#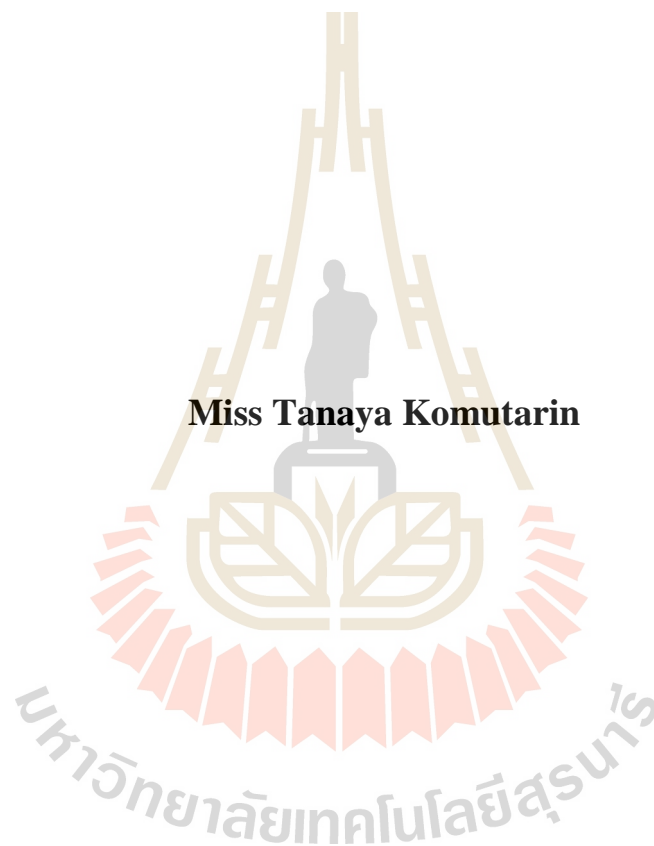 STUDIES OF CYTOTOXICITY ON TUMOR CELLS AND IMMUNOMODULATORY EFFECTS OF FLAVONOIDS EXTRACTED FROM SEED COAT OF *TAMARINDUS INDICA* L. IN MICE

Miss Tanaya Komutarin

การศึกษาความเป็นพิษต่อเซลล์เนื้องอกและผลการกระตุ้นระบบภูมิคุ้มกัน
ของสารสกัดฟลาโวนอยด์จากเปลือกหุ้มเมล็ดมะขามในหนู

นางสาวฐานยา โกมุทรินทร์

STUDIES OF CYTOTOXICITY ON TUMOR CELLS AND IMMUNOMODULATORY EFFECTS OF FLAVONOIDS EXTRACTED FROM SEED COAT OF *TAMARINDUS INDICA* L. IN MICE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Thesis Examining Committee

………………………………………

(Asst. Prof. Dr. Panee Wannitikul)

Chair person

………………………………………

(Asst. Prof. Dr. Benjamart Chitsomboon)

Member (Thesis Advisor)

………………………………………

(Prof. Dr. Maitree Suttajit)

Member

………………………………………

(Assoc. Prof. Dr. Korakod Indrapichate)

Member

………………………………………

(Assoc. Prof. Dr. Tassanee Sukosol)

Member

………………………………………   ………………………………………

(Assoc. Prof. Dr. Sarawut Sujitjorn)   (Assoc. Prof. Dr. Prasart Suebka)

Vice Rector for Academic Affairs   Dean of the Institute of Science

ฐานยา โกมุทรินทร์: การศึกษาความเป็นพิษต่อเซลล์เนื้องอกและผลการกระตุ้นระบบ ภูมิคุ้มกันของสารสกัดฟลาโวนอยด์จากเปลือกหุ้มเมล็ดมะขามในหนู (STUDIES OF CYTOTOXICITY ON TUMOR CELLS AND IMMUNOMODULATORY EFFECTS OF FLAVONOIDS EXTRACTED FROM SEED COATS OF *TAMARINDUS INDICA* L. IN MICE) อ. ที่ปรึกษา: ผศ. ดร. เบญจมาศ จิตรสมบูรณ์, 131 หน้า. ISBN 974-533-266-6

สารสกัดจากเปลือกหุ้มเมล็ดมะขามมีคุณสมบัติเป็นสารแอนติออกซิแดนท์ จากการศึกษา ความเป็นพิษของสารสกัดจากเปลือกหุ้มเมล็ดมะขามต่อเซลล์เนื้องอกทั้งหมดหกชนิด พบว่าสาร สกัดมีผลต่อเซลล์เนื้องอกในหลอดทดลองบางชนิด การศึกษาการยับยั้งการผลิตไนทริกออกไซด์ ในหลอดทดลองของสารสกัดในเซลล์ RAW 264.7 และการศึกษาในสัตว์ทดลอง โดยใช้เซลล์ แมโครฟาจของหนูเม้าส์ B6C3F1 พบว่าเซลล์ RAW 264.7 ที่กระตุ้นด้วย LPS และ IFN-γ และ ได้รับสารสกัดที่ความเข้มข้น 0.2-200 µg/mL ถูกยับยั้งการผลิตไนทริกออกไซด์ ตามลำดับความ เข้มข้น (p< 0.01) ได้มากถึง 68% ที่ความเข้มข้นสูงสุด ในหนูที่ป้อนสารสกัดความเข้มข้น 100-500 mg/kg ถูกยับยั้งการผลิตไนทริกออกไซด์เมื่อกระตุ้นด้วย TPA, LPS และ/หรือ IFN-γ ตามลำดับ ความเข้มข้น การศึกษาความปลอดภัยเบื้องต้นในหนู B6C3F1 พบว่าเฉพาะกลุ่มที่ได้รับสารสกัด สูงสุด (1000 mg/kg) มีน้ำหนักตัวลดลง แต่ไม่แสดงอาการเกิดพิษโดยทั่วไปด้านอื่น และไม่ เปลี่ยนแปลงการทำงานของเซลล์ NK นอกจากนี้การป้อนสารสกัดในหนู BALB/c ยังไม่แสดงผล ต่อภูมิคุ้มกันด้านเซลล์ต่อ DNFB หรือ HCA เมื่อทดสอบด้วย local lymph node assay และ ไม่มีผล ยับยั้งการระคายเคืองต่อผิวหนังที่ถูกกระตุ้นด้วย NA หรือ DNFB เมื่อทดสอบโดย iritancy assay

สาขาวิชาชีววิทยา	ลายมือชื่อนักศึกษา_____
ปีการศึกษา 2546	ลายมือชื่ออาจารย์ที่ปรึกษา_____
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

TANAYA KOMUTARIN: STUDIES OF CYTOTOXICITY ON TUMOR CELLS AND IMMUNOMODULATORY EFFECTS OF FLAVONOIDS EXTRACTED FROM SEED COAT OF *TAMARINDUS INDICA* L. IN MICE   THESIS ADVISOR: ASST. PROF. BENJAMART CHITSOMBOON, Ph.D. 131 PP. ISBN 974-2-533-266-6

The seed coat extract of *Tamarindus indica* has been shown to have antioxidant properties. *In vitro* cytotoxicity studies of six cell lines demonstrated that the extract exhibited selective cytotoxicity towards some cancer cells. *In vitro* exposure of RAW 264.7 cells to 0.2-200 μg/mL of *T. indica* significantly attenuated (as much as 68%) nitric oxide production induced by LPS and IFN-γ in a concentration-dependent manner (p< 0.01). Oral exposure of *T. indica* (100-500 mg/kg) dose-dependently suppressed TPA, LPS and/or IFN-γ induced production of nitric oxide in isolated peritoneal macrophages from B6C3F1 mice. The preliminary safety studies in mice demonstrated a decrease in body weight at only the highest dose tested (1000 mg/kg) without alteration of any general toxicities and effect on NK cell activity. Oral exposure to *T. indica* in BALB/c mice did not modulate T cell-mediated sensitization to DNFB or HCA as measured by local lymph node, and NA or DNFB as measured by irritancy assay.

School of Biology   Student's Signature _____

Academic Year 2003   Advisor's Signature _____

                                                    Co-advisor's Signature _____

                                                    Co-advisor's Signature _____

Dedication

This thesis is dedicated to my parents, for their unfaltering encouragement, and support, as well as their precious bequests of roots and wings.

ACKNOWLEDGEMENTS

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
ALT	alanine transferase
α	alpha
AST	aspartate aminotransferase
β	beta
BUN	blood urea nitrogen
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNFB	2,4-dinitrofluorobenzene
EDTA	ethylenediaminetetraacetic acid
gm	gram
HCA	α-hexylcinnamaldehyde
HCT	hematocrit
HGB	hemoglobin
hrs	hours
IFN-γ	interferon gamma
iNOS	inducible nitric oxide synthase

LIST OF ABBREVIATIONS (Continued)

i.p.	intraperitoneal
kg	kilogram
L	liter
LLNA	local lymph node assay
LPS	lipopolysacharide
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MTT	3(4,5-dimethyl thiazol-2-yl) 2,5-diphenyltetrazolium bromide
min	minute
mg	milligram
mM	millimolar
NA	nonanoic acid
NK	natural killer cell
nm	nanomolar
OD	optical density
PC	positive control
PI	propidium iodide
PBS	phosphate buffered saline

LIST OF ABBREVIATIONS (Continued)

RBC	red blood cell
RPMI	Roswell Park Memorial Institute
TPA	12-O-tetradecanoylphorbol-13-acetate
T. indica	*Tamarindus indica*
μg/mL	microgram per milliliter
μL	microliter
μM	micromolar
VH	vehicle
Vit.C	vitamin C (ascorbic acid)
Vit.E	vitamin E [(+)-α-tocopherol]
Beta-caro	Beta-carotene
WBC	white blood cell
wgt	weight
x	times

CHAPTER I

INTRODUCTION

Plant materials have long been used as traditional medicines for the treatment of a wide variety of ailments and diseases. Components of *Tamarindus indica*, a tree indigenous to India and South East Asia, have been used as a spice, food component, and snack. According to the Thai traditional medicine, the fruit of *T. indica* is regarded as a digestive, carminative, laxative, expectorant, and blood tonic. In addition, the seeds of *T. indica* are used as an anthelmintic, antidiarrheal, and an emetic, and the seed coat is used to treat burns and aid in wound healing as well as an antidysenteric (Farnsworth and Bunyapraphatsara, 1992).

Recently, Pumthong and colleagues (1999) have demonstrated the antioxidant activity of the seed coat extract of *T. indica*. The extract is composed of polyphenols including flavonoids, tannins, anthocyanidin, and oligomeric proanthocyanidins. Many of these flavonoids are also components of Pycnogenol®, the nutritional supplement which has been shown to have vasorelaxant activity, increase capillary permeability and participate in the cellular antioxidant network as indicated by its ability to regenerate the ascorbyl radical and to protect endogenous vitamin E and glutathione from oxidative stress (Packer, Rimbach, and Virgili, 1999; Rohdewald, 2002). Flavonoids found in various medicinal plants are natural antioxidants with free radical scavenging activity and they have also been shown to prevent free radical formation via inhibition of oxido-reductases (Middleton and Kandaswami, 1986;

Chen, Hwang, and Deng, 1993; Krol, Czuba, Threeadgill, Cunningham, and Pietsz, 1995, Carlo, Mascolo, Izzo, and Capasso, 1999).

Based on the reported antioxidant activity of the seed coat extract of *T. indica* which contains flavonoids among its major constituents, and the limited toxicological data available, the purposes of the present studies were to investigate the anti-inflammatory potential of this extract and begin to access its safety. *In vitro* studies using *T. indica* seed coat extract were conducted to evaluate the modulation of nitric oxide (NO) production by RAW 264.7 macrophages using LPS and IFN-γ as stimulants. Confirmation of the effect *in vivo* was tested by orally exposing B6C3F1 mice to *T. indica* for 14 days and evaluating NO production by freshly isolated peritoneal macrophages following stimulation *in vitro* with lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ), and *in vivo* or *in vitro* with 12-O-tetradecanolyphorbol-13-acetate (TPA). A 14 day toxicity study and studies to evaluate the effect of *T. indica* seed coat extract on components of innate and cellular immunity were performed to begin to assess safety.

CHAPTER II

LITERATURE REVIEW

2.1 Antioxidants and Immune Cells

In the last decade, there has been growing evidence that oxidative stress impairs immune responses and that antioxidant supplementation can reverse many aspects of oxidant-mediated immune suppression.

The immune system generates reactive oxygen metabolites as part of its regular defense functions. The most potent of the reactive oxygen species include the hydroxyl radical ($OH^\bullet$), superoxide anion radical ($O_2^\bullet$), hydroperoxyl radical ($HO_2^\bullet$), hyperchlorous acid (HOCl), hydrogen peroxide (H_2O_2), and nitric oxide (NO), all of which are generated during the oxidative burst by phagocytic neutrophils as well as macrophages. These reactive oxygen species play a critical role for immune cells to kill pathogens and clear dead tissues, however, prolonged overproduction of them as seen in a variety of pathologic conditions such as autoimmune and chronic inflammatory disorders can cause damage to the immune cells and compromise their functions. Likewise, immune cells are particularly sensitive to oxidative stress because of the high content of polyunsaturated fatty acids in their plasma membranes and a higher production of reactive oxygen species (ROS), which is a part of their normal function (Meydani, Wu, Santos, and Hayek, 1995). It is, therefore, conceivable that immune cells need more antioxidants to maintain optimal function. As a result, it has been well documented that the oxidant-antioxidant balance is an important determinant of immune cell function, not only for maintaining the intergrity

and functionality of membrane lipids, cellular proteins, and nucleic acids, but also for regulation of signal transduction and gene expression in immune cells. This is supported by the observation that immune cells in general have higher levels of antioxidant nutrients than do other cells (Hatman and Kayden, 1979; Coquette, Vray, and Vanderpas, 1986).

Antioxidants have the ability to stabilize highly reactive, potential harmful free radicals and protection of cells from oxidative damage. The ability of antioxidants to destroy highly reactive free radicals serves to protect the structural integrity of immune cells and prevents the loss of essential functions. Numerous animal experiments, clinical trials, and epidemiological investigations have provided evidence indicating a critical role of antioxidants in the regulation of immune cell functions, as well as in the pathogenesis of immune related diseases such as infection, inflammation, autoimmune, and neoplastic diseases. In general, deficiency of single or multi-antioxidant nutrients resulting in oxidant-antioxidant imbalance has been shown to correlate with impairment or abnormality of immune function. Supplementation with antioxidants can correct the deficiency and dysregulated the balance, and consequently reverse the impaired or abnormal immune function. Of the antioxidants linked to the modulation of immune function, vitamin E, β-carotene, and antioxidant enzymes have been the subject of intensive investigation.

2.2 Effects of Antioxidants on Immune Functions

The survival of aerobic organisms in an oxygen environment involves a complex interrelationship between the generation of very reactive chemical species, called free radicals, and the ability of the organism to control these substances. Increasing the oxygen available in the environment of an aerobic organism results in important biochemical changes which can produce cellular injury and death (Deneke and Fanburg, 1980).

Substances that neutralize the potential ill effect of free radicals are generally grouped in the so-called antioxidant defense system. Antioxidants interfere with the production of free radicals and/or inactivate them once they are formed. The antioxidant vitamins and minerals associated with antioxidant enzymes are important components of the overall antioxidant protection found within cells (Figure 1).
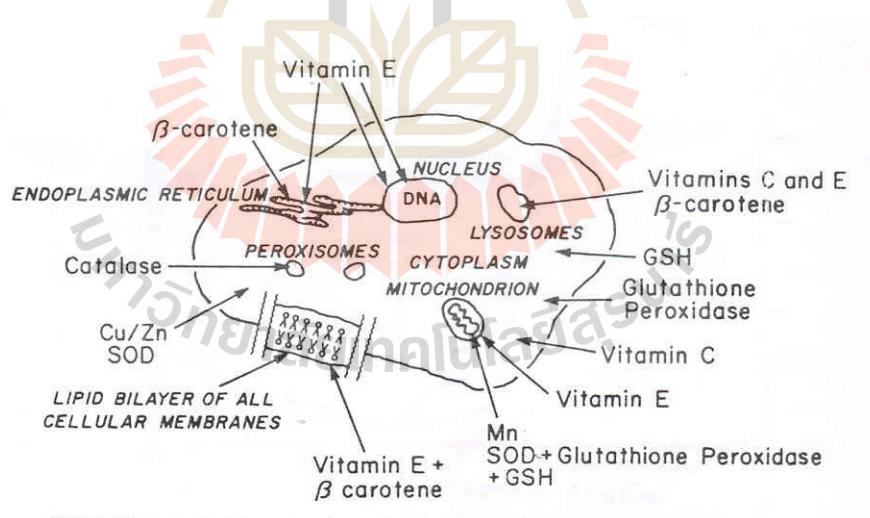
Figure 1. Antioxidant vitamins and mineral-containing antioxidant enzymes in cells (Deneke and Fanburg, 1980).

2.2.1 Antioxidant Vitamins

Only three essential nutrients can directly interfere with the propagation stage of free radical generation and scavenge free radicals. Vitamin E (alpha tocopherol), the major lipid soluble antioxidant present in all cellular membranes, protects against lipid peroxidation and prevents the loss of membrane fluidity. Vitamin E has been characterized as the most critical antioxidant in blood (Burton, Joyce, and Ingold 1983). Vitamin C (ascorbic acid), is water soluble and along with vitamin E, can quench free radicals as well as singlet oxygen (Frei, 1991). Ascorbate can also regenerate the reduced, antioxidant form of vitamin E (Bendich, Machlin, Scandurra, Burton, and Wayner, 1986). Beta-carotene, a pigment found in all photosynthetic plants, is an efficient quencher of singlet oxygen and can function as an antioxidant (Krinsky, 1989; Bendich, 1993). β-Carotene is the major dietary carotenoid precursor of vitamin A. Vitamin A, however, cannot quench singlet oxygen and has a very small capacity to scavenge free radicals.

Vitamin E

Vitamin E is the most widely distributed antioxidant in nature, being found in both the plant and animal kingdoms. The generic term vitamin E refers to at least eight structural isomers of tocopherol. Among these, α-tocopherol is the best known isomer and possesses the most potent antioxidant activity (Burton, Joyce, and Ingold, 1982). Because of the lipophilic property of the tocopherol molecule, vitamin E is the major free radical chain terminator. Within the phospholipid bilayer of plasma membrane, vitamin E is considered to protect the unsaturated bonds of fatty acids from oxidation. High levels of tocopherol are found in selected mammalian tissues

(e.g., adrenal glands, heart, testes, and liver), and this preferential distribution may result from its high lipid solubility. Intracellularly, vitamin E is associated with lipid-rich membranes such as mitochondria and endoplasmic reticulum. Thus the antioxidant action of tocopherol is expected to be highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals (Ross and Moldeus, 1991). In addition, the antioxidant form of vitamin E can be regenerated by electron donation from vitamin C, glutathione, and other antioxidants (Bendich, 1990). Like other critical micronutrients, vitamin E is commonly deficient in the elderly. Thus, low tissue vitamin E content or vitamin E deficiency enhance components of the inflammatory response and suppress components of the immune response, which lead to enhance production of immunosuppressors such as prostaglandins, however, supplementation readily recovers this problem (Meydani and Haytek, 1992). More specifically, vitamin E supplementation enhances cell-mediated immunity whereas vitamin E deficiency has been associated with both T and B lymphocyte dysfunctions. The relationship between vitamin E and immunological status was assessed in 100 healthy subjects over 60 years old. Subjects with higher than average serum vitamin E levels (> 1.35 mg/d) had significantly lower numbers of infections over a three year time span (Chavance et al., 1984). Recently, the study completed the first double-blind, placebo controlled study examining the effects of vitamin E supplementation on the immune responses of a healthy elderly population. Supplementation with 800 IU/day for approximately one month significantly (a) increased plasma and mononuclear cell vitamin E levels; (b) increased delayed hypersensitivity skin tests (a significant indicator of ability to combat infectious disease); (c) increased IL-2 production; (d)

increased mitogenic response to Con A; (e) decreased synthesis of prostaglandin (PGE2); and (f) decreased plasma lipoperoxide levels. No adverse side effects were noted in this well controlled study (Meydani et al., 1988; Meydani, Blumberg, Yogeeswaran, and Meydani, 1989).

Vitamin C

Ascorbic acid (vitamin C), unlike vitamin E, is hydrophilic and is important in decreasing free radical reactions in the intracellular and extracellular fluids. As a reducing and antioxidant agent, it directly reacts with $O_2^{-\bullet}$ and $^\bullet OH$ and various lipid hydroperoxides. In addition, vitamin C has recently been shown to restore the antioxidant properties of oxidized vitamin E, *in vitro* and *in vivo* models, suggesting that a major function of vitamin C is to regenerate the vitamin E radical (Bendich et al., 1986). Substantial evidence show that ascorbate can act also an oxidant, depending upon the concentration and the environment in which the molecule is present. Excess amounts (~1 mM) may act as a prooxidant in the presence of the transition metal Fe^{3+} or Cu^{2+} by generating cofactors of activated oxygen radicals during the promotion of lipid peroxidation (Bendich et al.).

The effects of vitamin C status on immune function has been extensively studies due to the high concentrations of the vitamin found in phagocytic cells and perceived importance of the vitamin as being anti-infective. Vitamin C enhances neutrophil chemotaxis. Neutrophils and mononuclear cells maintain high concentrations of vitamin C which are correlated with their functions. In addition, during the oxidative burst, increased levels of vitamin C are actively taken up by stimulated neutrophils. Nonspecific extracellular free radical damage which follows

oxidative burst activity is significantly reduced in the presence of supplemental ascorbate (Anderson, 1982). Vitamin C deficiency in animal models results in depressed cell mediated immune responses. Antibody production is not affected. Bacterial effectiveness is diminished (Goldschmidt, Masin, Brown, and Wyde, 1988). T and B lymphocyte proliferative responses are increased following vitamin C supplementation in some human studies and enhanced vitamin C status is associated with a lower rate of infections in an elderly population (Kennes, Dumont, Brochee, Hubert, and Neve, 1983). As mentioned earlier, ascorbic acid may enhance immune responses indirectly by maintaining optimal vitamin E levels.

Beta Carotene

Beta-carotene is one of approximately fifty carotenoids which can be metabolized to vitamin A. Carotenoids are red/yellow pigments found in all photosynthesizing organisms and have long been considered antioxidants because of their capacity to scavenge free radicals. Carotenoids protect lipids against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen (Krinsky, 1979). β-Carotene displays an efficient biological radical-trapping antioxidant activity through its inhibition of lipid peroxidation induced by the xanthine oxidase system (Krinsky and Deneke, 1982). Like vitamin C, β-carotene displays the function as both an antioxidant and a prooxidant. Under low O_2 partial pressures (<150 Torr), β-carotene exhibits excellent radical-scavenging activity, yet at higher oxygen partial pressures, its free radical-trapping capacity shows autocatalytic prooxidant effects with concomitant loss of its antioxidant activity (Burton and Ingold, 1984). There are over 500 carotenoids; only a small portion has provitamin A activity.

β-Carotene is unique among the carotenoids because it can form two molecules of vitamin A when centrally cleaved. In addition to its provitamin A activity, β-carotene can quench singlet oxygen (an energized form of oxygen capable of generating free radicals) and free radicals, whereas vitamin A is a relatively poor antioxidant compared to vitamin E or β-carotene, and vitamin A cannot inactivate singlet oxygen (Bendich and Olson, 1989).

Numerous experimental animal studies have demonstrated that carotenoids modulate host defense systems. For instance, Chew (1993) observed that β-carotene supplementation increased the (a) total number of circulating mononuclear cells; (b) number of helper T cells; (c) natural killer cell cytotoxicity; and (d) tumor necrosis factor alpha and interleukin-1. In humans, Alexander, Newmark, and Miller (1985) reported that β-carotene supplementation showed an increase in both the total lymphocytes and percent of T-helper cells (CD4$^+$) without affecting the percentage of T-cell subsets. More recently, it has been studied the effects of β-carotene on photosuppression of the immune response induced by ultraviolet light. The β-carotene supplemented group showed no significant change in delayed-type hypersensitivity (DTH) response to ultraviolet light, whereas the placebo group demonstrated a significant decrease in DTH response (Fuller, Faulkner, Bendich, Parker, and Roe, 1992). There are over a score of epidemiological studies which have shown a consistent association of high dietary carotenoid intake and beta carotene serum levels with significantly lower risk of cancer, especially lung cancer. In addition, several animal studies have found decreased tumor development in those given β-carotene. There is, therefore, growing evidence that carotenoids are

chemopreventive and that this activity is separate from any provitamin A activity and may be linked to the antioxidant properties of carotenoids (Bendich and Olson, 1989).

2.2.2 Antioxidant Enzymes

The antioxidant enzymes primarily interfere with the production of free radicals during the initiation phase by inactivating precursor molecules (Combs, 1987).

Superoxide Dismutase (SOD)

Dismutation of $O_2^{-\bullet}$ to H_2O_2 by SOD is often called the primary defense, because this enzyme prevents the further generation of free radicals. It exists in virtually all O_2-respiring organisms, and its major function is to catalyze the dismutative reaction

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

There are two types of superoxide dismutases which inactivate the superoxide anion; a Mn SOD localizes in mitochondria, and Cu/Zn localizes in cytoplasm. The activity of SOD varies among tissues. The highest levels are seen in the liver, adrenal gland, kidney, and spleen. Induction of SOD also occurs when stimulated by other chemical compounds, such as paraquat, known to generate $O_2^{-\bullet}$ (Yu, 1994).

Catalase (CT)

Catalase, the Fe-containing enzyme found in peroxisome, is a major primary antioxidant defense component that primarily works to catalyze the decomposition of H_2O_2 produced as a result of superoxide dismutation or by other reactions to H_2O and

O_2 as the end products as well as sharing this function with glutathione peroxidase. Glutathione peroxidase as well as catalase detoxify oxygen reactive radicals by catalyzing the formation of H_2O_2 derived from superoxide. At low H_2O_2 levels, organic peroxides are preferentially catalyzed by peroxidase. However, at high H_2O_2 concentrations, they are metabolized by CT. Like SOD, the tissue distribution of CT is widespread. The activity varies not only between tissues but within the cell itself. The liver, kidney, and red blood cell possess relatively high levels of CT. In hepatocytes, peroxisomes exhibit an expectedly high CT activity, although activity was found also in microsomes and in the cytosol (Thomas and Aust, 1985).

Glutathione Peroxidase (GSH-PX)

Most species exhibit GSH-PX, intracellularly located in the cytosol and mitochondrial matrix. Glutathione peroxidase catalyzes the reduction of H_2O_2 and organic hydroperoxides as follows

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

or

$$2GSH + ROOH \xrightarrow{GSH\text{-}PX} GSSG + H_2O + ROH$$
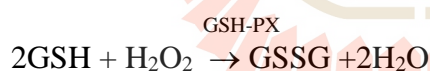
Both types of GSH-PX enzymes, selenium dependent and selenium independent, have been shown to catalyze the above reactions and thus protect against radical damage by reducing peroxides. However, they possess different substrate specificities. The selenium dependent peroxidase is found in the cytosol and exhibits a low capacity for reduction of H_2O_2. The selenium-independent peroxidase utilizes organic hydroperoxides as preferred substrates over H_2O_2.

Another form of selenium-dependent GSH-PX associated with membranes was reported by Ursini and colleagues (Ursini et al., 1995). This cytosolic protein exhibited GSH-PX activity in degradation of phospholipid hydroperoxide.

Circulating levels of antioxidant enzymes are very low compared to intracellular concentrations (Wayner, Burton, Ingold, Barclay, and Locke, 1987; Frei, Stocker, and Ames, 1988). The lack of antioxidant enzymes in extracellular fluids becomes important during inflammatory reactions such as seen in the arthritic joint or emphysemic bronchial fluid. The balance between free radicals formed and antioxidant protectors can be tipped and result in marked immune-mediated tissue degeneration.

2.3 The Role of Nitric Oxide in Immunity

Nitric oxide (NO) is a short-lived free radical and intercellular messenger that mediated a variety of biological functions, including vascular homeostasis, neurotransmission, cell-mediated cytotoxicity, antimicrobial defense, antitumor activities, vasodilation, immune responses and defense against infectious agents, smooth muscle relaxation, penile erection and platelet inhibition (Snyder and Bredt, 1996). Nitric oxide is formed when the guanido group of the essential amino acid L-arginine is cleaved. The reaction is catalyzed by nitric oxide synthase (NOS) and several cofactors, including flavones (FAD, FMN), tetrahydrobiopterin, and NADPH through a process that consumes five electrons and results in the formation of L-citruline (Figure 2). The reaction is stereospecific only L-arginine is cleaved by NOS to form L-citrulline and D-arginine does not interact with NOS (Moncada, Palmer, and Higgs, 1991; Nathan, 1992; Robbins and Sisson, 1996).

Nitric oxide synthases (NOS) is a heme-containing enzyme with a sequence similarity to cytochrome P-450 reductase. Although arginine transport into cells is probably necessary for continued NO production, NOS activity appears to be the major rate-limiting step of NO formation *in vivo* (Robbins and Sisson, 1996). Three isoforms of NOS have been identified: the neural form (nNOS), the endothelial form (eNOS), and the inducible form (iNOS). NOS are classified into two major categories, namely, constitutive and inducible NOS. The constitutive NOS (cNOS) that was first discovered in the vascular endothelium has been designated as eNOS, whereas that present in the brain, spinal cord and peripheral nervous system is termed nNOS. Both of them are mainly regulated posttranslationally by activated cytoplasmic calcium levels and calmodulin or phosphorylation by various protein kinases (Nathan, 1992; Nussler and Billiar, 1993). The form of NOS induced by immunological or inflammatory stimuli is known as iNOS. iNOS has been detected in several cell types including macrophages (Hibbs, Taintor, Vavin, and Rachlen, 1988), endothelial cells (Ignarro, Buga, Sood, Byrns, and Chandhuri, 1987), bone marrow (Punjabi, Laskin, Heck, and Laskin, 1992), hepatocytes (Curran, Billiar, Stuehr, Hofmann, and Simmons, 1989), messangial cells (Pfeilschifter and Schwarzenbach, 1990), and some tumor cells (Amber, Hibbs, Taintor, and Vavrin, 1988). However, iNOS is not usually expressed in resting cells, but is induced by lipopolysaccharide (LPS), interferon-γ (IFN-γ), picolinic acid, lipoarabinomannan, phorbol ester, hypoxia and various proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, interleukin-12 (IL-12), and other inducing agents including ultraviolet irradiation and ozone (Pendino, Laskin, Shuler, Punjabi, and Laskin, 1993; Warren, 1994). Among the most important stimuli for induction of iNOS is the

bacterial endotoxic LPS, and the level of stimulation can be augmented up to 100-fold by combining with IFN-γ (Lowenstein et al., 1993). Induction of iNOS results in the formation of much larger nanomolar amounts of NO than the picomolar amounts that result from eNOS and nNOS (Xia et al., 1992). Since calmodulin is already tightly bound, iNOS expression is largely independent of intracellular calcium (Marletta, 1994; Nathan and Xie, 1994).

NO formed by iNOS has been implicated to play a key role in host defense processes against infectious agents of almost every class including viruses, tumor cells, and alloantigens (Hibbs et al., 1988). However, owing to its potential cytotoxicity, an unregulated production of iNOS-derived free radicals causes tissue injury. iNOS is not detectable in healthy tissue but is expressed after immunologic challenge or injury. In addition, induction of iNOS by LPS contributes to the pathogenesis of septic shock (Petros, Bennett, and Vallance, 1991) and induction of iNOS by other stimuli leads to organ destruction (McCartney-Francis et al., 1993), arthritis, diabetes, transplanted organ rejection, sepsis, chronic inflammation and autoimmune diseases (Kleemann et al., 1993) including tumor growth, invasion, and angiogenesis (Jenkins et al., 1995; Klotz et al., 1999). It is well known that NO formation is increased during inflammation (e.g., arthritis), and several classic inflammatory symptoms are reversed by NOS inhibitors. However, these proinflammatory effects of NO are not evident under acute physiologic conditions, in which NO can mediate anti-inflammatory functions, such as inhibition of neutrophil adhesion, cyclooxygenase activity, and cytokine formation (Schmidt and Walter, 1994). In addition, other experimental evidence has led to the hypothesis that NO suppress inflammation. Lymphocytes can release NO, and murine macrophage

release of NO reduce lymphocyte activation (Albina, Abate, and Henry, 1991). These observations have led to the hypothesis that local NO production plays a suppressor role in allograft rejection (Langrehr et al., 1991). Also, iNOS knockout mice have markedly reduced defenses against microorganisms (e.g., Listeria and Leishmania), and against the proliferation of lymphoma tumor cells. These animals are, however, resistant to carrageenan inflammation and hypotension elicited by endotoxin (MacMicking et al., 1995; Wei et al., 1995).
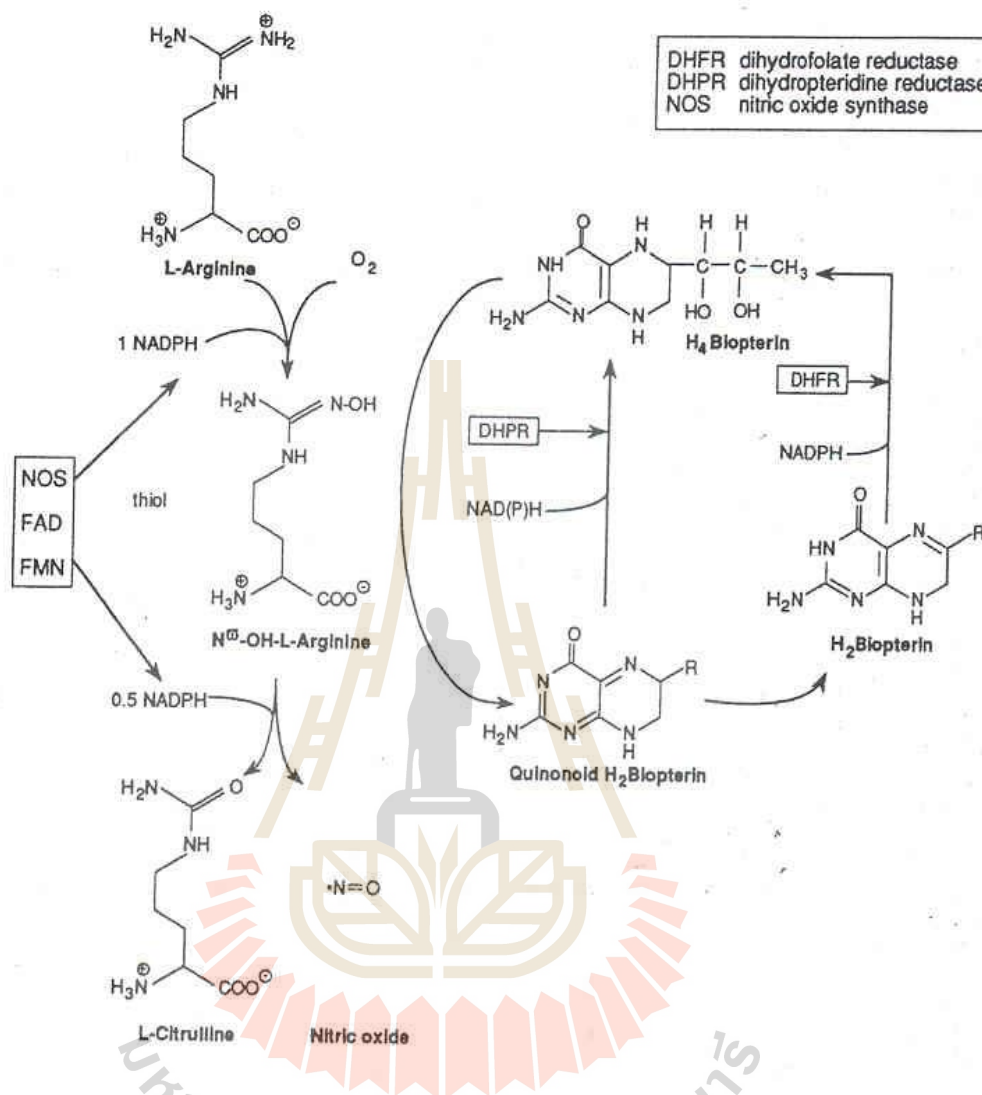
Figure 2. Biosynthesis of nitric oxide. A guanidino nitrogen of L-arginine undergoes a five-electron oxidation to yield the gaseous radical nitric oxide ($\cdot$NO) via an Nω-hydroxyl-L-arginine intermediate. NADPH donates two electrons for the formation of this intermediate and one electron for its further oxidation. Both steps are catalyzed by FAD- and FMN-containing enzyme, nitric oxide synthase (NOS). Molecular oxygen is incorporated both into the ureido group of product citrulline and into NO itself. Tetrahydrobiopterin is also required; the amounts needed are substoichiometric with respect to NO generated, provided that tetrahydrobiopterin can be regenerated from its oxidized form, quinonoid dihydrobiopterin. Regeneration of tetrahydrobiopterin proceeds both through the methotrexate-sensitive enzyme, dihydrofolate reductase and through the methotrexate-resistant enzyme, dihydropteridine reductase. Oxidative inactivation of NOS in cell lysates is retarded by inclusion of thiol (Nathan, 1992).

2.4 Antioxidant Effects of Plant Phenolic Compounds

Phenolic compounds are widely distributed in plants. One of the major groups of phenolic compounds is the flavonoids, which are important in contributing to the flavour and colour of many fruits and vegetables and products derived from them such as wine, tea and chocolate. The biological role of some of the other simple phenolic compounds is not well understood; they may play a role as building blocks for other compounds or in plant defense mechanisms. Dietary phenolic compounds have generally been considered as non-nutrients and their possible benefit to human health has just recently been considered. At present, there is much interest in the biological effects of phenolic compounds since evidence was found that diets rich in fruit and vegetables appear to protect against cardiovascular disease and some forms of cancer (Block, 1992; Hertog, Feskens, Hollman, Katan, and Kromhout, 1993; Block and Langseth, 1994). Since oxygen free radicals and lipid peroxidation are thought to be involved in several conditions such as atherosclerosis, cancer and chronic inflammation, the antioxidant activity of phenolic compounds has been of primary interest (Halliwell, 1994).

2.4.1   Chemistry and Biosynthesis

The term "phenolic compound" comprises a wide range of plant substanced which possess an aromatic ring bearing one or more hydroxyl substituents. They frequently occur attached to sugars (glycosides) and as such tend to be water soluble. The flavonoids are the largest single group of phenolic compounds. Flavonoids are C15 compounds composed of two phenolic rings connected by a three-carbon unit. The flavonoids are biosynthetically derived from acetate and shikimate (Mann, 1978)

such that the A ring has a characteristic hydroxylation pattern at the 5 and 7 position. The B ring is usually 4', 3'4' or 3'4'5' hydroxylated. Figure 3 shows these major structural features with examples of the chalcone, flavonol and flavone groups. The isoflavonoids are derived by cyclization of the chalcones such that B ring is located at 3 position (Figure 3). Other major groups of flavonoids include the catechins (often found as esters with gallic acid in tea) and the anthocyanidins, which are highly coloured pigments (Figure 4).

Of the simple phenolic acids, cinnamic acid and its derivatives are widespread in plants. They are derived primarily from the shikimate pathway via phenylalanine or tyrosine (Mann, 1978) and major examples are coumaric acid (single hydroxyl group) and caffeic acid (Figure 5). Oxidation of the side chain can produce derivatives of benzoic acid such as protocatechuic acid and gentistic acid. These compounds are usually found in nature as glucose ethers or in ester combination with quinic acid. Other phenolic compounds of interest include resveratol, a hydroxy stilbene found in red wine (Pace-Asciak, Hahn, Diamandis, Soleas, and Goldberg, 1995), oleuropein, a bitter principle of olives (Visioli and Galli, 1994), and complex compounds which may be derived by oxidative coupling of more simple phenolics, e.g. salvianolic acid isolated from *Salvia miltiorrhiza*, a plant used in traditional Chinese medicine (Lin, Zhang, and Liu, 1996).

Some major dietary sources of phenolic compounds are outlined in Table 1. The daily intake of flavonoids has been estimated at between 20 mg and 1 g (Hertog et al., 1993). The flavanols, particularly catechin and catechin-gallate esters and the flavonol quercetin, are found in beverages such as green and black tea (Stagg and Millin, 1975) and red wine (Frankel, Waterhouse, and Teissedre, 1995). Quercetin is
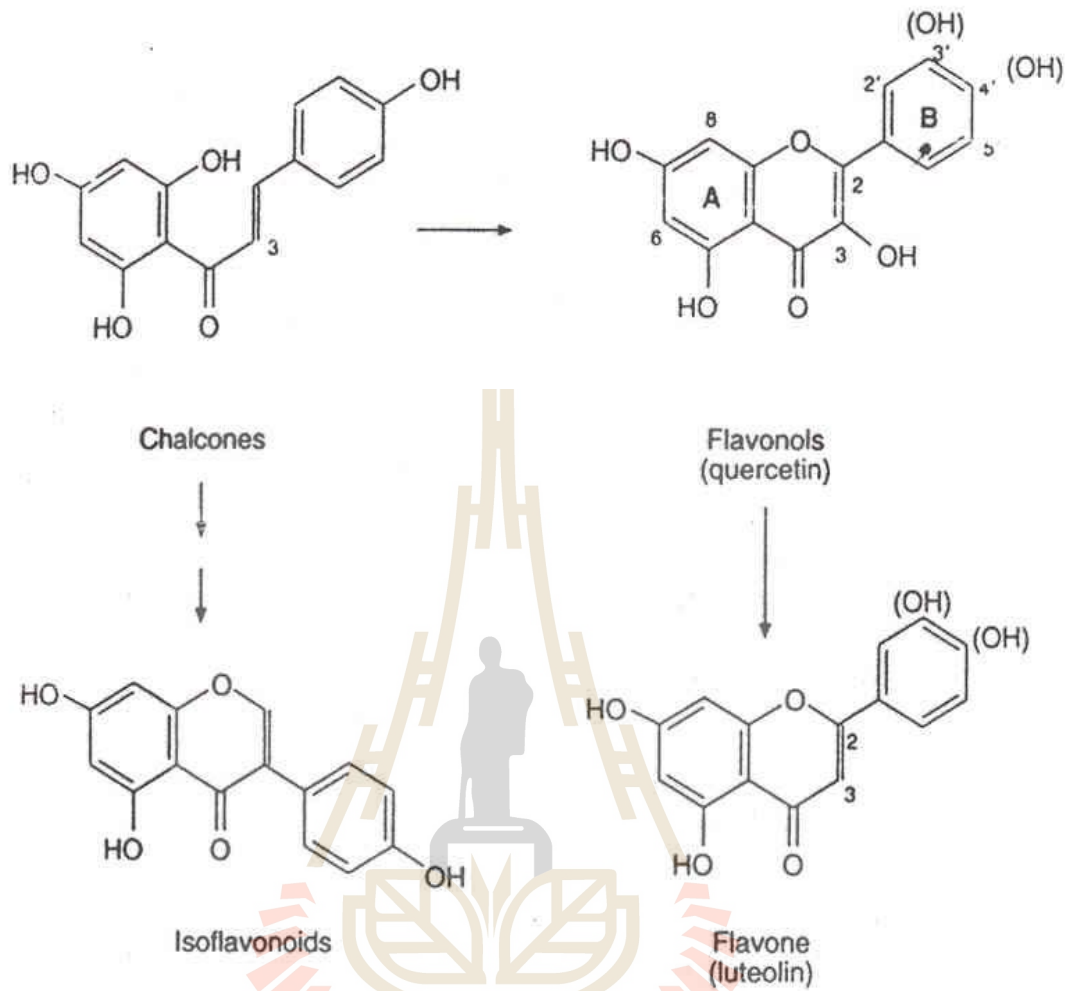
Figure 3. Structural features of chalcones and their biosynthetic product (Mann, 1978).
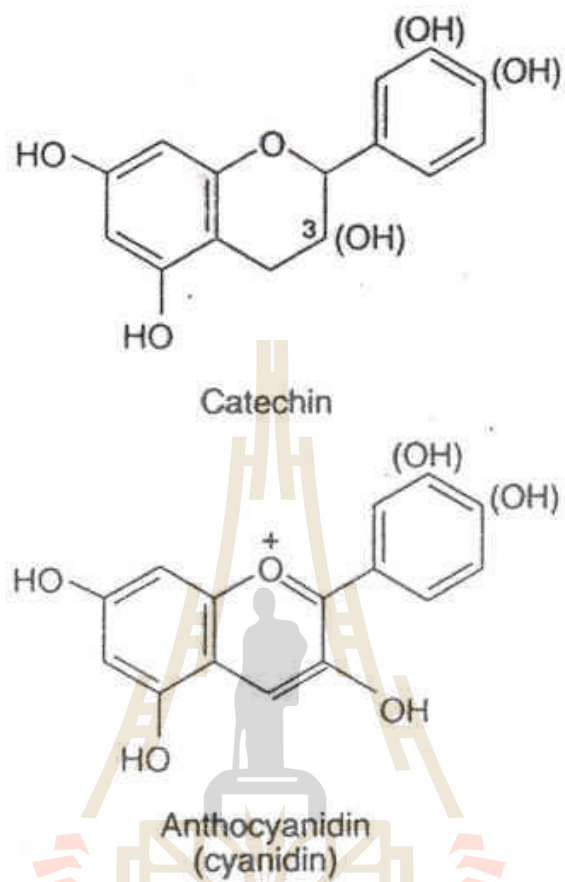
Figure 4. Structural features of catechin and anthocyanidin (Mann, 1978).
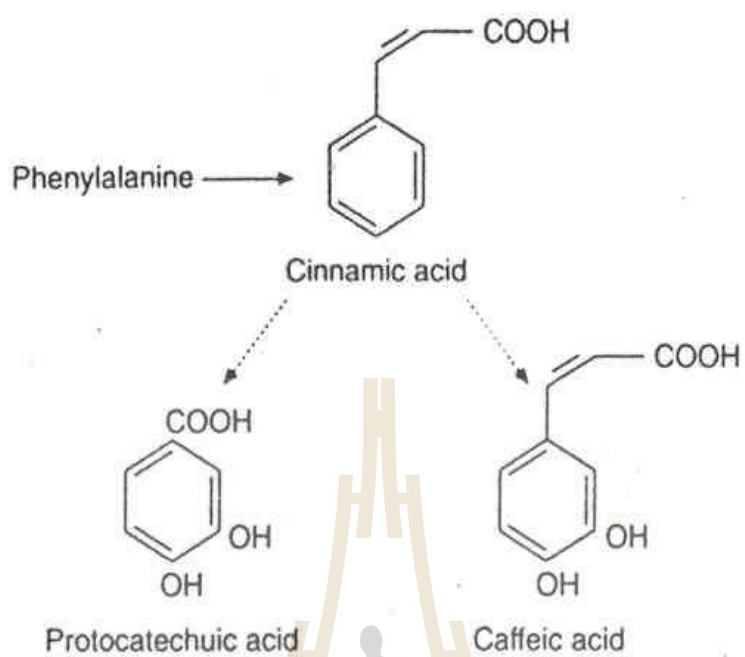
Figure 5. Cinnamic acid and its derivatives (Mann, 1978).

also a predominant component of onions, apples and berries. The flavanones, such as naringin, are mainly found in citrus fruits. The phenolic acids are widespread but are also found in red wine.

Table 1. Some dietary sources of flavonoids and phenolic acids

Flavonoid	Source
Catechins	Tea, red wine
Flavanones	Citrus fruits
Flavonols (e.g. Quercetin)	Onions, olives, tea, wine, apples
Anthocyanidins	Cherries, strawberries, grapes, coloured fruits
Caffeic acid	Grapes, wine, olives, coffee, apples, tomatoes, plums, cherries

2.4.2 Antioxidant Activity of Flavonoids and Phenolic Acids

Flavonoids and phenolic acids can act as antioxidants by a number of potential pathways. The most important is likely to be by free radical scavenging in which the polyphenol can break the free radical chain reaction. For a compound to be defined as an antioxidant it must fulfil two conditions: (i) when present at low concentrations compared with the oxidizable substrate it can significantly delay or prevent oxidation of the substrate; (ii) the resulting radical formed on the polyphenol must be stable so as to prevent it acting as a chain propagating radical (Halliwell, Aeschbach, Loliger, and Aruoma, 1995).   This stabilization is usually through delocalization, intramolecular hydrogen bonding or by further oxidation by reaction with another

lipid radical (Shahidi and Wanasundara, 1992). A number of studies have been carried out on the structure-antioxidant activity relationships of the flavonoids (Bors, Heller, Michel, and Saran, 1990; Chen, Chan, Ho, Fung, and Wang, 1996; Rice-Evans, Miller, and Paganda, 1996; van Acker et al., 1996; Cao, Sofic, and Prior, 1997). The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows:

1. An ortho-dihydroxy (catechol) structure in the B ring, for electron delocalization;

2. A 2,3 double bond in conjugation with a 4-keto function, provides electron delocalization from the B ring;

3. Hydroxyl groups at positions 3 and 5, provide hydrogen bonding to the keto group.

These structural features are illustrated in Figure 6.

The phenolic acids may also be good antioxidants, particularly those possessing the catechol-type structure such as caffeic acid (Laranjinha, Almeida, and Madeira, 1994; Nardini et al., 1995; Abu-Amsha, Croft, Puddey, Proudfoot, and Beilin, 1996). Recent studies have indicated that simple cell-derived phenolic acids such as 3-hydroxyanthranilic acid may also be efficient co-antioxidants for α-tocopherol, able to inhibit lipoprotein and plasma lipid peroxidation in humans (Thomas, Witting, and Stocker, 1996). The possible interaction between flavonoids and phenolic acids with other physiological antioxidants such as ascorbate or tocopherol is another possible antioxidant pathway for these compounds. The synergistic interaction of these antioxidants may be exemplified by the enhancement of the antiproliferative effect of quercetin by ascorbic acid, possibly due to its ability
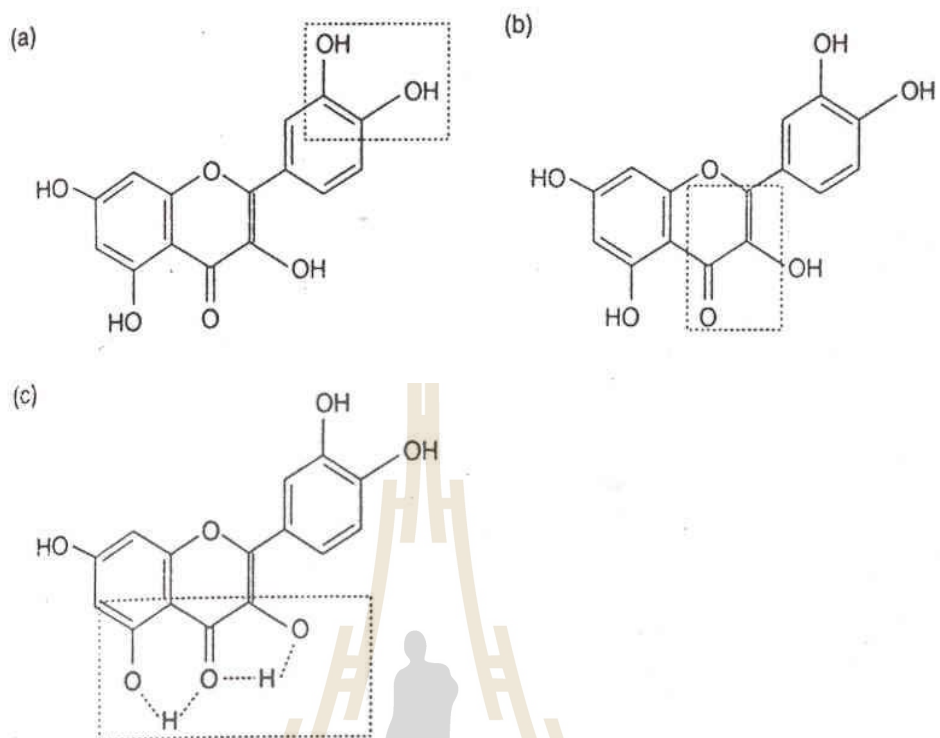
Figure 6. Structural groups of radical scavenging agent (Bors et al., 1990).

to protect the polyphenol from oxidative degradation (Kandaswami, Perkins, Soloniuk, Drzewiecki, and Middleton, 1993). In a similar manner, coincubation of low-density lipoprotein (LDL) with ascorbate and caffeic or coumaric acid resulted in a synergistic protection from oxidation promoted by ferrylmyoglobin (Vieira, Laranjinha, Madeira, and Almeida, 1998b). Another pathway of apparent antioxidant action of the flavonoids, particularly in oxidation systems using transition metal ions such as copper or iron, is chelation of the metal ions. Chelation of catalytic metal ions may prevent their involvement in Fenton-type reactions which can generate highly reactive hydroxyl radicals as follows (Halliwell et al., 1995):

$$H_2O_2 + Cu^+ \rightarrow {}^\bullet OH + OH^- + Cu^{2+}$$

$$Cu^{2+} + O_2{}^{-\bullet} \rightarrow Cu^+ + O_2$$
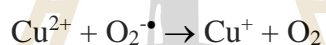
The ability of polyphenolics to react with metal ions may also render them pro-oxidant. For example, in a recent study by Cao et al. (1997) using three different oxidation systems, flavonoids had potent antioxidant activity against peroxyl radicals generated from AAPH and against hydroxyl radicals but were pro-oxidant with Cu^{2+}. Presumably flavonoids can reduce Cu^{2+} to Cu^+ and hence allow the formation of initiating radicals. Caffeic acid has also been shown to have pro-oxidant activity on Cu^{2+}-induced oxidation of LDL (Yamanaka, Oda, and Nagao, 1997).

The possible pro-oxidant effects of flavonoids may be important *in vivo* if free transition metal ions are involved in oxidation processes. In the healthy human body, metal ions appear largely isolated in forms that are unable to catalyse free radical reactions (Halliwell and Gutteridge, 1990). However, injury to tissues may release

iron or copper (Halliwell, Gutteridge, and Cross, 1992) and catalytic metal ions have been measured in atherosclerrotic lesions (Smith, Mitchinson, Arudma, and Halliwell, 1992). In these cases the potential for flavonoids to act as pro-oxidants is considered.

Other biological actions of phenolic compounds have been noted which may be relevant to their effects on human health. For example, caffeic acid may have cytoprotective effects on endothelial cells related not only to its antioxidant action but also to its ability to block the rise in intracellular calcium in response to oxidaized lipoproteins (Vieira et al., 1998a). Some phenolic compounds may also inhibit platelet aggregation (Pace-Asciak et al., 1995), while others may act as inhibitors of nuclear transcription factor NF-κB (Natarajan, Singh, Burke, Grunberger, and Aggarwal, 1996). The ability of phenolic compounds to trap mutagenic electrophiles such as reactive nitrogen species may also protect biologocal molecules from damage (Kato et al., 1997) and will be mentioned latter.

2.5 Flavonoids

Flavonoids (or bioflavonoids) are a group of over 4,000 naturally occurring phenolic compounds (polyphenol) sharing a similar chemical structure. The flavonoids are natural products widely distributed in fruits, vegetables, nuts, seeds, stems, flowers as well as tea and wine and are important constituents of the human diet. They are also found in several medicinal plants, and herbal remedies containing flavonoids have been used in traditional medicine around the world. (Carlo, Mascolo, Izzo, and Francesco, 1999).

Flavonoids can be divided into five categories (Harbone and Baxter, 1991).

1.   <u>Anthocyanidins, anthochlors and aurones.</u>   Anthocynidins are red-blue pigments in plants (such as found in red to blue of different parts in plants). Anthochlors and aurones are yellow pigments found in flowers.

2.   <u>Minor flavonoids.</u>   Minor flavonoids include flavanones, flavan-3-ols, dihydroflavones, and dihydrochalcones.   These are categorized as minor flavonoids due to their limited natural distribution.   Two flavan-3-ols (flavanol) that will be discussed below include (+)-catechin and epigallocatechin 3-gallate (EGCG).   Certain flavanols, or flavan-3-ols, are sometimes referred to by trademark term pycnogenols, coined by the French researcher Professor Jack Masquelier.   The term pycnogenol means "that which creates condensation," and refers to the tendency of flavanol to create dimers (two identical compounds joined together), oligomers (a few joined together), and polymers (many joined together).   One group of flavanol dimer and oligomers are termed proanthocyanidins, and are discussed in item 5.

3. <u>Flavones and flavonols.</u> Flavones and flavonols are the most widely occurring flavonoids. Although several hundred flavonol aglycones are known, only quercetin, kaempferol, and myricetin are widely distributed. More than 135 different glycosides of quercetin have been isolated, and the most common of which is rutin, a flavonol that has been used to treat capillary fragility. Flavones also occur as glycosides, but in a more limited fashion than flavonols. Baicalin, such as found in *Scutellaria baicalensis* (huang gin), is a common flavone O-glycoside used in Chinese medicine.

4. <u>Isoflavonoids.</u> Isoflavonoids are found mostly in the Leguminosae family (legumes), and can be divided into isoflavones, isoflavonones, pterocarpans, isoflavans, and rotenoids. Common isoflavonoids include genistein, daidzein, and biochanin A.

5. <u>Tannins.</u> Tannins include proanthocyanidins gallic acid phenolics (the gallo-and ellagi-tannins). They are characterized by their ability to bind with proteins. Proanthocyanidins are dimers of flavanols. In France, where much of the basic research on flavanols has taken place, they are termed "procyanidols" or in French "oligomeres proanthocyanidoliques" (oligomeric proanthocyanidins in English), or OPC for short. The abbreviation OPC is now usd in many countries to refer to proanthocyanidins.

The distribution of these compounds vary between plant species. For example, citrus fruits contain high levels of flavones and flavanones; green tea contains high levels of catechins (17 to 30% of dry weight) and gallic acid phenolics; red blue, and purple fruits such as berries, grapes contain high levels of anthocyanidins; and pine bark and grape seeds contain high levels of OPC. Small molecular weight flavonoids are responsible for the tartness and bitterness of many fruits, whereas large molecular weight flavonoids (tannins), are responsible for their astringency. The main classes of flavonoids and related compounds are shown in Figure 7.

Research on flavonoids began in 1936 when Szent-Gyorgi and colleagues discovered that crude extracts of vitamin C from lemon juice were more effective than pure vitamin C in treating guinea pigs with experimentally-induced scurvy. So far, a variety of *in vitro* and *in vivo* experiments have shown that flavonoids display a remarkable array of biochemical and pharmacological actions and they have been shown to affect a large variety of enzymes, to possess important enzyme-inducing activities, to possess free-radical scavenging activity, to chelate certain metal cations, to have antioxidant properties and, importantly, to affect cellular protein phosphorylation. In addition, the flavonoids have long been recognized to possess a

wide range of therapeutical activities such as antihepatotoxic, antiosteoporotic and vascular protective including antiallergic, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic activities as well as to affect some aspects of mammalian metabolism. They also act in the gastrointestinal tract as anti-ulcer, antispasmodic, antisecretory and antidiarrhoeal agents. These effects may have therapeutic uses, but this potential of the flavonoids has not yet been realized eventhough various researches support the possible utilization of these compounds in medicine because (i) flavonoids have not shown such a prominent and significant biological activity; (ii) flavonoids (catechin in particular) have shown severe side effects such as acute intravascular hemolysis, acute renal failure; (iii) flavonoids (quercetin, kaempferol, galangin) have produced genotoxic effects; (iv) the marketing regulations are very severe.
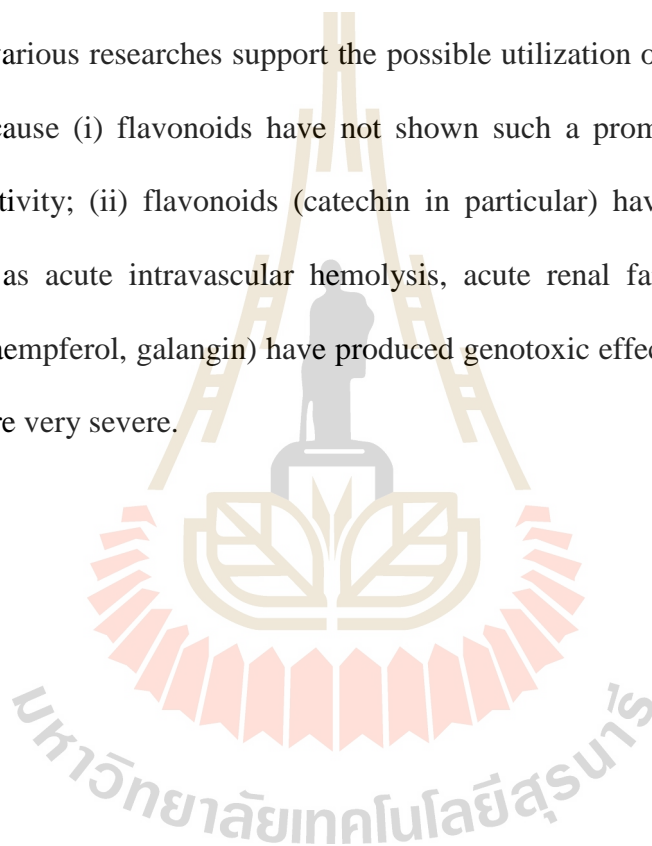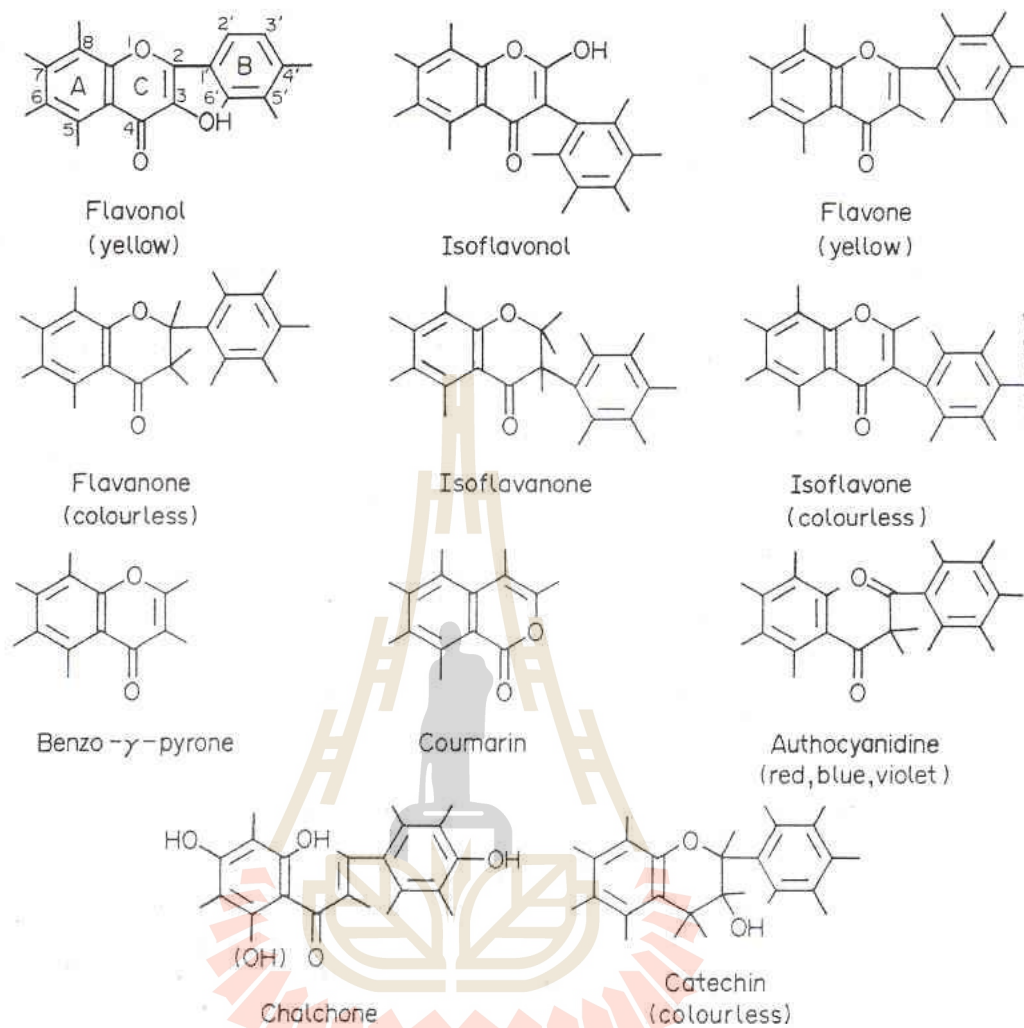
Figure 7. The main classes of flavonoids and related compounds (Harbone and Baxter, 1991).

2.5.1 Immunological Activities of Flavonoids

The immune system is a highly complex, complicately regulated group of cells whose integrated function is essential to health. Cells of the immune system may interact in a cognate cell-cell manner and may also respond to intercellular messages including hormones and cytokines elaborated by various cells. The immune system can be modified by diet, pharmacologic agents, environmental pollutants and naturally occurring food chemicals such as the flavonoids. Certain flavonoids significantly affect the function of the immune system and inflammatory cells. A number of flavonoids also affect the function of enzyme systems critically involved in the immune responses and the generation of inflammatory processes, namely, both tyrosine and serine-threonine protein protein kinases, phospholipase A2, phospholipase C, lipoxygenases and others. Recently, it has become evident that these enzymes are intimately involved in signal transduction and cell activation processes involving cells of the immune system as well as other cells activated by hormones, histamine, neurotransmitters and growth factors.

Antiallergic Properties

Flavonoids are also known for their anti-allergic effects. It is now well known that certain flavonoids, depending on structure, can inhibit the stimulated release of proinflammatory mast cell, basophil, and eosinophil granular constituents that participate in the pathogenesis of diseases such as asthma, allergic rhinoconjunctivitis, urticaria and others. These effects are in part attributed to the influence of flavonoids on the production of histamine (Berg and Daniel, 1988). In fact flavonoids inhibit enzymes that increase histamine release from mast cells and basophils: cyclic AMP

phosphodiesterase and calcium-dependent ATPase. Cyclic AMP phosphodiesterase degrades cAMP: large amounts of cAMP act by blocking intracellular reservoirs of histamine. Also calcium-dependent ATPase degrades ATP to release energy to facilitate the gating of calcium across the cell membrane: high intracellular calcium levels also cause histamine release from cellular storage granules. Khellin, a flavonoid isolated from fruits of the Egyptian plant *Ammi visnaga*, was used for the treatment of asthma and other disturbances (Samuelsson, 1993). It is currently little used because of side effects like nausea and vomiting. Studies to find better substances led to the development of sodium cromoglycate, a drug that prevents degranulation of the mast cells and consequently the release of histamine and other endogenous substances causing asthma. This drug has no effect on acute asthmatic attacks, but it acts prophylactically. It is now known that quercetin and other substances are more potent than sodium cromoglycate in inhibiting histamine release from mast cells (Amellal et al., 1985). Fisetin, quercetin, and luteolin were studied in basophil histamine release caused by tetradecanoly phorbol acetate (TPA), which activates protein kinase C directly and found that all of these were very active inhibitor of protein kinase C. Interestingly, the most active inhibitors of protein kinase C were also the most active inhibitors of TPA-induced basophil histamine release. Further kinetic studies with the rat brain preparation indicated that fisetin and quercetin caused inhibition of protein kinase C by means of blocking the ATP binding site in the catalytic portion of the enzyme. This could represent one fundamental mechanism of action of the flavonoids (Ferriola, Cody, and Middleton, 1989).

Antitumor Effect

It is now widely recognized that diets rich in fruits and vegetables appear to be associated with a reduced frequency of cancer of various organ systems. Many researchers have conducted *in vitro* studies on the potential antitumor activity of flavonoids. For example, the antitumor activity of catechin, a flavanol present in green tea, *Areca catechu*, *Crataegus oxyacatha*, *Cinnamomum cassia*, *Polygonum multiflorum*, *Rheum palmatum*, was examined using tumor invasion models (Bracke et al., 1988). This particular flavonoid inhibited such invasiveness and it was suggested that the activity of catechin may be related to its ability to bind tissue-type plasminogen activator (t-PA) to laminin, a molecule of extracellular matrix that play an important role during tumor cell adhesion, leading to partial inactivation of t-PA (Bracke et al., 1991). *Citrus* flavonoids (tangeretin) also exhibit anti-invasive activity, but appear to act by a different mechanism. They show a poor affinity for the extracellular matrix and do not bind enzymes to laminin (Bracke et al.). *In vitro* studies have been made on the antiproliferative effect of *Citrus* flavonoids quercetin, taxifolin, nobletin and tangeretin on human squamous cell carcinoma (Kandaswami, Perkins, Soloniuk, Drzewiecki, and Middleton, 1991). Whereas nobiletin and tageretin, which are both polymethoxylated flavonoids, markedly inhibited carcinoma cell growth at all concentrations tested, quercetin and taxifolin were ineffective. The difference in action may be due to the greater uptake by cell membrane of the less hydrophilic polymethoxylated flavonoids. However, ascorbic acid is able to potentiate the cytostatic effect of quercetin.

Flavonoids extracted from *Scutellaria baicalensis* (baicalein, baicalin and woogonin) exerted *in vitro* a concentration-dependent inhibition of the proliferative

response of cultured rabbit vascular smooth muscle cells upon exposure to 5% calf serum (Huang, Wang, and Hsieh, 1994). Beside from quercetin and baicalein, epigallocatechin gallate and green tea extract were also reported to inhibit tumor growth by inhibiting mitosis (Lea et al., 1993). Some flavonoids (quercetin, epigallocatechin) and green tea extract inhibit tumor growth both by inhibiting some phase of cell cycle and by blocking or competing for hormone receptor sites (Scambia et al., 1990). Quercetin was also reported to be effective in inhibiting of *in vitro* bromodeoxyuridine incorporation by cells from transition cell carcinoma of the bladder (Larocca et al., 1994). Other mechanisms by which flavonoids may inhibit tumor growth include: stabilizing collagen, altering gene expression and reducing free radicals. It is known that agents that inhibit collagen breakdown may inhibit invasion and metastasis. Catechin and its derivatives anthocyanins and proanthocyanidins promote collagen synthesis (Beretz and Cazenave, 1988; Murray, 1992), increase collagen resistance (Scutt, Meghji, Canniff, and Harvey, 1987) and inhibit collagenase activity (Makimura et al., 1993). Quercetin inhibits human breast cancer cells *in vitro* by inhibiting the expression of the mutated p53 plasma membrane protein: the p53 protein is a tumor suppressor protein that plays a key role in appoptosis (Avila, Velasco, Cansado, and Notario, 1994). Flavonoids also possess free radical scavenging effect and therefore inhibit tumor invasion and metastasis (Komori et al., 1993; Tanaka et al., 1997). Flavonoids were determined to have *in vivo* activity on experimental tumors. For example, quercetin and rutin administered by diet were reported as inhibiting azoxymethanol-induced colonic neoplasia in mice (Deschner, Ruperto, Wong, and Newmark, 1993). The flavonoids tricin and kaempferol-3-O-β-D-glucopyranoside derived from the traditional Chinese medicine plant *Wikstroemia*

indica, demonstrated antileukaemic activity in the P-388 leukemin mice (Lee et al., 1981) epidemiological studies indicated that diets containing linseed and soy (rich in isoflavonoids and lignans) may protect against colon, breast and prostatic cancer (Ren and Lien, 1997).

Also certain flavonoids actually inhibit adduct formation between carcinogens and DNA, and finally, certain flavonoids inhibit *in vivo* experimental carcinogenesis. A good example of the latter is an experiment where female rats developed mammary carcinoma in response to a particular carcinogen were found to have a 50% reduction in the number of tumors as compared to control when they consumed a diet containing 5% quercetin (Verma, Johnson, Gould, and Tanner, 1988). In addition, certain flavonoids possess antitumor-promoter activity, in which case the flavonoids inhibit the various activities of tumor promoters that are involved in the process of carcinogenesis. Moreover, antitumor activity of flavonoids has been described with a number of different hormone-dependent tumors and certain flavonoids turn out to be very active antiproliferative agents, inhibiting cancer cell proliferation *in vitro* (Middleton, 1996). Additionally, a rather extraordinary process that is stimulated by certain flavonoids is a prodifferentiation effect, that is, certain flavonoids can actually stimulate a malignant cell to develop into a mature phenotype (Middleton). Other properties of flavonoids beneficial to health include an inhibitory effect of certain flavonoids on the expression of multi-drug resistance gene and modulation of topoisomerase activity, which can be associated with reduced cancer growth. Finally, through effects on adhesion molecule expression and function certain flavonoids have an antimetastatic activity and reduce the development of metastases (Middleton).

Anti-inflammatory Activity

Anti-inflammatory properties of flavonoids have been studies both *in vitro* and *in vivo*. The studies of Middleton and Kandaswami suggested that flavonoids may have significant *in vivo* effects on homeostasis of the immune system and on the behaviour of secondary cell systems involved in the inflammatory response. Flavonoids were demonstrated as possessing *in vivo* anti-inflammatory properties. Some reports suggested that they had good anti-inflammatory activity without the ulcerogenic side-effects of other anti-inflammatory drugs. In contrast, flavonoids were described as having anti-ulcer effects.

Adhesion molecules are necessary in the initial process of inflammation by adhering leukocytes in the vascular system to the tissue that is becoming inflamed. Anne, Agarwal, Nair, Schwartz, and Middleton (1994) investigated the inhibitory role of quercetin in the expression of one particular endothelial cell adhesion molecule, known as intracellular adhesion molecule-1 (ICAM-1) following stimulation of the human umbilical vein endothelial cells (HUVEC) with endotoxin. Interestingly, quercetin caused a concentration-dependent inhibition of the expression of ICAM-1 in endotoxin-stimulated endothelial cells. Nepetin, a flavonoid obtained from *Nepeta hindostana*, was investigated in both acute and chronic models of inflammation in rats and found to possess significant activity in both proliferative and exudative phases of inflammation (Agarwal, 1982). Apigenin and luteolin from *Chamomilla recutita* were found to significantly inhibit the oedema caused by croton oil. This activity may have been due to a direct inhibition of arachidonic acid metabolism or to other mechanisms such as inhibition of histamine released or promotion of scavenging activity (Della Loggia et al., 1994). Studies on the *in vivo*

anti-inflammatory activity of the flavonoids, particularly hypolaetin-8-β-D-glucoside, sidertoflavone (isolated from the Spanish *Sideritis mugronensis*) and the flavonol glycosides gossypin and hibifolin (isolated from traditional Indian medicinal plants), showed a dose-dependent inhibition of both paw oedema and leucocyte accumulation in the peritoneum in the carrageenan-induced models of oedema and peritonitis (Ferrandiz and Alcaraz, 1991).

The macrophage/monocyte is a cell critical to the initiation of the immune response. These cells take up antigens and foreign particles, e.g. microbed and partially digest them and present them on the cell surface so that it can interact with the T-cell receptor to initiate an immune response. Some investigators have indicated that the process of antigen presentation can be inhibited in these cells by quercetin (Mookerjee, Lee, Lippes, and Middleton, 1986).

2.5.2 Flavonoids and Nitric Oxide

It is well known that some flavonoids are excellent scavengers of oxygen free radicals such as hydroxyl radical and superoxide anion radicals. More recently, they have also been found that the actions of some flavonoids may be correlated with their capacity to interact with nitric oxide (NO), which is a mediator of various biological systems (Moncada, Palmer, and Higgs, 1991).

Nitric oxide is formed by an enzyme called nitric oxide synthase (NOS), which is a type of diaphorase enzyme. Recent data suggest that flavonoids could inhibit *in vitro* brain NADPH diaphorase activity. These experiments showed that quercetin and apigenin markedly inhibited this enzyme's activity in a concentration-dependent fashion, suggesting that these flavonoids may also be able to inhibit the

production of nitric oxide in the brain (Tamura, Agawa, Tsuruo, Ishimura, and Morita, 1994). Chiesi and Schwaller (1995) studied the action of tannins and the flavonoid compound quercetin on constitutive endothelial NOS activity and found that both tannins and quercetin inhibited NOS activity, although the tannins were the more potent inhibitors. The flavonoids flavone, 3'-amino-4'-hydroxyflavone and genistein were investigated for their inhibitory activity of nitric oxide production in murine macrophage: 3'-amino-4'-hydroxyflavone showed the greatest potency. These data supported the suggestion that flavones could modulate the immune response and inflammatory reactions by controlling the production of nitric oxide (Krol, Czuba, Threadgili, Cunningham, and Pietsz, 1995). Carlo et al. (1993) examined the gastrointestinal activities of quercetin in animals pretreated with two inhibitors of NOS, NG-nitro-L-arginine methyl ester and NG-monomethyl-L-arginine (L-NAME and L-NMMA). This pretreatment potentiated the delay in transit, intestinal secretion and diarrhoea caused by quercetin, and it was thus suggested that nitric oxide was involved in the gastrointestinal activities of quercetin.

Furthermore, the reported antitumor properties of flavonoids were in part related to their activity against nitric oxide, which was reported to be carcinogenic. In particular it was shown that epigallocatechin gallate is able to inhibit the inducible nitric oxide synthase gene expression and enzyme activity (Chan, Fong, Ho, and Huang, 1997).

Taken together, part of the therapeutic effect of flavonoids has been ascribed to their free radical scavenging capacity. Because of the important role of nitric oxide ($^\bullet$NO) in various pathophysiological processes, the scavenging of $^\bullet$NO by therapeutic used flavonoids is contempated.

2.6 *Tamarindus indica* L. (*T. indica* L.)

Family: Caesalpiniaceae

Common Names: Tamarind, Makhaam, Taluup (Chaobon-Nakhon Ratchasima), Mong khlong (Karen-kanchanaburi), Mot-le, Saa-mo-kle (Karen mae Hong Son), Maak kaeng (Shan-Mae Hong Son), Am-pain (Khmer-Surin).

Botanical Description:

Tamarindus indica L. is a large tree, up to 30 m high, having spreading branches; bark brownish-gray, flaked. Leaves are even-pinnate, consisting of 10-18 pairs of small leaflets, rather closed together, petioles and rachis 5-12 cm long; leaflets oblong, 8-30 by 3-9 mm, opposite, pink or reddish when young, membranous, glabrous, apex obtuse or rounded, base unequal. Inflorescence is in terminal raceme, yellowish-orange or pale green; consisting of calyx-tube narrow turbinate, with 4 imbricate segments, 1 cm long; petals 3, unequal, upper cordate, about 1 cm long, 2 lateral ones, narrowed towards the base; fertile stamens 3, base connate; ovary linear, about 7 mm long, pubescent, on a stalk adnate to calyx-tube. Pods are oblong, slightly curved, 5-15 by 1-2.5 cm, reddish brown. Seed is glossy, dark brown, embedded in a thick, sticky and acid brown pulp.

Ecology and Distribution:

They are cultivated all over the tropics for their edible fruits, and as the ornamental trees.

Tamarind (*Tamaridus indica* L.) belongs to Leguminosae, and the place of its origin is said to be Africa. It grows naturally in tropical and subtropical regions now

and is one of the most important plant resources as food materials in tropical region particlarly in South India (Farnsworth and Bunyapraphatsara, 1992).

Pharmacological Activities and Its Usages:

Tamarind is regarded as a refrigerant, digestive, carminative, laxative and is useful in diseases supposed to be caused by deranged bile, increase bile flow rate and also biliary secretion of bile acids and cholesterol (Sambaiah and Srinivasan, 1991). The pulp is used in spices and seasoning, and it is accepted as a herb medicine in parts of the world. The flower and leaf are eaten as vegetables. The germ obtained from the seeds is used for manufacturing tamarind gum, and it has been added to many kinds of foods in Japan to improve their viscosity and use as food additive. However, the seed coat as byproduct of tamarind gum has been hardly used until the antioxidative activity of tamarind seeds was investigated.

In the method of ethanol extract prepared from the seed coat exhibited antioxidative activity as measured by the thiocyanate and thiobarbituric acid and the ethyl acetate extract prepared from seed coat had strong antioxidative activity. The 2-hydroxy-3',4'-dihydroxyacetophenone (TA0), methyl 3,4-dihydroxybenzoate (TA1), 3,4-dihydroxyphenyl acetate (TA2) and (-)-epicatechin (TA3) were isolated from the ethyl acetate extract and showed that TA0, TA1, and TA2 had strong antioxidative activity in the linoleic acid autoxidation system as measured by the thiocyanate and TBA method as well as α-tocopherol (Tsuda et al., 1994).

As reported, it is an established practice that these plant-food drug materials are co-administered with conventional modern pharmaceutical products indirectly as part of the normal meal or directly as medicinal preparation. *Tamarindus indica* L. fruit extract containing in the meal showed a statistically significant increase in the

plasma levels of aspirin and salicylic acid, repectively when it was administered with the aspirin tablets than when taken under fasting state or with the meal without the fruit extract. The study has indicated that *Tamarindus indica* L. fruit extract significantly increased the bioavailability of aspirin (Mustapha, Yakasai, and Aguye, 1996).

More recently, the effect of tamarind ingestion on excretion of floride in human was evaluated. The subjects consumed 10 g tamarind daily for 18 days and the result showed that tamarind intake caused significant increase in the excretion of fluoride in 24h urine as compared to excretion on control diet, hence, the tamarind intake might be help in delaying progression of fluorosis (Khandare, Rao, and Lakshmaiah, 2002). In addition, tamarind seed polysaccharide (TSP), a galactoxyloglucan isolated from seed kernel of *Tamarindus indica*, possesses properties like high viscosity, broad pH tolerance, adhesive and led to its application as stabilizer, thickener, gelling agent and binder in food and pharmaceutical industries (Sumathi and Ray, 2002). Furthermore, TSP also includes non-carcinogenicity (Sano et al., 1996), mucoadhesivity, biocompatibility (Burgalassi, Panichi, Saettone, Jacobsen, and Rassing, 1996), high drug holding capacity (Kulkarni, Ddwivedi, Sarin, and Singh, 1997), high thermal stability (Saettone et al., 1997) and possessed immunomodulatory effects by enhancing phagocytic cell, inhibiting leukocyte migration and cell proliferation (Sreelekha, Vijayakumar, Ankanthil, Vijayan, and Nair, 1993).

From the study of Pumthong (1999), tamarind seeds were obtained from ripen tamarind fruits after the edible parts. The good seeds were heated in a hot air oven at 140 °C, 45 minutes, cooled and readily cracked to separate their outside brown layer.

Only brown-red coats were collected and ground into fine powder. The coat parts of tamarind seeds were extracted with 70% ethanol followed by chloroform and the remaining extract of aqueous fraction was collected. The extract was dried and completely dissolved in methanol and this solution was used in following experiments. Chemical analysis as well as UV absorption and IR spectra of the extract compared with oligomeric proanthocyanidin (OPC), commercially extracted from pine barks and grape seeds, showed similar results indicating that the extract of tamarind seed coat contained polyphenolic compounds, so-called OPC. TLC chromatogram demonstrated that the extract contained at least six components. The extract had no peroxidase inhibitory effect and showed potent antioxidant scavenging activity against peroxyl radicals generated by $ABTS/H_2O_2$/peroxidase and $ABTS/H_2O_2$/metmyoglobin system, against hydroxyl radicals produced by $ABTS/H_2O_2/FeCl_3$ (Fenton reaction) and superoxide anions generated by hypoxanthine-xanthine oxidase (neotetrazolium) system. Antioxidant mechanism of the extract partially resembled curcumin playing a role as an iron-chelator and acted as a scavenger against free radical but quite differed from that of vitamin E, Trolox and vitamin C. In addition, the effect of the tamarind seed coat extract against oxidative damage of erythrocyte membrane generated by tert-Butylperoxide and $FeCl_3$, was measured by determining malondialdehyde (MDA). It was found that the extract could effectively prevent lipid peroxidation in a dose-dependent manner. The extract could also protect Ca^{2+}-ATPase in red blood cell membrane from oxygen free radical damage (Pumthong, 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

Tissue Culture Media

Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 4 mM glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90% of 4.5 g/L glucose, and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) was used for RAW 264.7 gamma NO(-) cells.

The supplemented culture medium for the human cell lines consisted of Eagle Minimum Essential Medium (Gibco) containing 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and supplemented with 90% of 0.01 mg/mL bovine insurin, and 10% of fetal bovine serum.

Supplemented complete culture medium for the murine peritoneal macrophages and splenocytes consisted of RPMI 1640 media (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS), and 0.05 U/mL penicillin and 0.05 mg/mL streptomycin (Gibco).

Flow Cytometry Reagents

100 μL of 1:20 dilution (in staining buffer) of propidium iodide (Sigma) were used for *in vitro* cytotoxicity studies.

MTT

MTT [3(4,5-dimethyl thiazol-2-yl) 2,5-diphenyltetrazolium Bromide] and Solubilization/Stop Solution in CellTiter 96 kit were purchased from Promega Corporation (Madison, WI) was used for cytotoxicity on tumor cells and % viability in nitrite studies.

3.1.2 Test Articles

Tamarindus indica **L.**- Extract (70% ethanol followed by chloroform) of seed coat prepared by Gannica Pumthong (Chiang Mai University) was dissolved in 10% dimethylsulphoxide (DMSO) in sterile complete Dulbecco's modified Eagle's medium (DMEM) for *in vitro* exposure or in PBS for oral exposure.

DNFB (2,4-Dinitrofluorobezene) (Sigma Chemical Company, St.Louis, MO), a potent irritant and contract sensitizer, was used as 0.15% solution in acetone to evaluate the anti-inflammatory potential of *T. indica* in local lymph node and irritancy assays and its effect on the development of sensitization.

HCA (α-Hexylcinnamaldehyde) (Sigma), a moderated contract sensitizer, was used as 30% solution in acetone to evaluate the anti-inflammatory potential of *T. indica* and its effect on the development of sensitization.

Nonanoic acid (Sigma), a potent irritant, was used as 60% solution in acetone to evaluate the anti-inflammatory potential of *T. indica* in irritancy assay.

Anti-Asialo GM 1 was used in natural killer cell activity.

LPS (lipopolysaccharide: *Escherichia coli* serotype 0111: B4) (Sigma) was used as 5 μg/mL in complete DMEM to stimulate NO production in RAW 264.7 gamma NO(-) cells and peritoneal macrophages.

IFN-γ (interferon gamma) (Research Diagnostics, Flanders, NJ) was used as 10 ng/mL in complete DMEM to fully stimulate NO production in RAW 264.7 gamma NO(-) cells and peritoneal macrophages.

Vitamin C (ascorbic acid) (Sigma), **vitamin E (α-tocopherol)** (Sigma), and **β-carotene** (Sigma) were used for comparison with the seed coat extract of *T. indica*.

TPA (12-O-tetradecanolyphorbol-13-acetate) (Sigma), an inducer of nitric oxide production, was used as 0.1 μg/mL in sterile PBS in intraperitoneal injection (i.p.) for *in vivo* exposure and in RPMI 1640 culture media for *in vitro* exposure.

Con A (concanavalin A) (Sigma) was used to stimulate splenocyte proliferation.

3.1.3 Vehicles

DMSO (Sigma) was used as 10% Dimethyl Sulfoxide (DMSO) solution in sterile PBS for preparing the various concentrations of the seed coat extract of *T. indica* and vitamin C.

Acetone (Sigma) was used to make the 0.1% DNFB solution, 30% HCA, and 60% nonanoic acid.

Corn oil was used as solvent for vitamin E and β-carotene.

3.2 Methods

3.2.1 Animals

Female B6C3F1 mice were used for dose range finding and toxicity *in vivo* studies and as a source of splenocytes for *in vitro* studies, natural killer (NK) cell activity, *in vitro* and *in vivo* TPA-induced peritoneal macrophages. Mice were

obtained from Taconic Farms (Germantown, NY) or Charles Rivers Laboratories in Wilmington, MA. Female BALB/c mice were used to evaluate the anti-inflammatory effects in local lymph node assay and irritancy assay of the extract and were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were maintained in the NIOSH animal facility under conditions specified within NIH guidelines (NIH, 1996) and experiments were conducted under an approved NIOSH Animal Care and Use Committee protocol. Mice were 8-12 weeks of age upon arrival and were quarantined for at least one week prior to use and were housed in polycarbonate "shoebox" cages with hardwood bedding. Animals were weighed and assigned into homogeneous weight groups (n=5). All mice were provided tap water, and Agway Prolab Animal Diet (5% fat) *ad libitum*. Animal rooms were maintained between 18-26°C and 40-70% relative humidity with light/dark cycles of 12-hour intervals.

3.2.2 Cell Lines

AGS human gastric adenocarcinoma, HaCat human epidermal keratinocyte, MCF-7 human mammary adenocarcinoma, A427 human lung carcinoma, B16-F10 murine melanoma and L1210 murine lymphocytic leukemia cell lines were purchased from the American Type Culture Collection (Bethesda, MD).

The L1210, B16-F10 and HaCat cell lines were maintained *in vitro* in supplemented Dulbecco's modified Eagle's medium containing 10% horse serum at 37°C and 5% CO_2. For cell culturing purposes, newly received cells, if frozen, were thawed, removed from shipping vial, washed with Dulbecco's modified Eagle's medium, and spun at 300xg for 10 minutes, to remove any freezing chemicals. The

cells were then reconstituted, and counted using a hemocytometer and trypan blue exclusion method to exclude nonviable cells. The cells were then seeded into 25 cm^2 flasks at a concentration of approximately 250,000 cells/mL and a total volume of 5 mL. Upon reaching concentrations of 500,000 to 1x 10^6 cells/mL, approximately 50,000 cells/mL were transferred to 75 cm^2 (total volume of 25 mL) or 162 cm^2 (total volume of 50 mL) flasks for maintenance purposes. Cells were harvested as necessary, depending on the tumor line doubling time, and cell numbers were cut back to maintain the tumor lines at sub-confluent, logarithmic growth. A 0.25% trypsin/0.03% EDTA (Gibco) solution in PBS was used to subculture these cell lines. Briefly, all of the media was removed from the culture flask, 0.5 mL to 2 mL of the trypsin solution was added to the flask, and the flask was rested horizontally in the hood. After 2 minutes, the flask was reconstituted with Dulbecco's modified Eagle's medium, the cap tightly sealed, and then the flask was shaken. Cell scrapers were then used to scrape the flask bottoms. The cell/media mixture was then removed, and spun at 300xg for 10 minutes. The supernatant was poured off and the cells were reconstituted in flasks with Dulbecco's modified Eagle's medium. For freezing purposes, cells were spun at 300xg for 10 minutes and reconstituted in a 1:20 DMEM:DMSO solution at a concentration of 1x10^6 cells/mL 1.8 mL of the cell mixture was added to 2 mL freezer vials (Costar) and the vials were placed in the-20°C freezer for 30 to 60 minutes. The vials were transferred to the -80°C freezer for long term storage.

The A427 and MCF-7 cell lines were maintained *in vitro* in supplemented Eagle Minimum Essential Medium containing 10% fetal bovine serum and the AGS was maintained *in vitro* in supplemented Ham's F-12K medium containing 10% fetal

bovine serum. For cell culturing purposes, the same method was used as described above for L1210, B16-F10 and HaCat.

The murine macrophage cell line RAW 264.7 gamma NO(-) (ATCC CRL-2278) used in nitrite assay and was kindly provided by Dr. Gabriel K. Harris (National Institute for Occupational Safety and Health, Morgantown, WV). Cells were cultured and maintained in DMEM containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and grown at 37°C with 5% CO_2 in humidified air. Exponentially growing cells were used for experiments when they reached 80% confluency.

3.2.3 Splenocyte Suspension

Splenocyte single cell suspensions were prepared from B6C3F1 mice. Mice were sacrificed by CO_2 asphyxition. The spleens were aseptically excised and placed in 12x75 snap cap tubes containing 3 mL RPMI 1640 culture media. Under a lamina flow hood the spleens were pressed between the frosted ends of two microscope slides into a 60x15 petri dish with RPMI from the collection tube. Cells were counted on a Coulter counter using 20 μL of splenocyte suspension in 10 mL of Isoton (1:500) with 2 drops of Zap solution and plated for assay.

3.2.4 *In Vitro* Cytotoxicity Studies

3.2.4.1 Cell Viability on Splenocytes

Single cell suspensions of splenocytes from B6C3F1 mice were used to evaluate the *in vitro* toxicity of *T. indica*. Cells were diluted in RPMI to a concentration of $5x10^6$ cells/mL. 100 μL of cells were plated in each well at a

concentration of $5x10^5$ cells per well, in a 96-well plate. 10^{-5}-10^3 μg/mL in 20 μL of the seed coat extract of *T. indica* were used. The viability of the splenocytes was evaluated by Trypan blue exclusion and propidium iodide.

Trypan Blue Exclusion Cell Viability Determination:

A 100 μL of the cell suspension was tranferred to a 1.5 mL microfuge tube. 100 μL (an equal volume) of 0.4% (w/v) trypan blue was added to the tube and mixed throughly. A pasteur pipet or pipetman was used to transfer a small (~20 μL) amount of this diluted sample to one or both chambers of the hemocytometer and allow the chamber to fill by capillary action (do not underfill or overfill the chamber). The 10X objective was used to focus on the grid lines of the chamber. The viable cells (exclude trypan blue) and nonviable cells (blue) were counted in the four 1 mm^2 corner squares in one chamber. Cells that lie on the lines should only be counted if they are touching the top and left hand lines of each corner square. The percent viable cells were calculated according to the following formula:

$$\% \text{ Viable cells} = \frac{\text{Total viable cell per mL}}{\text{Total cells per mL}} \times 100$$

Propidium Iodide Uptake:

Cells were exposed to PI (0.1mg/mL) for 5 minutes, washed, and processed on a flow cytometer to determine the percent of viable cells.

3.2.4.2 Comparison of MTT and ^{3}H-thymidine incorporation

5×10^5 splenocytes were seeded into a culture plate. Different concentrations of Concanavalin A were added to each well. Duplicate wells were plated for each concentration. The cells were incubated at 37°C and 5% CO_2 for 48, 72, and 96 hours. For MTT assay 4 hrs. prior to harvesting, 15 μL of MTT dye solution was added to each well. After 4 hrs. 100 μL of solubilization/stop solution was added to each well. Cells were incubated at room temperature for 1 hr. The solution in each well was mixed thoroughly, and the absorbance at 570/730 nm was recorded using a 96 well plate reader. Results were reported as OD. For radioactive assay 24 hrs. prior to harvesting of the culture, 20 μL of thymidine (50 μCi/mL of HBSS) was added to each well to obtain 1 μCi/well. On the next day the plate was harvested using Packard cell harvester and the samples were counted on Beta liquid scintillation counter. The results were reported as CPM/5×10^5 cells.

3.2.4.3 Cytotoxicity on Tumor Cells

HaCat, and AGS, MCF-7, A427, B16-F10, L1210 tumor cell lines were used. 500 μL of the cells were added to the wells of a 48 well plate at the concentration of 5×10^4 cells/well and then 20 μL of the extract was added to each well at the concentration of 0.1-20 μg/well. Cells were incubated at 37°C and 5% CO_2 for 48 hrs. Concentrations were chosen based on data from the *in vitro* splenocyte study. The proliferation of the cells was evaluated by MTT as mentioned in 3.2.4.2.

3.2.5 *In Vivo* Studies

3.2.5.1 Fourteen Days Toxicity Study

Oral Exposure

For all *in vivo* studies, animals were exposured daily through oral gavage for 14 days. Animals were manually restrained and dosed with 0.2 mL/ 20 g mouse of different concentration of *T. indica* seed coat extract. The dosing solutions were administered through 18 guage feeding tube and 1 cc syringe. Animals were exposed daily for the number of day indicated in each study.

B6C3F1 were weighed and assigned into homogenous weight groups. Animals were dosed for 14 days with 50, 100, 500, and 1000 mg/kg concentrations of *T. indica* seed coat extract in 0.2 mL PBS per 20 g via oral gavage and weighted daily and observed for mortality. On the final day of study, 24 hours after the last dose, animals were weighed and sacrificed by CO_2 inhalation. Blood was collected for hematology (white blood cell count, red blood cell count, hematocrit, and hemoglobin) and serum chemistry analysis (alkaline phosphatase, alanine transferase, aspartic transferase, blood urea nitrogen, uric acid, bilirubin, and creatine). Selected organ weights were taken including liver, brain, heart, spleen, kidneys, lungs, and thymus.

3.2.5.2 Natural Killer (NK) Cell Activity

Following 14 days of oral exposure to *T. indica* or vitamin C (n=5)*,* an *in vitro* cytotoxicity assay as described previously (Holsapple, White, McCay, Bradley, and Munson, 1988; Duke, Schook, and Holsapple, 1985) was used to evaluate NK cell function. Positive control animals were administered 0.2 mL of a 1:10 dilution of anti-asialo GM1 by i.p. injection 24 hrs prior to assay. All animals were sacrificed

and spleens were collected 24 hrs following the final exposure. Single cell suspensions were made and splenocytes were adjusted to 2×10^7 cells per mL in complete media (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μg streptomycin). Splenocyte and ^{51}Cr-labeled Yac-1 cells (500 μCi as sodium chromate per 1.5-2 x 10^7 cells) (ATCC, TIB 160, Manassas, VA) were prepared in ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1 and replicate wells plated for each concentration. After a 4 hrs incubation at 37°C and 5% CO_2, 100 μL of supernatant was transferred to tubes and counted on a gamma counter (Packard Cobra II Auto-Gamma). Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X in complete media. Spontaneous release was determined by incubating Yac-1 tumor cells in complete media. The results were expressed in percent lysis for each of the effector to target ratios using the following equation:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

The study design for the natural killer cell assay was shown in Table 2.

Table 2. The study design for the natural killer cell assay

Treatment	N
VH (10% DMSO in PBS)	5
T. indica (50 mg/kg)	5
T. indica (250 mg/kg)	5
T. indica (500 mg/kg)	5
Vit. C (100 mg/kg)	5
Anti Asialo GM 1 (200 μg/mouse)	5

3.2.5.3 Local Lymph Node Assay

The LLNA was conducted in animals following oral exposure to *T. indica* to evaluate the modulatory effect of *T. indica* on the development of a T cell-mediated contact hypersensitivity response.

Five mice per treatment group received vehicle or increasing concentrations of *T. indica* by oral gavage for 14 consecutive days. On days 10, 11 and 12, separate groups of mice were dosed with 50 µL of either 30% HCA or 0.15% DNFB on the dorsal surface of both ears (25 µL on each ear). On day 15, the mice were injected intraveneously via the lateral tail vein with 0.2 mL (20 µCi) of ^{3}H-thymidine (specific activity of 0.2 µCi/mL). Five hours later the mice were sacrificed by CO_2 inhalation. The left and right draining cervical lymph nodes of each animal, located at the bifurcation of the jugular vein, were excised and placed into 4 mL cold phosphate-buffered saline (PBS). A single cell suspension was generated by dissociating the lymph nodes between the frosted ends of two microscope slides. Cells were washed twice with 10 mL PBS and precipitated in 3 mL 5% trichloroacetic acid (TCA) overnight at 4°C. Following resuspension in 1 mL of 5% TCA, the cells were transferred to 5 mL of scintillation cocktail. ^{3}H-thymidine incorporation was determined with a Packard, Tri-carb 2500TR Beta Counter. Samples were counted for 5 min each and mean DPM-background was calculated for each dose group. The stimulation index for each test group was calculated by dividing as the group mean by the mean for the vehicle control group. The study design for the local lymph node assay was shown in Table 3.

Table 3. The study design for the local lymph node assay

T. indica Treatment	LLNA Treatment	N
VH (10% DMSO)	VH (acoetone)	5
VH (10% DMSO)	0.15% DNFB, 30% HCA	5
T. indica (50 mg/kg)	0.15% DNFB, 30% HCA	5
T. indica (100 mg/kg)	0.15% DNFB, 30% HCA	5
T. indica (200 mg/kg)	0.15% DNFB, 30% HCA	5

3.2.5.4 Irritancy Assay

An irritancy study was performed to investigate the effects of oral exposure to the seed coat extract of *T. indica* on the development of dermal irritation to the moderate irritant nonanoic acid (NA) and the potent irritant 2,4-dinitrofluorobenzene (DNFB).

Animals were administered concentrations of the seed coat extract of *T. indica* (50, 100 or 200 mg/kg) by oral gavage once a day for 14 days. On day 11, the ears of mice were measured using a modified Mitutoyo micrometer (Mitutoyo Corporation, Japan). Because remeasurement of exactly the site on each ear could not be assured following treatment, all readings at each time point were done in duplicate to obtain a mean value per ear. See Figure 8 for measurement sites. On days 12, 13, and 14 separate groups of animals were dosed dermally with 50 µL of 60% NA or 0.15% DNFB in acetone on the dorsal surface of each ear (25 µL/ear). Twenty-four after the final exposure (day 15), the ears of the mice were again measured to evaluate irritancy. Percent ear swelling was calculated for individual animals at each time point using the following equation:

$$\% \text{ Ear swelling } = \frac{(\text{Post-measurement} - \text{Pre-measurement})}{\text{Pre-measurement}} \times 100$$
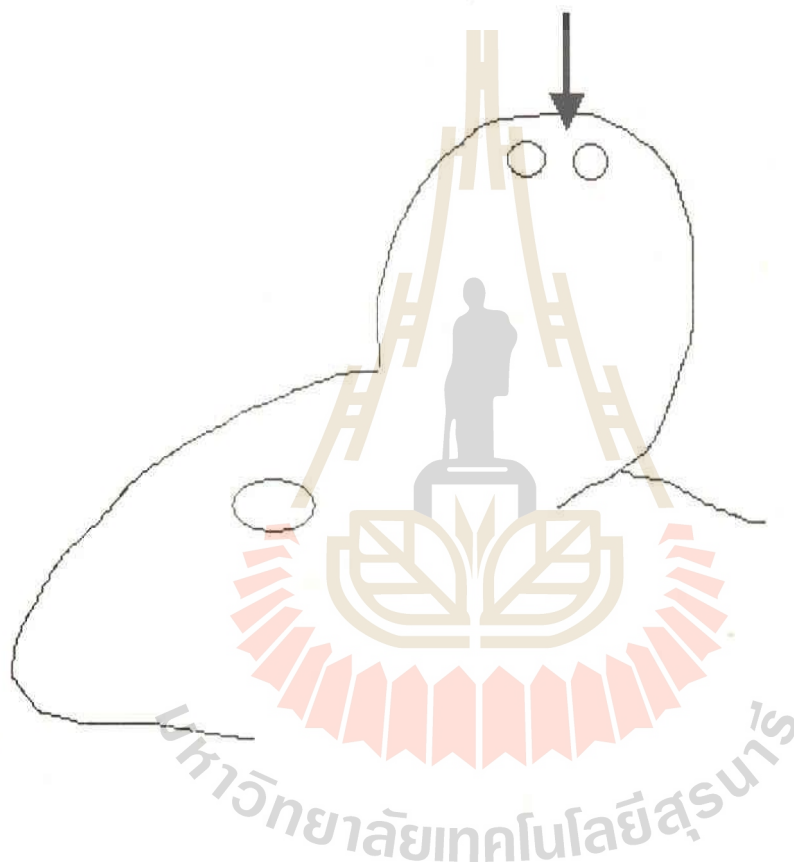
Figure 8. Schematic representation showing the area in which mouse ear
measurement and topical exposure should be performed.

The study design for the irritancy assay was shown in Table 4.

Table 4. The study design for the irritancy assay

T. indica Treatment	Dermal treatment	N
VH (10% DMSO in PBS)	VH (acetone)	5
VH (10% DMSO in PBS)	60% NA, 0.15% DNFB	5
T. indica (50 mg/kg)	60% NA, 0.15% DNFB	5
T. indica (100 mg/kg)	60% NA, 0.15% DNFB	5
T. indica (200 mg/kg)	60% NA, 0.15% DNFB	5

3.2.6 Nitrite Studies

3.2.6.1 *In Vitro* Exposure of Seed Coat Extract of *T. indica* and *In Vitro* Stimulated NO Production with LPS and IFN-γ in RAW 264.7 Cells

For *in vitro* RAW 264.7 cells studies, cells were plated at a concentration of 2×10^5 cells per well and incubated with test article or other antioxidant controls at 37°C for 24 hrs in the presence or absence of NO stimulants. The following stimulants were used; LPS (5 µg/ml) and IFN-γ (10 ng/mL). Vitamin C (500 µM), vitamin E (141.3 µM) and β-carotene (2.5 mM) were used as other antioxidant controls. The concentrations of *T. indica* tested ranged from 0.2 to 200 µg/mL. The NO production was determined by measuring nitrate concentration using the Griess reagent as described below in 3.2.6.5

3.2.6.2 *In Vivo* **Exposure of Seed Coat Extract of** *T. indica* **and** *In Vivo* **Stimulated NO Production with TPA in Peritoneal Macrophages.**

B6C3F1 mice (5 animals/group) were administered increasing concentrations of *T. indica* seed coat extract (50, 250, 500 or 1000 mg/kg), vitamin C (100 mg/kg), vitamin E (100 mg/kg) or β-carotene (50 mg/kg) by oral gavage once a day for 14 days. Twenty-four hours following the final exposure, animals were sacrificed by CO_2 inhalation and peritoneal macrophages were collected. In studies where peritoneal macrophages were stimulated *in vivo* with TPA, animals were injected intraperitoneally with 0.1 μg TPA diluted in 1 mL of PBS 2 hrs prior to sacrifice and measured the NO production as described in 3.2.6.5. The study design was shown in Table 5.

Table 5. The study design for *in vivo* exposure to *T. indica* and *in vivo* TPA stimulant for nitric oxide production

Treatment	*In Vivo* Exposure	N
VH (10% DMSO in PBS)	PBS	5
VH (10% DMSO in PBS)	TPA	5
T. indica (50 mg/kg)	TPA	5
T. indica (250 mg/kg)	TPA	5
T. indica (500 mg/kg)	TPA	5
Vit. C (100 mg/kg)	TPA	5
VH (Corn oil)	TPA	5
Vit. E (100 mg/kg)	TPA	5
β-carotene (50 mg/kg)	TPA	5

3.2.6.3 Collection of Peritoneal Macrophages

Murine peritoneal cells were removed by peritoneal lavage with 7 mL of ice cold PBS with 10% fetal calf serum. Differentials of the peritoneal cells indicated an average of 70% macrophages. Cells were kept cold on ice until transfer to a complete medium consisting of RPMI-1640 with 10% FCS, and penicillin (0.05 U/mL)-streptomycin (0.05 mg/mL). Cells were counted on a Coulter counter and $2x10^5$ cells were plated in 96-well flat-bottom plates and incubated for 2 hrs at 37°C. Non-adherent cells were removed by washing with pre-warmed (37°C) culture medium.

3.2.6.4 *In Vivo* Exposure of Seed Coat Extract of *T. indica* and *In Vitro* Stimulated NO Production with LPS, IFN-γ, LPS plus IFN-γ or TPA in Peritoneal Macrophages.

In this studies, B6C3F1 mice were administered with the seed coat extract of *T. indica* or vitamin C (100 mg/kg) as mention in the 3.2.6.2, cells were plated at a concentration of $2x10^5$ cells per well and incubated with test article at 37°C for 24 hrs in the presence or absence of NO stimulants. The following stimulants were used; LPS (5 µg/mL), IFN-γ (10 ng/mL), LPS (5 µg/ml) plus IFN-γ (10 ng/mL), or TPA (0.1 µg/mL). Vitamin C (100 mg/kg) was used as antioxidant control in only *in vitro* TPA stimulant.   The NO production was measured by determining nitrite concentration as described in 3.2.6.5. The study design was shown in Tables 6 and 7.

Table 6. The study design for *in vivo* exposure to *T. indica* and *in vitro* LPS, IFN-γ, and LPS plus IFN-γ stimulants for nitric oxide production

Treatment	In Vitro exposure			N
	LPS	IFN-γ	LPS + IFN-γ	
VH (10% DMSO in PBS)	LPS	IFN-γ	LPS + IFN-γ	5
T. indica (50 mg/kg)	LPS	IFN-γ	LPS + IFN-γ	5
T. indica (250 mg/kg)	LPS	IFN-γ	LPS + IFN-γ	5
T. indica (500 mg/kg)	LPS	IFN-γ	LPS + IFN-γ	5

Table 7. The study design for *in vivo* exposure to *T. indica* and *in vitro* TPA stimulant for nitric oxide production

T. indica Treatment	In Vitro exposure	N
VH (10% DMSO in PBS)	TPA	5
T. indica (50 mg/kg)	TPA	5
T. indica (250 mg/kg)	TPA	5
T. indica (500 mg/kg)	TPA	5
Vit. C (100 mg/kg)	TPA	5

3.2.6.5 Measurement of Nitrite

Nitrite concentration was determined using the Griess reagent as described by Padgett and Pruett (1992). Following the 24 hrs incubation with the stimulant and test article, 100 µL of cell-free supernatant from each well was transferred to another 96-well flat-bottom plate. Greiss reagent (100 µL, 1% sulfanilamide in 30% glacial acetic acid and 0.1% napthylethylenediamine dihydrochloride in 60% glacial acetic acid) was added. The absorbance of samples was measured at 545 nm with a

spectrophotometric microplate reader (Spectra MAX 340). Nitrite in the samples was quantitated by comparison with a standard curve. A nitrite multi point linear standard curve was determined using known concentrations of $NaNO_2$ ranging from 1.56 μM/mL to 200 μM/mL added to the complete culture media and analysed with softmax 3.3.3 ELISA software.

3.2.6.6 Cell Viability

Once 100 μL cell-free supernatant was removed from each well to determine nitrite concentration, cell viability was evaluated in the original plates by the additional of 15 μL of Dye Solution- MTT for 4 hrs. After washing 2 times in DMEM, the supernatant was removed and the formed insoluble formazan product was dissolved with 100 μL of Solubilization/Stop Solution. Then, the optical densities of the culture wells were measured using a spectrophotometric microplate reader (Spectra MAX 340) at reference wavelengths of 570 and 730 nm. The optical density of formazan formed in control (untreated) cells was taken as 100%. Percent viability of test samples were determined as:

% Viability = (average OD for test group/average OD for control group) x 100

The summary of the study designs for nitrite studies was shown in Table 8.

Experimental Number	Cell Source	Exposure to *T. indica*	Stimulants for nitric oxide production
1	RAW 264.7	*in vitro*	LPS + IFN-γ (*in vitro*)
2	peritoneal macrophage	*in vivo*	TPA (*in vivo*)
3	peritoneal macrophage	*in vivo*	LPS, IFN-γ, LPS + IFN-γ (*in vitro*)
4	peritoneal macrophage	*in vivo*	TPA (*in vitro*)

Table 8. Summary of the study designs for nitrite studies

3.2.7 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism version 3.0 (Graph Pad Software Inc., San Diego, CA). For multiple comparisons, data were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($p \leq 0.05$). Comparison between two groups was made using a Student *T*-test. Linear regression was used to determine dose-responsiveness. Data were considered statistically significant at $p \leq 0.05$.

CHAPTER IV

RESULTS

4.1 *In Vitro* Cytotoxicity Studies

Initial studies were conducted to investigate the toxicity of the seed coat extract of *T. indica* in an *in vitro* system. Initial dose range-finding studies were conducted using single cell splenocyte cultures from B6C3F1 mice. Figure 9 shows cell counts derived from 2 separate studies which cells were exposed to concentrations ranging from 10^{-5} μg/well to 10^3 μg/well of the seed coat extract of *T. indica*. No change in cell number was observed in any concentration tested. Viability was evaluated by two methods including PI uptake using flow cytometry and Trypan blue exclusion. Data from these studies are shown in Figure 10A and B. Cell viability was seen to decrease at concentrations higher than 10 μg/well. Based on this data a concentration range from 0-20 μg/well was chosen to be used in further cytotoxicity studies.

MTT was used to assess proliferation of cells in response to the seed coat extract of *T. indica*. An initial study was conducted to compare the results where proliferation to concanavalin A was measured by MTT or ^{3}H-thymidine incorporation. Figure 11 showed a similar bell shaped curve of a peak response observed with exposure to 10 μg/well whether measured by MTT or ^{3}H-thymidine. The seed coat extract of *T. indica* had no effect on splenocyte proliferation at concentrations ranging from 0-20 μg/well (Figure 12).

MTT was then used to evaluate the cytotoxicity of six cell lines including tumor cell lines (L1210, B16-F10, MCF-7, AGS, A427) and immortalized human keratinocytes (HaCat) to the seed coat extract of *T. indica*. In the L1210 cell line, a slight increase in cell proliferation occurred following exposure to 1-20 μg/well. A decrease in cell number was observed in HaCat, AGS and A427 cell lines at concentrations of 5 μg/well and higher, and in B16-F10 melanoma cells at concentration of 20 μg/well. The seed coat extract of *T. indica* exposure had no effect on MCF-7 cell proliferation (Figure 13).
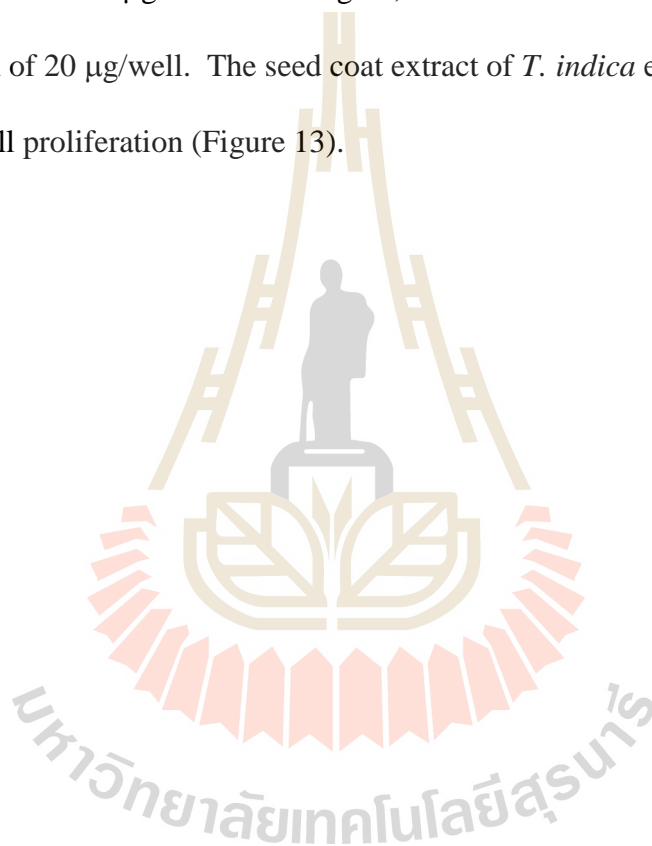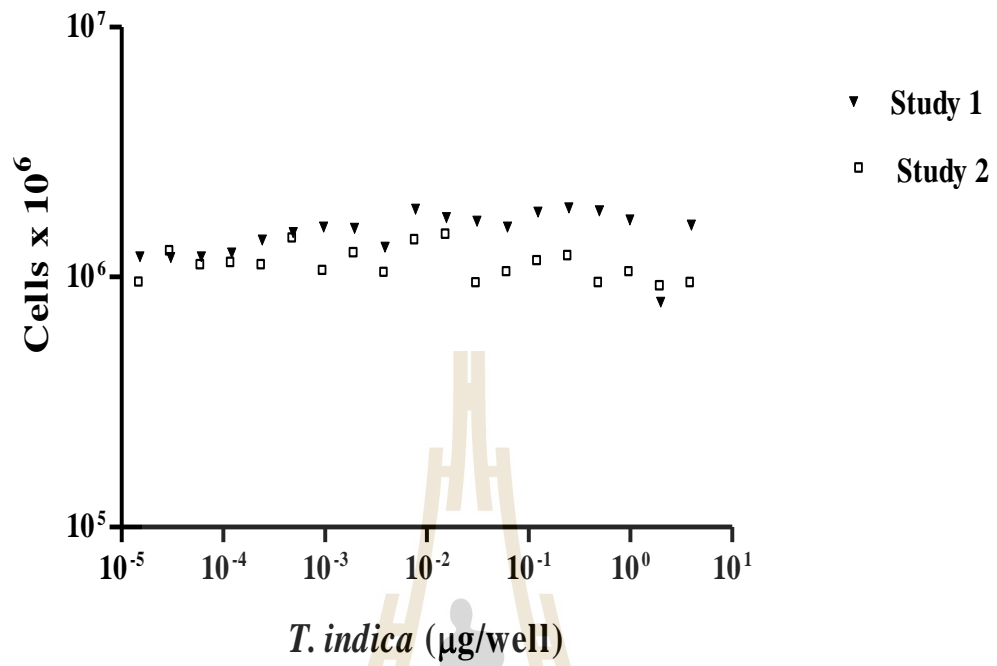
Figure 9. Cell counts from two different studies. Cell numbers were determined using a Coulter Counter.
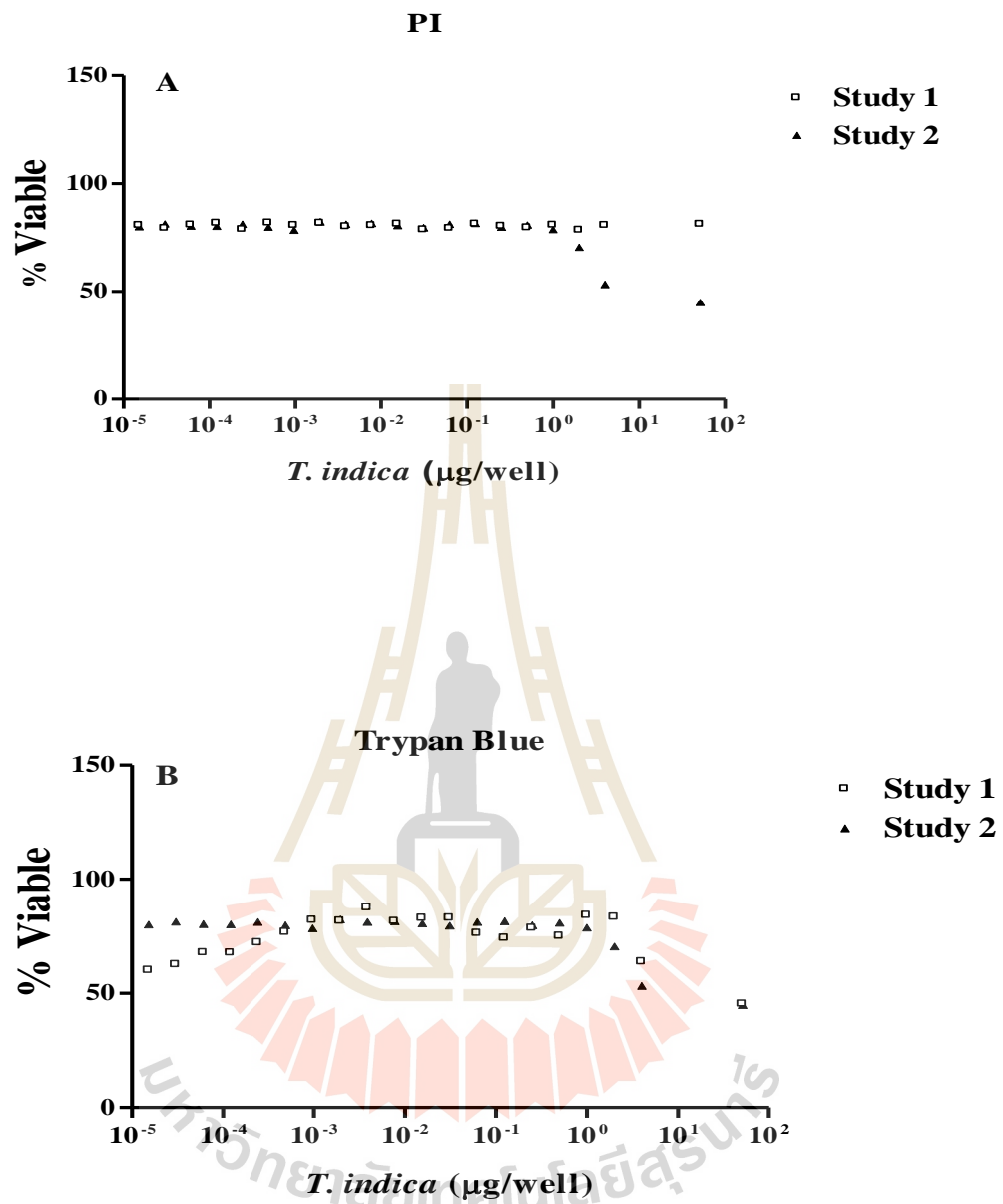
Figure 10. Percent viability determination using PI uptake (A) and Trypan Blue exclusion (B).
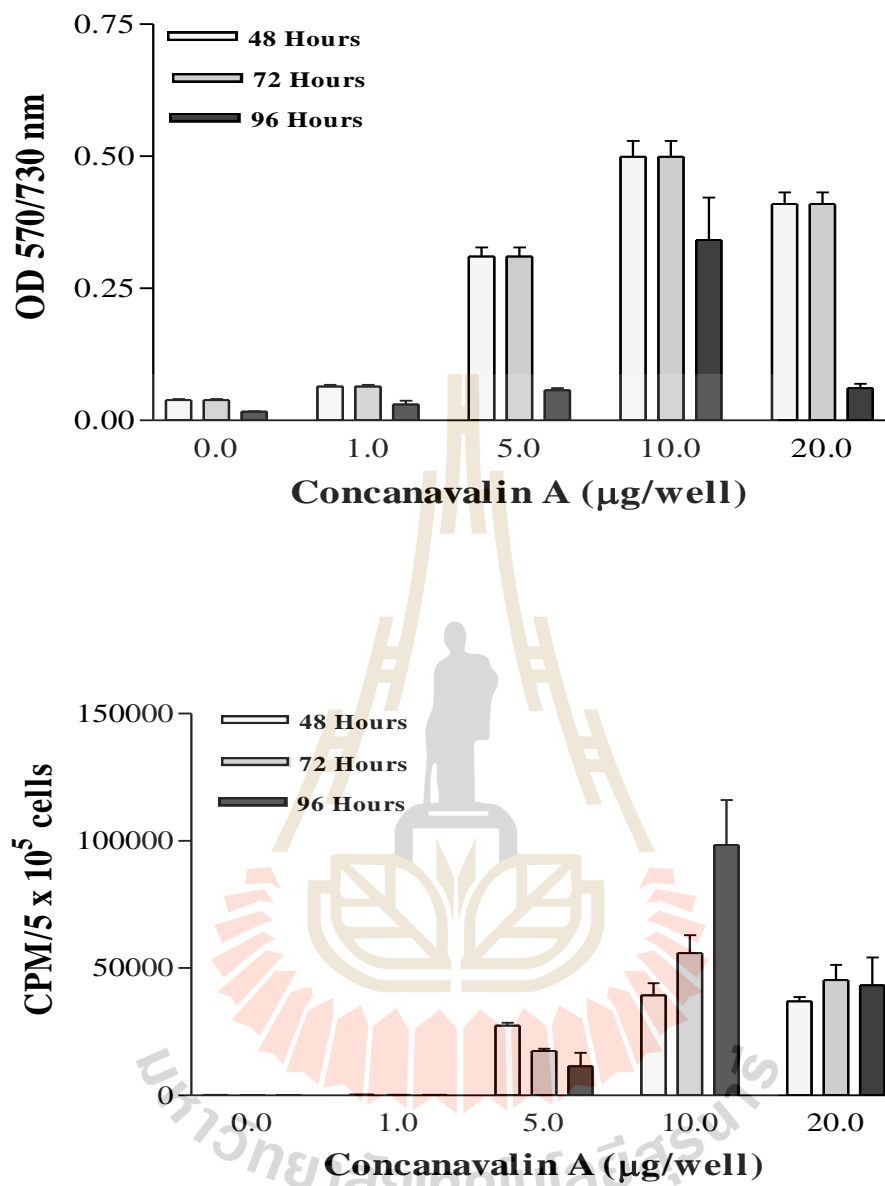
Figure 11. The comparison of MTT and ^{3}H-thymidine incorporation using Concanavalin A stimulated splenocytes.
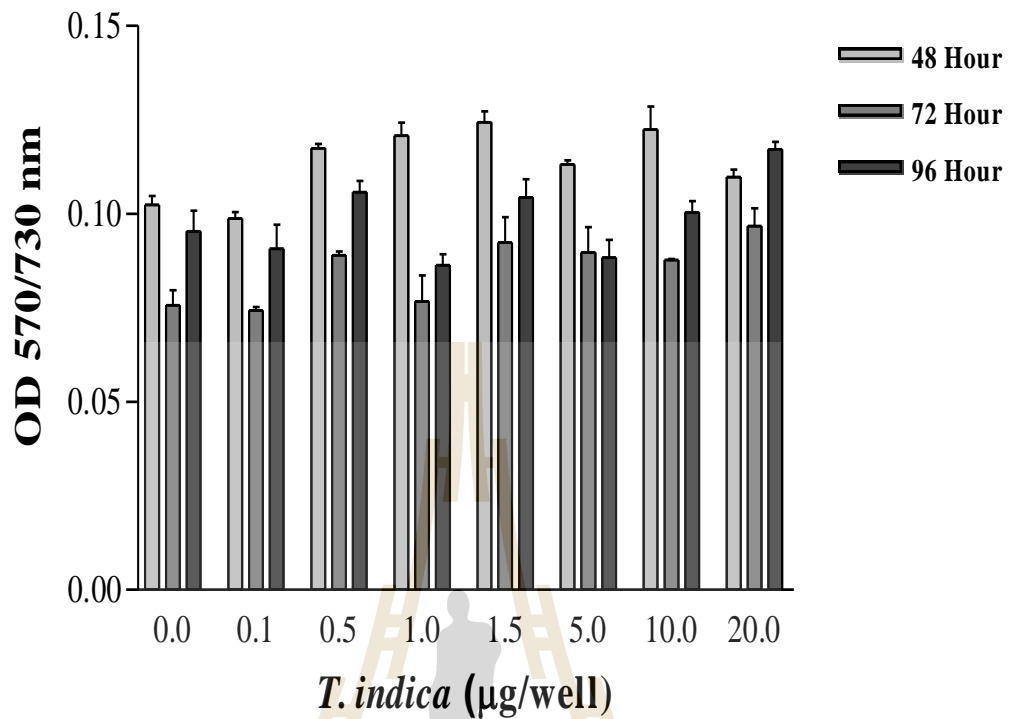
Figure 12. The comparison of different incubation time of increasing concentration of *T. indica* added to splenocytes.
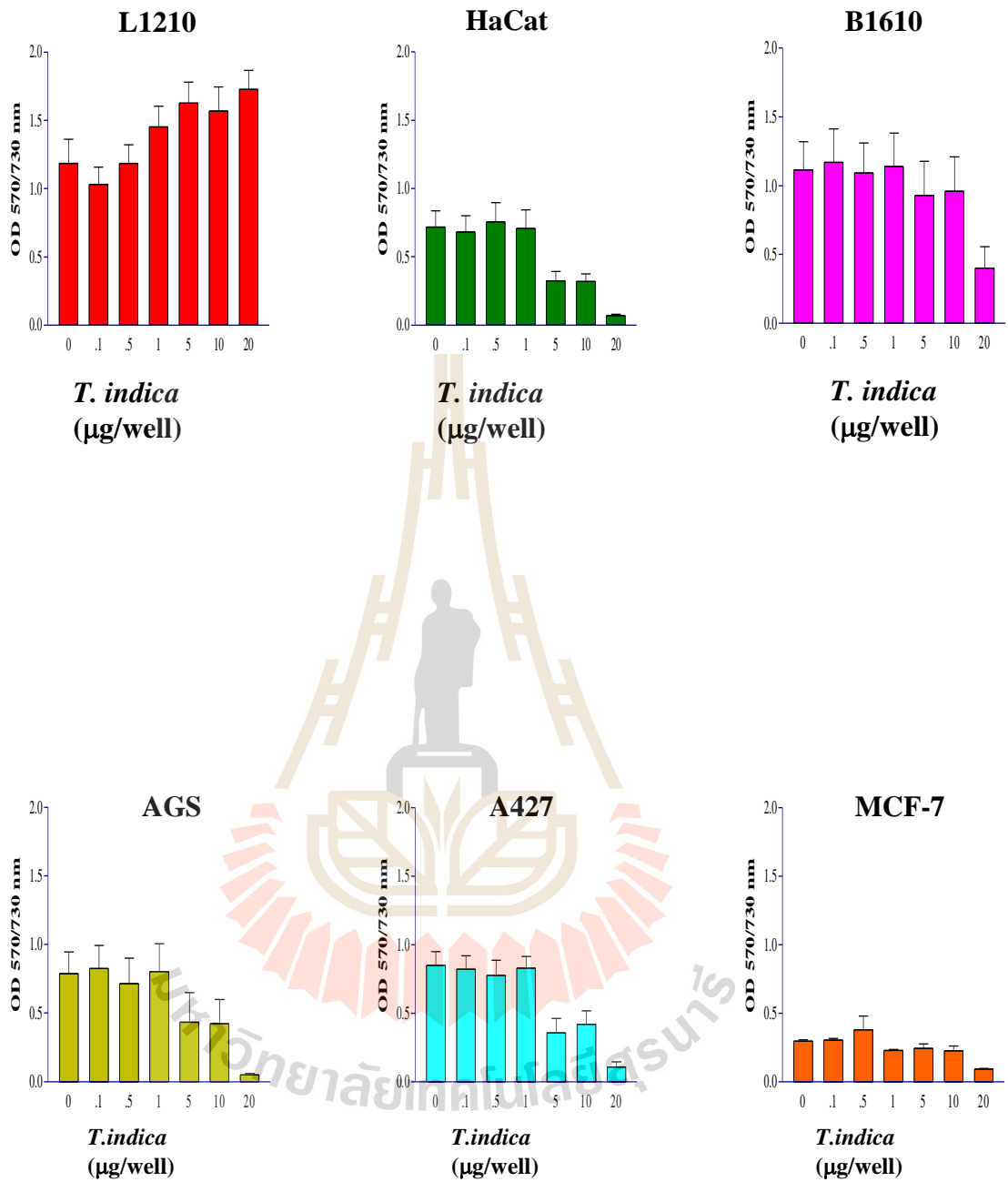
Figure 13. The cytotoxicity effects of *T. indica* on six tumor cell lines.

4.2 *In Vivo* Toxicity Study

Prior to evaluating the anti-inflammatory and immunological effects of *T. indica*, it was necessary to determine the toxicity of the extract. An initial range-finding study was conducted to determine concentration to be used in future studies and evaluate the systemic toxicity of the seed coat extract of *T. indica*. Mice were exposed by oral gavage for 14 days. Body weights were recorded daily prior to the first exposure and then daily following gavage. Twenty-four hours after the final exposure mice were sacrificed, blood was collected by cardiac puncture for hematology [white blood cell count (WBC), red blood cell count (RBC), hematocrit (HCT), and hemoglobin (HGB)] and serum chemistry analysis [alkaline phosphatase (ALP), alanine transferase (ALT), aspartic transferase (AST), blood urea nitrogen (BUN), uric acid, bilirubin, and creatine], and selected organ weights were taken including liver, brain, heart, spleen, kidneys, lungs, and thymus.

Figure 14A and B show the body weight and body weight changes for animals during this study. No deaths, except the high (500 mg/kg) and highest dose tested (1000 mg/kg) or abnormal clinical signs were observed in any of the mice treated with the seed coat extract of *T. indica* throughout the 14 days of the study. Exposure of B6C3F1 mice to 1000 mg/kg of the extract resulted in a decrease in body weight, reaching a maximum of 14% weight loss in day 11 and recovered some of it by day 14. There were no exposure-related changes in brain, spleen, kidney, lung or thymus weights. The liver weight was slightly increased in the 100 mg/kg dose group only and heart weights were slightly decreased in animals receiving 50, 500 and 1000 mg/kg (Table 9).
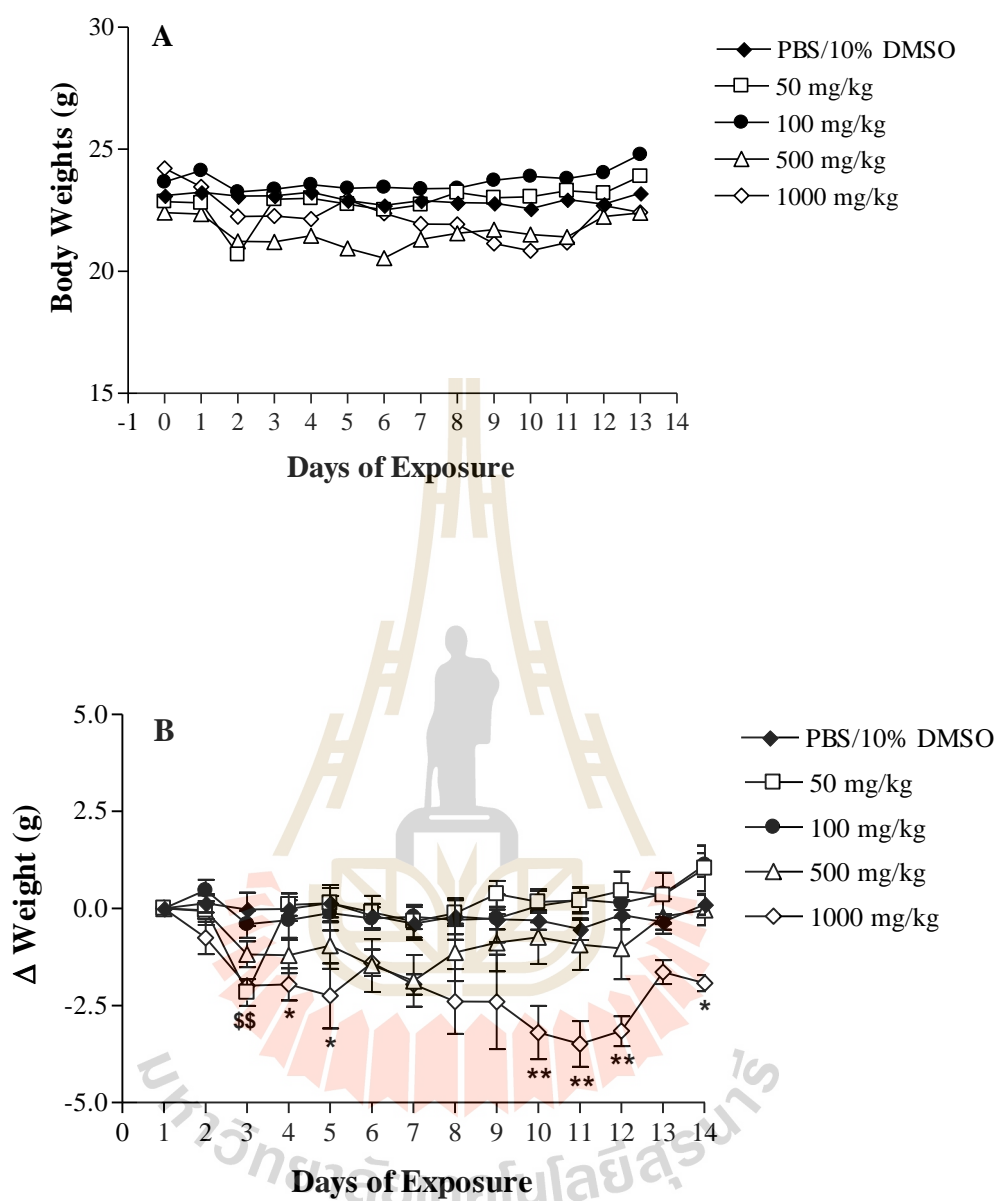
Figure 14. Body weights (A) and body weight changes (B) for animals during the 14 days toxicity study. Maximum weight loss was observed in animals dosed with 1000 mg/kg on day 11 (approximately 3.0 g).

No effects of *T. indica* exposure were seen on serum chemistries including ALP, ALT, AST, BUN, uric acid, bilirubin and creatine (Table 10). There were no dose related effects seen on erythroid parameters to include RBC count, HCT, HGB, mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) (Table 11, 12). The mean corpuscular volume (MCV) for animals exposed to 50 and 100 mg/kg was statistically increased. No significant difference was seen in total WBC and platlet counts. However, a trend toward an increase in neutrophils and a decrease in lymphocytes was observed. No effects were seen on monocytes, eosinophils, and basophils (Table 13). Based on the results of the toxicity study, doses of 50, 250, and 500 mg/kg were chosen for further studies.

To evaluate the effect of *T. indica* seed coat extract on components of innate immunity, natural killer cell activity that are directly involved with tumor surveillance and killing was assessed.

4.3 Modulation of NK Cell Activity Following *In Vivo* Exposure to Seed Coat Extract of *T. indica*

Natural killer cell activity in vehicle exposed B6C3F1 mice demonstrated a 20% lysis at the 200:1 effector to target ratio. A 72% suppression in NK activity was observed in animals exposed to the control, anti-asialo GM1. Fourteen day oral exposure to the seed coat extract of *T. indica* at doses up to 500 mg/kg or to the antioxidant control, vitamin C (100 mg/kg), had no effect on NK activity (Figure 15).

To evaluate the effect of *T. indica* seed coat extract on components of cellular immunity, a combined local lymph node and irritancy assay was conducted using BALB/c mice.

Table 9. Selected organ weights of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*

Exposure	Brain (mg)	Liver (mg)	Spleen (mg)	Kidneys (mg)	Thymus (mg)	Heart (mg)	Lung (mg)	N
Vehicle	395.2 ± 5.8	1067.2 ± 2.6	70.0 ± 3.4	304.6 ± 2.2	51.4 ± 2.5	125.2 ± 2.9	203.8 ± 3.1	5
50 mg/kg	423.2 ± 6.2	1088.2 ± 3.7	69.8 ± 2.5	304.6 ± 3.2	64.4 ± 2.7	107.4 ± 3.4	192.4 ± 4.2	5
100 mg/kg	417.4 ± 4.9	1199.0 ± 2.4	74.4 ± 6.4	280.0 ± 4.1	58.0 ± 3.5	119.60 ± 2.1	185.4 ± 3.2	5
500 mg/kg	425.0 ± 5.1	1015.5 ± 5.4	72.0 ± 3.3	292.5 ± 1.5	55.0 ± 4.4	107.25 ± 1.5	189.25 ± 4.9	4
1000 mg/kg	436.67 ± 8.9	979.0 ± 4.1	67.33 ± 5.1	308.33 ± 1.7	48.33 ± 2.3	107.33 ± 2.4	186.67 ± 2.4	3

Data is representative of the mean ± SE of selected organ weights of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*. In these studies n=5 , except for when n=4, n=3 due to toxic deaths at 500 mg/kg and 1000 mg/kg of *T. indica*, respectively.

Table 10. Selected serological parameters of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*

Exposure	ALP (U/L)	ALT (U/L)	AST (U/L)	BUN (mg/dl)	Uric Acid (mg/dl)	Bilirubin (mg/dl)	Creatine (mg/dl)	N
PBS	153.8 ± 4.6	36.0 ± 1.7	75.0 ± 5.8	26.4 ± 0.9	1.2 ± 0.7	0.22 ± 0.02	0.56 ± 0.07	5
50 mg/kg	121.8 ± 7.0	31.6 ± 10.5	82.2 ± 3.0	15.8 ± 6.4	0.9 ± 0.5	0.12 ± 0.06	0.15 ± 0.05	5
100 mg/kg	138.6 ± 4.8	64.8 ± 17.3	96.4 ± 34.3	26.9 ± 1.0	0.8 ± 0.1	0.16 ± 0.02	0.36 ± 0.07	5
500 mg/kg	122.8 ± 18.8	36.0 ± 1.7	30.3 ± 15.3	17.0 ± 5.6	0.6 ± 0.2	0.05 ± 0.03	0.33 ± 0.03	4
1000 mg/kg	116.7 ± 4.8	160.3 ± 121.8	53.7 ± 19.5	9.0 ± 8.8	0.5 ± 0.3	0.07 ± 0.007	0.50 ± 0.00	3

Data is a representative of the mean ± SE of selected serological parameters of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*. In these studies n=5 , except for when n=4, n=3 due to toxic death at 500 and 1000 mg/kg of *T. indica*, respectively.

Table 11. Selected hematologic parameters of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*

Exposure	Leukocytes ($10^3/\mu$L)	Erythrocytes ($10^6/\mu$L)	Hematocrit (%)	Hemoglobin (g/dl)	Platelets (10^5/mm)	N
Saline	5.0 ± 0.9	10.7 ± 0.2	48.9 ± 0.6	16.1 ± 0.2	955 ± 22	5
50 mg/kg	4.8 ± 0.5	10.5 ± 0.2	49.9 ± 0.8	15.9 ± 0.2	983 ± 123	5
100 mg/kg	5.3 ± 0.7	10.6 ± 0.1	49.8 ± 0.5	16.0 ± 0.1	1107 ± 29	5
500 mg/kg	6.5 ± 0.4	10.6 ± 0.1	48.7 ± 0.5	15.8 ± 0.2	1112 ± 39	4
1000 mg/kg	5.3 ± 1.0	10.8 ± 0.3	49.3 ± 1.0	15.9 ± 0.3	1137 ± 80	3

Data is a representative of the mean $\pm$ SE of selected hematologic parameters of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*. In these studies n=5 , except for when n=4, n=3 due to toxic death at 500 and 1000 mg/kg of *T. indica*, respectively.

Table 12. Erythrocyte indices of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*

Exposure	MCV (fl)	MCHC (g/dl)	MCH (pg)	N
Vehicle	45.7 ± 0.3	32.9 ± 0.3	15.1 ± 0.1	5
50 mg/kg	$47.4 \pm 0.2^{**}$	31.9 ± 0.5	15.1 ± 0.3	5
100 mg/kg	$46.9 \pm 0.3^{*}$	32.1 ± 0.3	15.1 ± 0.1	5
500 mg/kg	46.1 ± 0.3	32.5 ± 0.4	15.1 ± 0.1	4
1000 mg/kg	45.8 ± 0.4	32.4 ± 0.8	14.8 ± 0.3	3

Data is a representative of the mean $\pm$ SE of erythrocyte indices of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*. In these studies n=5 , except for when n=4, n=3 due to toxic deaths at 500 and 1000 mg/kg of *T. indica*, respectively. * denotes $p < 0.05$ as compared to control group. ** denotes $p < 0.01$ as compared to control group.

Table 13. Differentials of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*

Exposure	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)	N
Vehicle	10.7 ± 1.2	85.3 ± 2.1	2.9 ± 1.3	1.0 ± 0.03	0.01 ± 0.01	5
50 mg/kg	10.3 ± 1.0	84.5 ± 2.3	4.8 ± 2.3	0.4 ± 0.2	0.05 ± 0.05	5
100 mg/kg	12.2 ± 2.0	82.2 ± 1.4	4.8 ± 1.4	0.8 ± 0.5	0.03 ± 0.03	5
500 mg/kg	13.9 ± 1.5	82.4 ± 1.7	3.3 ± 1.3	0.4 ± 0.2	0.01 ± 0.01	4
1000 mg/kg	20.1 ± 5.3	$73.1 \pm 3.9^{**}$	5.9 ± 2.7	0.9 ± 0.4	0.03 ± 0.03	3

Data is a representative of the mean $\pm$ SE of differentials of female B6C3F1 mice following fourteen days dosing with the seed coat extract of *T. indica*. In these studies n=5 , except for when n=4, n=3 due to toxic death at 500 and 1000 mg/kg of *T. indica*, respectively. ** denotes $p < 0.01$ as compared to control group.
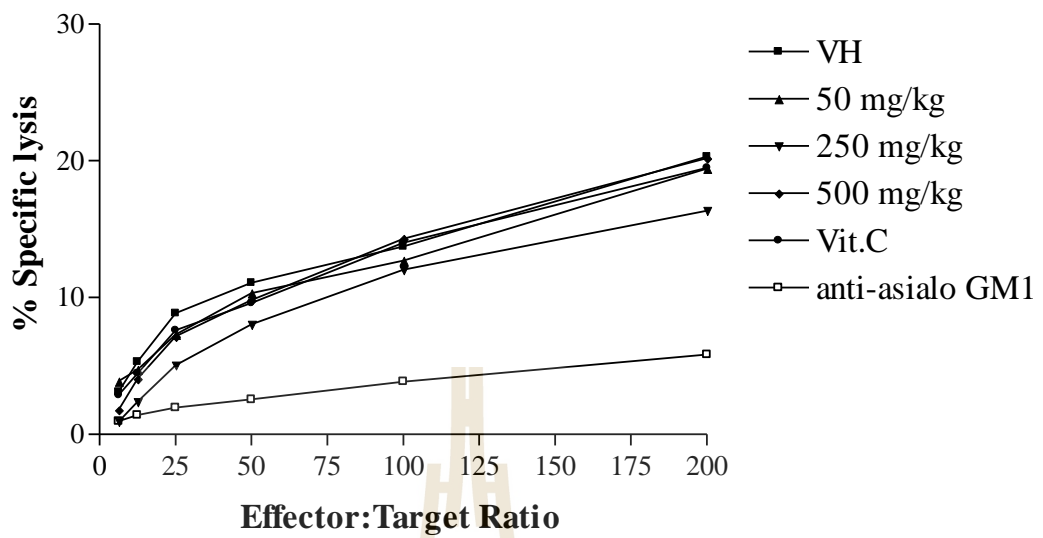
Figure 15. Natural killer cell activity in female B6C3F1 mice (n=5) following a fourteen days exposure to the seed coat extract of *T. indica* or vitamin C. Data are represented as mean ± SE. Anti-asialo GM1 was used as a control to assess the % lysis was mediated by NK population.

4.4 Modulation of The Induction of T-cell Mediated Sensitization Following *In Vivo* Exposure to Seed Coat Extract of *T. indica*

Topical exposure to 0.15% DNFB, a potent sensitizer, induced lymphocyte proliferation in the draining lymph nodes of BALB/c mice with a stimulation index of 31. Figure 16 A and B show the body and body weight change for these animals on days 0, 7, and 14 of the study. A dose response decrease in body weights was seen with animals exposed to 200 mg/kg losing an average of 2.5 g by day 14.

Mice were exposed to increasing concentrations of the seed coat extract of *T. indica* orally for 14 days. On days 10-12 mice were exposed on each ear with 0.1% DNFB. Prior to the first dermal exposure the thickness of each mouse was pre-measured. The ear thickness was post-measured 24 hours following the last dermal exposure, and % ear swelling was calculated as described in the method section. One group of mice were exposed to VH orally and on the ears, and one group received VH orally and 0.15% DNFB on the ear as shown in Table 3.

Fourteen days of oral exposure to the seed coat extract of *T. indica*, at concentrations up to 200 mg/kg, or vitamin C (100 mg/kg) did not have any modulatory effect on this response (Figure 17). A similar study was conducted using the more moderate sensitizer HCA at a concentration of 30% with no modulatory effect on lymph node cell proliferation observed following oral exposure to *T. indica* (Figure 18).
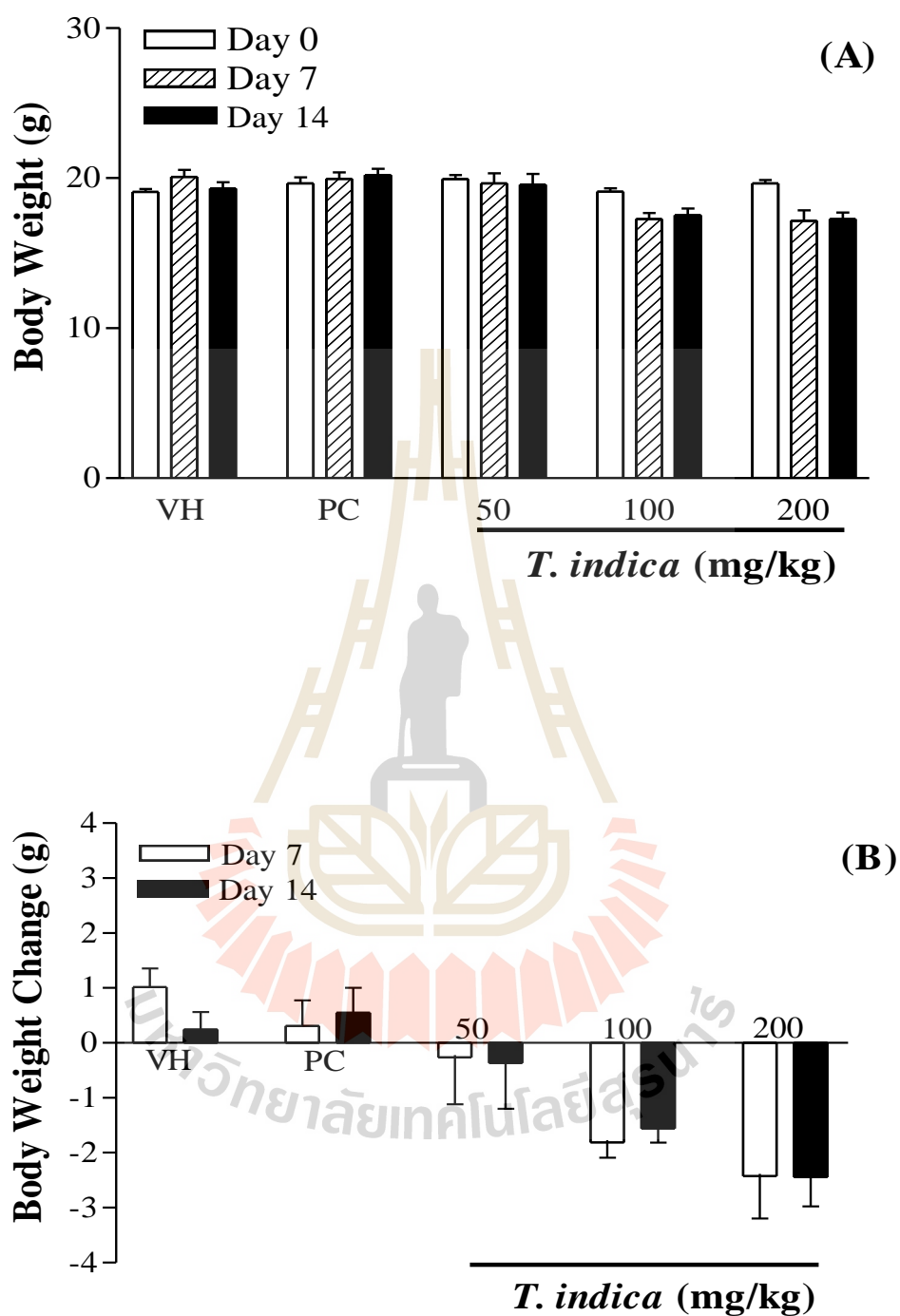
Figure 16. Body weights for each dose group on days 0, 7, and 14 (A), and body weight changes for mice in each group on days 7 and 14 compared to day 0 (B).
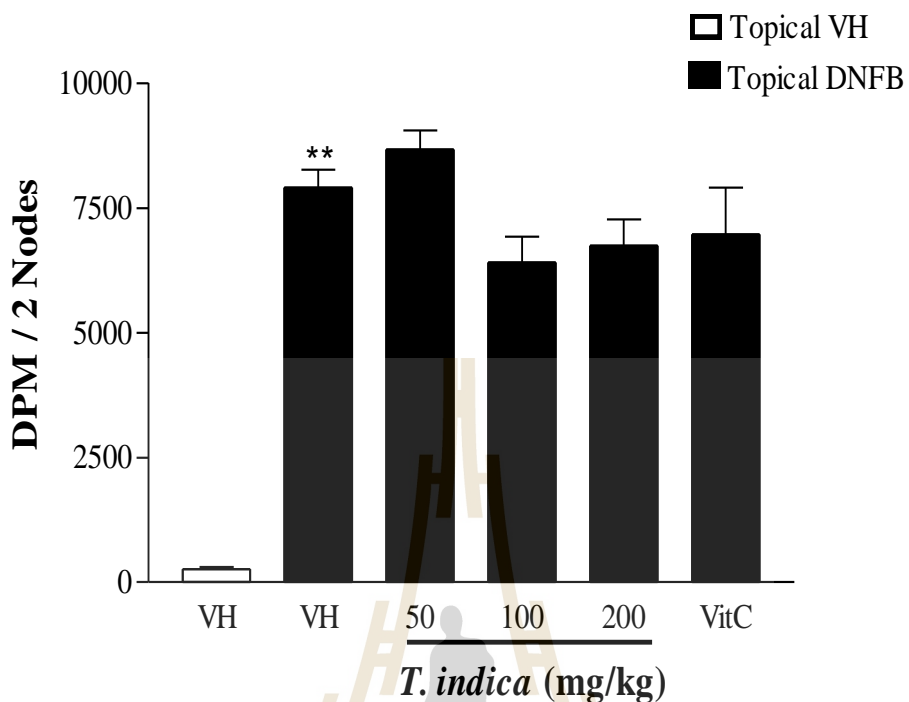
Figure 17. Modulatory effect of *T. indica* on the lymphoproliferative response to 0.15% DNFB in the local lymph node assay. BALB/c mice (n=5) were dosed orally with either vehicle (PBS/10% DMSO) or increasing concentrations of the seed coat extract of *T. indica* as shown. Open bar indicates topical exposure to vehicle (acetone), and closed bars represent topical exposure to 0.15% DNFB. Bars represent mean ± SE. ** represents $p < 0.01$ as compared to the open bar by Student's t-test.
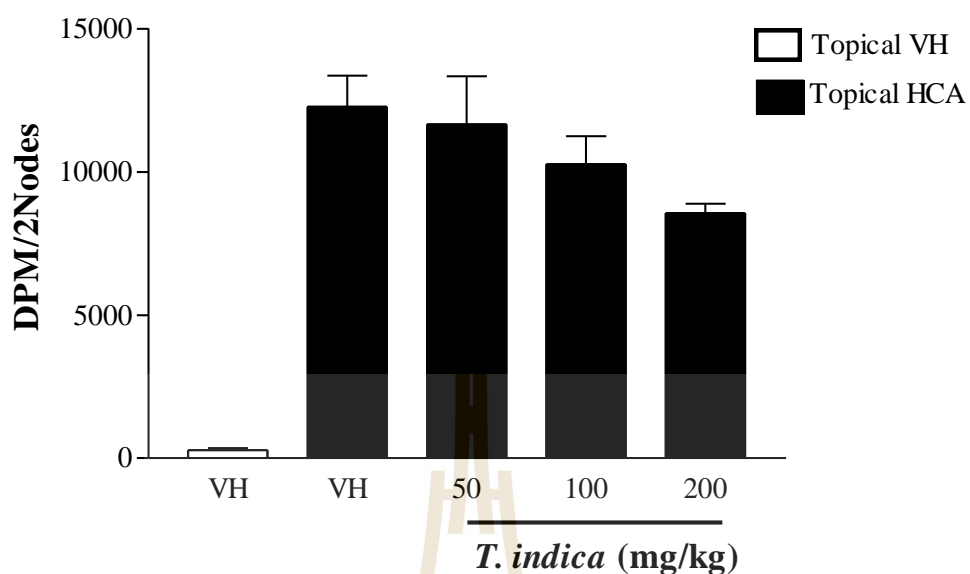
Figure 18. Modulatory effect of *T. indica* on the lymphoproliferative response to 30% HCA in the local lymph node assay. BALB/c mice (n=5) were dosed orally with either vehicle (PBS/10% DMSO) or incresing concentrations of the seed coat extract of *T. indica* as shown. Open bar indicates topical exposure to vehicle (acetone), and closed bars represent topical exposure to 30% HCA. Bars represent mean ± SE.

4.5 Modulation of The Dermal Irritant Response to Nonanoic Acid and DNFB

Following *In Vivo* Exposure to Seed Coat Extract of *T. indica*

An irritancy assay was conducted using BALB/c mice. Topical exposure to the moderate irritant nonanoic acid (60%) induced an approximate 38% increase in ear swelling in BALB/c mice. Mice were orally gavaged with 50, 100 or 200 mg/kg of the seed coat extract of *T. indica* for 14 days. The high dose was decreased to 200 mg/kg because of the observed weight loss in the previous study at the 250 mg/kg dose (data not shown).

Fourteen days of oral exposure to the seed coat extract of *T. indica* (up to 200 mg/kg) did not have any modulatory effect on this response (Figure 19). Likewise, no effect was seen following 14-days oral exposure to *T. indica* in a similar study using 0.15% DNFB as the irritant (Figure 20).

4.6 Optimization of Lipopolysaccharide (LPS) and Interferon Gamma (IFN-γ)

**for Nitrite Studies Using RAW 264.7 Cells**

Prior set up the nitrite assay, various concentrations of LPS and IFN-γ were chosen. 0.5, 1, 2, 5, 10, and 20 μg/mL of LPS and 1, 2.5, 5, 10, 20, 30, and 40 ng/mL of IFN-γ were used to stimulate RAW 264.7 cells for optimization of the assay. The data demonstrated that 5 μg/mL of LPS (Figure 21A) and 10 ng/mL of IFN-γ (Figure 21B) are appropriate to use in the futher *in vitro* and *in vivo* nitrite assays.
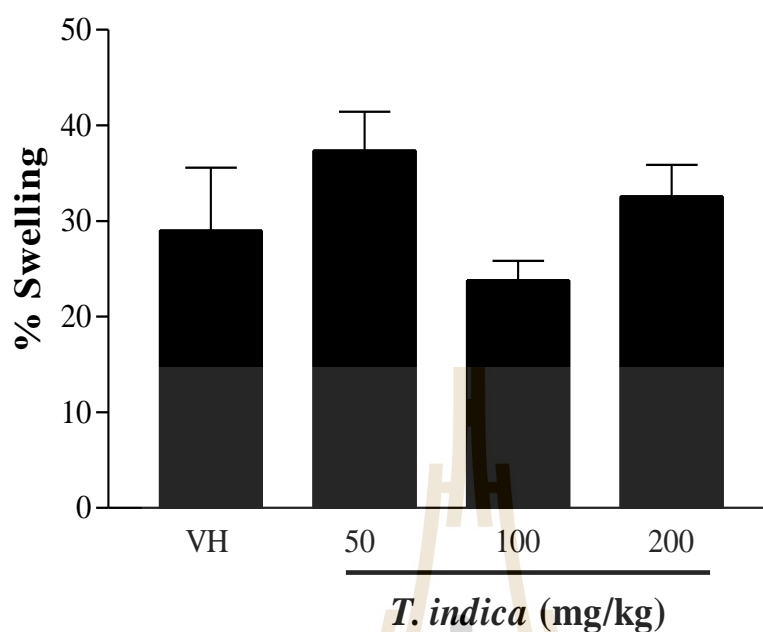
Figure 19. Modulatory effect of *T. indica* on the dermal irritant response to 60% nonanoic acid in the irritancy assay. BALB/c mice (n=5) were orally exposed to either VH (PBS/10% DMSO) or the concentration of the seed coat extract of *T. indica* shown for 14 days. Starting on the 12th day following initial oral exposure, animals were exposed topically with 60% nonanoic acid for 3 consecutive days. Ear thickness was measured pre-exposure and 24 hrs post-final exposure. Bars represent mean ± SE of the calculated percent ear swelling.
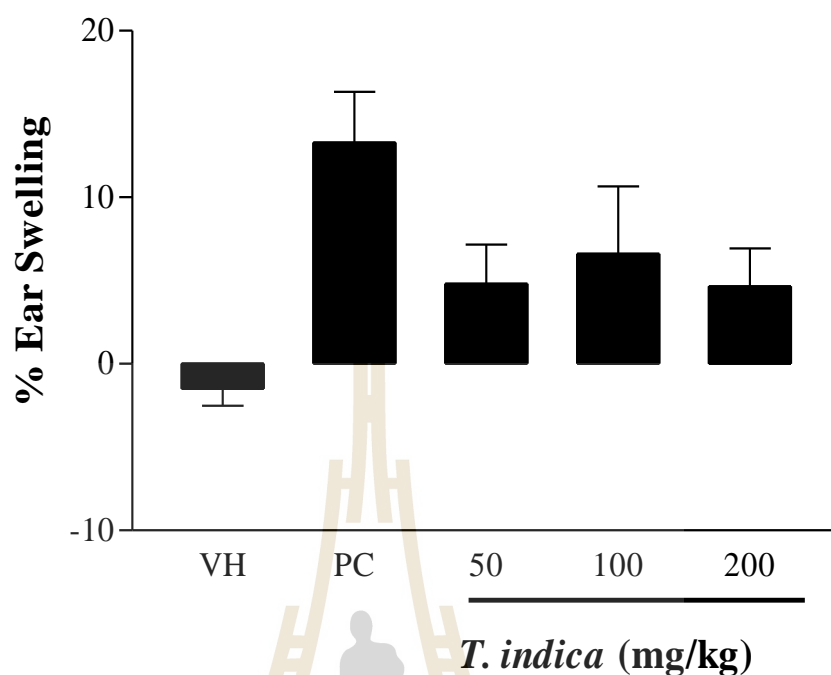
Figure 20. Modulatory effect of *T. indica* on the dermal irritant response to 0.1% DNFB in the irritancy assay. BALB/c mice (n=5) were orally exposed to either VH (PBS/10% DMSO) or the concentration of the seed coat extract of *T. indica* shown for 14 days. Starting on the 12th day following initial oral exposure, animals were exposed topically with 0.1% DNFB for 3 consecutive days. Ear thickness was measured pre-exposure and 24 hrs post-final exposure. Bars represent mean ± SE of the calculated percent ear swelling.

A
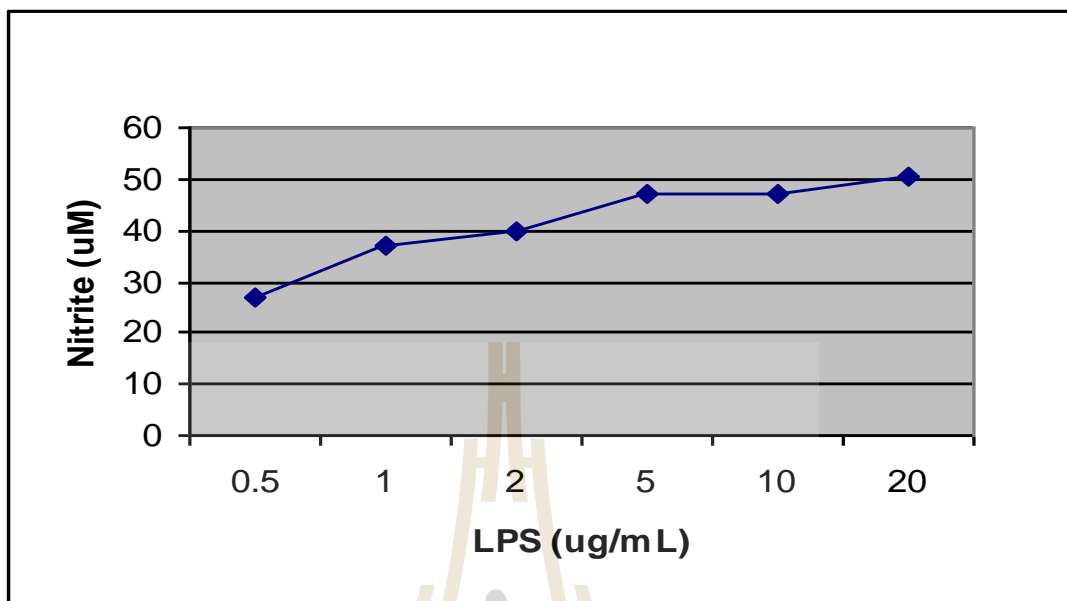
B
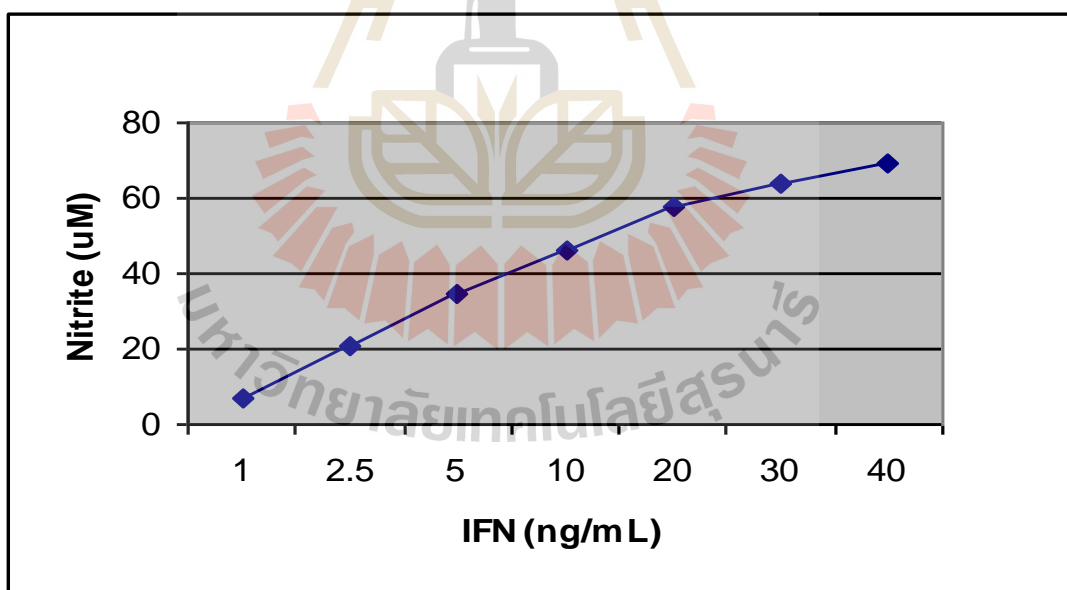
Figure 21. Optimization of LPS and IFN-γ for nitrite studies. RAW 264.7 cells were plated in 96-well plate at a concentration of 2×10^5 cells per well and incubated with different concentration of LPS (0.5, 1, 2, 5, 10, and 20 μg/mL) and IFN-γ (1, 2.5, 5, 10, 20, 30, and 40 ng/mL) for 24 hrs. Nitrite concentration was determined using the Griess reagent as described in Materials and Methods.

4.7 Modulation of NO Production by LPS and IFN-γ Stimulated RAW 264.7 Cells Following *In Vitro* Exposure to Seed Coat Extract of *T. indica*.

To investigate the effect of the seed coat extract of *T. indica* on NO production, the accumulation of nitrite, the stable metabolite of NO, was measured in the culture media of RAW 264.7 cells using Greiss reagent. Resting RAW 264.7 cells were stimulated with LPS (5 μg/mL) and/or IFN-γ (10 ng/mL) to stimulate NO production. Cells were simultaneously treated with increasing concentrations of the seed coat extract of *T. indica* or with vitamin C (500 μM), vitamin E (141.3 μM), or beta-carotene (2.5 μM) for 24 hrs. As shown in Figure 22A**,** *in vitro* exposure to seed coat extract of *T. indica* at concentrations ranging from 0.2 to 200 μg/mL inhibited nitrite production in a concentration-dependent manner reaching a 68% suppression at the high dose. An approximate 54% suppression in NO production was observed in cells treated with other antioxidant controls, vitamin C (500 μM), vitamin E (141.3 μM), or beta-carotene (2.5 μM). Based on the MTT assay, the observed decrease in NO production by mice exposed to *T. indica*, vitamin E or β-carotene occurred in the absence of any effect on cell viability (Figure 22B). An approximate 10% reduction in cell viability was seen following exposure to vitamin C.

4.8 Modulation of NO Production by Peritoneal Macrophages Following *In Vivo* Exposure to Seed Coat Extract of *T. indica.*

To assess whether *T. indica* extract can modulate NO production *in vivo*, female B6C3F1 mice were treated with vehicle, 10% DMSO in PBS, or increasing concentrations of the extract (50, 250, 500 mg/kg) by oral gavage for 14 days. On day 15 animals were sacrificed and peritoneal macrophages were collected and treated
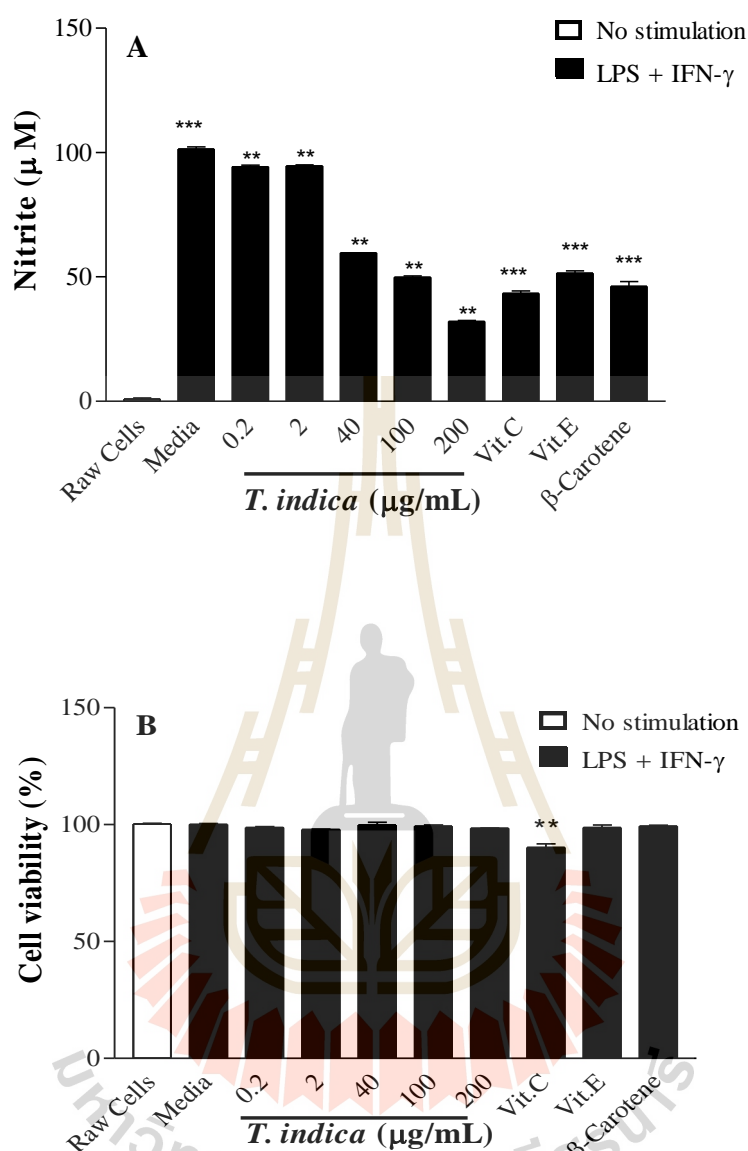
Figure 22. Nitrite production (A) and percent cell viability (B) of RAW 264.7 cells treated with increasing concentrations of *T. indica*, Vit C (500 μM), Vit E (141.3 μM) or β-carotene (2.5 mM) and simultaneously stimulated with LPS (5 μg/mL) and IFN-γ (10 ng/mL) for 24 hrs. Results are expressed as mean ± SE for groups of 5 mice. Nitrite production by LPS and IFN-γ stimulated RAW cells was compared to non-stimulated cells by student's t-test (*** represents $p < 0.0001$). For LPS and IFN-γ stimulated cells, data from *T. indica* exposed groups were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected ($p < 0.05$) ** represents $p < 0.01$ as compared to VH control. Vit C, Vit E, and β-carotene exposed group were individually compared to the media exposed RAW cells using t-test (** represents $p < 0.001$, *** represents $p < 0.0001$).

with LPS (5 μg/mL), IFN-γ (10 ng/mL), or LPS and IFN-γ for 24 hrs. As shown in Figure 23A, B, and C, treatment with LPS and/or IFN-γ induced a significant increase in nitrite production when compared with unstimulated peritoneal macrophages. Combined exposure to LPS and IFN-γ increased nitrite production greater than exposure to either LPS or IFN-γ alone (p < 0.05). Exposure to *T. indica* dose-dependently inhibited NO production by IFN-γ and LPS and IFN-γ stimulated cells reaching statistical significance in the highest *T. indica* exposed group (500 mg/kg). No effect on the viability of peritoneal macrophages due to test article exposure was observed in this study. Cell viability was greater than 90% for all groups (Figure 24A, B, and C).

Following oral exposure to *T. indica* extract, when TPA was used as the *in vitro* stimulant, NO production by peritoneal macrophages was significantly inhibited in a dose-dependent manner reaching 55% inhibition at the 500 mg/kg dose, a level comparable to that seen following exposure to 100 mg/kg Vit. C (Figure 25A). As shown in Figure 26A, nitrite production induced by intraperitoneal exposure to TPA was also inhibited dose-dependently by oral exposure to the seed coat extract of *T. indica*, reaching statistical significance at 250 mg/kg dose and showing 70% inhibition at the 500 mg/kg dose. Cell viability was not affected by exposure to either *T. indica* or TPA in either of these studies (Figure 25B, 26B).
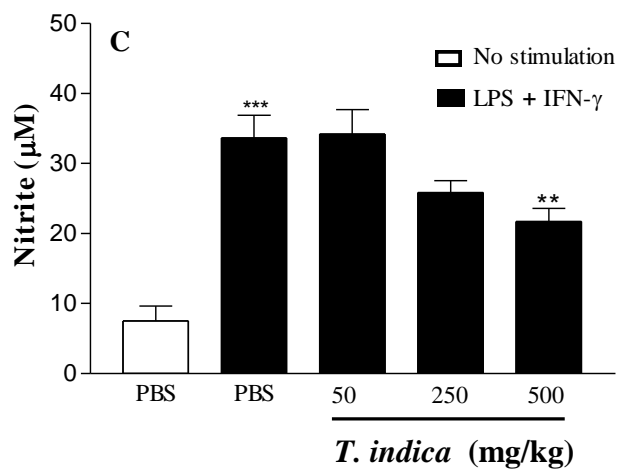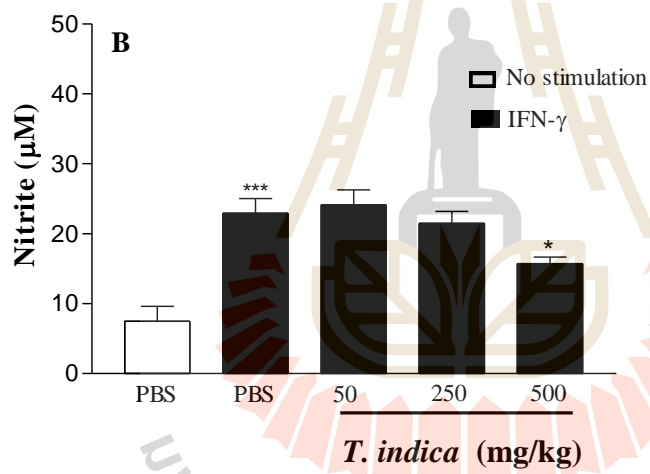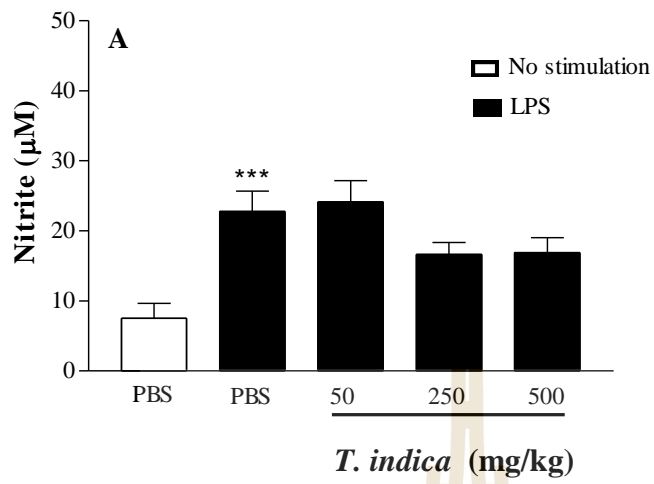
Figure 23. Nitrite production by peritoneal macrophages from mice treated with PBS (PBS/10% DMSO) or increasing concentrations of *T. indica* and stimulated *in vitro* with LPS (A), IFN-γ (B), or LPS and IFN-γ (C) for 24 hrs. Results are expressed as mean ± SE for group of 5 mice. Levels of nitrite from IFN-γ and/or LPS stimulated macrophages from PBS control animals were compared with levels from non-stimulated macrophages using student's t-test (*** represents $p < 0.0001$). Levels of nitrite from *T. indica* treatment groups and IFN-γ and/or LPS stimulated macrophages from PBS control data were compared by one-way ANOVA followed by Dunnett's test when significant differences were detected ($p < 0.05$). (* represents $p < 0.05$, *** represents $p < 0.0001$).
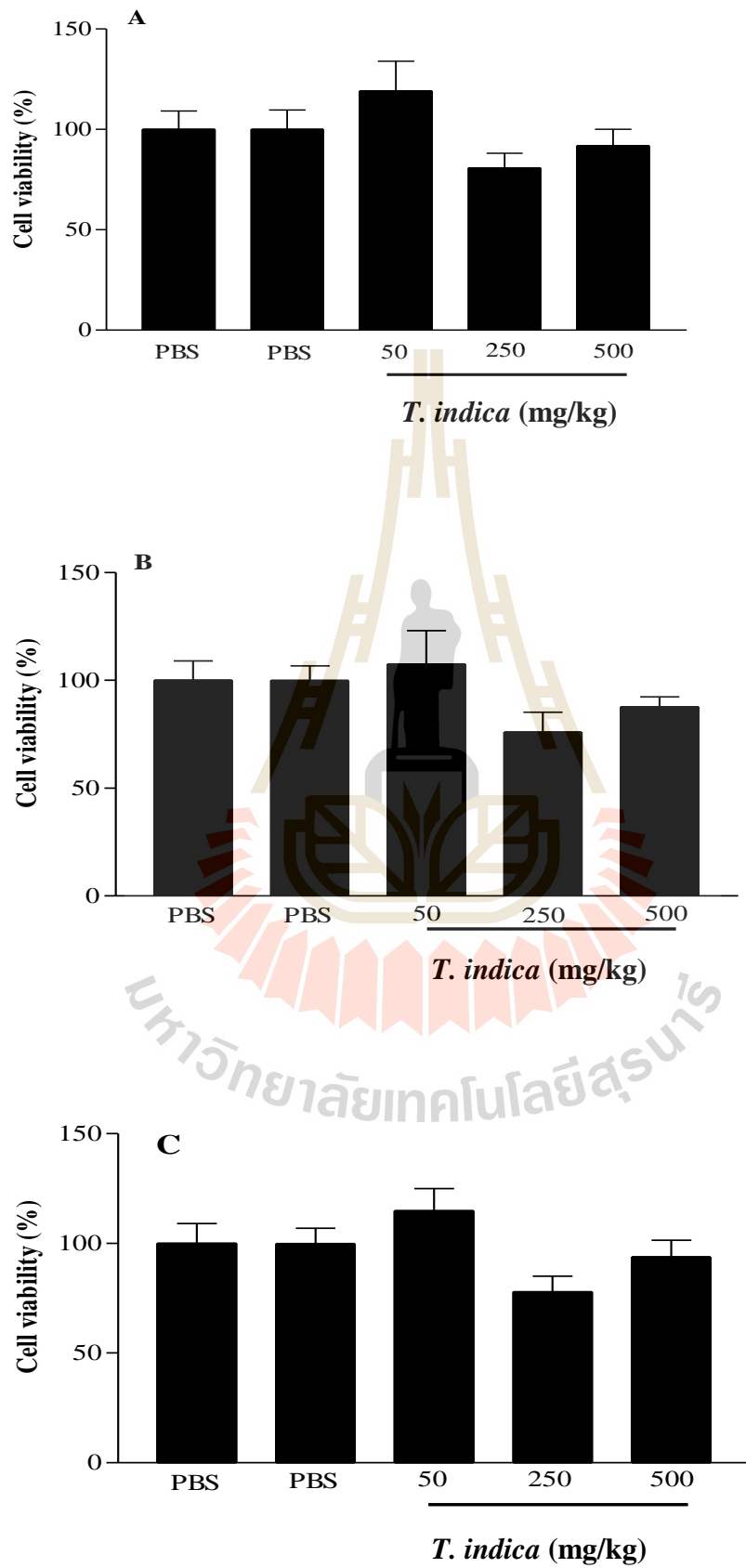
Figure 24. Percent cell viability of peritoneal macrophages from mice treated with PBS (PBS/10% DMSO) or increasing concentrations of *T. indica* and stimulated *in vitro* with LPS (A), IFN-γ (B), or LPS and IFN-γ (C) for 24 hrs. Results are expressed as mean ± SE for group of 5 mice.
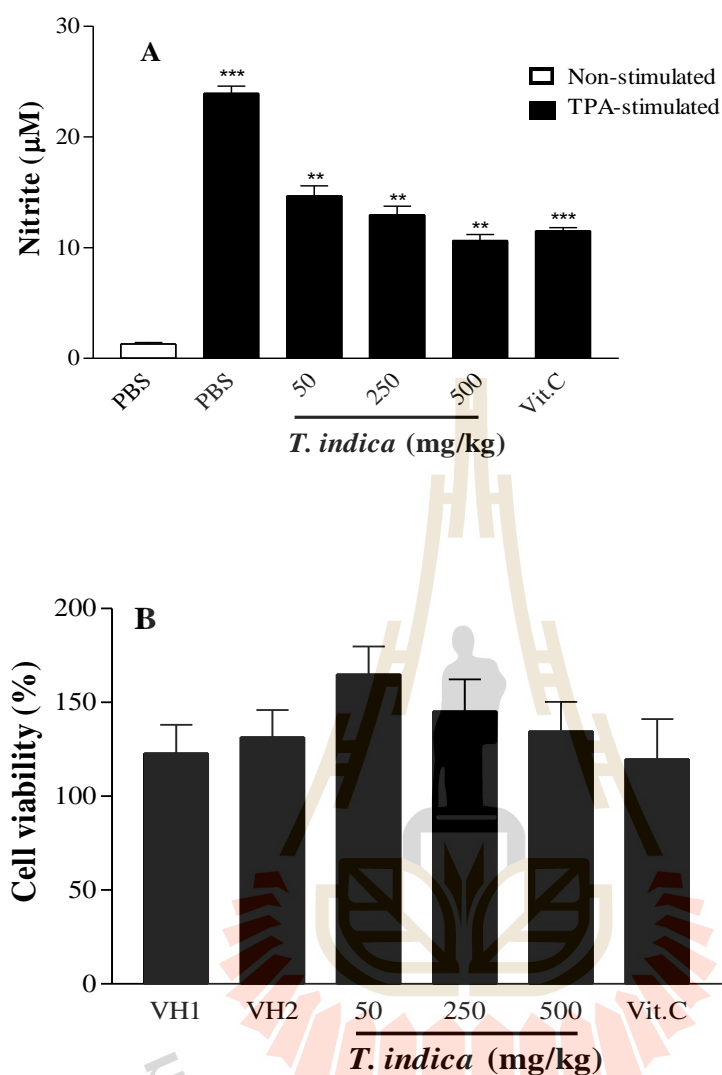
Figure 25. Nitrite production (A) and percent cell viability (B) of peritoneal macrophages from mice treated with PBS (PBS/10% DMSO), increasing concentrations of *T. indica*, or Vit.C (100 mg/kg) and stimulated *in vitro* with 0.1 μg/mL of TPA. Results are expressed as mean ± SE for groups of 5 mice. The PBS exposed (TPA stimulated and non-stimulated) mice were compared using a t-test (***$p < 0.0001$). For the TPA stimulated groups, *T. indica* exposed groups and the PBS exposed group were compared using ANOVA followed by Dunnett's post test when significant differences ($p < 0.05$) occurred. ** represents $p < 0.01$. The Vit.C exposed group was compared with the PBS exposed group using a student's t-test (*** represents $p < 0.0001$).
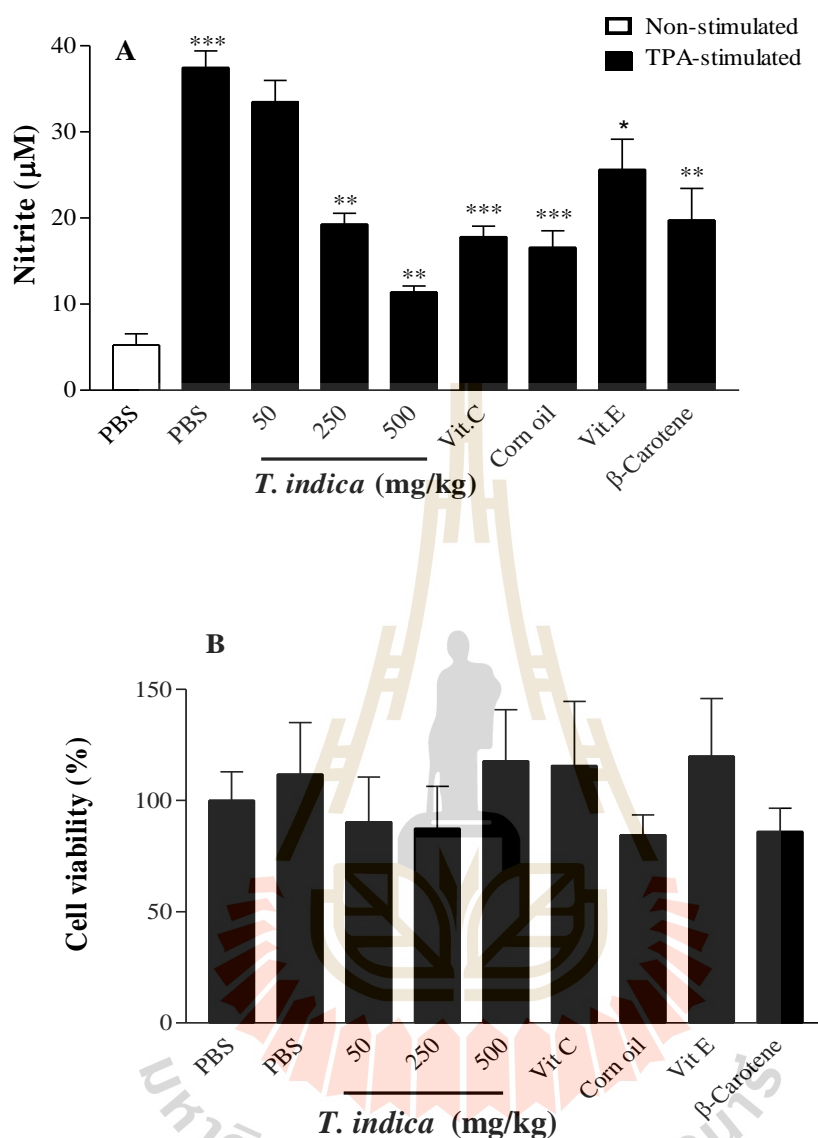
Figure 26. Nitrite production (A) and percent cell viability (B) of peritoneal macrophages from mice treated with PBS (PBS/10% DMSO), increasing concentrations of *T. indica*, Vit.C (100 mg/kg), Corn oil, Vit.E (100 mg/kg) , or β-carotene (50 mg/kg) and intraperitoneally injected with PBS or 0.1 μg/mL of TPA. Results are expressed as mean ± SE for groups of 5 mice. The PBS exposed (TPA stimulated and non-stimulated) mice were compared using a t-test (***p < 0.0001). For the TPA stimulated groups, *T. indica* exposed groups and the PBS exposed group were compared using ANOVA followed by Dunnett's post test when significant differences (p < 0.05) occurred. TPA stimulated-Vit.C, -Vit.E, and -β-carotene exposed groups were individually compared with the PBS exposed-TPA stimulated group using a t-test (*p < 0.01, **p < 0.001, ***p < 0.0001).

CHAPTER V

DISCUSSION

The present studies have investigated the therapeutic potential and safety of components of *T.indica*. A polysaccharide isolated and purified from the seeds of *T.indica* has been shown *in vitro* to have immunomodulatory activity such as phagocytic enhancement and inhibition of leukocyte migration and cell proliferation (Sreelekha et al., 1993). Tamarind seed polysaccharide has been tested in B6C3F1 mice with results demonstrating the absence of carcinogenicity in mice of either sex following long-term dietary exposure (Sano et al., 1996).

The seed coat extract of *T.indica*, contains high amounts of polyphenolic flavonoids, which are shown to exhibit strong antioxidant scavenging activity against peroxyl radicals generated by $ABTS/H_2O_2/peroxidase$ and $ABTS/H_2O_2/myoglobin$ systems, hydroxyl radicals produced by $ABTS/H_2O_2/FeCl_3$ (Feton reaction) and superoxide anions generated by hypoxanthine-xanthine oxidase (neotetrazolium) system (Pumthong, 1999).

In this study, toxicological evaluations were made to investigate the toxicity of the seed coat extract of *T.indica.* Animal receiving the highest dose of the extract (1000 mg/kg) lost an average of 3.0 g body weight by day 11 and was partially recovered at the end of the study. Some of the weight loss could be due to the stress of daily gavage, and not related to exposure to *T.indica.* The trends of increasing in liver weight and decreasing heart weight were observed in most of treated groups. Given the lack of dose response relationships these changes are not expected to be

biologically relevant. Difference in heart weight might be due to variation in volume of blood remaining in the heart following blood collection by cardiac puncture. By comparing the organ weights to the body weights, it was possible to distinguish overall body weight change from the change of weight in a specific target organ. Statistically increased MCV for animals exposed to 50 and 100 mg/kg but no the biological relevance is questioned due to the lack of dose response. Also, trends toward an increase in neutrophils and a decrease in lymphocytes were observed which correlates with the body weight loss in these animals and may be associated with stress.

In the study of direct cytotoxicity on tumor cells, the seed coat extract of *T.indica* demonstrated slightly increase cell proliferation in L1210 murine lymphocytic leukemia cell lines, in contrast, a decrease in proliferation was observed in HaCat human epidermal keratinocyte, AGS human gastric adenocarcinoma, A427 human lung carcinoma, and B16-F10 melanoma cell lines. However, no cytotoxic or growth inhibitory effects of the seed coat extract of *T.indica* was observed on MCF-7 human mammary adenocarcinoma. Hence, these experiments demonstrated that the seed coat extract of *T.indica* exhibited selective cytotoxicity towards some cancer cells. The difference cell susceptibility effect and the mechanisms of cytotoxicity mediated by this extract need to be further investigated.

It is well known that owing to the multiple phenolic groups in their structure flavonoids are potentially able to quench free radicals by forming more stable oxidized products. Among the variety of free radical mediators released by activated macrophages, the simple gas radical NO has been identified as a potent bioregulator that plays an important role in diverse physiological and pathological processes,

including smooth muscle relaxation, penile erection, anti-platelet aggregation, neurotransmission, immunomodulation, inflammation and autoimmunity (Moncada et al., 1991; Nathan, 1992; Kolb and Kolb-Bachofen, 1992; Robbins and Sisson, 1996). Various other naturally occurring polyphenolic flavonoids such as rutin, quercetin, apigenin, wogonin, luteolin, tectorigenin, galangin, morin, naringenin (van Acker et al., 1995; Kim et al., 1999; Raso, Meli, Carlo, Pacilio, and Carlo, 2001), epigallocatechin gallate from tea (Lin, 1997; Chan et al., 1997), *Ginkgo biloba* extract (Egb 761) (Kobuchi, Droy-Lefaix, Christen, and Packer, 1997), silymarin (Kang, Jeon, Kim, Han, and Yang, 2002) have been reported to inhibit NO production. Therefore, the present studies were also designed to investigate the potential for the seed coat extract of *T.indica* in modulation of NO production.

NO production by activated macrophages is known to be up regulated by various stimuli, including LPS, IFN-γ, tumor necrosis factor alpha (TNF-α), IL-12, cAMP-elevating agents, UV light and ozone (Lorsbach, Murphy, Lowenstein, Snyder, and Russell 1993; Xie, Kashiwabara, and Nathan, 1994; Nathan and Xie, 1994), and down regulated by transforming growth factor-β (TGF-β), IL-4, and IL-10 (Hinz, Brune, and Pahl, 2000). Biosynthesis of NO is formed from L-arginine to L-citrulline by a family of enzyme nitric oxide synthases (NOSs) that require oxygen (O_2), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), and tetrahydrobiopterin (Marleteta, Yoon, Iyengar, Leaf, and Wishnok, 1988). The cellular concentration and activity of inducible nitric oxide synthase (iNOS) are the rate-limiting steps for NO synthesis in macrophages (Weisz, Oguchi, Cicatiello, and Esumi, 1994; Robbins and Sisson, 1976).

Low concentrations of NO from activated macrophages are beneficial as, along with other reactive nitrogen intermediates, they are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in the function of natural killer cells and the expression of cytokines such as IFN-γ or transforming growth factor-β (Bogdan, Rollinghoff, and Diefenbach, 2000). However, overproduction of NO has been found to be associated with various diseases such as septic shock, autoimmune diseases, and chronic inflammation by increasing vascular permeability and the extravasations of fluid and proteins at the inflammatory site (Moncada et al., 1991; Kolb and Kolb-Bachofen, 1992; Ialenti, Ianaro, Moncada, and Rosa, 1992). Excess NO can react with DNA, causing mutations and eventually carcinogenicity (Ohshima and Bartsch, 1994; Tamir and Tannenbaum, 1996). Furthermore, NO can react with superoxide to form peroxynitrite (ONOO$^-$), a more potent oxidant and cytotoxic agent (Ischiropoulos et al., 1992). Therefore, inhibition of high-output NO production could be useful strategy for treatment of various inflammatory diseases.

These studies demonstrated *in vivo* and *in vitro* the suppressive effect of the seed coat extract of *T.indica* on NO production using both a murine macrophage cell line and freshly isolated peritoneal macrophages. The RAW 264.7 gamma NO(-) used in this study was derived from the RAW 264.7 mouse macrophage cell line which requires LPS for full activation (ATCC CRL-2278). However, both LPS and IFN-γ were chosen as stimulators in this study due to the synergistic effect reported previously of both agents on NO synthesis in RAW 264.7 (Lorbach et al., 1993). The present study clearly indicated that the seed coat extract of *T.indica* induces a dose dependent suppression of *in vitro* production of nitrite, the measurement index of NO,

in LPS and IFN-γ activated RAW 264.7 cells. The inhibitory effect occurred at concentrations below which cytotoxicity occurred *in vitro* and the absence of systemic toxicity *in vivo*.

Noticeably, the control vitamin C treated culture at 500 µM gave 10% reduction in cell viability compared with the activated-media control (p<0.001). This slightly toxic effect might be caused by the pro-oxidant role of vitamin C since it has been demonstrated both *in vitro* and *in vivo* that vitamin C at high concentration or under certain conditions has pro-oxidant effects causing oxidative damage in contrast to its known antioxidative properties (Galloway and Painter, 1979; Speit, Wolf, and Vogel, 1980).

In this study, the highest inhibition of 68% of NO production was observed at 200 µg/mL of *T.indica*, whereas other antioxidant controls gave lower inhibition of about 54% at the selected concentrations providing the highest antioxidant activities. From the present study, *T.indica* was a more potent inhibitor than β-carotene as it induced stronger inhibition at the lower concentration.

Results from these studies were comparable to those reported for more well known natural dietary supplements. Using a similar model of LPS and IFN-γ stimulated RAW 264.7 cells, at 100 µg/mL of the extract, NO production was demonstrated to be inhibited approximately 50% by the seed coat extract of *T.indica* whereas Pycnogenol or *Ginkgo biloba* extract approximately inhibited 70% and 55% respectively (Virgili, Kobuchi, and Packer, 1998; Kobuchi et al., 1997). Also, *in vivo* treatment of *T.indica* provided the same inhibitory effect on NO production. The peritoneal macrophages collected from *T.indica* treated mice suppressed NO

production in a dose-dependent manner following *in vitro* LPS and/or IFN-γ activation, compared with the untreated group.

Results from the present study showed the inhibitory role of *T.indica* on NO production by peritoneal macrophages activated with TPA both *in vitro* and *in vivo* suggesting the inhibition might be mediated through the protein kinase C (PKC) pathway. As previously reported, the signal transduction through the PKC activation by TPA is sufficient for iNOS expression in peritoneal macrophages and the induction of iNOS activity in RAW 264.7 (Hortelano, Genaro, and Bosca, 1993; Paul, Pendreigh, and Plevin, 1995). Whether the mechanisms of NO inhibition by the seed coat extract of *T.indica* are mediated through iNOS expression and/or its activity, or through the regulation of lymphokines involving iNOS regulation need further investigation. Unexpectedly, data from NO production by peritoneal macrophages following *in vivo* TPA stimulation showed that the vehicle control for vitamin E and β-carotene, corn oil, inhibited stronger nitrite production than both vitamins. This observation might be caused by the presence of % of vitamin E and butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which can also act as antioxidants in the corn oil.

Since several antioxidants can modulate immunological activities *in vivo* and *in vitro* (Knight, 2000), we investigated the *in vivo* effects of the seed coat extract of *T.indica* on innate and cellular immunity. A combined local lymph node and irritancy assay was conducted using BALB/c mice. After one day of dosing, a decision was made to reduce the high dose of the seed coat extract of *T.indica* from 500 mg/kg to 200 mg/kg. This decision was based on the fact that BALB/c mice are possibly more sensitive to chemical stress than the hybrid B6C3F1 mice.

This study clearly showed that at the concentrations required to suppress NO production, no effects was seen on alteration of natural killer (NK) cell activity or the development of T cell-mediated sensitization response in the studies of local lymph node assay (LLNA) towards DNFB and HCA, and irritancy test against nonanoic acid. As mentioned above, evaluation of 14 day toxicity study suggested no toxicity of the seed coat extract of *T.indica* in B6C3F1 mice at doses ranging from 50 to 1000 mg/kg. Only some animals in the highest exposed group display slightly weight loss which recovered at the end of the study and no alteration of other toxicological parameters were observed. Therefore, the toxicological and immunological studies indicated that the seed coat extract of *T.indica* is relatively nontoxic and does not affect innate and cellular immune response in the NK, LLNA, and IR assay.

In view of the fact that excess NO production has been associated many diseases such as autoimmunity, rheumatoid arthritis, inflammatory bowel disease and septic shock (Moncada et al., 1991; Nathan, 1992), the suppression of NO production at apparently safe concentrations of the seed coat extract of *T.indica* suggests that this compound possibly further investigation for its potential health-promoting and food supplement value.

REFERENCES

Abu-Amsha, R., Croft, K.D., Puddey, I.B., Proudfoot, J.M., and Beilin, L.J. (1996). Phenolic content of various beverages determines the extent of inhibition of human serum and low density lipoprotein oxidation *in vitro*: identification and mechanism of action of some cinnamic acid derivatives from red wine. **Clinical Science** 91: 449-58.

Agarwal, O.P. (1982). The anti-inflammatory action of nepitrin, a flavonoid. **Agents Actions** 12: 298-302.

Albina, J.E., Abate, J.A., and Henry, W.L. (1991). Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation; role of IFN-γ in the induction of the nitric oxide-synthesizing pathway. **Journal of Immunology** 147: 144-8.

Alexander, M., Newmark, H., and Miller, R.G. (1985). Oral beta-carotene can increase the number of OKT4+ cells in human blood. **Immunology Letter** 9: 221-4.

Amber, I.J., Hibbs, J.B., Taintor, R.R., and Vavrin, Z. (1988). The L-arginine dependent effector mechanism is induced in murine adenocarcinoma cells by culture supernatant from cytotoxic activated macrophages. **Journal of Leukocyte Biology** 43: 187-92.

Amella, M., Bronner, C., Briancon, F., Haag, M., Anton, R., and Landry, Y. (1985). Inhibition of mast cell histamine release by flavonoids and biflavonoids. **Planta Medica** 1: 16-20.

Anderson, R. (1982). Ascorbic acid and immune functions: mechanism of immunostimulation. In J.N. Counsell and D.H. Hornig (eds.). **Vitamin C Ascorbic Acid** (249-272). London: Applied Science.

Anne, S., Agarwal, R., Nair, M.P., Schwartz, A., and Middleton, E. (1994). Inhibition of Endothelial cells by the plant flavonol quercetin. **Journal of Allergy and Clinical Immunology** 93: 276 (abstract).

Avila, M.A., Velasco, J.A., Cansado, J., and Notario, V. (1994). Quercetin mediates the downregulation of mutant p53 in the human breast cancer cell line MDA-MB468. **Cancer Research** 54: 2424-8.

Bendich, A., Machlin, L.J., Scandurra, O., Burton, G.W., and Wayner, D.D.M. (1986). The antioxidant role of vitamin C. **Free Radicals Biology and Medicine** 2: 419-44.

Bendich, A. (1993). Clinical importance of beta carotene. **Perspective Applied Nutrition** 1: 14-22.

Bendich, A. (1990). Antioxidant micronutrients and immune responses. In A. Bendich, and R.K. Chandra. **Micronutrients and Immune Functions** (pp. 168-180). New York: New York Academy of Sciences.

Bendich, A., and Olson, J.A. (1989). Biological actions of carotenoids. **FASEB Journal** 3:1927-32.

Beretz, A., and Cazenave, J.P. (1988). Plant Flavonoids in Biology and Medicine II. In V. Cody, E. Middleton, and J.B. Harborne (eds.). **Progress in Clinical and Biological Research** 280: 187-200. New York: Alan R. Liss Inc.

Berg, P.A., and Daniel, P.T. (1988). Plant Flavonoids in Biology and Medicine II.
In V. Cody, E. Middleton, and J.B. Harborne (eds.). **Progress in Clinical and Biological Research** 280: 157-1. New York: Alan R. Liss Inc.

Block, G. (1992). A role for antioxidants in reducing cancer risk**. Nutrition Review** 50 : 207-13.

Block, G., and Langseth, L. (1994). Antioxidant vitamins and disease prevention. **Food Technology July** 24: 80-4.

Bochner, B. (1998). Cellular adhesion in inflammation. In E. Middleton, C.E. Reed, E.F. Ellis, N.F. Adkinson, J.W. Yunginger, W.W. Busse (eds.). **Allergy: Principles and Practice**. Mosby Yearbook: St. Louis, MO.

Bogdan, C., Rollinghoff, M., Diefenbach, A. (2000). The role of nitric oxide in innate immunity. **Immunological Reviews** 173: 17-26.

Bors, W., Heller, W., Michel, C., and Saran, M. (1990). Flavonoids as antioxidants: Determination of radical-scavenging efficiences. **Methods in Enzymology** 186 : 343-55.

Bracke, M.E., De Pestel, G., Castronovo, V., Vyncke, B., Foidart, J.M., Vakaet, L.C.A., and Marcel, M.M. (1988). Plant Flavonoids in Biology and Medicine II. In V. Cody, E. Middleton, and J.B.Liss (eds.). **Progress in Clinical and Biological Research** New York: Alan R. Liss Inc.

Bracke, M., Vyncke, B., Opdenakker, G., Foidart, J.M., De Pestel, G., Mareel, M. (1991). Effect of catechins and citrus flavonoids on invasion *in vitro*. **Clinical and Experimental Metastasis** 9: 13-25.

Bryant, J., Day, Roger, Whiteside, T.L., and Herberman, R.B. (1992). Calculation of lytic units for the expression of cell-mediated cytotoxicity. **Journal of Immunological Methods** 146: 91-103.

Burgalassi, S., Panichi, L., Saettone, M.F., Jacobsen, J., and Rassing, M.R. (1996). Development and *in vitro*/ *in vivo* testing of mucoadhesive buccal patches releasing benzydamine and lidocaine. **International Journal of Pharmaceutics** 133: 1-7.

Burton, G.W., and Ingold, K.U. (1984). β-Carotene is an unusual type of lipid antioxidant. **Science Washiton DC** 224: 569-73.

Burton, G.W., Joyce, A., and Ingold, K.U. (1982). First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. **Lancet** 2: 327.

Burton, G.W., Joyce, A., and Ingold, K.U. (1983). Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? **Archives of Biochemistry and Biophysics** 221: 281-90.

Cao, G., Sofic, E., and Prior, R.L. (1997). Antioxidant and pro-oxidant bahaviour of flavonoids: structure-activity relationships. **Free Radical Biology and Medicine** 22: 749-60.

Carlo, G.D., Autore, G., IZZOAA, Moiolino, P., Mascolo, N., Viola, P., Diumo, M.V. and Capawa, F. (1993). Inhibition of intestinal motility and secretion by flavonoids in mice and rats; structure-activity relationships. **Journal of Pharmacology** 45: 1045-59.

Carlo, G.D., Mascolo, N., Izzo, A.A., and Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. **Life Sciences** 65: 337-53.

Chan, M.M.Y., Fong, D., Ho, C.T., and Huang, H.I. (1997). Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. **Biochemical Pharmacology** 54: 1281-6.

Chavance, M., Brubacher, G., Herbeth, B., Vernhes, G., Mikstacki, T., Dete, F., Fournier, C., Janot, C. (1984). Immunological and nutritional status among the elderly. In A.L. de Weck (ed.). **Lymphoid Cell Functions in Aging** (pp 231-237). Eurage: Rijswijk.

Chen, Z.Y., Chan, P.T., Ho, K.Y., Fung, K.P., and Wang, J. (1996). Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. **Chemistry and Physics of Lipids** 76: 157-63.

Chen, S., Hwang, J., Deng, P.S.K. (1993). Inhibition of NAD(P)H: Quinone acceptor oxidoreductase by flavones: a structure activity study. **Archives of Biochemistry and Biophysics** 302: 72-7.

Chew, B.P. (1993). Role of carotenoids in the immune response. **Journal of Dairy Science** 76: 2804-11.

Chiesi, M., and Schwaller, R. (1995). Inhibition of constitutive endothelial NO synthase activity by tannin and quercetin. **Biochemical Pharmacology** 49: 495-501.

Combs, G.F.J. (1987). Protective roles of minerals against free radical tissue damage, AIN Symposium Proceedings. **Nutrition** 87: 55-9.

Coquette, A., Vray, B., and Vanderpas, J. (1986). Role of vitamin E in the protection of the resident macrophage membrane against oxidative damage. **Archives International Physiology and Biochemistry** 94: 529-34.

Curran, R.D., Billiar, T.R., Stuehr, D.J., Hofmann, K, and Simmons, R.L. (1989). Hepatocytes produce nitric oxide from L-arginine in response to inflammatory products of Kupffer cells. **Journal of Experimental Medicine** 170: 1769-74.

Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, S., and Isaac, O. (1994). The role of triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* flowers. **Planta Medica** 60: 516-20.

Deneke, S.M., and Fanburg, B.L. (1980). Normobaric oxygen toxicity of the lung. **New England Journal of Medicine** 303: 76-86.

Deschner, E.E., Ruperto, J.F., Wong, G.Y., and Newmark, H.L. (1993). The effect of dietary quercetin and rutin on AOM-induced acute colonic epithelial abnormalities in mice fed a high-fat diet. **Nutritional Cancer** 20: 199-204.

Duke, S.S, Schook, L.B., and Holsapple, M.P. (1985). Effects of N-nitrosodimethylamine on tumor susceptibility. **Journal of Leukocyte Biology** 37: 383-94.

Farnsworth, N.R., and Bunyapraphatsara, N. (1992). **Thai medicinal plants recommended for primary health care system**. Bangkok: Prachachon.

Ferrandiz, M., and Alcaraz, M.J. (1991). Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. **Agents Actions** 32: 283-8.

Ferriola, P.C., Cody, V., and Middleton, E. (1989). Protein kinase C inhibition by plant flavonoids, kinetic mechanisms and structure-activity relationships. **Biochemical Pharmacology** 38: 1617-24.

Fidler, I. (1975). Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. **Cancer Research** 35: 218-24.

Frankel, E.N., Waterhourse, A.L., and Teissedre, P.L. (1995). Principle phenolic phytochemicals in selected Californian wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. **Journal of Agricultural and Food Chemistry** 43: 890-4.

Frei, B. (1991). Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. **American Journal of Clinical Nutrition** 54: 1113S-8S.

Frei, B., Stocker, R., and Ames, B.N. (1988). Antioxidant defences and lipid peroxidation in human blood plasma. **Proceedings of National Academy of Sciences** 85: 9748-52.

Fuller, C.J., Faulkner, H., Bendich, A., Parker, R.S., and Roe, D.A. (1992). Effect of beta-carotene supplementation on photosuppression of delayed-type hypersensitivity in normal young men. **American Journal of Clinical Nutrition** 56: 684-90.

Galloway, S.M., and Painter, R.B. (1979). Vitamin C is positive in the DNA synthesis inhibition and sister-chromatid exchange tests. **Mutation Research** 60: 321-7.

Goldschmidt, M.C., Masin, W.J., Brown, L.R., and Wyde, P.R. (1988). The effect of ascorbic acid deficiency on leukocyte phagocytosis and killing of *Actinomyces viscosus*. **International Journal for Vitamin and Nutrition Research** 58: 326-34.

Halliwell, B. (1994). Free radicals, antioxidants and human disease: curiosity, cause or consequence?. **Lancet** 344: 721-4.

Halliwell, B., Aeschbach, R., Loliger, J., and Aruoma, O.I. (1995). The characterization of antioxidants. **Food Chemical Toxicity** 33: 601-17.

Halliwell, B., and Gutteridge. J.M.C. (1990). The antioxidants of human extracellular fluids. **Archives of Biochemistry and Biophysics** 280: 1-8.

Halliwell, B., Gutteridge, J.M.C., and Cross, C.E. (1992). Free radicals, antioxidants and human disease: where are we now? **Journal of Laboratory Clinical Medicine** 119: 598-620.

Harbone, J.B., and Baxter, H, editors. (1991). **Phytochemical dictionary: A handbook of bioactive compounds from plants.** Bristol, Taylor and Frances, Inc.

Hatman, L.J., and Kayden, H.J. (1979). A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. **Journal of Lipid Research** 20: 639-45.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B., and Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease. The Zutphen elderly study. **Lancet** 342: 1007-11.

Holsapple, M.P., White, K.L.JR, McCay, J.A., Bradley, S.G., and Munson, A.E. (1988).

    An immunotoxicological evaluation of 4,4'-thiobis-(6-t-butyl-m-cresol) in female

    B6C3F1 mice. 2. Humoral and cell-mediated immunity, macrophage function,

    and host resistance. **Fundamental and Applied Toxicology** 10: 701-16.

Hortelano, S., Genaro, A.M., and Bosca, L. (1993). Phorbal esters induce nitric oxide

    synthase and increase arginine influx in cultured peritoneal macrophages.

    Federation of European Biochemical Societies 320: 135-9.

Hibbs, J., Jr., Taintor, R., Vavin, Z., and Rachlen, E. (1988). Nitric oxide: A cytotoxic

    activated macrophage effector molecule. **Biochemical Biophysical Research**

    Communication 157: 87-94.

Hinz, B., Brune, K., and Pahl, A. (2000). Nitric oxide inhibits inducible nitric oxide

    synthase mRNA expression in RAW 264.7 macrophages. **Biochemical**

    Biophysical Research Communication 271: 353-7.

Huang, H.C., Wang, H.R., and Hsieh, L.M. (1994). Antiproliferative effect of baicalein,

    a flavonoid from a Chinese herb, on vascular smooth muscle cell. **European**

    Journal of Pharmacology 251: 91-3.

Ialenti, A., Ianaro, A., Moncada, S., and Rosa, M.D. (1992). Modulation of acute

    inflammation by endogenous nitric oxide. **European Journal of Pharmacology**

    211: 177-82.

Ignarro, L.J., Buga, G.M., Sood, K.S., Byrns, R.E., and Chandhuri, G. (1987).

    Endothelium-derived relaxing factor produced and released from artery and vein

    is nitric oxide. **Proceedings of the National Academy of Sciences** 84: 9265-9.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.C., Smith, C.D., and Beckman, J.S. (1992). Peroxynitrite mediated tyrosine nitration catalyzed by superoxide dismutase. **Archives of Biochemistry and Biophysics** 298: 431-7.

Jenkins, D.C., Charles, I.G., Thomsen, L.L., Moss, D.W., Holmes, L.S., Rhodes, P., Westmore, K., Emson, P.C., and Moncada, S. (1995). Roles of nitric oxide in tumor growth. **Proceedings of the National Academy of Sciences of the United States of America** 92: 4392-6.

Kandaswami, G., Perkins, E., Soloniuk, D.S., Drzewiecki, G., and Middleton, E. (1991). Antiproliperative effects of Citrus flavonoids on human squamous cell carcinoma *in vitro*. **Cancer Letters** 56: 147-52.

Kandaswami, G., Perkins, E., Soloniuk, D.S., Drzewiecki, G., and Middleton, E. (1993). Ascorbic acid enhanced antiproliferative effect of flavonoids on squamous cell carcinoma *in vitro*. **Anticancer Drugs** 4: 91-6.

Kang, J.S., Jeon, Y.J., Kim, H.M., Han, S.H., and Yang, K.H. (2002). Inhibition of inducible nitric-oxide synthase expression by silymarin in lipopolysaccharide-stimulated macrophages. **Journal of Pharmacology and Experimental Therapeutics** 302: 138-44.

Kato, Y., Ogino, Y., Aoki, T., Uchida, K., Kawakishi, S., and Osawa, T. (1997). Phenolic antioxidants prevent peroxynitrite-derived collagen modification *in vitro*. **Journal of Agricultural and Food Chemistry** 45: 3004-9.

Kennes, B., Dumont, I., Brochee, D., Hubert, C., and Neve, P. (1983). Effect of vitamin C supplements on cell-mediated immunity in old people. **Gerontology** 29: 305-10.

Khandare, A.L., Rao, G.S., and Lakshmaiah, N. (2002). Effect of tamarind ingestion on fluoride excretion in humans. **European Journal of Clinical Nutrition** 56: 82-5.

Kim, H.K., Cheon, B.S., Kim, Y.H., Kim, S.Y., and Kim, H.P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure–activity relationships. **Biochemical Pharmacology** 58: 759-65.

Kleemann, R., Rothe, H., Kolb-Bachofen,V., Xie,Q.W., Nathan, C., Martin, S., and Kolb H. (1993). Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. **FEBS Letters** 328: 9-12.

Klotz, T., Bloch, W., Jacobs, G., Niggemann, S., Engelmann, U., and Addicks, K. (1999). Immunolocalization of inducible and constitutive nitric oxide synthases in human bladder cancer. **Urology** 54: 416-9.

Knight, J.A. (2000). Review: Free radicals, antioxidants, and the immune system. **Annals of Clinical & Laboratory Science** 30: 145-58.

Kobuchi, H., Droy-Lefaix, M.T., Christen, Y., and Packer, L. (1997). Ginkgo biloba extract (Egb761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. **Biochemical Pharmacology** 53: 897-903.

Kolb, H., and Kolb-Bachofen, V. (1992). Nitric oxide: A pathogenic factor in

    autoimmunity. **Immunological Today** 13: 157-60.

Komori, A., Yatsunami, J., Okabe, S., Abe, S., Hara, K., Suganuma, M., Kim, S.J., and

    Fujiki, H. (1993). Anticarcinogenic activity of green tea polyphenols.

    Japanease Journal of Clinical Oncology 23: 186-90.

Krinsky, N.I. (1979). Carotenoid protection against oxidation. **International Union of**

    Pure and Applied Chemistry 51: 649-60.

Krinsky, N.I., and Deneke, S.M. (1982). Interaction of oxygen and oxy-radicals with

    carotenoid. **Journal of the National Cancer Institute** 69: 205-9.

Krinsky, N.I. (1989). Carotenoids in medicine. In N.I. Krinsky, M.M. Mathews-Roth,

    and R.F. Taylor (eds.). **Chemistry and Biology** (pp 279-291). New York :

    Plenum Press.

Krol, W., Czuba, Z.P., Threadgill, M.D., Cunningham, B.D.M., and Pietsz, G. (1995).

    Inhibition of nitric oxide (NO$^\bullet$) production in murine macrophages by flavones.

    Biochemical Pharmacology 50: 1031-5.

Kukarni, D., Ddwivedi, D.K., Sarin, J.P.S., and Singh, S. (1997). Tamarind seed

    polyose: A potential polysacharide for sustained release of verapamil

    hydrochloride as a model drug. **Indian Journal of Pharmaceutical Sciences** 59

    : 1-7.

Langrehr, J.M., Hoffman, R.A., Billiar, T.R., Lee, K.K.W., Schraut, W.H., Simmons,

    R.L. (1991). Nitric oxide synthesis in the *in vivo* allograft response; a possible

    regulatory machanism. **Surgery** 110: 335-42.

Laranjinha, J.A.N., Almeida, L.M., and Madeira, V.M.C. (1994). Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. **Biochemical Pharmacology** 48: 487-94.

Larocca, L.M., Giustacchini, M., Maggiano, N, Ranelletti, F.O., Piantelli, M., Alcini, E., and Capelli, A. (1994). Growth-inhibitory effect of quercetin and presence of type II estrogen binding sites in primary human transitional cell carcinomas. **Journal Urology** 152: 1029-33.

Law, H., Dunn, T., Boyle, P., and Miller, J. (1949). Observations of the effect of a folic acid antagonist on transplantable lymphoid leukemias in mice. **Journal of the National Cancer Institute** 10: 179-92.

Lea, M.A., Xiao, Q., Sadhukhan, A.K., Cottle, S, Wang, Z.Y., and Yang, C.S. (1993). Inhibitory effects of tea extracts and (-)-epigallocatechin gallate on DNA synthesis and proliferation of hepatoma and erythroleukemia cells. **Cancer Letters** 68: 231-6.

Lee, K.H., Tagahara, K., Wu, R.Y., Haruna, M., Hall, I.H., Huang, H.C., Ito, K., Ida, I., and Lai, J.S. (1981). Antitumor agents 49: Daphnoretin and Kaempferol-3-0-β-D-glucopyranoside, antitumor dicoumaryl ether and flavone glucoside from Wikstroemia indica. **Journal Natural Product** 44: 530-5.

Lin, T.J., Zhang, K.J., and Liu, G.T. (1996). Effects of salvianolic acid A on oxygen radicals released by rat neutrophils and on neutrophil function. **Biochemical Pharmacology** 51: 1237-41.

Lin, Y.L., and Lin, J.K. (1997). (-)-Epigallocatechin-3-gallate blocks the induction of
  nitric oxide synthesis by down-regulating lipopolysaccharide-induced activity of
  transcription factor nuclear factor-κB. **Molecular Pharmacology** 52: 464-72.

Lorsbach, R.B., Murphy, W.J., Lowenstein, C.J., Snyder, S.H., and Russell, S.W. (1993).
  Expression of the nitric oxide synthase gene in mouse macrophages activated for
  tumor cell killing. Molecular basis for the synergy between interferon-gamma and
  lipopolysaccharide. **Journal of Biological Chemistry** 268: 1908-13.

Lowenstein, C.J., Alley, E.W., Raval, P, Snowman, A.M., Snyder, S.H., Russell, S.W.,
  Murphy, W.J. (1993). **Proceedings of the National Academy of Sciences** 90:
  9730-4.

MacMicking, J.D., Nathan, C, Hom, G., Chartrain, N., Fletcher, D.S., Trumbauer, M.,
  Stevens, K., Xie, Q.W., Sokol, K., Hutchinson, N., Chen, H., and Mudgett, J.S.
  (1995). Altered responses to bacterial infection and endotoxic shock in mice
  lacking inducible nitric oxide synthase. **Cell** 81: 641-50.

Makimura, M., Hirasawa, M., Kobayashi, K., Indo, J., Sakanaka, S., Taguchi, T., and
  Otake, S. (1993). Inhibitory effect of tea catechins on collagenase activity.
  Journal of Periodontology 64: 630-6.

Mann, J. (1978). **Secondary Metabolism**. Oxford Chemistry Series. Oxford :
  Clarendon Press.

Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D., and Wishnok, J.S. (1988).
  Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an
  intermediate. **Biochemistry** 27: 8706-11.

Marletta, M.A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. **Cell** 78: 927-30.

McCartney-Francis, N., Allen, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W., Nathan, C.F., and Wahl, S.M. (1993). Suppression of arthritis by an inhibitor of nitric oxide synthase. **Journal of Experimental Medicine** 178: 749-54.

Meydani, S.N., Barklund, M.P., Liu, S., Meydani, M., Miller, R., Cannin, J., Morrow, F., Rocklin, R., and Blumberg, J. (1988). Effect of vitamin E supplementation on immune responsiveness of healthy elderly subjects. **Federation of American Societies for Experimental Biology Journal** 3: A1057.

Meydani, S.N., Blumberg, J.B., Yogeeswaran, G., and Meydani, M. (1989). Antioxidant and the aging immune system. In A. Bendich, M. Phillips, and R. Tengerdy (eds.). **Antioxidant Nutrients and Immune Functions**. Plenum Press: New York.

Meydani, S.N., and Haytek, M. (1992). Vitamin E and the immune response. In R.K. Chandra (ed.). **International Conference on Nutrition, Immunity, and Illness in the the Elderly** (pp 105-128). St. Johns, Newfoundland: ARTS Biomedical Publ.

Meydani, S.N., Wu, D., Santos, M.S., and Hayek, M.G. (1995). Antioxidants and immune response in aged persons: Overview of present evidence. **American Journal of Clinical Nutrition.** 62 (suppl): 1465S-76S.

Middleton, E. (1996). The flavonoids as potential therapeutic agents. In E.S. Kimball (ed.). **ImmunoPharmaceuticals** (pp. 227-57). Boca Raton, FL: CRC Press.

Middleton, E., Kandaswami, C. (1986). The impact of plant flavonoids on mammalian

biology: Implications for immunity, inflammation and cancer. In: Harborne, J.B.

(ed.). **The Flavonoids: Advances in research since 1986** (pp. 619-652).

London: Chapman & Hall.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991). Nitric oxide: physiology,

pathophysiology, and pharmacology. **Pharmacological Reviews** 43: 109-42.

Mookerjee, B.K., Lee, T.P., Lippes, H.A., and Middleton, E. (1986). Some effects of

flavonoids on lymphocyte proliferative processes. **Journal**

Immunopharmacology 8: 371.

Moore, G., Sandberg, A., and Ulrich, K. (1966). Suspension cell culture and *in vivo* and

in vitro chromosome constitution of mouse leukemia L1210. **Journal of the**

National Cancer Institute 36: 405-21.

Mulsch, A., Schray-Utz, B., Mordvintcey, P.I., Hauschildt, S., and Busse, R. (1993).

Diethyldithiocarbamate inhibits induction of macrophage NO synthase. **FEBS**

Letters 321: 215-8.

Murray, M.T. (1992). **The Healing Power of Herbs, Rocklin**. Prima Publishing.

Mustapha, A., Yakasai, I.A., and Aguye, I.A. (1996). Effect of *Tamarindus indica* L. on

the bioavailability of aspirin in healthy human volunteers. **European Journal of**

Drug Metabolism and Pharmacokinetics 21: 223-6.

Nardini, M., D'Aquino, M., Tomassi, G., Gentili, V., Di Felice, N., and Scaccini, C. (1995). Inhibition of human LDL oxidation by caffeic acid and other hydroxycinnamic acid derivatives. **Free Radical Biology and Medicine** 19: 541-52.

Natarajan, K., Singh, S., Burke, T.R., Grunberger, D., and Aggarwal, B.B. (1996). Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB. **Proceedings of the National Academy of Sciences** 93: 9090-5.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. **FASEB Journal** 6: 3051-64.

Nathan, C, and Xie, Q.W. (1994). Regulation of biosynthesis of nitric oxide. **Journal of Biological Chemistry** 269: 13725-8.

NIH (1996). **Guide for the Care and Use of Laboratory Animals**. Washington, D.C.: National Academy Press.

Nussler, A.K. and Billiar, T.R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. **Journal of Leukocyte Biology** 54: 171-8.

Ohshima, H., and Bartsch, H. (1994). Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. **Mutation Research** 305: 253-64.

Pace-Asciak, C.R., Hahn, S., Diamandis, E., Soleas, G., and Goldberg, D.M. (1995). The red wine phenolics trans-resveritrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. **Clinica Chimica Acta** 235: 207-19.

Packer, L., Rimbach, G., Virgili, F. (1999). Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, Pycnogenol. **Free Radical Biology & Medicine** 27: 704-24.

Padgett, E.L. and Pruett, S.B. (1992). Evaluation of nitrite production by human monocyte-derived macrophages. **Bichemical Biophysical Research Communication** 186: 775-81.

Palmer, R.M., Ashton, D.S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. **Nature (Lond)** 333: 664-6.

Paul, A., Pendreigh, R.H., and Plevin, R. (1995). Protein kinase C and tyrosine kinase pathways regulate lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 murine macrophages. **British Journal of Pharmacology** 114: 482-8.

Pfeilschifter, J., and Schwarzenbach, H. (1990). Interleukin 1 and tumor necrosis factor stimulates cGMP formation in rat renal mesangial cells. **FEBS Letters** 273: 185-7.

Pendino, K.J., Laskin, J.D., Shuler, R.L., Punjabi, C.J., and Laskin, D.L. (1993). Enhance production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increase expression of nitric oxide synthase. **Journal of Immunology** 151: 7196-205.

Petros, A., Bennett, D., and Vallance, P. (1991). Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. **Lancet** 338: 1557-8.

Pumthong, G. (1999). **Antioxidative activity of polyphenolic compounds extracted from seed coat of *Tamarindus indica* Linn**. M.S. thesis, Chiang Mai University, Thailand.

Punjabi, C.J., Laskin, D.L., Heck, D.E., and Laskin, J.D. (1992). Production of nitric oxide by murine bone marrow cells. **Journal of Immunology** 149: 2179-84.

Raso, G.M, Meli, R., Carlo, G.D., Pacilio, M., and Carlo, R.D. (2001). Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. **Life Sciences** 68: 921-31.

Ren, S., and Lien, E.J. (1997). Natural products and their derivatives as cancer chemopreventive agents. **Progress in Drug Research** 48: 147-71.

Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1996). Structure –antioxidant activity relationships of flavonoids and phenolic acids. **Free Radical Biology and Medicine** 20: 933.

Robbins, R.A., and Sisson, J.H. (1996). Nitric Oxide. In R.G., Townley and D.K., Agrawal (eds). **Immunopharmacology of Allergic Disease** (pp 415-432). New York: Marcel Dekker.

Rohdewald, P. (2002). A review of the French maritime pine bark extract (Pycnogenol®), a herbal medication with a diverse clinical pharmacology. **International Journal of Clinical Pharmacology and Therapeutics** 40: 158-68.

Ross, D., and Moldeus, P. (1991). Antioxidant defense systems and oxidative stress. In C. Vigo-Pelfrey, and F.L. Boca Raton. **Membrane Lipid Oxidation** (pp 151-170). CRC.

Saettone, M.F., Burgalassi, S., Giannaccini, B., Boldrini, E., Bianchini, P., and Luciani, G. (1997). **Ophthalmic solutions viscosified with tamarind seed polysaccharide.** WO. PCT Int. Appl.

Sambaiah, K. and Srinivasan, K. (1991). Effect of cumin, cinnamon, ginger, mustard and tamarind in induced hypercholesterolemic rats. **Die Nahrung** 35: 47-51.

Samuelsson, G. (1993). **Drugs of Natural Origin. Textbook of Pharmacognosy**. Swedish Pharmaceutical Press Editor.

Sano, M., Miyata, E., Tamano, S., Hagiwara, A., Ito, N., and Shirai, T. (1996). Lack of carcinogenicity of tamarind seed polysaccharide in B6C3F1 mice. **Food and Chemical Toxicology** 34: 463-7.

Scambia, G., Ranelletti, F.O., Benedetti, P.P., Bonanno, G, De Vincenzo, R., Piantelli, M., and Mancuso, S. (1990). Synergistic antiproliferative activity of quercetin and cisplatin on ovarian cancer cell growth. **Anticancer Drugs** 1: 45-8.

Schmidt, H.H.H.W., and Walter, U. (1994). NO at work. **Cell** 78: 919-25.

Scutt, A, Meghji, S., Canniff, J.P., and Harvey, W. (1987). Stabilisation of collagen by betel nut polyphenols as a mechanism in oral submucous fibrosis. **Experientia** 43: 391-3.

Shahidi, F., and Wanasundara, P.K.J.P.D. (1992). Phenolic antioxidants. **Critical Review of Food Science and Nutrition** 32: 67-103.

Smith, C., Mitchinson, M.J., Arudma, O.I., and Halliwell, B. (1992). Stimulation of lipid peroxidation and hydroxyl radical generation by the contents of human atherosclerotic lesions. **Biochemical Journal** 286: 901-5.

Snyder, S.H., and Bredt, D.S. (1992). Biological roles of nitric oxide. **Scientific American** 266: 68-77.

Speit, G., Wolf, M., and Vogel, W. (1980). The SCE-inducing capacity of vitamin C: investigations *in vitro* and *in vivo*. **Mutation Research** 78: 273-8.

Sreelekha, T.T., Vijayakumar, T., Ankanthil, R., Vijayan, K.K., and Nair, M.K. (1993). Immunomodulatory effects of a polysaccharide from *Tamarindus indica*. **Anti-Cancer Drugs** 4: 209-12.

Stagg, G.V., and Millin, D.J. (1975). The nutritional and therapeutic value of tea – a review. **Journal of Science Food and Agriculture** 26: 1439-59.

Sumathi, S., and Ray, A.R. (2002). Release behaviour of drugs from tamarind seed polysaccharide tablets. **Journal of Pharmacology and Pharmaceutical Science** 5: 12-8.

Tamir, S., and Tannenbaum, S.R. (1996). The role of nitric oxide (NO) in the carcinogenic process. **Biochemical Biophysical Acta** 1288: F31-6.

Tamura, M., Kagawa, S., Tsuruo, Y., Ishimura, K., and Morita, K. (1994). Effects of flavonoid compounds on the activity of NADPH diaphorase prepared from the mouse brain. **Japanese Journal of Pharmacology** 65: 371-3.

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara, A, Sumida, T., Fukutani, K., Tanaka, T., and Ogawa, H. (1997). Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. **Cancer Research** 57: 246-52.

Thomas, C.E., and Aust, S.D. (1985). Rat liver microsomal NADPH-dependent release of iron from ferritin snd lipid peroxidation. **Free Radical Biological and Medicine** 1: 293-300.

Thomas, S.R., Witting, P.K., and Stocker, R. (1996). 3-Hydroxyanthranilic acid is an efficient, cell-derived co-antioxidant for α-tocopherol, inhibiting human low density lipoprotein and plasma lipid peroxidation. **Journal of Biological Chemistry** 271: 32714-21.

Tsuda, T., Watanabe, M., Ohshima, K., Yamamoto, A., Kawakishi, S., and Osawa, T. (1994). Antioxidative components isolated from the seed of tamarind (*Tamarindus indica* L.). **Journal of Agricultural and Food Chemistry** 42: 2671-4.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K.D., Roveri, A., Schomburg, D, and Flohe, L. (1995). Diversity of glutathione peroxidases. **Methods in Enzymology** 252: 38-53.

van Acker, S.A.B.E., Tromp, M.N.J.L., Haenen, G.R.M.M., van der Vijgh, W.J.F. and
Bast, A. (1995). Flavonoids as scavenger of nitric oxide radical. **Biochemical
and Biophysical Research Communication** 214: 755-9.

van Acker, S.A.B.E., van Denberg, D.J., Tromp, M.N.J.L., Griffioen, D.H., van
Bennekom, W.P., van Dervijgh, W.J.F., and Bast, A. (1996). Structural aspects
of antioxidant activity of flavonoids. **Free Radical Biology and Medicine** 20:
331-42.

Verma, A.K., Johnson, J.A., Gould, M.N., and Tanner, M.A. (1988). Inhibition of 7,12-
dimethylbenz[a]anthracene and N-nitroso methylurea-induced rat mammary
cancer by dietary flavonol quercetin. **Cancer Research** 48: 5754-8.

Vieira, O., Escargueil-Blanc, I., Meilhac, O., Basile, J.P., Laranjinha, J., Almeida, L.,
Salvayre, R., and Negre-Salvayre, A. (1998a). Effect of dietary phenolic
compounds on apoptosis of human cultured endothelial cells induced by oxidized
LDL. **British Journal of Pharmacology** 123: 565-73.

Vieira, O., Laranjinha, J., Madeira, V., and Almeda, L (1998b). Cholesteryl ester
hydroperoxide formation in myoglobin-catalysed low density lipoprotein
oxidation; concerted antioxidant activity of caffeic and courmaric acids with
ascorbate. **Biochemical Pharmacology** 55: 333-40.

Virgili, F., Kobuchi, H., and Packer, L. (1998). Procyanidins extracted from *Pinus
maritima* (PYCNOGENOL®): scavengers of free radical species and modulators
of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages.
Free Radical Biological and Medicine 24: 1120-9.

Visioli, F., and Galli, C. (1994). Oleuropein protects low density lipoprotein from

oxidation. **Life Science** 55: 1965-71.

Warren, J.B. (1994). Nitric oxide and human skin blood flow response to acetylcholine

and ultraviolet light**. FASEB Journal** 8: 247-51.

Wayner, D.D.M., Burton, G.W., Ingold, K.U., Barclay, L.R.C., and Locke, S. (1987).

The relative contributions of vitamin E, urate, ascorbate and proteins to the total

peroxyl radical-trapping antioxidant activity of human blood. **Biochemical**

Biophysical Acta 924: 408-19.

Wei, X.Q., Charles, I.G., Smith, A., Ure, J., Feng, G.J., Huang, F.P., Xu, D., Muller, W.,

Moncada, S., and Liew, F.Y. (1995). Altered immune responses in mice lacking

inducible nitric oxide synthase. **Nature** 375: 408-11.

Weisz, A., Oguchi, S., Cicatiello, L., and Esumi, H. (1994). Dual mechanism for the

control of inducible-type NO synthase gene expression in macrophages during

activation by interferon-γ and bacterial lipopolysaccharide: Transcriptional and

post-transcriptional regulation. **Journal of Biological Chemistry** 269: 8324-33.

Xie, Q.W., Cho, H.J., Calaycay, J, Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A,

Troso, T, and Nathan, C. (1992). Cloning and characterization of inducible nitric

oxide synthase from mouse macrophages. **Science** 256: 225-8.

Xie, Q.W., Whisnant, R., and Nathan, C. (1993). Promoter of the mouse gene encoding

calcium–independent nitric oxide synthase confers inducibility by interferon

gamma and bacterial lipopolysaccharide. **Proceedings of National Academy of**

Sciences 90: 9730-4.

Xie, Q.W.Y., Kashiwabara, and Nathan, C. (1994). Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. **Journal of Biological Chemistry** 269: 4705-8.

Yamanaka, N., Oda, O., and Nagao, S. (1997). Pro-oxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu^{2+}-induced low density lipoprotein oxidation. **FEBS Letters** 405: 186-90.

Yu, B.P. (1994). Cellular defenses against damage from reactive oxygen species. **Physiological Reviews** 74: 139-61.
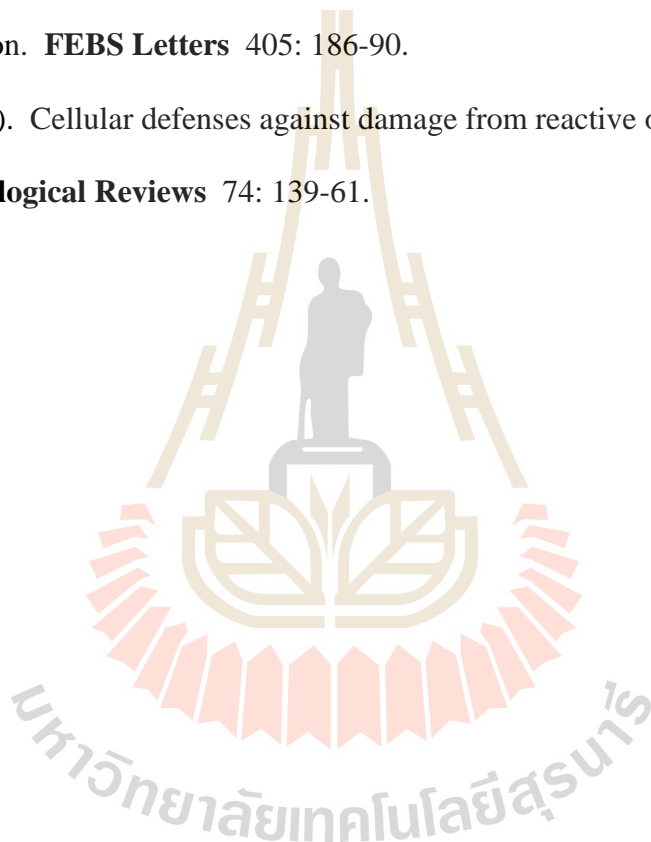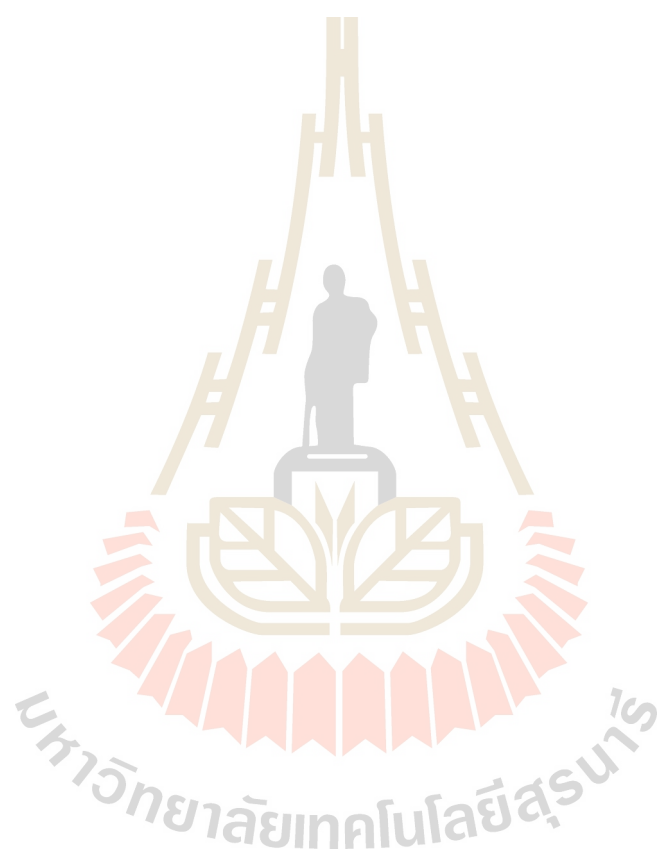
APPENDIX I

EXTRACTION METHOD

1. Preparation of Tamarind Seed Coat Powder

Tamarind seeds were obtained from ripen tamarind fruits after removing the edible parts. The good seeds were heated in a hot air oven at 140°C, 45 minutes, cooled and readily cracked to separate their outside brown layer. Only brown-red seed coats were collected and ground into fine powder.

2. Extraction of Antioxidative Substances from Tamarind Seed Coat Powder

The tamarind seed coat powder weighed 0.5 gram were added to 10 mL of 70% ethanol in a separating funnel. The mixture was vigorously shaken for 10 minutes and then filtered through a filter paper. The extraction was repeatedly done until the filtrate became colorless all. The filtrates were pooled, and 5 mL of the pooled filtrate, so-called Fraction 1 or ethanol fraction, were further fractionated in separating funnel by addition 5 mL of chloroform. The mixture was shaken well and left it until the 2 layer were separated apart. The lower layer or chloroform layer, called Fraction 2, was firstly collected approximately 8 mL of volume, and the rested upper layer of aqueous layer in funnel, so-called Fraction 3 having amount as equal as 2 mL, was added 5 mL of ethyl acetate. The mixture was shaken and left to separate into 2 layers. The lower layer or Fraction 4

containing a large amount of ethyl acetate was collected at 5 mL, and the remaining layer was totally dissolved in 5 mL of methanol and was called Fraction 5 (6.5 mL) of volume.

APPENDIX II

CELL LINES

L1210 murine lymphocytid leukemia cell lines

The L1210 murine leukemia originated in 1949 in mouse #234 of subline 212 of the DBA/2 strain 8 months of age by painting with 0.2% methylcholanthrene in ethyl ether. The L1210 cells grew *in vivo* and were transferred by subcutaneous (s.c.) and intramuscular (i.m.) inoculation (Law, Dunn, Boyle, and Miller, 1949). The *in vitro* growth of the lines was first characterized by Moore et al. (1966) as a stationary suspension with doubling times in culture of 8-10 hours (Moore, Sandberg, and Ulrich, 1966).

HaCat human epidermal keratinocyte

The HaCat human epidermal keratinocyte cell line was established in 1996. Cells from the scalp of a 65 year old male were transfected with plasmid p1321 (Plasmid p1321 contains human papillomavirus (HPV) 16 E6 and E7 genes. HEK001 cells express keratin 14 but not keratin 10 suggesting that they are proliferating basal-type keratinocytes. A culture at passage 80 was submitted to the ATCC in June of 1998 (Moore, Sandberg, and Ulrich, 1966).

B16F10 murine melanoma

The B16F10 melanoma occurred spontaneously in a C57B1/6 mouse in 1954. The selection of tumor cells capable of forming pulmonary nodules in syngeneic mice was described in 1975 by Fidler. B16F10 tumor cells grow in vitro, as astationary adherent culture, with doubling times in the range 17-22 hours (Fidler, 1975).

AGS human gastric adenocarcinoma

The AGS cell line was derived from fragments of a tumor resected a patient who had received no prior therapy. The selection of this tumor cells cause tumorigenicity in athymic BALB/c mice. AGS tumor cells grow *in vitro*, as a stationary adherent culture, with doubling time 20 hours (Moore, Sandberg, and Ulrich, 1966).

A427 lung carcinoma

The A427 line was derived by D.J. Giard, as indicated in the description for ATCC HTB-41. This cell line forms an undifferentiated tumor suggestive of adenocarcinoma in nude mice (Moore, Sandberg, and Ulrich, 1966).

MCF-7 human mammary adenocarcinoma

The MCF-7 line retains several characteristics of differentiated mammary epithelium including ability to process esteadiol via cytoplasmic estrogen receptors and the capability of forming domes and contain Tx-4 oncogene. The growth of MCF-7 cells is inhibited by tumor necrosis factor alpha (TNF-α). The secretion of insulin-like growth factor binding proteins (IGFBP) can be modulated by treatment with antiestrogens (Moore, Sandberg, and Ulrich, 1966).

RAW 264.7 gamma NO(-) murine macrophage

The RAW 264.7 gamma NO(-) was derived from RAW 264.7 mouse monocyte/macrophage cell line ordinally obtained in 1978 from Dr. Peter Palph. The cells were not intentionally cloned but were serendipitously obtained during routine culture. Unlike the parental line, RAW 264.7 gamma NO(-) does not produce nitric oxide upon treatment with interferon gamma (IFN-γ) alone, but requires LPS for full activation (the iNOS promoter linked to a luciferase reporter gene is also unresponsive to IFN-γ alone). This property makes its behavior more like that of normal macrophages from some commonly used strains of mice (e.g., C3H/HeN) (Moore, Sandberg, and Ulrich, 1966).
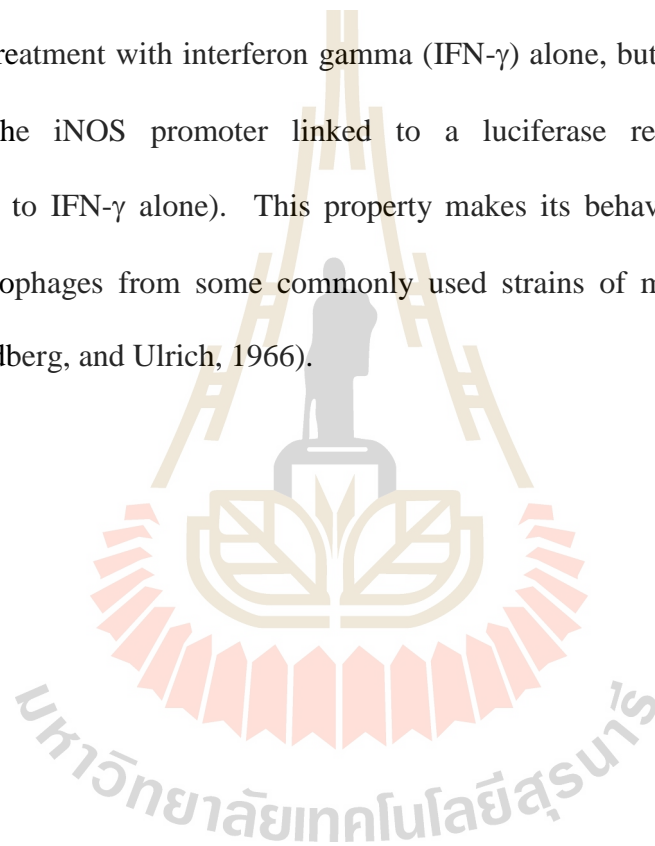
CURRICULUM VITAE

Tanaya Komutarin was born on October 24 [th], 1973, in Nakhon Ratchasima. She graduated with a Bachelor of Science degree in Public Health from Khon Kaen University in 1994. She received her Master of Science degree in Medical Microbiology from Khon Kaen University in 1998. During her master's degree, she was supported by the Research Fund of the Faculty of Medicine Khon Kaen University. She received the Royal Golden Jubilee Ph.D. Scholarship from the Thailand Research Fund at Suranaree University of Technology in 1999 and did her research at the National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA, in 2002. Upon acceptance of this thesis, she will graduate with a Doctor of Philosophy in Environmental Biology from Suranaree University of Teachnology in 2003.