1 Reagents for ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay

9.2.1.1 7 mM ABTS and 2.45 mM potassium persulfate

ABTS 0.038409 g and 0.006623 g potassium persulfate were dissolved in 10 ml of distilled water. This solution was kept in the dark at room temperature for 12–16 h before use.

9.2.1.2 2.5 mM Trolox

Trolox 0.15643 g was dissolved in 250 ml of phosphate bufferred saline.

9.2.2 Reagents for analyzing inhibitory effects of curcuminoids on human erythrocyte hemolysis

9.2.2.1 150 mM AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride**)**

AAPH 8.136 g were dissolved in 200 ml of 5 mM phosphate bufferred saline, pH 7.4.

9.3. Reagents for malondialdehyde assay

9.3.1 Isosmotic phosphate- bufferred saline, pH 7.4

Na_2HPO_4 .$7H_2O$ 1.5917 g, NaH_2PO_4 .H_2O 0.21561 g and NaCl 3.9447 g were mixed in distilled water 450 ml. The solution was then adjusted pH to 7.4 and adjusted volume to 500 ml. This solution was kept at 4°C and used within 6 months.

9.3.2 4 mM Sodium azide

Sodium azide 0.026 g was added to 200 ml of isosmotic phosphate bufferred saline, pH 7.4 and mixed with magnetic stirrer. This solution was kept at 4°C and used within 3 months.

9.3.3 0.068% hydrogen peroxide

22.67 µl of 30% hydrogen peroxide was pipetted to 10 ml of isosmotic phosphate-bufferred saline, pH 7.4 in 10 ml volumetric flask. The solution was mixed gently. This solution was prepared freshly before assay about 5-10 minutes.

9.3.4 TCA-arsenite

Trichloroacetic acid (TCA) 28 g was added to 40 ml of distilled water, mixed and sodium m-arsenite 1.299 g was then also added. The TCA solution and the sodium m-arsenite were mixed together and then adjusted volume to 100 ml with distilled water. This solution was kept at room temperature in dark bottle. This solution was used within 3 months.

9.3.5 1 M Stock NaOH

Sodium hydroxide 4 g was added to distilled water 100 ml and kept it as stock solution at room temperature.

9.3.6. 0.05 M NaOH

25 ml of 1 M stock NaOH was mixed in 500 ml volumetric flask containing distilled water and then mixed it well. This solution was used as solvent to prepare 1% thiobarbituric acid.

9.3.7 1%TBA in 0.05 M NaOH

Thiobarbituric amcid (TBA) 1 g was added to 90 ml of 0.05 M NaOH. The solution was stirred on heat magnetic stirrer with turn on heat level 1. After that, this reagent was adjusted volume to 100 ml in volumetric flask. The solution was kept in dark bottle at room temperature. This solution was used within 2 months.

9.4 Reagents for determining reactive oxygen species

9.4.1 5 mM phosphate buffered saline (PBS)

$Na_2HPO_4.7H_2O$ 1.0616 g, $NaH_2PO_4.H_2O$ 0.14352 g, and NaCl 8.5 g were mixed with 800 ml of distilled water. The mixture pH was adjusted to 7.4 and volume adjusted to 1000 ml with distilled water. This solution was kept at 4ºC and used within 3 months.

9.4.2 2 mM hydrogen peroxide

31.60 µl of 33% H_2O_2 was diluted with 10 ml PBS. This solution was prepared freshly before assay about 5-10 minutes.

9.4.3 2′-7′dichlorofluorescin diacetate (DCFH-DA)

10 mg of 2′-7′dichlorofluorescin diacetate was added to 1 ml methanol.

9.5. Reagents for reduced glutathione assay

9.5.1 Precipitating solution

30 g of sodium chloride (NaCl) was dissolved in 80 ml of distilled water. Glacial metaphosphoric acid (a mixture of HPO_3 and $NaPO_3$) 1.67 g and disodium ethylenediamine tetrachloroacetic acid (EDTA) 0.2 g was added to the NaCl solution. The mixture was adjusted volume to 100 ml in volumetric flask. This solution is stable for approximately 3 weeks at 4°C.

9.5.2 0.3 M Phosphate solution

Na_2HPO_4 .$7H_2O$ 16.0842 g was prepared in 200 ml of distilled water. This solution is stable for 6 months.

9.5.3 DTNB reagent

5,5'-Dithiobis-(2-nitrobenzoic acid) 40 mg was added into 100 ml of 1% sodium citrate solution. One percent sodium citrate was prepared by adding sodium citrate dihydrate 1.13948 g in 100 ml of distilled water. The DTNB reagent is stable for at least 13 weeks at 4ºC.

10. Sample analysis

10.1 The method for measuring antioxidant capacity of curcuminoid.

Principle

Generation of the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances (108), aqueous mixtures and beverages (170). This method involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. This has absorption maxima at wavelengths 734 nm. Addition of antioxidants to the pre-formed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus the extent of decolorization as percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) as a standard, under the same conditions (171).

Procedure

0.038409 g of ABTS and 0.006623 g of potassium persulfate were dissolved in 10 ml of distilled water. ABTS radical cation ($ABTS^{\bullet+}$) was produced and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Stock solutions of antioxidant compounds (curcumin was dissolved in 70%DMSO, ascorbic acid and glutathione was dissolved in distilled water and α -tocopherol was dissolved in 80% methanol) were diluted in various concentrations. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution (A_{734nm} = 0.700 ± 0.020) to 10 µl of antioxidant compounds or Trolox standards in PBS, the absorbance reading was taken at 30°C. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

Blood plasma of volunteers at various collection times were measured antioxidant capacity by the same method as described above. 10 µl of blood plasma were used instead of antioxidant compound.

10.2 Determination of free radical scavenging activity by DPPH method

Principle

The H-donor activity of curcumin was measured by 2,2-diphenyl-1-Picrylhydrazyl (DPPH) method. The DPPH is a stable radical, its methanolic solution shows maximum absorbency at 517 nm. The H-donor molecules reduce DPPH radical and thus decrease its absorbance at 517 nm.

Briefly, the reaction mixture contained 2 ml of dilutions of the curcumin solution (curcumin was dissolved in 80% methanol), 1 ml of 0.5 mM DPPH (in methanol) and 2 ml of distilled water. After standing for 30 minutes, the absorbance of the mixture was measured at 517 nm. The curcumin solution can interact with DPPH. The percentage of DPPH radical scavenging activity was calculated by the following formula.

$$\% \text{ radical scarvenging activity} = [\ 1- (\ A_{\text{curcumin solution}} / A_{\text{control}}\ )\ ] \text{ X } 100$$

The DPPH scavenging activity was plotted against the concentration of the curcumin solution. A logarithmic regression curve was established in order to calculate the IC_{50}, which is the amount of concentration of curcumin solution necessary to decrease by 50% the absorbance value of the control.

10.3 Inhibitory effect of curcuminoid on human erythrocyte hemolysis

Principle

Erythrocyte oxidative hemolysis was induced by 2,2'-azobis (2-methylpropionamidine) dihydrochoride (AAPH), a peroxyl radical initiator. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipid and proteins, resulting in hemolysis.

Procedure

0.5 ml of RBC suspension in PBS was mixed with 1 ml of PBS solution containing varying amounts of curcuminoids or PBS as a control. The reaction mixture was preincubated at 37°C in a shaking water bath for 30 minutes. 1ml of 150 mM AAPH in PBS was added to the mixture. The reaction mixture was incubated at 37°C in a shaking water bath for 1 hour. After incubation, the reaction mixture (0.1 ml) was withdrawn into 0.7 ml of ice-cold PBS and centrifuged at 7,200 x g for 5 minutes. The supernatant of mixture was read at 540 nm. L-Ascorbic acid was added instead of curcuminoids in a refrence tube. The percentage of hemolysis was calculated according to the formula below;

$$\% \text{ Hemolysis} = (A_{cur} / A_{control}) \text{ X } 100$$

while, A_{cur} is the absorbance value of the reaction mixture containing curcuminoids,

$A_{control}$ is the absorbance value of the reaction mixture without curcuminoids.

The percentage of hemolysis inhibition is plotted against the concentration of antioxidant substance to find out the IC_{50}, which is the amount of concentration of antioxidant substance necessary to decrease by 50% the absorbance value of the control.

10.4 Reactive oxygen species (ROS)

Principle

2′-7′dichlorofluorescin diacetate (DCFH-DA) is used for measuring ROS generation by the fluorescence of their oxidized derivatives. When dichlorofluorescin diacetate crosses red cell membrane, it undergoes deacetylation by intracellular esterases, producing the non-fluorescence compound (DCFH) that is trapped inside the cells. Oxidation by ROS produced the highly fluorescent 1,2 dichlorofluorescein. (DCF). To quantify fluorescence, the rate and extent of ROS production can be measured by flow cytometry (172).

Procedure

2 μl of blood sample was diluted with ion - free phosphate buffered saline (PBS) 9 ml to a concentration 1×106 RBCs/ml. Then, 20 μl of 2′-7′dichlorofluorescin diacetate (DCFH-DA) dissolved in methanol (10mg/ml) was added to 1 ml cell suspension. After incubation at 37° C for 15 minutes in a humidified atmosphere of 5 % CO_2 in air, the RBC suspension was washed with PBS and resuspended in PBS to the original cell concentration. The samples were further incubated at room temperature with (stimulated) or without (unstimulated) 2 mM hydrogen peroxide (H_2O_2), which was freshly prepared before each experiment by diluting a 33% stock solution with PBS.

Flow cytometry: RBCs were analyzed by fluorescene activated cell sorter excitation (FACS). The arithmetic mean fluorescence channel (MFC) was derived by the CellQuest software.

10.5 Reduced glutathione assay

Principle

Glutathione is a tripeptide of glycine, glutamic acid and cysteine. In the blood, almost all of the reduced glutathione is found within the red blood cells. The sulfhydryl group of glutathione react with DTNB to give a relatively stable yellow color (173).

Procedure

400 μl of 50% red cell suspension was added to 1600 μl of distilled water to obtain hemolysate. Three milliliters of precipitating solution was mixed with the hemolysate. The mixture was allowed to stand for approximately 5 minutes and then filtered with the filter paper Whatman no.1. 500 μl of filtrate was added to 2.0 ml of 0.3 M phosphate solution. Then, 250 μl of DTNB reagent was added and let stand for 5 minutes before measuring at 412 nm. A blank is prepared with 2 ml of 0.3 M phosphate solution and 500 μl of diluted precipitating solution (3:2 of precipitating solution: distilled water), and 250 μl of DTNB reagent.

Calculation

The value of GSH was calculated from the difference of the absorption between before and after adding DTNB reagent. GSH content was calculated by the equation (121):

GSH Content (mg/dl) = 310.4 x (OD*after* -OD*before*)

10.6. Malonyldialdehyde assay

Principle

Lipid peroxidation was assayed by measuring the formation of malondialdehyde (MDA). The formation of MDA was exposed by hydrogen peroxide. Then, MDA was extracted by trichloroacetic acid (TCA), and reacts with thiobarbituric acid (TBA) in boiling condition to MDA-TBAR complex. This complex gives pink color and measuring at 532 nm (173, 174).

Procedure

50% Red cells were hemolyzed and adjusted hemoglobin concentration to 10 g/dl. 1.5 ml of samples was pre-incubated with 1.5 ml of 4 mM sodium azide at 37°C for 1 hour. The samples were incubated with 3 ml of 0.068 % H_2O_2 at 37°C for 1 hour. The reaction in mixtures (4 ml) were stopped by adding trichloroacetic acid (2 ml) and then centrifuged at 2300 x g at 37°C for 10 minutes. Supernatant (4 ml) was removed and added 1 ml of TBA. After boiling for 15 min, the sample was cooled under tap water, and determined at the difference of the absorbance 532 and 600 nm (174).

Calculation

From the equation $A = \varepsilon bc$

When A = the different of the absorbance 532 and 600 nm

ε = molar exinction of MDA at 532 nm =1.56×10^5

b = slit wide of cuvette 1 cm

c = the concentration of MDA (nmoles/g Hb).

Therefore;

MDA (nmoles/g Hb) = $6.4102 \times 10^3 \times (OD_{532}-OD_{600})$

11. Hematological parameters

Complete blood count (CBC) was read in 2 ml of the EDTA blood by automated cell counter. Number of white cells, red blood cells, hemoglobin concentration, percent hematocrit, MCV, MCH, MCHC, platelet counts, and reticulocytes count were recorded in both whole blood and 50% red cell suspension. Hemoglobin was also analyzed.

12. Statistical analysis

Data were analyzed by using two-way analysis of variance (ANOVA) using SPSS. Fisher least significance difference test was used to examine differences between group means. A p-value < 0.05 was considered statistically significant.

CHAPTER IV
RESULTS

1. Hematological data of normal subjects

Hematological data of 20 normal subjects, 13 males and 7 females, was shown in Table 3. All subjects were healthy on clinical assessment and wrote informed consents. The mean of age, height, weight, and body mass index was 28 ± 7 years, 163.53 ± 9.72 cm., 58.83 ± 8.30 kg. and 21.89 ± 2.12 kg/m^2. Other hematological and biochemical parameters such as complete blood count, hemoglobin typing, liver function test, renal function test and lipid profiles were determined (Table 3). They were healthy as determined by various parameters.

2. The antioxidant capacity of curcuminoids

This method determined by the decolorization of the $ABTS^+$, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. Curcuminoids decolorized 50% at 0.39 mM (IC_{50} = 0.39 mM) while IC_{50} of glutathione (GSH) was 0.8 mM, vitamin E was 1.1 mM, and IC_{50} of vitamin C was 1.2 mM (Figure 7). Figure 8 showed percent inhibition of the $ABTS^{\bullet+}$ radical cation of antioxidant compounds at 1mM., curcumin inhibited at 77.05%, glutathione inhibited at 50.79%, alpha-tocopherol inhibited at 30.44% and ascorbic acid inhibited at 37.97% (n = 10 and done in triplicate).

Curcuminoids inhibited human erythrocyte hemolysis from 2,2′-azinobis –2-methylpropionamidine dihydrochloride (AAPH) induction. It inhibited erythrocyte hemolysis 50% at 16 μM (Figure 9). (for all determination n = 10 and in triplicate)

3. Determination of free radical scavenging activity using DPPH method

The data demonstrated that the ability of curcumin to scavenge the DPPH radicals measured as IC_{50}. The results were shown in Figure 10. IC_{50} value was calculated by a logarithmic regression curve. The IC_{50} of curcumin was 32.47 μM.

Table 3. Hematological and clinical chemistry data of normal subjects before receiving treatment (mean±SD)

Parameters	Healthy Reference	Male (n = 13)	Female (n = 7)	Total (n = 20)
Weight (kg)	-	62.62 ± 7.99	51.79 ± 1.23	58.83 ± 8.30
Height (cm)	-	169.31 ± 6.02	152.79 ± 4.45	163.53 ± 9.72
Age	-	26 ± 6	33 ± 7	28 ± 7
Body Mass Index (kg/m^2)	-	21.71 ± 2.40	22.21 ±1.57	21.89 ± 2.12
Blood Pressure(Systolic)	-	116 ± 8	106 ± 13	112 ± 11
Blood Pressure (Diastolic)	-	75 ± 6	69 ± 11	73 ± 9
Pulse Rate (cpm)	-	68 ± 11	73 ± 9	70 ± 10.4
Respiratory Rate(cpm)	-	20 ± 2	21 ± 2	21 ± 2
Temperature (°C)	-	36.56 ± 0.29	36.61 ± 0.22	36.58 ± 0.26
Hb typing	-	A_2A	A_2A	A_2A
WBC ($10^3/\mu l$)	4-11	5.78 ± 1.59	5.26 ± 1.23	5.60 ± 1.46
RBC ($10^6/\mu l$)	4.2-5.4	5.16 ± 0.32	4.55 ± 0.59	4.94 ± 0.51
Hemoglobin (g/dl)	12-18	14.82 ± 0.97	13.34 ± 1.94	14.31 ± 1.52
Hematocrit (%)	37-52	43.73 ± 2.58	41.03 ± 5.51	42.79 ± 3.94
MCV (fl)	80-99	84.86 ± 3.30	90.13 ± 2.30	86.71 ± 3.90
MCH (pg)	26-32	28.77 ± 1.44	29.30 ± 1.36	28.96 ± 1.4
MCHC (g/dl)	31-35	33.89 ± 1.24	32.47 ± 0.78	33.40 ± 1.29
Platelet ($10^3/\mu$)	150-440	237.54 ± 43.5	228.14 ± 55.85	234.25 ± 46.91
Glucose (mg/dl)	76-110	85.31 ± 6.30	85.29 ± 5.56	85.3 ± 5.9
Creatinine (mg/dl)	0.5-1.5	1.04 ± 0.13	0.66 ± 0.08	0.91 ± 0.22

Table 3. Hematological and clinical chemistry data of normal subjects before receiving treatment (mean±SD). (continued)

Parameters	Healthy Reference	Male (n = 13)	Female (n = 7)	Total (n = 20)
Urea nitrogen (mg/dl)	7-20	15.08 ± 4.73	11 ± 1.29	13.65 ± 4.32
Cholesterol (mg/dl)	100-200	190.08 ± 20.77	203.86 ± 43.9	194.9 ± 30.44
Triglyceride (mg/dl)	50-200	79.69 ± 56.14	65.01 ± 14.69	74.55 ± 45.94
HDL-cholesterol (mg/dl)	35-100	56.15 ± 9.19	67.57±16.49	60.15±13.06
Albumin (g/dl)	3.5-5.5	4.48 ± 0.25	4.37 ± 0.38	4.45 ± 0.30
Aspartate aminotransferase (AST) (U/l)	0-37	19.85 ±5.96	18.29 ± 6.10	19.3 ± 5.89
Alanine aminotransferase(ALT) (U/l)	0-40	19.85 ± 8.94	16.71±12.26	18.75±10.02
Alkaline phosphatase (ALP) (U/l)	39-117	70.46±18.09	55.14±9.63	65.1±17.09
Total bilirubin (mg/dl)	0.3-1.2	0.71 ± 0.28	0.57 ± 0.22	0.66 ± 0.26
Uric acid (mg/dl)	2.4-7	6.12 ± 1.33	4.27 0± .58	5.48 ± 1.43
Ferritin (ng/ml)	8-140	85.92±48.83	37.71±15.91	68 ± 47.05
Folate (ng/ml)	4.2-19.9	6.22 ± 2.18	6.34 ± 1.14	6.26 ± 1.85

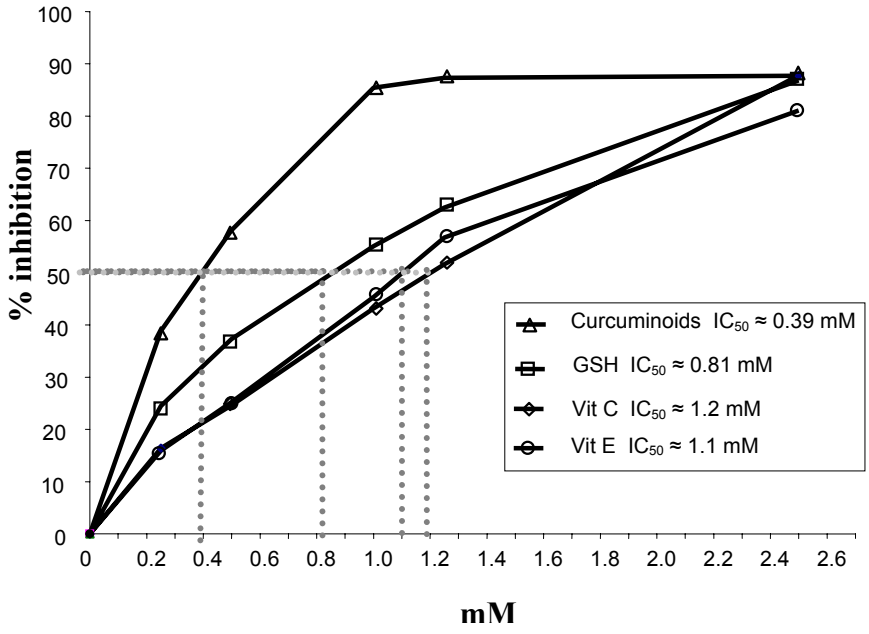

Figure 7. Percent inhibition of the $ABTS^{\bullet+}$ radical cation and IC_{50} of curcuminoids (Δ) , GSH (□), Vitamin C (◊) and Vitamin E (○).

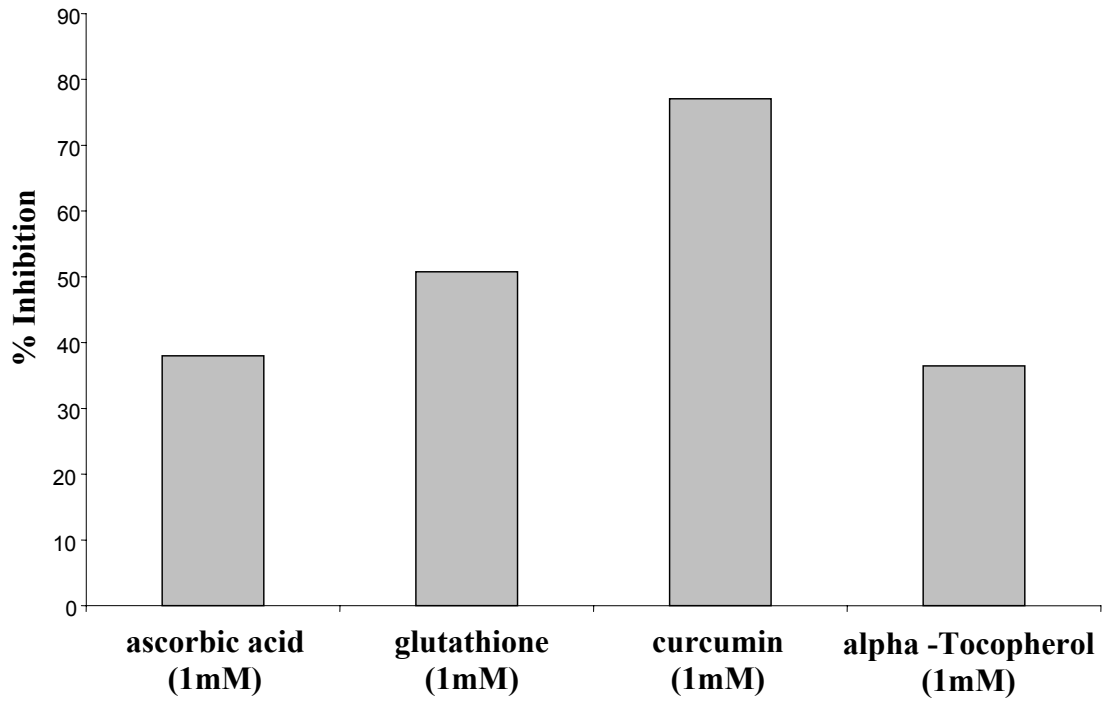

Figure 8. Percent inhibition of the $ABTS^{\bullet+}$ radical cation of antioxidant compounds at 1mM

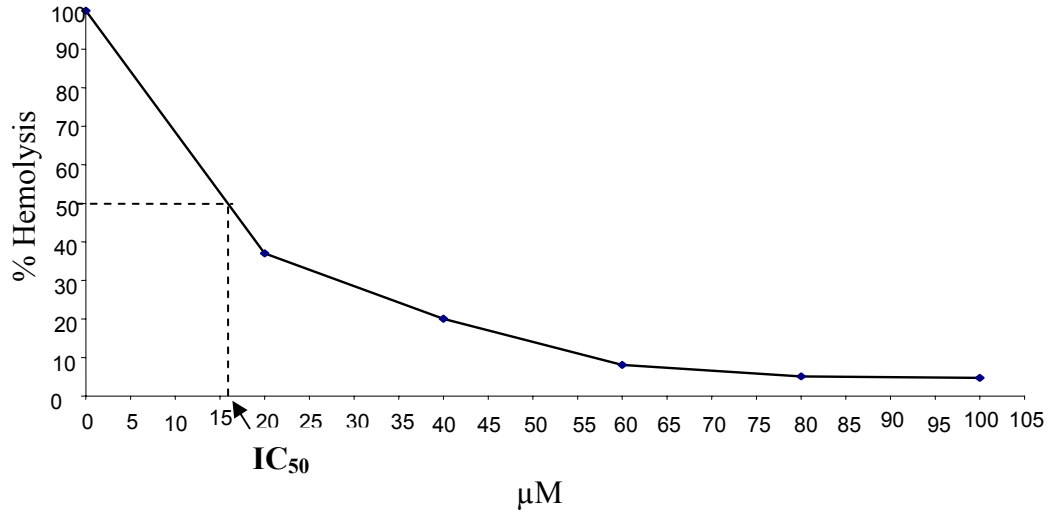

Figure 9. Percentage of erythrocyte hemolysis at different concentration of curcuminoids.

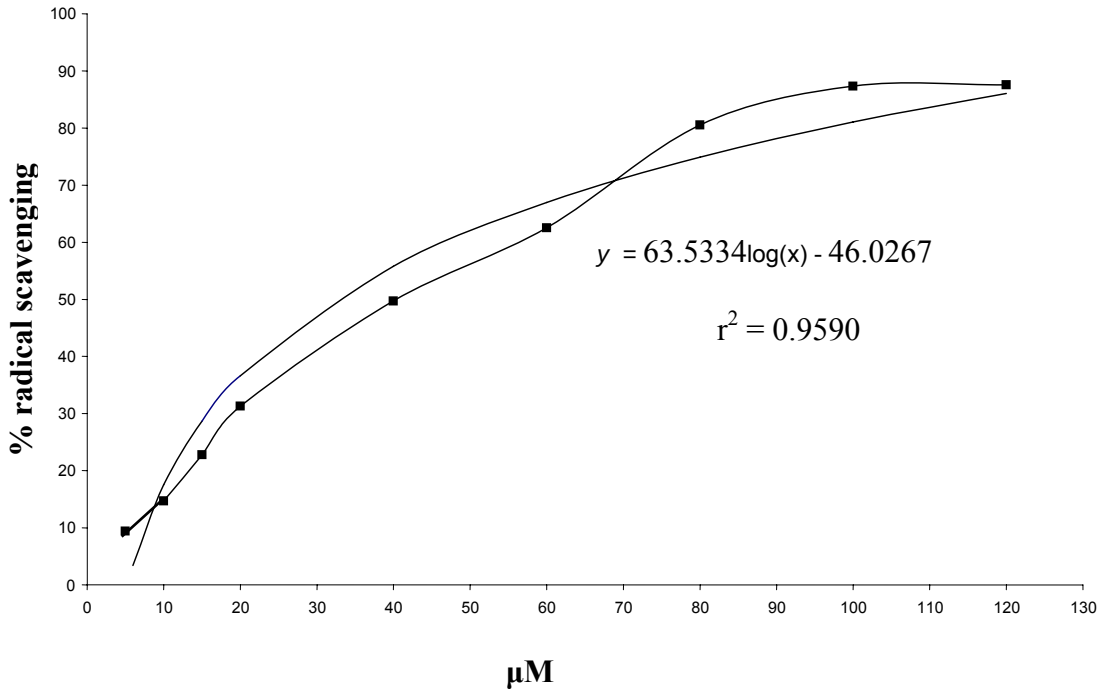

Figure 10 Percent DPPH radicals scarvenging and a logarithmic regression curve of curcumin.

4. The effect of curcuminoids on oxidative stress.

Parameters of oxidative stress and antioxidant statuses in this research were malondialdehyde, reactive oxygen species, and reduced glutathione.

4.1 Oxidative stress

4.1.1 Malondialdehyde

Lipid peroxidation was assayed by measuring the formation of malondialdehyde (MDA). MDA level in erythrocyte was demonstrated in Tables 4 and 5. It was measured at different time 15, 30, 45, 60, 75 min, 1.5, 2, 4, 8 and 24 hours after drug administration. MDA levels at baseline were approximately 760 nmoles/gHb. It slightly decreased within 1 hour after receiving curcuminoids and MDA level was stable for 8 hours (Figure 11). Then it returned to the same level as baseline at 24 hours. However, MDA levels at various time were not significantly different from the baseline ($p>0.05$). Similarly MDA levels at visit 2 (Tables 4 and 5) were almost similar at every time points. The MDA levels before and after receiving folic acid did not show significantly difference ($p>0.05$).

4.1.2 Reactive oxygen species

2′-7′dichlorofluorescin diacetate (DCFH-DA) was used for measuring ROS generation by the fluorescence of their oxidized derivatives. When dichlorofluorescin diacetate cross red cell membrane, it underwent deacetylation by intracellular esterases, producing the non-fluorescence compound (DCFH) that was trapped inside the cells. Oxidation by ROS produced the highly fluorescent 1, 2 dichlorofluorescein (DCF). To quantify fluorescence, the rate and extent of ROS production could be quantified by flow cytometry.

Tables 6 and 7 demonstrated the percent change of the fluorescent intensities of dichlorofluororescien (DCF) in red blood cells (RBCs) during treatment with curcuminoids at different times. ROS gradually decreased from baseline (0 minute) approximately 30% at 75 minutes after curcuminoids ingestion, It was then increased at 24 hours (Figure 12). There was no significantly difference in ROS at each time point and between before and after receiving folic acid.

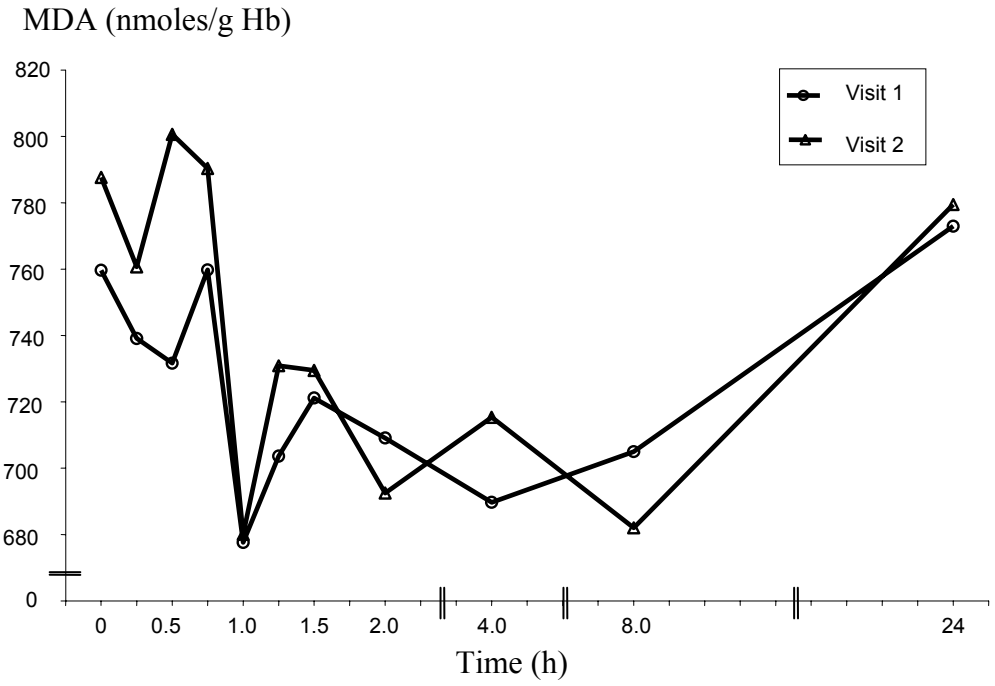

Figure 11. The erythrocyte MDA in normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) and visit 2 (after receiving folic acid).

Table 4. The erythrocyte MDA in normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) (mean ± SEM).

Time (h.) After curcuminoids administration	Malondialdehyde (nmoles/g Hb)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	738.28 ± 25.31	799.31 ± 25.03	759.64 ± 19.41
0.25	760.10 ± 31.46	700.17 ± 34.33	739.12 ± 24.07
0.5	710.92 ± 31.65	770.15 ± 15.79	731.65 ± 21.93
0.75	746.86 ± 28.11	783.76 ± 29.88	759.78 ± 20.96
1	685.81 ± 30.02	662.48 ± 40.29	677.64 ± 23.58
1.25	656.90 ± 27.45	790.43 ± 15.77	703.63 ± 23.46
1.5	698.38 ± 31.84	763.46 ± 49.89	721.16 ± 27.24
2	703.18 ± 22.49	720.10 ± 56.75	709.10 ± 23.81
4	677.81 ± 11.47	711.85 ± 40.95	689.72 ± 15.91
8	684.60 ± 29.45	742.84 ± 60.54	704.98 ± 28.31
24	742.33 ± 21.25	829.86 ± 44.47	772.97 ± 22.26

Table 5. The erythrocyte MDA in normal subjects after receiving curcuminoids at visit 2 (after receiving folic acid) (mean ± SEM).

Time (h.) After curcuminoids administration	Malondialdehyde (nmoles/g Hb)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	798.96 ± 18.62	766.66 ± 27.02	787.66 ± 15.35
0.25	777.02 ± 26.17	730.39 ± 42.67	760.70 ± 22.55
0.5	790.20 ± 27.85	819.89 ± 39.25	800.59 ± 22.34
0.75	792.61 ± 48.53	786.16 ± 28.50	790.35 ± 32.51
1	685.96 ± 36.29	669.21 ± 25.20	680.10 ± 24.78
1.25	721.05 ± 31.29	749.09 ± 12.99	730.86 ± 20.74
1.5	753.20 ± 32.59	685.38 ± 43.28	729.46 ± 26.42
2	689.17 ± 33.02	698.74 ± 26.13	692.52 ± 22.90
4	719.08 ± 25.96	708.45 ± 47.28	715.36 ± 22.91
8	715.92 ± 43.61	619.01 ± 28.87	682.04 ± 31.39
24	771.05 ± 24.41	795.10 ± 40.90	779.47 ± 20.89

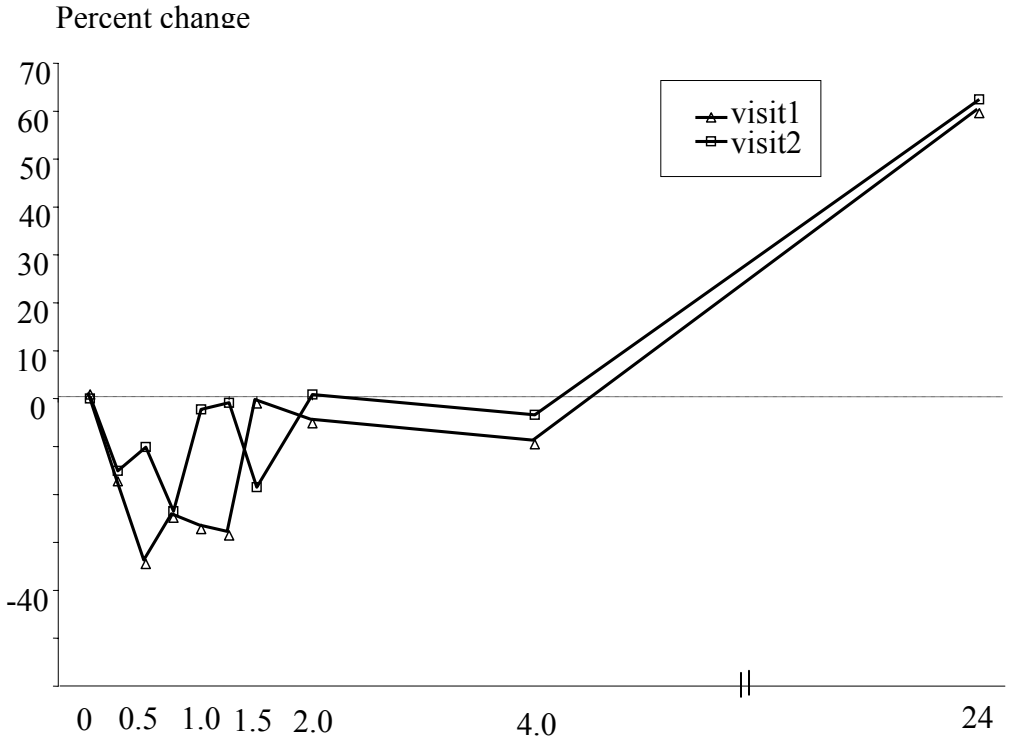

Figure 12. Percent change of reactive oxygen species (ROS) in erythrocyte of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) and visit 2 (after receiving folic acid).

Table 6. Percent change of reactive oxygen species (ROS) in erythrocyte of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) (mean ± SEM).

Time (h.) After curcuminoids administration	Percent change of Reactive Oxygen Species		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0.0	0.0	0.0
0.25	-24.40 ± 4.17	-5.23 ± 8.41	-17.69 ± 4.40
0.5	-41.50 ± 4.54	-22.78 ± 5.61	-34.95 ± 4.02
0.75	-39.08 ± 8.86	0.30 ± 15.41	-25.30 ± 8.77
1	-37.68 ± 9.18	-9.07 ± 10.95	-27.67 ± 7.59
1.25	-38.60 ± 11.85	-19.34 ± 21.67	-28.97 ± 10.50
1.5	-5.18 ± 11.04	5.45 ± 13.19	-1.46 ± 8.40
2	-11.78 ± 11.15	6.03 ± 18.38	-5.55 ± 9.60
4	-15.42 ± 11.30	0.18 ± 15.07	-9.96 ± 8.97
24	62.94 ± 25.49	52.13 ± 22.64	59.16 ± 18.02

Table 7. Percent change of reactive oxygen species (ROS) in erythrocyte of normal subjects after receiving curcuminoids at visit 2 (after receiving folic acid) (mean ± SEM).

Time (h.) After curcuminoids administration	Percent change of Reactive Oxygen Species		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0.00	0.00	0.00
0.25	-22.11 ± 5.34	-3.22 ± 13.62	-15.50 ± 6.04
0.5	14.86 ± 12.33	-2.64 ± 11.12	-10.58 ± 8.82
0.75	-40.12 ± 7.07	6.05 ± 9.84	-23.96 ± 7.53
1	-0.25 ±13.22	-7.32 ± 10.88	-2.72 ± 9.24
1.25	-20.75 ± 8.87	34.78 ± 17.55	-1.31 ± 10.16
1.5	-23.18 ± 11.67	-14.72 ± 11.73	-18.95 ± 9.48
2	-10.30 ± 10.69	20.30 ± 17.62	0.41 ± 9.62
4	-13.31 ± 13.85	13.70 ± 14.97	-3.86 ± 10.60
24	75.17 ± 29.11	37.63 ± 42.79	62.03 ± 23.82

4.2 Antioxidant activity

4.2.1 ABTS radical cation decolorization assay

After normal subjects received curcuminoids at different time. (15, 30, 45, 60, 75 min, 1.5, 2, 4, 8 and 24 hours after drug administration). Plasma was determined antioxidant activity by ABTS radical cation decolorization assay at different time (Figure 13, Tables 8 and 9). The reduction of the radical cation of plasma was not significantly different at different time ($p>0.05$). There was no sigficanttly difference ($p>0.05$) between before and after receiving folic acid.

4.2.2 Reduced glutathione content

The levels of reduced glutathione was shown as mean$\pm$SEM in Figure 14 (tables 10 and 11). It was not significantly different at different times ($p>0.05$). There was no sigficanttly difference ($p>0.05$) between before and after receiving folic acid.

5. Hematological data after all treatment

After all treatment, hematological data were analyzed again, data shown in Table 12. All parameters were not significantly different ($p>0.05$) from before treatment (Table 3), except the level of folate. After washing out period for one week, volunteers took 5 mg folic acid for 14 days. The level of folate at visit 2 increased from 6.26 ± 1.85 to 27.79 ± 3.4 ng/ml.

Tables 13 and 14 showed all parameters in normal subjects at visit 1 and visit 2. The level of Curcumin ,demethoxycurcumin and bis-demethoxycurcumin concentrations were showen (data from Miss Yuwadee Saejeong , M.s. Thesis) Statistical analysis showed p-value of all determinations in Table 15.

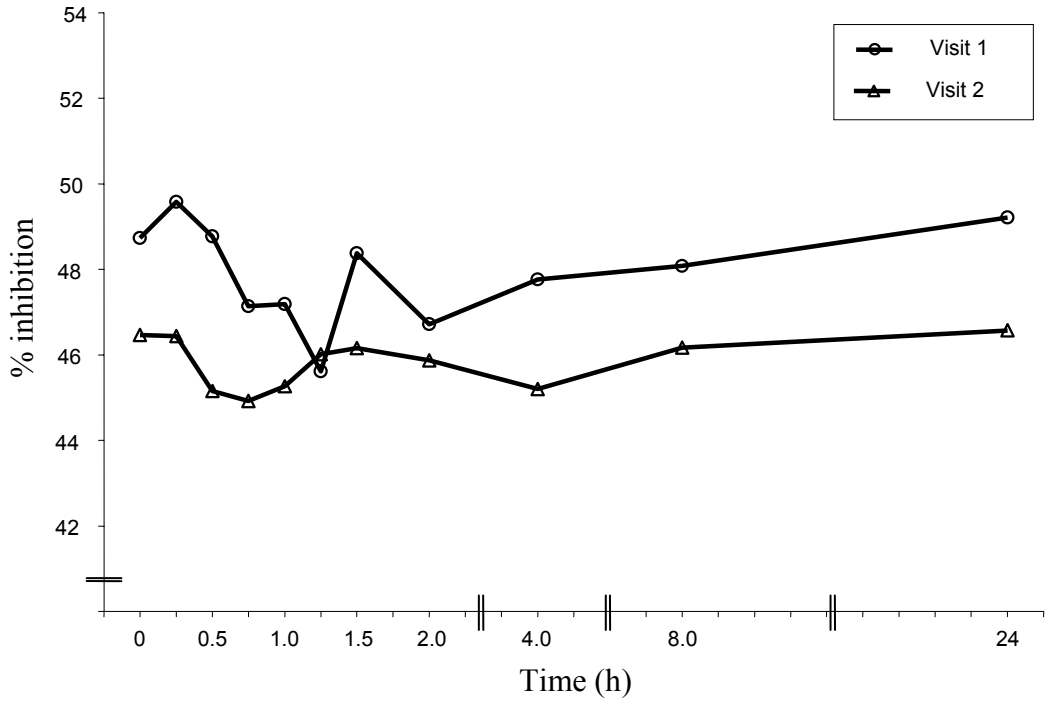

Figure 13. Percent inhibition of the $ABTS^{\bullet+}$ radical cation in plasma of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) and visit 2 (after receiving folic acid).

Table 8. Percent inhibition of the $ABTS^{\bullet+}$ radical cation in plasma of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid, mean ± SEM).

Time (h.) After curcuminoids administration	%ABTS inhibition		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	48.95 ± 1.17	48.32 ± 1.91	48.73 ± 0.98
0.25	49.62 ± 1.74	49.50 ± 1.55	49.58 ± 1.23
0.5	48.93 ± 1.21	48.48 ± 2.61	48.77 ± 1.16
0.75	47.26 ± 1.40	46.94 ± 2.68	47.14 ± 1.27
1	47.40 ± 2.08	46.80 ± 4.71	47.19 ± 2.06
1.25	45.78 ± 2.29	45.31 ± 4.00	45.62 ± 1.98
1.5	48.22 ± 1.08	48.68 ± 1.06	48.38 ± 0.78
2	47.14 ± 1.25	45.94 ± 1.77	46.72 ± 1.00
4	49.07 ± 1.59	45.34 ± 1.60	47.77 ± 1.22
8	48.84 ± 1.56	46.68 ± 2.81	48.08 ± 1.39
24	50.03 ± 1.14	47.69 ± 1.16	49.21 ± 0.86

Table 9. Percent inhibition of the $ABTS^{\bullet+}$ radical cation in plasma of normal subjects after receiving curcuminoids at visit 2 (after receiving folic acid, mean ± SEM).

Time (h.) After curcuminoids administration	%ABTS inhibition		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	46.77 ± 1.48	45.89 ± 2.97	46.46 ± 1.37
0.25	45.48 ± 2.25	48.20 ± 3.34	46.43 ± 1.84
0.5	44.86 ± 1.39	45.69 ± 3.13	45.15 ± 1.78
0.75	45.60 ± 2.57	43.54 ± 2.63	44.92 ± 1.27
1	46.04 ± 1.23	44.64 ± 3.10	45.27 ± 1.95
1.25	45.90 ± 1.32	45.98 ± 0.17	46.02 ± 0.79
1.5	45.05 ± 1.08	46.66 ± 1.31	46.16 ± 0.96
2	44.92 ± 1.82	47.39 ± 1.03	45.87 ± 0.81
4	46.03 ± 1.38	45.73 ± 2.70	45.20 ± 1.47
8	46.38 ± 1.42	46.43 ± 3.91	46.17 ± 1.57
24	45.70 ± 1.67	46.92 ± 2.51	46.57 ± 1.21

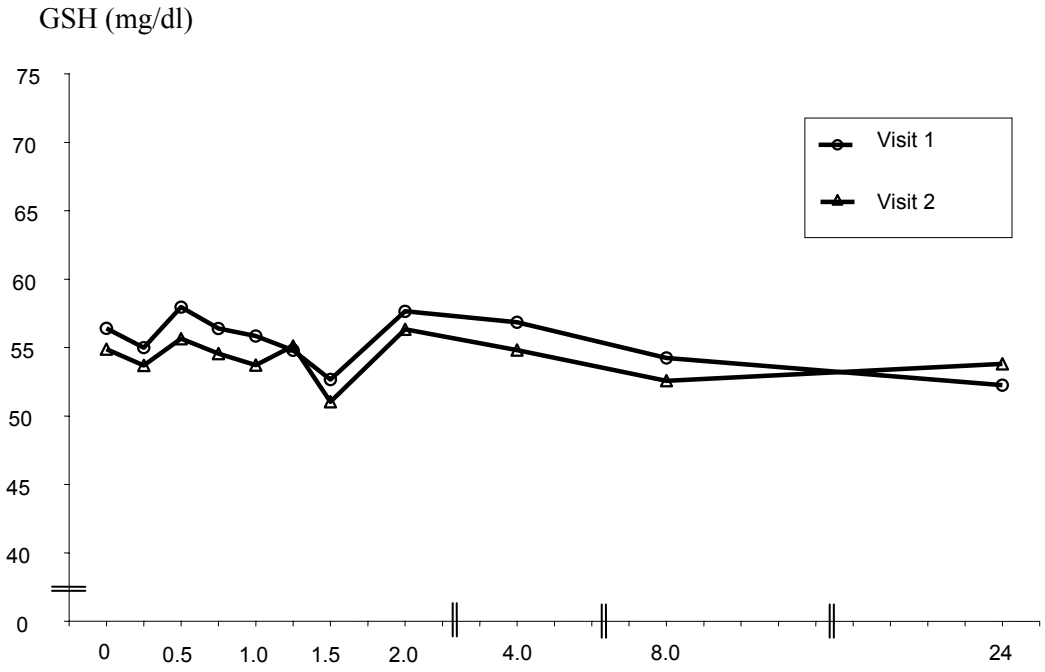

Figure 14. Reduced glutathione(GSH) level in erythrocyte of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid) and visit 2 (after receiving folic acid).

Table 10. Reduced glutathione(GSH) level in erythrocyte of normal subjects after receiving curcuminoids at visit 1 (before receiving folic acid mean ± SEM).

Time (h.) After curcuminoids administration	GSH (mg/dl)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	54.14 ± 2.60	60.62 ± 3.46	56.41 ± 2.16
0.25	53.18 ± 1.59	58.35 ± 1.99	54.99 ± 1.34
0.5	55.31 ± 2.44	62.85 ± 3.25	57.95 ± 2.07
0.75	55.14 ± 2.70	58.71 ± 2.25	56.39 ± 1.92
1	53.38 ± 1.91	60.41 ± 3.84	55.84 ± 1.93
1.25	50.72 ± 2.01	62.39 ± 5.38	54.80 ± 2.55
1.5	51.20 ± 1.87	55.40 ± 2.68	52.67 ± 1.56
2	55.81 ± 3.10	61.07 ± 4.04	57.65 ± 2.47
4	55.94 ± 3.39	58.54 ± 2.42	56.85 ± 2.33
8	52.71 ± 2.17	57.08 ± 4.92	54.24 ± 2.20
24	50.82 ± 2.13	54.95 ± 5.51	52.26 ± 2.33

Table 11. Reduced glutathione(GSH) level in erythrocyte of normal subjects after receiving curcuminoids at visit 2 (after receiving folic acid mean ± SEM).

Time (h.) After curcuminoids administration	GSH (mg/dl)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	52.37 ± 3.45	59.58 ± 2.97	54.89 ± 2.55
0.25	50.55 ± 2.48	59.53 ± 2.09	53.69 ± 2.00
0.5	53.42 ± 3.02	59.79 ± 3.22	55.65 ± 2.32
0.75	53.15 ± 3.80	57.18 ± 3.52	54.56 ± 2.74
1	50.72 ± 2.67	59.30 ± 2.82	53.72 ± 2.17
1.25	53.51 ± 4.21	58.14 ± 3.05	55.13 ± 2.93
1.5	48.65 ± 2.22	55.51 ± 1.91	51.05 ± 1.73
2	53.57 ± 3.29	61.46 ± 3.16	56.33 ± 2.51
4	53.00 ± 3.59	58.21 ± 2.63	54.82 ± 2.53
8	49.28 ± 3.61	58.73 ± 2.57	52.59 ± 2.67
24	54.64 ± 3.70	52.28 ± 3.04	53.82 ± 2.59

Table 12 Hematological and clinical chemistry data of normal subjects after receiving treatment (mean±SD).

Parameters	Healthy Reference	Male (n= 13)	Female (n=7)	Total (n=20)
Weight (kg)	-	62.85 ± 7.54	51.86 ± 1.46	59 ± 8.09
Height (cm)	-	169.31 ± 6.02	152.79 ± 4.45	163.53 ±9.72
Age	-	26 ± 6	33 ± 7	28 ± 7
Body Mass Index (kg/m^2)	-	21.91 ± 2.12	22.27 ± 1.56	22.04 ± 1.91
Blood Pressure(Systolic)	-	120 ± 10	110 ± 13	117 ± 12
Blood Pressure (Diastolic)	-	77 ± 6	71 ± 6	75 ± 6
Pulse Rate (cpm)	-	67 ± 10	73 ± 5	69 ± 9
Respiratory Rate (cpm)	-	22 ± 2	21 ± 2	22 ± 2
Temperature (°C)	-	36.37 ± 0.27	36.5 ± 0.16	36.42 ± 0.24
Hb typing	-	A_2A	A_2A	A_2A
WBC ($10^3/\mu l$)	4-11	5.86 ± 1.6	5.99 ± 1.4	5.91 ± 1.5
RBC ($10^6/\mu l$)	4.2-5.4	5.19 ± 0.57	4.45 ± 0.51	4.93 ± 0.65
Hemoglobin(g/dl)	12-18	14.94 ± 1.42	12.89 ± 1.62	14.22 ± 1.76
Hematocrit(%)	37-52	44.93 ± 4.44	40.57 ± 4.81	43.41 ± 4.93
MCV (fl)	80-99	86.75 ± 3.44	91.24 ± 2.62	88.32 ± 3.8
MCH (pg)	26-32	28.91 ± 1.51	28.97 ± 1.10	28.93 ± 1.35
MCHC (g/dl)	31-35	33.25 ± 1.13	31.76 ± 0.46	32.73 ± 1.19
Platelet ($10^3/\mu l$)	150-440	205.23±35.53	212.71±50.70	207.85±40.28
Glucose (mg/dl)	76-110	89.92 ± 5.88	86.57 ± 4.86	88.75 ± 5.66
Creatinine (mg/dl)	0.5-1.5	1.02 ± 0.16	0.61 ± 0.07	0.88 ± 0.24

Table 12. Hematological and clinical chemistry data of normal subjects after receiving treatment (mean±SD). (continued)

Parameters	Healthy Reference	mean (Male) (n=7)	mean (Female) (n=13)	mean (Total) (n=20)
Urea nitrogen (mg/dl)	7-20	13.23±3.03	10.14 ± 2.12	12.15 ±3.08
Cholesterol (mg/dl)	100-200	187.54 ± 21.24	184.14 ± 28.20	186.35 ± 23.21
Triglyceride (mg/dl)	50-200	70 ± 30.99	61 ± 28.07	66.85±29.57
HDL-cholesterol (mg/dl)	35-100	57.54±9.38	60.14±14.66	58.45±11.18
Albumin (g/dl)	3.5-5.5	4.38 ± 0.18	4.06 ± 0.31	4.27 ± 0.28
Aspartate aminotransferase (AST) (U/l)	0-37	16 ± 2.48	16.43 ± 3.26	16.15 ± 2.7
Alanine aminotransferase (ALT) (U/l)	0-40	17.54 ± 6.85	12.86 ± 3.13	15.9 ± 6.16
Alkaline phosphatase(ALP) (U/l)	39-117	66.92±18.02	49.86 ± 8.9	60.95±17.31
Total bilirubin (mg/dl)	0.3-1.2	0.69 ± 0.18	0.34 ± 0.17	0.57 ± 0.24
Uric acid (mg/dl)	2.4-7	6.17 ± 1.13	3.73 ± 0.66	5.32 ± 1.54
Ferritin (ng/ml)	8-140	54.46 ± 28.5	23.43±20.19	43.6 ± 29.54
Folate (ng/ml)	4.2-19.9	22.35 ± 3.10	20.74 ± 4.08	27.79 ± 3.45

Table 13. Curcuminoids levels and biochemical parameters in normal subjects after receiving curcuminoids at visit 1 (before receiving Folic acid, mean±SEM)

Time (h.)	Curcumin (ng/ml)	Demethoxycurcumin (ng/ml)	Bis-demethoxycurcumin (ng/ml)	Malondialdehyde (nmoles/g Hb)	Percent changed of Reactive Oxygen Species	%ABTS inhibition	GSH (mg/dl)
	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)
0	0	0	0	759.64 ± 19.41	0.0	48.73 ± 0.98	56.41 ± 2.16
0.25	0.052 ± 0.026	0.197 ± 0.108	0.023 ± 0.029	739.12 ± 24.07	-17.69 ± 4.40	49.58 ± 1.23	54.99 ± 1.34
0.5	0.161 ± 0.023	0.487 ± 0.235	0.116 ± 0.031	731.65 ± 21.93	-34.95 ± 4.02	48.77 ± 1.16	57.95 ± 2.07
0.75	0.242 ± 0.029	0.685 ± 0.315	0.227 ± 0.176	759.78 ± 20.96	-25.30 ± 8.77	47.14 ± 1.27	56.39 ± 1.92
1	0.469 ± 0.080	1.058 ± 0.445	0.680 ± 0.176	677.64 ± 23.58	-27.67 ± 7.59	47.19 ± 2.06	55.84 ± 1.93
1.25	0.588 ± 0.094	1.441 ± 0.608	1.399 ± 0.431	703.63 ± 23.46	18.89 ± 10.50	45.62 ± 1.98	54.80 ± 2.55
1.5	0.394 ± 0.058	1.005 ± 0.394	0.600 ± 0.149	721.16 ± 27.24	-1.46 ± 8.40	48.38 ± 0.78	52.67 ± 1.56
2	0.286 ± 0.05	0.550 ± 0.249	0.199 ± 0.054	709.10 ± 23.81	-5.55 ± 9.60	46.72 ± 1.00	57.65 ± 2.47
4	0.198 ± 0.036	0.455 ± 0.226	0.054 ± 0.023	689.72 ± 15.91	-9.96 ± 8.97	47.77 ± 1.22	56.85 ± 2.33
8	0.116 ± 0.026	0.359 ± 0.175	0.020 ± 0.007	704.98 ± 28.31	59.16 ± 18.02	48.08 ± 1.39	54.24 ± 2.20
24	0.045 ± 0.019	0.257 ± 0.159	0.005 ± 0.003	772.97 ± 22.26		49.21 ± 0.86	52.26 ± 2.33

Table 14. Curcuminoids levels and biochemical parameters in normal subjects after receiving curcuminoids at visit 2 (after receiving folic acid, mean±SEM)

Time (h.)	Curcumin (ng/ml)	Demethoxycurcumin (ng/ml)	Bis-demethoxycurcumin (ng/ml)	Malondialdehyde (nmoles/g Hb)	Percent changed of Reactive Oxygen Species	%ABTS inhibition	GSH (mg/dl)
	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)	Total (n = 20)
0	0	0	0	787.66 ± 15.35	0.00	46.46 ± 1.37	54.89 ± 2.55
0.25	0.039 ± 0.013	0.077 ± 0.058	0.074 ± 0.029	760.70 ± 22.55	-15.50 ± 6.04	46.43 ± 1.84	53.69 ± 2.00
0.5	0.156 ± 0.039	0.180 ± 0.090	0.210 ± 0.036	800.59 ± 22.34	-10.58 ± 8.82	45.15 ± 1.78	55.65 ± 2.32
0.75	0.311 ± 0.078	0.308 ± 0.141	1.098 ± 0.686	790.35 ± 32.51	23.96 ± 7.53	44.92 ± 1.27	54.56 ± 2.74
1	0.564 ± 0.119	0.807 ± 0.389	0.905 ± 0.119	680.10 ± 24.78	-2.72 ± 9.24	45.27 ± 1.95	53.72 ± 2.17
1.25	0.632 ± 0.117	1.019 ± 0.420	0.957 ± 0.143	730.86 ± 20.74	-1.31 ± 10.16	46.02 ± 0.79	55.13 ± 2.93
1.5	0.418 ± 0.095	0.628 ± 0.355	0.534 ± 0.111	729.46 ± 26.42	10.70 ± 9.48	46.16 ± 0.96	51.05 ± 1.73
2	0.256 ± 0.067	0.486 ± 0.288	0.318 ± 0.064	692.52 ± 22.90	0.41 ± 9.62	45.87 ± 0.81	56.33 ± 2.51
4	0.173 ± 0.042	0.438 ± 0.331	0.189 ± 0.047	715.36 ± 22.91	-3.86 ± 10.60	45.20 ± 1.47	54.82 ± 2.53
8	0.132 ± 0.037	0.183 ± 0.140	0.073 ± 0.028	682.04 ± 31.39	62.03 ± 23.82	46.17 ± 1.57	52.59 ± 2.67
24	0.036 ± 0.025	0.006 ± 0.005	0.029 ± 0.016	779.47 ± 20.89	0.00	46.57 ± 1.21	53.82 ± 2.59

Table 15. p-value of all determination

parameters	p value	
	visit1 & visit2	male & female
MDA	0.844	0.430
ROS by flow cytometry	0.611	0.433
ABTS assay	0.406	0.998
GSH	0.999	1.000

CHAPTER 5

DISCUSSION

The interest in curcumin has grown in recent years based on its putative beneficial pharmacological effects including antioxidant, anti-inflammatory, and cancer chemopreventive actions. However, therapeutic effectiveness of curcumin is limit due to its poor absorption from the gastrointestinal tract (15, 46). It is necessary to study clearly for pharmacokinetics and pharmacodynamics of curcuminoids in human. We, therefore, evaluated their antioxidant effects *in vitro* and pharmacodynamics of curcuminoids in 20 normal volunteers taking a single oral dose of 6 gram curcuminoid capsules. Twenty normal subjects enrolled in this study were 13 males and 7 females. They were healthy and had normal hemoglobin typing (Hb A_2A).

Curcuminoid capsules were donated from The Government Pharmaceutical Organization. Each 250 mg curcuminoid capsule consists of curcumin: demethoxycurcumin: bis-demethoxycurcumin in the ratio 1 : 0.3 : 0.1, respectively under optimized HPTLC. Mass Spectra revealed no endogenous substance and curcuminoid metabolite peaks. The *in vitro* results showed that curcuminoid capsules exhibited antioxidant properties. ABTS radical cation decolorization assay and determination of free radical scavenging activity using DPPH method showed that curcuminoids had better antioxidant property than vitamin C and vitamin E. Curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals, which are the initiators of lipid peroxidation. Curcumin is a good antioxidant and inhibits lipid peroxidation (11, 50). Moreover, curcuminoids can protect red blood cell lysis induced by 2,2′ azobis (2-methylpropioarnidine) dihydrochloride (AAPH). Shui-Ling Deng, *et al*. (175) reported that curcumin and its analogues protected oxidative hemolysis of human red blood cells. Curcumin and its analogues are significantly more effective than vitamin E in their anti-hemolysis effects, this result was in good agreement with ours.

The reactive oxygen species determined by flow cytometer in our study revealed that ROS decreased at the first hour after taking curcuminoids and then increased to maximum level at 24 h. The result gets along well with the result of malondialdehyde which is the end product of lipid peroxidation. Levels of malondialdehyde (MDA) are widely used as an index of oxidative injury induced by oxygen free radicals on lipid membranes (176). Thus, many analytical methods (177) have been developed for the quantitative evaluation of this dialdehyde in biological matrices where MDA exists both free and bound to SH and NH_2 groups of macromolecules as proteins and nucleic acids (178). Since only low amounts of free MDA are present in biological samples and highly sensitive methods are needed for its detection, usually the total (free and bound) MDA is evaluated. Thus the biological samples are reacted with thiobarbituric acid (TBA) and then the formed MDA-TBA adduct is detected by spectrophotometry (179). The malondialdehyde level was slightly decreased after curcuminoids administration for 1 h and then returned to baseline level at 24 hours while percent inhibition of the ABTS radical in plasma of normal subjects were not different at every time points. The level of reduced glutathione (GSH) was not different before (visit 1) and after (visit 2) receiving folic acid. All parameters analyzing at visit 1 and visit 2 were not significantly different. The parameters of oxidative stress and antioxidation status in this study were malondialdehyde, reactive oxygen species analysed by flow cytometry, and reduced glutathione. They were not significantly different at every time points after curcuminoids administration because normal subjects did not have high oxidative stress.

Durgaprasad S, *et al.* (180) studied the antioxidant effect of curcumin in tropical pancreatitis. They found that twenty consecutive patients with tropical pancreatitis were randomized to receive 500 mg of curcumin with 5 mg of piperine, or placebo for 6 weeks, and the effects on the pattern of pain, and on red blood cell levels of malonyldialdehyde (MDA) and glutathione (GSH) were assessed. There was a significant reduction in the erythrocyte MDA levels following curcumin therapy compared with placebo; but the corresponding increase in the GSH level was not statistically significant.

After oral administration of curcuminoids, the pharmacokinetic properties of curcuminoids by LC/MS-MS were investigated (the study of pharmacokinetics was concomitantly done by Miss Yuwadee Saejeong, Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University), 0.588 ng/ml of curcumin was maximum at 1.25 hours (visit 1), it was not significantly difference between male and female ($p>0.05$). After receiving folic acid curcumin level was 0.632 ng/ml. Demethoxycurcumin was detected highest amount among other curcuminoids in the level of 1.44 ng/ml and 1.019 ng/ml before and after receiving folic acid, respectively. Whereas bis-demethoxycurcumin was 1.399 ± 0.431 ng/ml and 0.96 ± 0.143 ng/ml at visit 1 and 2, respectively. It can be implied that demethoxycurcumin was slightly stable in blood more than another curcuminoids. All curcuminoids (curcumin, demethoxycurcumin and bis-demethoxycurcumin) were found in very low amount in blood circulation.

Analysis of pharmacokinetic parameters (table in appendix) revealed that there was no significantly difference ($p>0.05$) in all parameters between male and female healthy volunteers after taking a single oral 6 gram dose. Plasma half-life of curcumin showed the highest followed by demethoxycurcumin and bis-demethoxycurcumin.

Curcuminoids reached time to maximum plasma concentration (Tmax) at 1.24 h and maximum plasma concentration (Cmax) of demethoxycurcumin and bis-demethoxycurcumin were 1.64 ± 0.6 and 1.54 ± 0.4 ng/ml whereas Cmax of curcumin was 0.65 ± 0.09 ng/ml. The normal volunteers then received folic acid for 14 days and followed by another pharmacokinetic study of single oral 6 gram curcuminoids. The results did not differ from the previous experiment implying that folic acid did not have any effect on pharmacokinetic parameters of curcumin. Mean residence time (MRT) of curcuminoids was about 5-6 hours. The level of curcuminoids was very low in blood plasma showing that it was bound to tissue. Volume of distribution (Vd/F/kg) of curcumin and demethoxycurcumin were higher than bis-demethoxycurcumin indicating that they were bound to tissue. Bis-demethoxycurcumin was found in blood plasma higher than other curcuminoids because it was more polar. The pharmacokinetics study showed that curcuminoids were quickly metabolized and bound to adipose tissue.

Curcuminoids were found in very low amount in blood circulation. This correlated well with our finding that the effects of curcuminoids as antioxidant were not significantly different from baseline and every time points.

In mouse, curcumin is first biotransformed to dihydrocurcumin (DHC) and tetrahydrocurcumin (THC) and these compounds are subsequently converted to monoglucuronide conjugates including curcumin-glucuronide, dihydrocurcumin-glucuronide and tetrahydrocurcumin-glucuronide (181). In human and rat hepatocytes, curcumin is metabolized into curcumin glucuronide, curcumin sulfate, THC, hexahydrocurcumin (HHC) and octahydrocurcumin (OHC) (17, 42). Antioxidant activities of THC have already been studied both *in vitro* and *in vivo*. Venkatesan P, *et al.* (182) reported that THC had higher activity than curcumin in protecting the nitrite induced oxidation of haemoglobin and lysis of erythrocytes. Somparn P, *et al*. compared the antioxidant activities of curcumin, its demethoxy derivertive (demethoxycurcumin and bis-demethoxycurcumin), and hydrogenated derivatives (THC, HHC and OHC) using three *in vitro* models: radical scavenging activity by DPPH assay, AAPH induced linoleic acid oxidation and AAPH induced red blood cells hemolysis. They found that all of the metabolite hydrogenated derivatives of curcumin (THC, HHC and OHC) showed greater DPPH scavenging activity, inhibition of linoleic acid peroxidation and free radical induced red blood cell hemolysis than their curcumin parent compound (183).

Several studies have addressed the absorption, metabolism, and tissue distribution of curcumin both in animal (rat) and human. Experiment involving rats, when orally administered curcumin in dose of 1 to 5 g/kg found that 75% of curcumin was excreted in the feces while only traces appeared in the urine (15). Oral and intraperitoneal doses of [^{3}H] curcumin led to the fecal excretion of most of radioactivity but intravenous administration were well excreted in the bile of cannulated rats (16), whereas measurement of plasma levels with radioactivity have shown that curcumin is poorly absorbed from the gut (17-19). After oral administration of 400 mg curcumin to rats, about 60% of the dose was absorbed and could not detect curcumin colorimetrically in heart blood and only traces (<5 µg/ml) in portal blood and negligible quantities in liver and kidney (<20 µg/tissue) were observed (39). These studies indicated that curcumin is transformed during absorption

from the intestines, and the transformed products, which is more polar and colorless than curcumin, enters the serosal side.

On the other hand, after i.v. injection of 50 mg/kg [^{3}H] curcumin in rats, the majority of radioactivity in bile was present in glucuronide conjujate of tetrahydrocurcumin (THC) and hexahydrocurcumin, which are the hydrogenated metabolite (16). After i.p. injection of curcumin into mice found that curcurmin was first biotransformed to dihydrocurcumin and THC and that these compounds subsequently were converted to monoglucuronide conjugates (40). To investigate the absorption and metabolism of orally administered curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), HPLC and LC-MS analyses were performed after enzymatic hydrolyses of rat plasma. The predominant metabolites found were glucuronides and glucuronide/sulfates (conjugates with both glucuronide and sulfate) of curcuminoids (184). These results indicate that orally administered curcuminoids are absorbed from the alimentary tract and present in the blood circulation after largely being metabolized to the form of glucuronide and glucuronide/sulfate conjugates.

In human study, the plasma concentration of curcumin usually peaked at 1 to 2 hours after oral intake of curcumin and gradually declined within 12 hours by which the peak plasma concentration of curcumin was only 1.77 μM even at the highest dose of 8000 mg/day (34). It may be caused poor bioavailability of curcumin or it may be due to its rapid metabolism in the liver and intestinal wall. At oral doses 440 to 2200 mg/day, neither curcumin nor its metabolites were detected in blood or urine, but curcumin recovered from feces. Curcumin was poorly available, following oral administration, with nanomolar levels of the parent compound and its glucuronide and sulfate conjugates found in the peripheral or portal circulation. Curcumin was not found in liver tissue, trace levels of products of its metabolite reduction were detected (47). Also, the effect of combining piperine, a known inhibitor of hepatic and intestinal glucuronidation, was evaluated on the bioavailability of curcumin in rats and health human volunteers. When curcumin was given alone, in the dose 2 g/kg to rats, moderate serum concentrations were achieved over a period of 4 h. Concomitant administration of piperine 20 mg/kg increased the serum concentration of curcumin for a short period of 1-2 h. Time to maximum was significantly increased while

elimination half-life and clearance significantly decreased, and the bioavailability was increased by 154%. On the other hand in humans after a dose of 2 g curcumin alone, serum levels were either undetectable or very low. Concomitant administration of piperine 20 mg produced much higher concentration from 0.25 to 1 h later and the increase in bioavailability was 2000%. The study shows that in the dosages used, piperine enhances the serum concentration, extent of absorption, and bioavailability of curcumin in both rat and human with no adverse effects (57). These studies indicate that curcumin is rapidly metabolized in circulation.

All parameters of oxidative stress and antioxidation status in this study were not significantly different at every time points after curcuminoids administration because normal subjects did not have high oxidative stress. Since the levels of curcuminoids in blood were low and curcuminoids were rapidly metabolized and bound to adipose tissue. Therefore, they did not exert antioxidant activity at the time we examined. It would be beneficial if we could treat subjects with high oxidative stress for longer period of time.

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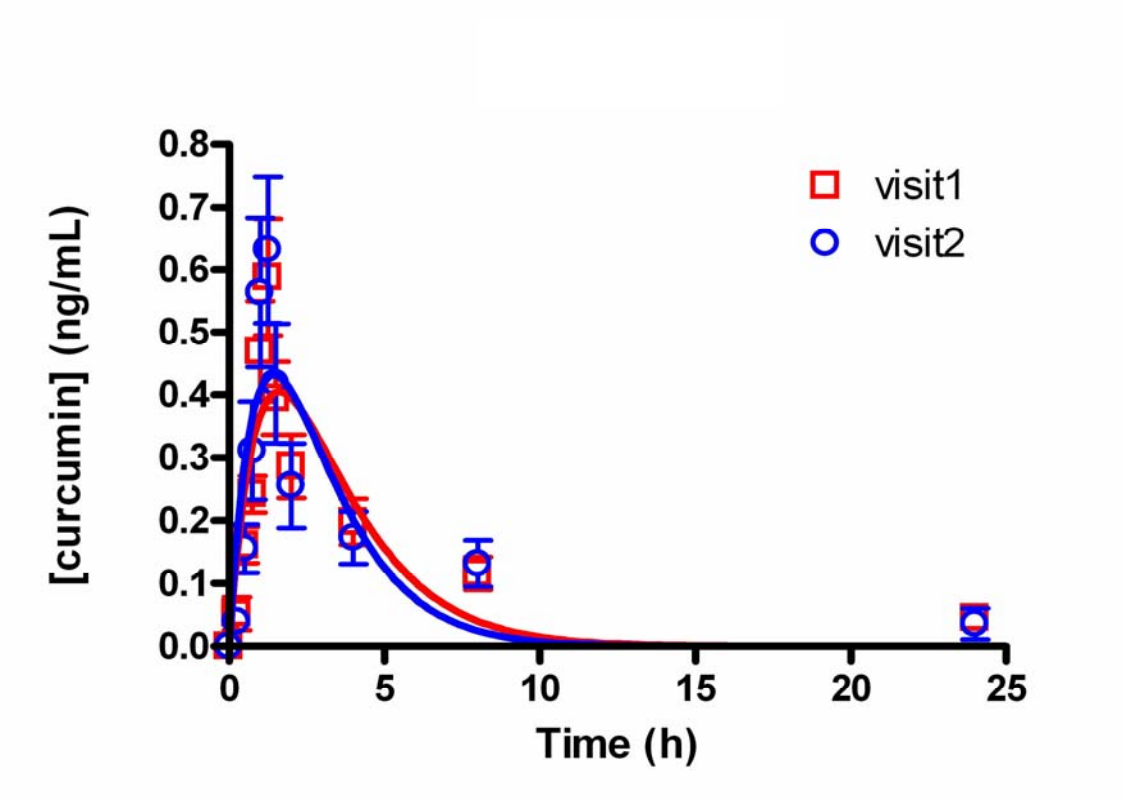
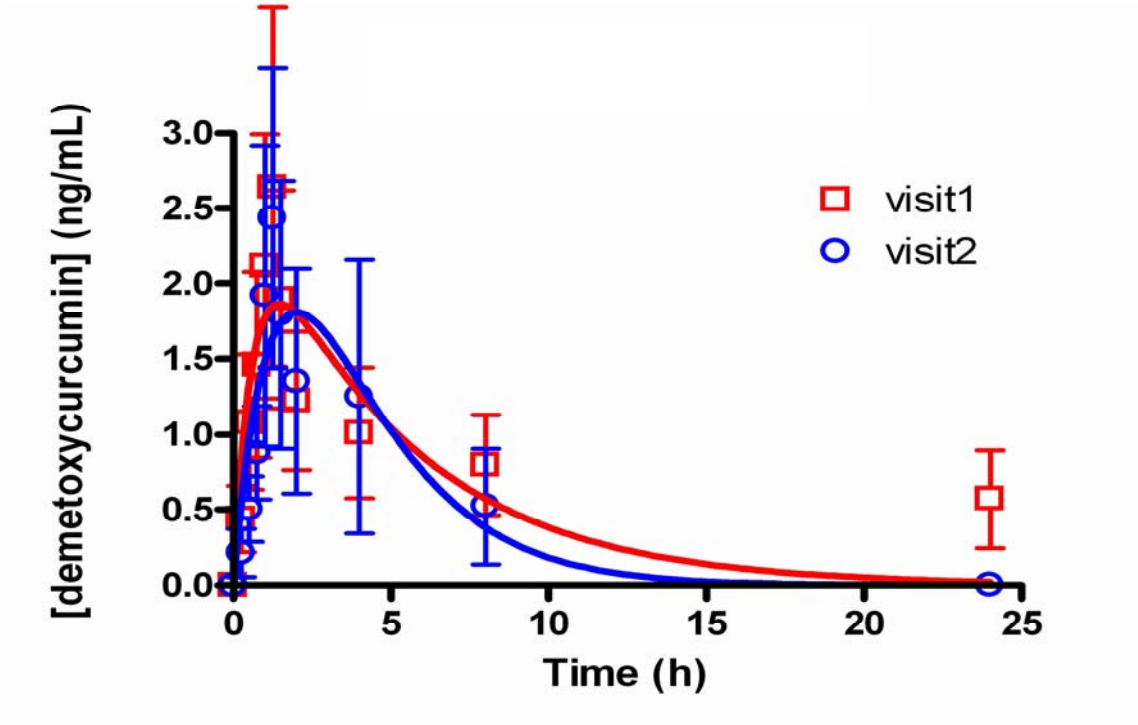
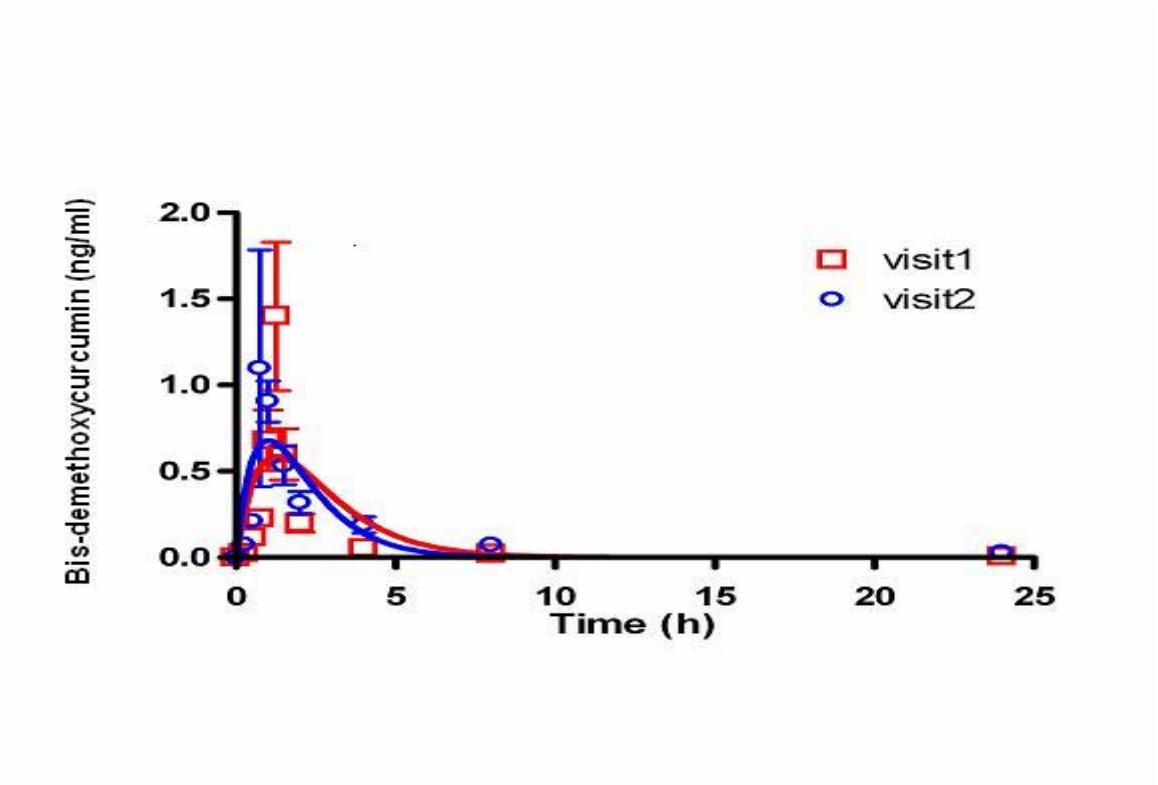
APPENDIX



Appendix 1 plasma concentration of curcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 and visit 2 (mean ± SEM).



Appendix 2 plasma concentration of demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 and visit 2 (mean ± SEM).



Appendix 3 plasma concentration of bis-demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 and visit 2 (mean ± SEM).

Appendix 4 plasma concentration of curcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 (before receive folic acid , mean ± SEM).

Time (h.)	Curcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.069 ± 0.038	0.020 ± 0.02	0.052 ± 0.026
0.5	0.182 ± 0.039	0.121 ± 0.037	0.161 ± 0.023
0.75	0.252 ± 0.034	0.224 ± 0.058	0.242 ± 0.029
1	0.484 ± 0.116	0.442 ± 0.09	0.469 ± 0.080
1.25	0.589 ± 0.118	0.585 ± 0.166	0.588 ± 0.094
1.5	0.343 ± 0.077	0.489 ± 0.078	0.394 ± 0.058
2	0.298 ± 0.070	0.264 ± 0.064	0.286 ± 0.05
4	0.192 ± 0.047	0.209 ± 0.06	0.198 ± 0.036
8	0.116 ± 0.029	0.117 ± 0.056	0.116 ± 0.026
24	0.020 ± 0.009	0.091 ± 0.047	0.045 ± 0.019

Appendix 5 plasma concentration of demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 (before receive folic acid , mean ± SEM).

Time (h.)	demethoxycurcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.196 ± 0.133	0.199 ± 0.199	0.197 ± 0.108
0.5	0.497 ± 0.241	0.538 ± 0.525	0.487 ± 0.235
0.75	0.567 ± 0.293	0.827 ± 0.756	0.685 ± 0.315
1	0.778 ± 0.355	1.578 ± 1.118	1.058 ± 0.445
1.25	1.008 ± 0.584	2.245 ± 1.381	1.441 ± 0.608
1.5	0.616 ± 0.350	1.728 ± 0.906	1.005 ± 0.394
2	0.570 ± 0.293	0.596 ± 0.486	0.550 ± 0.249
4	0.454 ± 0.243	0.520 ± 0.484	0.455 ± 0.226
8	0.383 ± 0.193	0.368 ± 0.368	0.359 ± 0.175
24	0.205 ± 0.142	0.382 ± 0.382	0.257 ± 0.159

Appendix 6 plasma concentration of bis-demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 1 (before receive folic acid , mean ± SEM).

Time (h.)	Bis-demethoxycurcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.035 ± 0.106	0.001 ± 0	0.023 ± 0.029
0.5	0.123 ± 0.039	0.121 ± 0.053	0.116 ± 0.031
0.75	0.223 ± 0.461	0.236 ± 0.113	0.227 ± 0.176
1	0.776 ± 0.251	0.501 ± 0.191	0.680 ± 0.176
1.25	1.182 ± 0.466	1.803 ± 0.912	1.399 ± 0.431
1.5	0.422 ± 0.129	0.931 ± 0.332	0.600 ± 0.149
2	0.175 ± 0.052	0.245 ± 0.125	0.199 ± 0.054
4	0.041 ± 0.014	0.079 ± 0.063	0.054 ± 0.023
8	0.018 ± 0.007	0.024 ± 0.016	0.020 ± 0.007
24	0.006 ± 0.005	0.005 ± 0.003	0.005 ± 0.003

Appendix 7 plasma concentration of curcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 2 (after receive folic acid , mean ± SEM).

Time (h.)	Curcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.037 ± 0.016	0.042 ± 0.022	0.039 ± 0.013
0.5	0.189 ± 0.055	0.093 ± 0.040	0.156 ± 0.039
0.75	0.335 ± 0.109	0.267 ± 0.099	0.311 ± 0.078
1	0.578 ± 0.133	0.536 ± 0.251	0.564 ± 0.119
1.25	0.677 ± 0.154	0.547 ± 0.186	0.632 ± 0.117
1.5	0.460 ± 0.129	0.342 ± 0.136	0.418 ± 0.095
2	0.282 ± 0.099	0.207 ± 0.061	0.256 ± 0.067
4	0.187 ± 0.055	0.146 ± 0.067	0.173 ± 0.042
8	0.143 ± 0.052	0.111 ± 0.049	0.132 ± 0.037
24	0.042 ± 0.037	0.024 ± 0.016	0.036 ± 0.025

Appendix 8 plasma concentration of demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 2 (after receive folic acid , mean ± SEM).

Time (h.)	demethoxycurcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.029 ± 0.017	0.165 ± 0.165	0.077 ± 0.058
0.5	0.169 ± 0.095	0.201 ± 0.201	0.180 ± 0.090
0.75	0.327 ± 0.170	0.270 ± 0.270	0.308 ± 0.141
1	0.882 ± 0.582	0.669 ± 0.330	0.807 ± 0.389
1.25	1.106 ± 0.611	0.859 ± 0.446	1.019 ± 0.420
1.5	0.848 ± 0.534	0.221 ± 0.192	0.628 ± 0.355
2	0.654 ± 0.433	0.173 ± 0.173	0.486 ± 0.288
4	0.619 ± 0.507	0.101 ± 0.101	0.438 ± 0.331
8	0.255 ± 0.214	0.050 ± 0.050	0.183 ± 0.140
24	0.007 ± 0.007	0.004 ± 0.004	0.006 ± 0.005

Appendix 9 plasma concentration of bis-demethoxycurcumin by LC/MS-MS in normal subjects subjects after receive curcuminoids at visit 2 (after receive folic acid , mean ± SEM).

Time (h.)	bis-demethoxycurcumin (ng/ml)		
	Male (n = 13)	Female (n = 7)	Total (n = 20)
0	0	0	0
0.25	0.075 ± 0.038	0.070 ± 0.046	0.074 ± 0.029
0.5	0.185 ± 0.037	0.257 ± 0.078	0.210 ± 0.036
0.75	0.446 ± 0.079	2.309 ± 1.963	1.098 ± 0.686
1	0.898 ± 0.151	0.918 ± 0.208	0.905 ± 0.119
1.25	1.013 ± 0.206	0.853 ± 0.160	0.957 ± 0.143
1.5	0.448 ± 0.086	0.693 ± 0.279	0.534 ± 0.111
2	0.345 ± 0.085	0.267 ± 0.097	0.318 ± 0.064
4	0.234 ± 0.063	0.106 ± 0.059	0.189 ± 0.047
8	0.091 ± 0.037	0.039 ± 0.039	0.073 ± 0.028
24	0.044 ± 0.023	0	0.029 ± 0.016

Appendix 10 Pharmacokinetic parameters of curcumin in normal subject before recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	5.072 ± 0.94	11.001 ± 5.532	7.147 ± 2.041
MRT (h)	7.072 ± 1.491	17.127 ± 8.068	10.591 ± 3.052
$AUC_{0-\infty}$ (ng.h/ml)	3.191 ± 0.675	10.382 ± 5.547	5.708 ± 2.051
Vd/F/kg (L/h/kg)	359296 ± 340335	322118 ± 135904	346284 ± 75602
C_{max} (ng/ml)	0.646 ± 0.125	0.658 ± 0.142	0.650 ± 0.093
T_{max} (h)	1.19 ± 0.04	1.32 ± 0.04	1.24 ± 0.03

Appendix 11 Pharmacokinetic parameters of demethoxycurcumin in normal subject before recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	4.321 ± 2.125	0.846 ± 0.345	3.235 ± 1.451
MRT (h)	6.619 ± 2.903	3.023 ± 1.622	5.294 ± 1.953
$AUC_{0-\infty}$ (ng.h/ml)	18.625 ± 12.154	13.526 ± 12.535	16.840 ± 8.848
Vd/F/kg (L/h/kg)	213604 ± 114511	3510.72 ± 2184.66	147950 ± 78667
C_{max} (ng/ml)	1.187 ± 0.589	2.4841 ± 1.351	1.641 ± 0.604
T_{max} (h)	1.15 ± 0.04	1.25 ± 0.08	1.18 ± 0.04

Appendix 12 Pharmacokinetic parameters of bis-demethoxycurcumin in normal subject before recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	1.186 ± 1.71	0.769 ± 0.351	1.032 ± 0.321
MRT (h)	2.676 ± 2.354	2.307 ± 0.588	2.547 ± 0.463
$AUC_{0-\infty}$ (ng.h/ml)	1.318 ± 1.293	1.868 ± 0.938	1.510 ± 0.392
Vd/F/kg (L/h/kg)	21918 ± 37097	5105.7 ± 4128.5	15724 ± 6893
C_{max} (ng/ml)	1.303 ± 1.634	1.986 ± 0.881	1.542 ± 0.419
T_{max} (h)	1.19 ± 0.11	1.32 ± 0.07	1.237 ± 0.03

Appendix 13 Pharmacokinetic parameters of curcumin in normal subject after recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	2.726 ± 0.552	3.902 ± 0.944	3.138 ± 0.490
MRT (h)	5.135 ± 1.062	6.747 ± 1.730	5.699 ± 0.908
$AUC_{0-\infty}$ (ng.h/ml)	3.920 ± 1.625	2.828 ± 0.960	3.538 ± 1.096
Vd/F/kg (L/h/kg)	164964 ± 48204	246850 ± 56883.60	193625 ± 37307.75
C_{max} (ng/ml)	0.811 ± 0.179	0.783 ± 0.219	0.801 ± 0.136
T_{max} (h)	1.15 ± 0.05	1.32 ± 0.13	1.21 ± 0.06

Appendix 14 Pharmacokinetic parameters of demethoxycurcumin in normal subject after recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	13.052 ± 6.912	0.716 ± 0.214	2.935 ± 0.579
MRT (h)	4.514 ± 1.348	4.518 ± 2.296	4.516 ± 1.136
$AUC_{0-\infty}$ (ng.h/ml)	6.234 ± 4.712	1.683 ± 1.406	4.641 ± 3.095
Vd/F/kg (L/h/kg)	102670.80 ± 58097.44	7918.96 ± 444.62	4.64 ± 3.09
C_{max} (ng/ml)	1.137 ± 0.610	0.859 ± 0.440	1.040 ± 0.419
T_{max} (h)	1.275 ± 0.08	1.20 ± 0.04	1.25 ± 0.06

Appendix 15 Pharmacokinetic parameters of bis-demethoxycurcumin in normal subject after recieiving folic acid. (mean ± SEM)

Parameters	Male (n = 13)	Female (n = 7)	Total (n = 20)
$T_{½}$ (h)	5.322 ± 2.331	2.152 ± 1.096	4.203 ± 1.568
MRT (h)	8.293 ± 3.436	2.082 ± 0.629	6.005 ± 2.282
$AUC_{0-\infty}$ (ng.h/ml)	5.878 ± 2.357	2.411 ± 0.665	4.601 ± 1.551
Vd/F/kg (L/h/kg)	15172.42 ± 2323.06	22204.44 ± 17788.45	0.04 ± 0.01
C_{max} (ng/ml)	1.234 ± 0.197	2.962 ± 1.865	1.839 ± 0.660
T_{max} (h)	1.18 ± 0.08	1.21 ± 0.10	1.19 ± 0.06

BIOGRAPHY

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