INHIBITORY EFFECT OF NITRIC OXIDE PRODUCTION ON INFLAMMATION AND APOPTOSIS IN MACROPHAGE RAW 264.7 BY EXTRACT FROM SEED COAT OF *TAMARINDUS INDICA* L.

MISS JINDAWAN WIBULOUTAI

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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ผลการยับยั้งของการสร้างไนตริกออกไซด์ต่อการอักเสบ และอะพอพโตซีสใน แมคโครฟาจ RAW 264.7 โดยสารสกัดจากเปลือกหุ้มเมล็ดมะขาม

นางสาวจินดาวัลย์ วิบูลย์อุทัย

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

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จินดาวัลย์ วิบูลย์อุทัย : ผลการยับยั้งของการสร้างไนตริกออกไซด์ต่อการอักเสบและอะพอพโตซีสในแมคโครฟาจ RAW 264.7 โดยสารสกัดจากเปลือกหุ้มเมล็ดมะขาม (INHIBITORY EFFECT OF NITRIC OXIDE PRODUCTION ON INFLAMMATION AND APOPTOSIS IN MACROPHAGE RAW 264.7 BY EXTRACT FROM SEED COAT OF *TAMARINDUS INDICA* L.) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. เบญจมาศ จิตรสมบูรณ์, 143 หน้า.

การตรวจสอบฤทธิ์ทางชีวภาพของสารสกัดจากเปลือกหุ้มเมล็ดมะขามประเมินจากความสามารถในการต้านอนุมูลอิสระ ต้านการอักเสบ และการยับยั้งการตายของเซลล์แบบอะพอพโตซีส ภายหลังการสกัดเปลือกหุ้มเมล็ดมะขามด้วย 50% อะซิโตนได้ปริมาณสารสกัดร้อยละ 45.8 ของน้ำหนักแห้ง โดยมีสารประกอบฟีนอลิกเทียบเท่าน้ำหนักกรดแกลิค 178$\pm$3.8 มิลลิกรัมต่อกรัม และมีคุณสมบัติซึ่งแปรผันตามปริมาณสารในการต้านอนุมูลอิสระและการรีดักชั่นสูงกว่าวิตามินซีและสารสกัดจากเมล็ดองุ่น เมื่อประเมินโดยวิธีทดสอบ DPPH และ FRAP สารสกัดยับยั้งการผลิตไนตริกออกไซด์ได้สูงถึงร้อยละ 60 ในเซลล์ RAW 264.7 เมื่อถูกกระตุ้นด้วย LPS และ IFN-γ ที่ความเข้มข้น 10 ไมโครกรัมต่อมิลลิลิตรของสารสกัด ซึ่งเป็นระดับสารที่ไม่ก่อให้เกิดพิษต่อเซลล์ นอกจากนั้นความเข้มข้นเดียวกันของสารสกัดสามารถลดระดับการแสดงออกของเอมไซม์ iNOS และ COX-2 และลดการเกิดอะพอพโตซีสของเซลล์ RAW 264.7 ที่ถูกกระตุ้นด้วยอีโทโพไซด์ได้ร้อยละ 10 เมื่อประเมินโดยวิธี annexin-V-PI ซึ่งผลที่ได้สอดคล้องกับการลดการแตกหักของดีเอ็น-เอที่แปรผันตามปริมาณของสารสกัด เมื่อป้อนสารสกัดที่ 400 มิลลิกรัมต่อกิโลกรัมในหนูเมาส์ ICR พบว่าสารสกัดช่วยบรรเทาความเจ็บปวดโดยลดการหดเกร็งของกล้ามเนื้อหน้าท้องที่ถูกชักนำโดยกรดอะซิติกได้ร้อยละ 44 และลดความเจ็บปวดร้อยละ 50 เมื่อทดสอบด้วยวิธีม้วนหางหนีความร้อน ดังนั้นสารสกัดจึงมีผลทั้งต่อระบบประสาทส่วนกลางและส่วนปลาย โดยรวม การศึกษาชี้ชัดว่าสารสกัดจากเปลือกหุ้มเมล็ดมะขามเป็นแหล่งสำคัญของสารต้านอนุมูลอิสระตามธรรมชาติซึ่งมีประสิทธิภาพสูง ราคาถูก และมีฤทธิ์ทางเภสัชวิทยาที่ควรค่าต่อการพัฒนาให้เป็นยาธรรมชาติเพื่อการป้องกันหรือผลิตภัณฑ์เสริมอาหารต่อไปในอนาคต

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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JINDAWAN WIBULOUTAI : INHIBITORY EFFECT OF NITRIC OXIDE PRODUCTION ON INFLAMMATION AND APOPTOSIS IN MACROPHAGE RAW 264.7 BY EXTRACT FROM SEED COAT OF *TAMARINDUS INDICA* L. THESIS ADVISOR : ASST. PROF. BENJAMART CHITSOMBOON, Ph.D. 143 PP.

TAMARIND/NITRIC OXIDE/INDUCIBLE NITRIC OXIDE SYNTHASE/ CYCLOOXYGENASE-2/ANTI-APOPTOSIS/ ANTI-INFLAMMATION

Effects of seed coat extract of *Tamaridus indica* Linn. (TAM) on the biological activities, including antioxidant capacity, anti-inflammatory, and anti-apoptotic activities were investigated. After 50% acetone extraction, the yield of TAM extract was 45.8% and the total phenolic content was 178±3.8 mg/g gallic acid equivalent. TAM extract showed a higher dose dependent radical scavenging activity and power of reduction than vitamin C and grape seed extract as evaluated by the DPPH and FRAP assays. TAM extract induced a high suppression (≈60%) of NO production by LPS plus IFN-γ activated RAW 264.7 cells at 10 μg/ml, the concentration that had no cytotoxicity. In addition, TAM at the same concentration induced a dose-dependent inhibition of iNOS and COX-2 protein expressions. TAM at this concentration also reduced the etoposide-induced apoptosis of RAW 264.7 cells by 10% as evaluated by the annexin V-PI binding. The decreased apoptotic result was also confirmed by a dose-dependent reduction of DNA fragmentation. TAM exerts its pain-relieving effect in a dose-dependent manner by anti-nociceptive activity in ICR mice. The maximal inhibition of the nociceptive response assessed by

acetic acid-induced writhing test was 44% at 400 mg/kg, p.o., whereas the same dose significantly increased the analgesic activity by 50% in the tail flick response to thermal-nociceptive stimuli. Therefore, the TAM extract possesses both peripheral and central analgesic activities in mice. Overall, the present data suggest that TAM extract has a high and real potential as a safe, effective, low-cost source of natural antioxidants with many pharmaceutical properties and is worthwhile to be developed as natural chemopreventive products or nutraceuticals in the future.

School of Biology

Academic Year 2006

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Jindawan Wibuloutai

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LIST OF ABBREVIATIONS

Ab	Antibody
Abs	Absorbance
ATCC	American Type Culture Collection
Bp	Basepair
COX-1, COX-2	cyclooxygenases-1, cyclooxygenases-2
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
ECL	Enhanced chemiluminescence
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing antioxidant power
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IFN-γ	Interferon-gamma
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
eNos	endothelial nitric oxide synthase
iNos	inducible nitric oxide synthase
nNos	neuron nitric oxide synthase
kDa	Kilodalton

LIST OF ABBREVIATIONS (Continued)

LPS	Lipopolysaccharide
µg/ml	Microgram per milliliter
µL	Microliter
µM	Micromolar
mg/ kg	milligram per kilogram
min	minute
ml	milliliter
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
$NO^{\bullet}$	Nitric oxide
NF-κB	Nuclear factor-kappa B
$^{1}O_2$	Singlet oxygen
$O_2^{\bullet}$-	Superoxide anion
$OCl^{\bullet}$	Hypochlorite
O.D.	Optical Density
$OH^{\bullet}$	Hydroxyl radical
OONO-	Peroxynitrite
OPCs	Oligomeric proanthocyanidins
PARP	Poly (ADP-ribose) glycohydrolase
PBS	Phosphast buffered saline
PI	Propidium Iodide

LIST OF ABBREVIATIONS (Continued)

PGE_2	Prostaglandin E_2
PS	Phosphatidylserine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute number 1640
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAM	*Tamarindus indica* Linn.
TB	Trypan blue exclusion
TLC	Thin layer chromatography
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV	Ultraviolet
VH	Vehicle

CHAPTER I

INTRODUCTION

1.1 Introduction

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) include both radical and non-radical molecules with unpaired orbital electrons derived from nitrogen (e.g. nitric oxide) and oxygen (e.g. peroxyl radical), respectively. Both RNS and ROS have important roles in the host defense against infection. In acute inflammation, however, overproduction of ROS and RNS results in tissue damage and vascular leakage common to conditions such as septicaemia, rheumatoid arthritis and inflammatory bowel disease (Darley *et al*., 1995). Moreover, the interaction between ROS and RNS can also lead to the production of highly reactive non-radical species such as peroxynitrite, a product of nitric oxide and superoxide; commonly generated by macrophages under pathological conditions (Ischiropoulos *et al*., 1992). ROS are also able to induce apoptosis in various cell types (Li *et al*., 1997; Slater *et al*., 1995; Stangel *et al*., 1996). The biological production of nitric oxide occurs from enzymatic oxidation reaction associated with L-arginine (MacMicking *et al*., 1997). The two types of nitric oxide synthase (NOS) responsible for the biological synthesis of nitric oxide under both physiological and pathophysiological conditions are constitutive NOS (cNOS) and inducible NOS (iNOS). The latter is induced in various cells exposed to endotoxin stimulation. The low-output nitric oxide pathway by cNOS

provides a physiological function for nitric oxide in the healthy host, while an iNOS-controlled high-output pathway is engaged during inflammation and infection (MacMicking *et al*., 1997). In fact, the expression of iNOS has been found in several human tumor tissues and inflammatory disorders (Surh *et al*., 2001), with the extent of nitric oxide production by iNOS often reflects the degree of inflammation (Chen *et al*., 2001). Similarly, constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2) are also present. In addition to iNOS, macrophages will overproduce COX-2, which is involved in transforming arachidonic acid to prostaglandin H_2, a precursor of prostaglandin E_2 (PGE_2), prostacyclin and thromboxane upon endotoxin stimulation. Compounds interfering with both iNOS and COX-2 generally act as potential inhibitors of NF-κB activation (Bremneret *et al*., 2002), a transcription factor required for the induction of iNOS (Lin *et al*., 1999 and Hu *et al*., 2003). Products of arachidonic acid metabolism as well as released arachidonic acid itself plays critical roles in many biological processes, such as the development of aging, inflammation, platelet aggregation, angiogenesis, atherosclerosis and cancer (Rao *et al*., 1994). High levels of released arachidonic acid and its products are found in cancer cell such as breast, colon and lung (Wolff *et al*., 1998 and Wu *et al*., 2001).

Apoptosis is involved in many normal biological processes, such as embryonic and T-cell development, metamorphosis, and hormone-dependent atrophy. Apoptosis can also be induced by a variety of cytotoxic processes. Moreover, apoptosis is of major importance in the pathogenesis of several diseases (Thompson *et al*., 1995) such as cancer (Dive, 1997), neurological disorders such as Alzheimer's and Parkinson's disease (Carson and Ribeiro, 1993), cataracts and macular degeneration

(Meyer, 2005), cardiovascular disease, cognitive impairment (Valko, 2004) and immune dysfunction (Ryan-Harshman, 2005).

The flavonoid family contains more than 4000 individual compounds found in the plant kingdom (Heim *et al*., 2002), and accounts for two-thirds of human total dietary intake of polyphenol, mainly from fruit and vegetable sources (Scalbert *et al*., 2002). Phytochemicals in the flavonoid family have been noted for the bioactivities to suppress both ROS and RNS (Surh *et al*., 2001; Chen *et al*., 2001; Hu *et al*., 2003 and Wang *et al*., 2002). They can act as free radical scavengers, reducing agents, potential complexers of prooxidant metals, and quenchers from the formation of singlet oxygen (Shahidi and Naczk, 1995).

Tamarindus indica Linn. (TAM) is a tree-type of plant belonging to the Caesalpiniaceae Sub family. The seeds of TAM have been long used as an anthelmintic, antidiarrheal, and an emetic, and the seed coat is used to treat burns, wound healing and antidysenteric (Farnsworth and Bunyapraphatsara, 1992). With a particular astringent taste, tamarind seed coat contains high amount of polyphenolic compounds which exhibit strong antioxidative activity (Luengthanapol *et al*., 2003). The main active antioxidant isolated was chemically proved to be oligomeric proanthocyanidins (OPCs), which function as a scavenger against peroxyl radicals, hydroxyl radicals and superoxide anions *in vitro*. It also acts as a protective agent against lipid peroxidation and oxidative damage of Ca^{2+}-ATPase in red blood cell membrane (Pumthong, 1999). Furthermore the seed coat of TAM significantly attenuated nitric oxide production *in vitro* using Lipopolysaccharide (LPS) and Interferon gamma (IFN-γ) activated RAW264.7 cells, the murine macrophages cell line, and *in vitro* and *in vivo* using isolated peritoneal macrophages from B6C3F1

mice. The safety studies in B6C3F1 mice demonstrated that the extract at 100-500 mg/kg can modulate the NO production without inducing any clinical signs of toxicity. Neither the development of a T-cell mediated sensitization responses to DNFB or HCA nor the dermal irritation to nonanoic acid or DNFB is modulated by the *T. indica* treatment (Komutarin *et al*., 2004).

In theory, supplementation with antioxidants may help maintain the oxidant/antioxidant balance and consequently reverse or improve inflammation related abnormalities especially in production of inflammatory compounds (Meydani *et al*., 1986; Furukawa *et al*., 1987 and Hayek *et al*., 1994). It is suggested that the reduction in NO production might result from scavenging of NO radicals, direct inhibition of iNOS enzyme activity, and/or inhibition of iNOS gene expression (Sheu *et al.*, 2001; Park *et al.*, 2000). Therefore, suppression of NO level by phytochemicals may be a new and efficient approach for the treatment of inflammation and cancer.

Based on the reported antioxidant activity of TAM which contains flavonoids among its major constituents, and the suppression of NO production in activated macrophages both *in vitro* and *in vivo*, but inhibitory mechanism of NO has not yet studied. Therefore, the present studies further investigate the inhibitory mechanism of NO production as well as the attenuating effects of TAM extract on inflammation and apoptotic cell death *in vitro* in activated RAW 264.7 cells. In addition, anti-inflammatory effect of TAM extract *in vivo* was also explored.

1.2 Research objectives

To investigate the effects of TAM extract on the followings:

1. Inhibition of iNOS expression as the inhibitory mechanism of nitric oxide production in activated RAW 264.7 cells.

2. Inhibition of COX-2 expression as the *in vitro* anti-inflammatory effect in activated RAW 264.7 cells.

3. Anti-inflammation by using an *in vivo* writhing murine model.

4. Anti-apoptosis in activated RAW 264.7 cells by flow cytometric analysis of annexin V-PI and DNA fragmentation by visualization of DNA ladders in the agarose gel electrophoresis.

1.3 Scope and limitation of the study

The studies used *in vitro* model of mouse macrophage cell line RAW 264.7 cells activated with LPS and IFN-γ to induce nitric oxide production and inflammatory response, and used etoposide to induce apoptosis. The inhibitory mechanism of NO production and the *in vitro* anti-inflammation by the TAM extract was limited to the measurement of iNOS and COX-2 protein levels, respectively. Anti-apoptosis was assessed by DNA ladders formation in agarose gel and the annexin V-PI staining using flow cytometry. Anti-nociceptive activity of TAM extract was investigated by using acetic acid-induced writhing and the tail flick tests. The scope of the study was summarized in Figure 1.

1.4 Expected results

1. Information on biological activities of the seed coat extract of *T. indica* which can be used as a basic pharmacological data for a consideration of its therapeutic potential in the future.

2. Data on inhibitory mechanism of nitric oxide production by the seed coat extract of *T. indica* on RAW 264.7 cells.

3. Scientific data that help to support and strengthen the knowledges and practices of Thai traditional medicine.

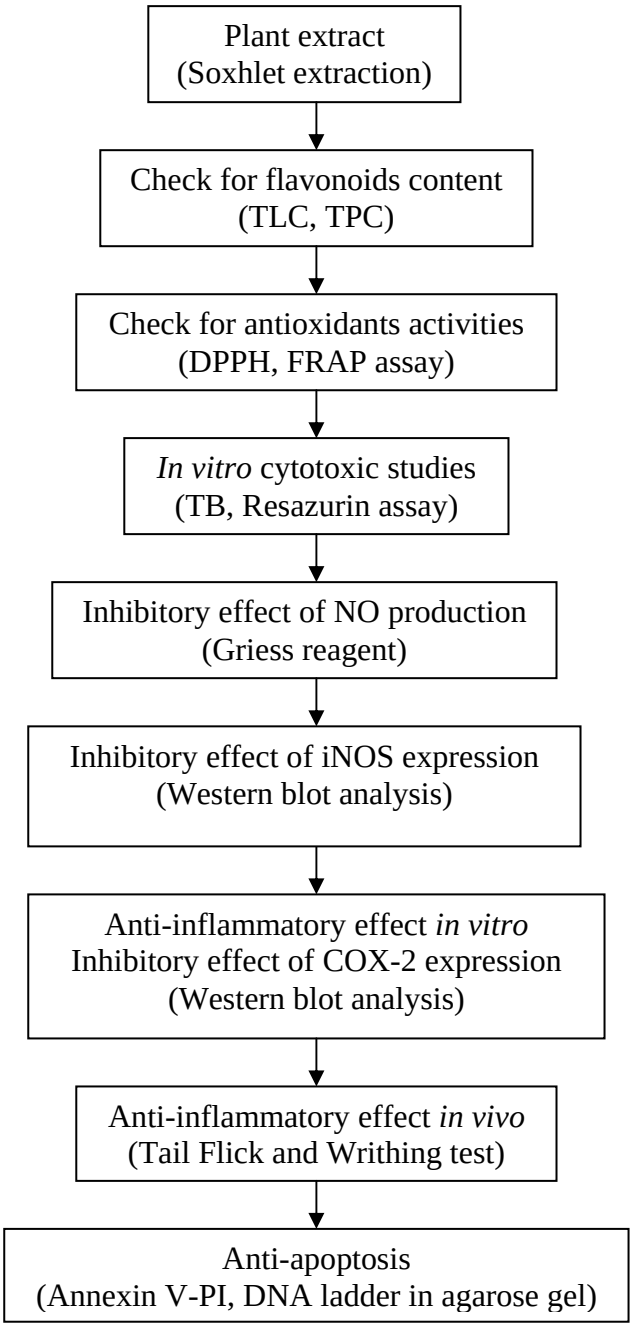


Figure 1 Scope and limitation of the studies.

CHAPTER II

LITERATURE REVIEWS

2.1 Biological significance of nitric oxide

Nitric oxide (NO, formula $^{\bullet}N=O$) is a simple, inorganic, gaseous free radical whose predominant functions are that of a messenger and effector molecules. In mammals, NO is synthesized by a family of enzymes referred as the nitric oxide synthases (NOS). NO is physiologically significant for its role in regulating vascular tone and in signaling neurotransmission (Furchgott and Zawadzki, 1980; Moncada *et al*., 1991). Nitric oxide is also an important component of the antineoplastic and antimicrobial armament of macrophages (Coleman *et al*., 2001). This highly labile and noxious gas is produced in large and sustained quantities by macrophages following exposure to a variety of immunologic and inflammatory mediators. The high-output production of nitric oxide is dependent on induction and expression of the inducible nitric oxide synthase (iNOS) expressed in macrophages (Fang *et al*., 1997). Endothelial cells and neurons also express unique forms of nitric oxide synthase as eNOS and nNOS, respectively. However, the expressions of both enzymes are constitutive and nitric oxide is produced at lower steady state levels than iNOS (Hevel *et al*., 1991). It is this low level of production that is biological significance, while overproduction may lead to circulatory shock, chronic inflammation and carcinogenesis (Hidaka *et al*., 1997). NO and its functions have been shown to be

more and more complex in physiological and pathological processes, for example, NO can regulate vascular tone, smooth muscle cell relaxation, neurotransmission, neuromodulation, apoptosis (Kim *et al*., 2004) and modulate mitochondrial energy generation (Moncada and Erusalimsky, 2002). NO also has been implicated in different mechanisms of diseases such as atherosclerosis (Barton and Haudenschild, 2001), asthma (Fischer *et al*., 2002), neurologic disorders and septic shock (Thiemermann *et al*., 1997). The isoforms of nitric oxide synthase and their major physiological functions and implications in various diseases are summarized in Figures 2 and 3.

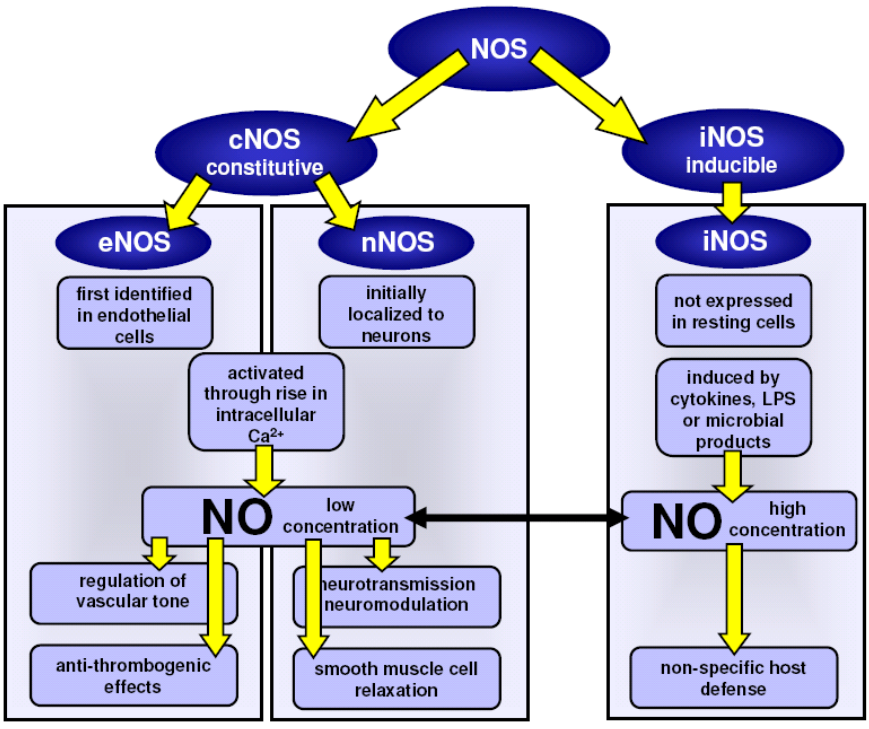


Figure 2 The isoforms of nitric oxide synthases. Two cNOS enzymes (eNOS, nNOS) are contrasted by a third, inducible NOS (iNOS) (Hemmrich *et al*., 2003).

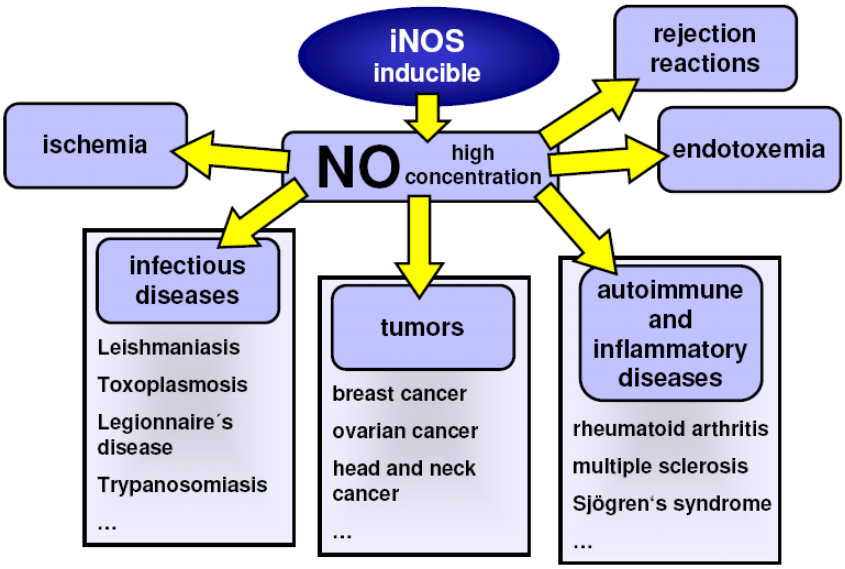


Figure 3 Implication of iNOS-derived NO in various human diseases. NO demonstrates its key roles in the human defense against adverse factors from the environment. Furthermore, many chronic inflammatory diseases are associated with sustained iNOS expression (Hemmrich *et al*., 2003).

The iNOS gene is located on chromosome 17 (Warpeha *et al*., 1999) with a sequence similarity between iNOS and nNOS of 53%, and iNOS and eNOS of 51%. Murine and human iNOS share a similarity of some 80% (Gonzalez-Gay *et al*., 2004). In contrast to the constitutively active isoforms, iNOS exerts its functions independent of Ca^{2+}. Calmodulin remains non-covalently bound to the iNOS complex and therefore constitutes an essential subunit of this isoform (Park *et al*., 2000). In common with other isoforms, consensus binding sites for NADPH, FMN and FAD exist (Cutruzzola, 1999). iNOS activity has been demonstrated in a wide array of cells and tissues, e.g. macrophages, chondrocytes, Kupffer cells, hepatocytes, neutrophils, pulmonary epithelium and vasculature (Bogdan, 2001). iNOS produces NO in response to a wide array of stimuli, most prominently endotoxin and endogenous pro-

inflammatory mediators. Regulation of NO production via iNOS necessarily occurs during transcription and translation, for once active, iNOS synthesizes NO until substrate depletion (Alderton *et al*., 2001). However, recent evidence suggested a constitutive expression of iNOS in the pulmonary epithelium and the small intestine (Guo *et al*., 1995), although these findings may also be explained by ongoing immune mechanisms in these critical contact barriers. Furthermore, sustained iNOS expression occurs in virus-infected lymphocytes (De Groote and Fang, 1995). iNOS gene expression and subsequent mRNA translation is controlled by an ever increasing number of agonists, especially pro-inflammatory mediators. The most prominent cytokines involved in iNOS stimulation are tumor necrosis factor-γ (TNF-γ), interleukin-1γ (IL-1γ) and IFN-γ. Furthermore, endotoxin LPS has been extensively investigated. Other stimuli include hypoxia (Bouton, 1999) and phorbol esters (Bouton and Demple, 2000). One important intracellular signal transduction pathway of these stimuli is the activation of NF-κB. An alternative pathway involves the janus tyrosine kinase (JAK) – signal transducers and activators of transcription (STAT) (Collins *et al*., 1995). In addition, the mitogen-activated protein kinases (MAPK) pathway leading to the activation of transcription factors such as activator protein 1 (AP1), activating transcription factor 2 (ATF2), cAMP-responsive elements, NF-κB, most likely contributes to iNOS gene expression. In most *in vitro*-studies, only a combination of multiple cytokines was able to elicit a profound iNOS gene expression, whereas a single stimulus exhibited only a moderate effect in specific cell types (Bourke and Moynagh, 1999). This suggests that two or more signal transduction pathways are necessary to fully upregulate iNOS expression.

Although numerous *in vitro* and *in vivo* studies suggest that endogenous NO synthesis inhibits tumor growth and metastases, recent data support the view that iNOS-derived NO promotes rather than inhibits growth and progression of solid tumors. Tumor cell expression of iNOS has even been found to correlate with the grading of some human tumors, such as breast, ovarian and head and neck cancer. In addition, iNOS-derived NO also plays an important role in ischemia, endotoxemia, and rejection reactions.

2.1.1 Monitoring iNOS expression and activity

Precisely monitoring of iNOS expression and activity are important for implementation of research results into clinical practice. Measurement of NO generation can be performed by different experimental setups. The rate of conversion of ^{3}H- or ^{14}C- labeled L-arginine into the respective labeled L-citrulline and subsequent spectrophotometry is a standard procedure for indirect confirmation of NO production. L-citrulline can be readily detected using ion-exchange procedures to separate substrate and product (Chan *et al*., 1997). Since NO activates guanylate cyclase, production of cGMP has been used as another indirect approach for measuring NO (Chan *et al*., 1997 and Gow *et al*., 1999). In addition, there are many spectrophotometric assays available for the determination of NO metabolites. In oxygenated solution, NO reacts with O_2 to form nitrite and nitrate which can be measured using the Griess reaction (Figure 4) (Kelm *et al*., 1997). Alternatively, nitrite can be processed with 2,3-diaminonaphthelene to generate the fluorescent product 1-(H)-naphthotriazole, resulting in a 50-100-fold higher sensitivity for detection of NO as compared to the classic form of the Griess reaction. Since NO

reacts directly with the ferrous iron of hemoglobin to yield the ferric form, methemoglobin concentrations can be measured spectrophotometrically to evaluate rate of NO synthesis *in vitro* (Kelm *et al*., 1997). Using a more sophisticated equipment, NO can be measured by chemiluminescence following the reaction with ozone (Moncada *et al*., 1991) by using a NO electrode since NO is electrochemically active in aqueous solution (Tsuda *et al*., 1994), or by electron paramagnetic resonance spectroscopy using hemoglobin as a spin-trap (Andree *et al*., 2001).

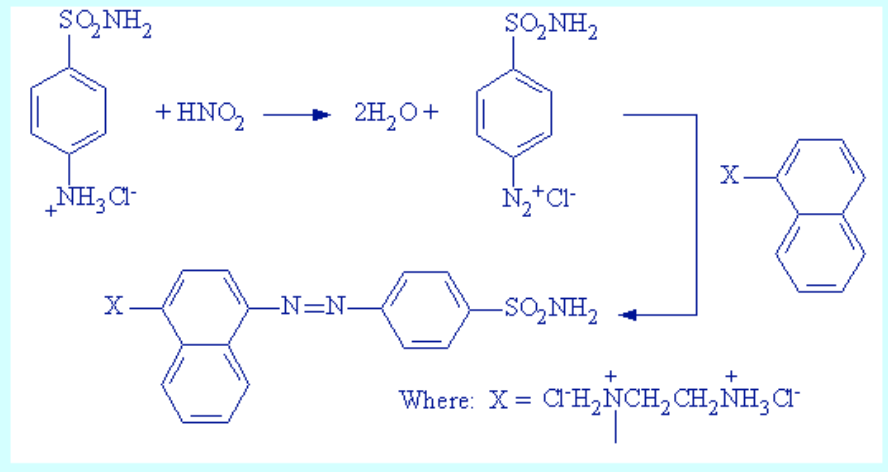


Figure 4 Mechanism of nitrite detection by Griess reagent. These reagents form an azo dye after about 20 min. The color intensity in an acidic solution (read at 540 nm) is directly proportional to the amount of nitrite. (available online at http://www.idealibrary.com)

Each of the above-mentioned techniques has its advantages and disadvantages. The choice of method depends on the application. The Griess reaction, for example, is brief, simple and inexpensive, but not very specific and sensitive making it useful for

the detection of iNOS activity but not for the detection of small amounts of NO produced by eNOS.

2.1.2 Activation of macrophages and ROS production

Macrophages play a central role in immune response as phagocytic cells capable of targeting and ingesting invading organisms. During infection, macrophages have the capacity to become activated by lymphokines and different bacterial products. LPS is a component of the outer membrane of gram-negative bacteria. As one of the most potent activators of monocytes, LPS can activate several signal transduction pathways through induction of G-proteins, cAMP dependent kinase, protein kinase C, tyrosine kinase, PI3 kinase, and the ERK, p38, and JNK mitogen activated kinase (MAPK) families (Furukawa *et al*., 1987). In addition, LPS stimulated macrophages produce TNFα which initiates a cascade of gene transcription and transcriptional de-repression (Goodwin and Ceuppens, 1983). The culmination of this broad signaling response is the synthesis and secretion of inflammatory mediators such as complement components, prostaglandins, interleukins, and pro-inflammatory cytokines (Goodwin and Ceuppens, 1983). The combination of LPS (20 ng/mL) with different concentrations of IFN-γ resulted in a synergistic effect, which enhanced the amounts of NO released by macrophages compared to the endotoxin alone (Scuro *et al*., 2004).

To enhance their microbicidal and tumoridical functions, activated macrophages undergo a respiratory burst that produces such reactive oxygen and nitrogen species as $^{\bullet}O_2$, H_2O_2, NO and $ONOO^-$ (Grimble, 1996). It is important to note that stimulated macrophages release these reactive species in quantities that are orders of magnitude greater than non-phagocytic cells. The large amount and broad

reactivity of the ROS produced by activated macrophages assist these cells with their essential protective duties, but unfortunately, also contribute to the onset of pathological conditions like bacterial sepsis, ischemia/reperfusion injury, and chronic inflammatory diseases (Harris *et al*., 2002). The capacity of the macrophage to regulate and defend itself against oxidant-induced damage is therefore very important, not only to protect and preserve its own function, but also to protect the surrounding tissue parenchyma from oxidative damage. As protection from the deleterious effects of reactive species, macrophages are equipped with a multi-tier antioxidant enzyme system, consisting of catalase, superoxide dismutase, and selenoproteins TrxR and GPx, to name a few (Heiss *et al*., 2001). Without optimal antioxidant protection, the macrophage would be exposed to excessive levels of reactive oxidant species, which may prolong the activated state and contribute to the development of DNA mutations, tissue damage and a state of chronic inflammatory conditions (Brown *et al*., 1993).

2.1.3 Nitric oxide in infection and inflammation

While all cells produce reactive oxygen and nitrogen species (ROS/RNS) such as superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2) NO and peroxynitrite ($ONOO^{-}$) during normal respiration, activated macrophages produce elevated levels of these species as components of the immune respiratory burst. In addition, ROS/RNS are important members of cellular signaling pathways, particularly those leading to the inducible activation of nuclear factor kappa B (NF-κB). NF-κB is a redox sensitive transcription factor, which is associated with the expression of various immediate-early genes including COX-2 and iNOS. The products of these enzymes, prostaglandins and NO, together with the acute phase cytokines regulate the initiation and duration of the inflammatory response. Thus, the excess production of ROS/RNS

by macrophages may contribute significantly to the pathophysiology of inflammatory disorders if it is not carefully controlled by cellular antioxidant defense systems.

2.1.3.1 Mediators of Inflammation

The redox regulation of transcription factors, their target genes and gene products are important processes in the normal inflammatory response, as well as in the development of immune disorders. As previously discussed, NF-κB is the major redox responsive transcription factor and it binds with a consensus sequence located in the promoters of nearly all genes involved in the initiation of an inflammatory response (Kleinert *et al*., 1996). COX-2 and iNOS are two such genes, whose products exert broad effects in inflammation and the development of inflammatory disease (De Groote and Fang, 1995).

Prostaglandins, the products of the COX-2 pathway, promote inflammation by increasing vascular permeability, vasodilation, and the secretion of proinflammatory cytokines that direct cellular migration to the site of injury (Issekutz and Movat, 1982). Sustained, elevated prostaglandin levels are connected with the onset of chronic inflammation and cancer (Ochoa *et al*., 1992). Cyclooxygenase catalyzes the first committed step in the formation of prostaglandins and thromboxane from free arachidonic acid (Herschman, 1996). It exists in two isoforms: COX-1 and COX-2. COX-1, for the most part, is constitutively expressed in all cell types and is involved in normal kidney, gastrointestinal and reproductive functions (Smith and Langenbach, 2001). COX-2 may be basally expressed in some tissues, but is largely inducible by a wide variety of mitogens, hormones, cytokines and other stimuli and is thus associated with inflammation and disease (Krakauer, 2004). Macrophages secrete prostaglandins following stimulation with LPS, primarily due to the induced

transcription of the COX-2 gene and production of COX-2 enzyme (Ishimura *et al*., 2004). In addition to responding to stimulation from LPS and cytokines, COX-2 is also subject to redox regulation at two levels. First, low concentrations of peroxides are required for activation, while higher amounts may affect enzyme activity (Surh *et al*., 2001). In addition, the gene encoding for COX-2 protein is under the regulation of NF-κB, which, again is influenced by oxidant status. The COX-2 promoter in both human and mouse genes contains two κB enhancer elements, to which NF-κB binds to initiate transcription (Heinrich, 2002).

NO is a central mediator of innate (nonspecific) host defense. It is involved in various responses to infection and tissue damage. Resistance to bacteria, viruses, parasites, and fungi is often conferred via iNOS induced by a spectrum of pro-inflammatory cytokines and bacterial products, including TNF-β, IFN-γ, IL-1β or LPS (Collins *et al*., 1995). In contrast, some diseases like influenza virus pneumonia or intestinal helminth infections are exacerbated by NO-induced "inappropriate" tissue damage similar to COX-2. Transcriptional activation of iNOS is considered to be the major means for regulating nitric oxide production and is also subject to redox modulation (Steinbach *et al*., 2000). Antioxidant enzymes inhibit iNOS mRNA synthesis, while hydrogen peroxide generated by LPS activated macrophages up regulates iNOS protein expression (Collins *et al*., 1995). Several transcription factors have been identified as important in the activation of the murine iNOS gene, including NF-κB. Similar to the COX-2 promoter, the human and mouse iNOS gene promoters both contain two NF-κB binding sites (Surh *et al*., 2001) (Figure 5).

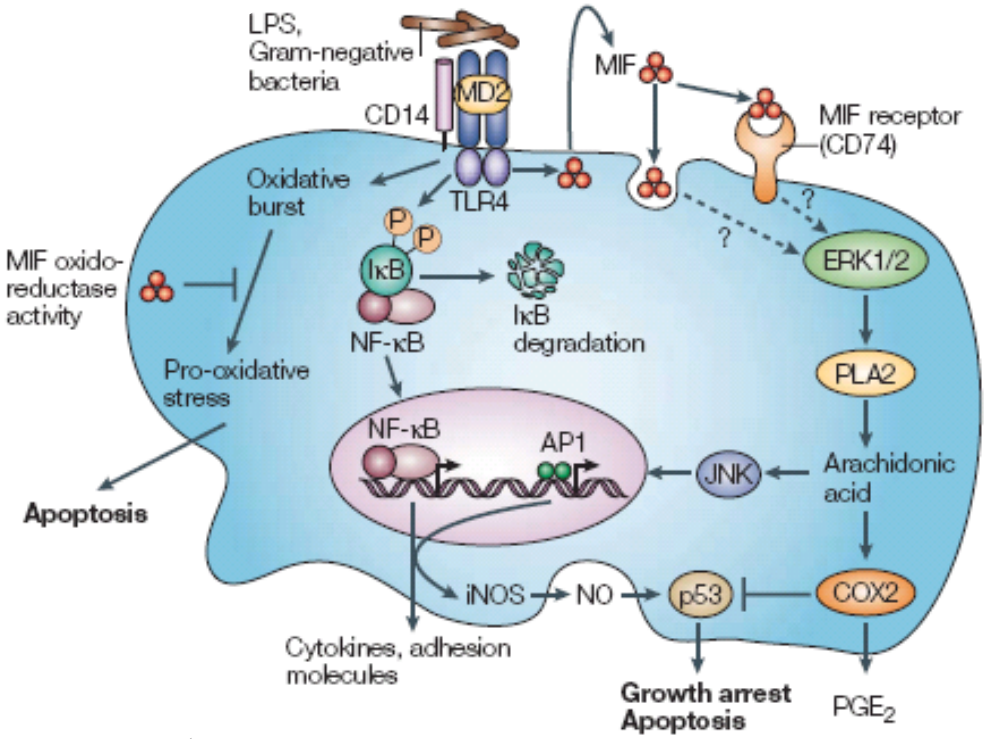


Figure 5 The induction and regulation of inflammatory responses of innate immune cells by migration inhibitory factor (MIF). MIF upregulates the expression of TLR4 by macrophages allowing rapid recognition of endotoxin-containing bacteria, which promotes the production of cytokines (including MIF), nitric oxide (NO) and other mediators. After it is released, MIF activates a cascade of events consisting of the phosphorylation of ERK1/ERK2, the induction of cytoplasmic phospholipase A2 (PLA2), arachidonic acid, JUN N-terminal kinase (JNK) activity and prostaglandin E2 (PGE2). Through the generation of oxidoreductase activity and cyclooxygenase 2 (COX-2), MIF prevents activation-induced apoptosis mediated by the oxidative burst and by p53 (Surh *et al*., 2001).

2.1.4 NO as an activator in apoptotic pathway

$NO^{\bullet}$ is a mediator of a lot of physiological processes. It is involved in muscle energetics through stimulating glucose delivery with its vasodilatory effect, and promoting glucose uptake. However, it down-regulates glycolysis, inhibits CK154, and modulates the electron transport chain and ATP-synthesis as well. Some of the actions of NO depend on its concentration, being anti-apoptotic at low, physiological concentrations, while promoting cell death at high concentrations.

In contrast to its role as an antioxidant providing protection against ROS and preserving cross-bridge function at physiological levels, at high intracellular levels, $NO^{\bullet}$ acts as an oxidizing agent. At moderate levels, inhibition of the electron transport chain by $NO^{\bullet}$ leads to increased electron transport chain derived superoxide. At still higher levels, $NO^{\bullet}$ reacts with these superoxide molecules to form peroxynitrite. Among the other harmful effects of peroxynitrite are damage to proteins, cell membrane (lipid peroxidation), endothelium, and nucleic acids, thereby activating poly (ADP-ribose) glycohydrolase (PARP). It can decrease the GSH concentration in the cell and disturb the anti-oxidant system, or stimulate the leakage of protons from mitochondria and interfere with the coupling between oxidative phosphorylation and ATP synthesis. Through stimulation of several Ca^{2+} channels. $NO^{\bullet}$ induces an increase in $[Ca^{2+}]$. Peroxynitrite as well as NO can also stimulate mitochondrial permeability transition pore opening, and cytochrome c release, initiating the mitochondrial apoptotic pathway. $NO^{\bullet}$ can also activate caspases, stimulate mitochondrial phospholipid oxidation, upregulate pro-apoptotic gene expression, transcription factors such as NFκB, apoptosis inducing ligands like FasL, and pro-

apoptotic proteins like Bax, and down-regulate anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1/A1.

2.2 Apoptosis (Programmed Cell Death)

2.2.1 Morphologic features of apoptotic cells

Apoptosis which is a term that describes regulated cell death is fundamental feature of many processes including normal development, homeostasis, and disease. Apoptosis can occur in a single cell surrounded by healthy neighbors. Characteristics of apoptotic cells are cell shrinkage, DNA condensation and fragmentation, and maintenance of organelle integrity. The cell membrane remains largely intact, but phosphatidylserine is translocated to the outer leaflet to make apoptotic cells recognizable by macrophages. Finally, cell membrane blebbing occurs, an active process thought to be driven by contractile forces generated by the actin-myosin filaments, and the cell fragments into smaller parts, termed apoptotic bodies, which are phagocytosed by macrophages (Allen *et al*., 1997). How much apoptosis contributes to pressure ulcer formation remains a question, since apoptotic cells are normally orderly removed. However, this neat way of cell death requires energy. Therefore, apoptosis cannot occur when cells are deprived of both oxygen and glucose. But Hockenbery (2002) found that supply of only glucose, allowing anaerobic glycolytic ATP generation to occur, was already sufficient to execute apoptosis. After an insult that does not deplete the cell's energy, apoptosis is activated either extrinsically or intrinsically. In the first case, an external signal induces receptor aggregation in the cell membrane, forming a death-inducing signaling complex. The intrinsic pathway is activated by an apoptotic stimulus inside the cell (Figure 6).

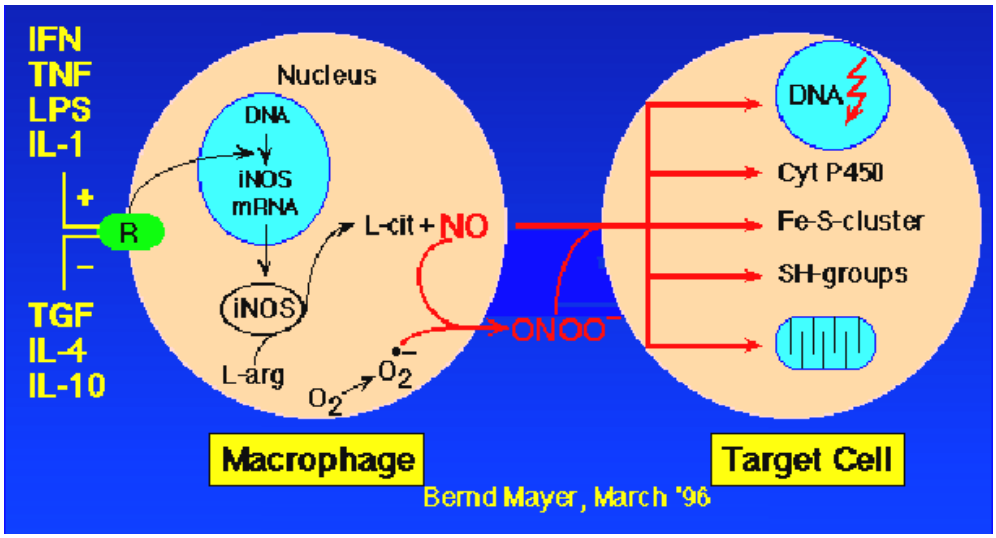


Figure 6 Nitric oxide as one of mediators that activate the intrinsic pathway of apoptosis (Mayer *et al*., 1996).

2.2.2 Detection or quantitation of apoptosis

Apoptosis was initially characterized by morphological changes of dying cells. Phosphatidyl serine exposure in the outer leaflet of the cell membrane is the first evidence of morphological changes which are later seen as shrinkage, condensation of the nucleus and membrane blebbing. Cells undergoing apoptosis fragmented into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or other neighboring cells without generating an inflammatory response. This is in contrast to necrosis, which results from gross insult to the cell, characterized by cell swelling, release of lysosomal enzymes, cellular disintegration and inflammation (Cotran *et al*., 1999). Apoptosis has now established its importance in numerous areas of biology particularly its major constitution in health and disease. This fact underlies the critical need for learning the most efficient and effective methods to study it. Although the morphology of death during development had been

recognized as being different from necrosis for many years, characterization of this difference remained elusive. However, studies which detailed the cytological description of changes in cells undergoing "apoptosis" and its critical role in maintenance of tissue turnover, size, and shape in both normal and pathological processes (Kerr *et al*., 1972 and Wyllie *et al*., 1985) have led the way in establishing a need to further investigate this essential mode of cell death. It is now clear that apoptosis is a distinct mode of programmed cell death defined by a set of characteristic morphological and biochemical features. It is known to serve many critical functions, such as cell deletion during embryonic development, balancing cell numbers in continuously renewing tissues, hormone–dependent involution in the adult, immune system development, selective immune cell deletion, and many other physiologic processes. Furthermore, numerous pathologically induced conditions such as Alzheimers, autoimmune disease, cancer, and AIDS, often show varying levels of apoptosis, with greatest significance lying in whether dysregulation of apoptosis is a primary event in the pathology and subsequent clinical sequelae.

In this review, we will discuss the morphology of apoptosis and discriminate it from necrosis, discuss relevant biochemical events integral to the apoptotic process, and, finally, describe how to identify characteristic morphological and biochemical features in cells undergoing apoptosis using selective and specific techniques. The assays used in this study were DNA fragmentation and annexin V and propidium iodide (PI) binding

1) DNA Fragmentation

Cells that undergoing apoptosis display their DNA fragmented into oligonucleosome and can be analyzed by separation in agarose gel electrophoresis,

stained with ethidium bromide (EtBr) and visualized under UV light. Therefore, DNA fragmentation analysis by agarose gel electrophoresis is considered a biochemical hallmark for the apoptotic cells. This assay is highly specific but not very sensitive, often requiring DNA from a large number of cells to analyze the fragmentation pattern. This method in not suitable for every cell type, since apoptosis in some cell types can occur in the absence of internucleosomal cleavage and in the presence of fragmentation of DNA about 300 bp and/or 50 kbp. If a DNA ladder is present, then death by apoptosis is almost a surety (Figure 7); however, if absent, then death by apoptosis cannot be ruled out since larger fragments have not been tested for. To do this, one can use pulse-field gel electrophoresis techniques, such as field-inversion gel electrophoresis, which can accurately evaluate 300 bp or 50-kbp fragments (Walker *et al*., 1991; Martin *et at*., 1996; Brown *et al*., 1993). Therefore, at least one other biochemical assay, such as TUNEL or annexin-V labeling, should be conducted with the morphological assessment either by DNA fragmentation or by microscopy.

If a characteristic ladder pattern cannot be seen, cleavage of DNA into large fragments might be occurring and caused the exclusive of internucleosomal cleavage. Then, adjustments may need to be made to the DNA extraction procedure. Alternatively, there is always a possibility that exogenous nucleases are degrading the DNA if all equipments are not entirely sterile. In contrary, DNA can be lost in the interphase during extraction. Therefore, one needs to be extremely careful when removing the upper aqueous phase of DNA during extraction. Another way is to use more cells during extraction which should increase the extracted DNA content (Allen *et al*., 1997).

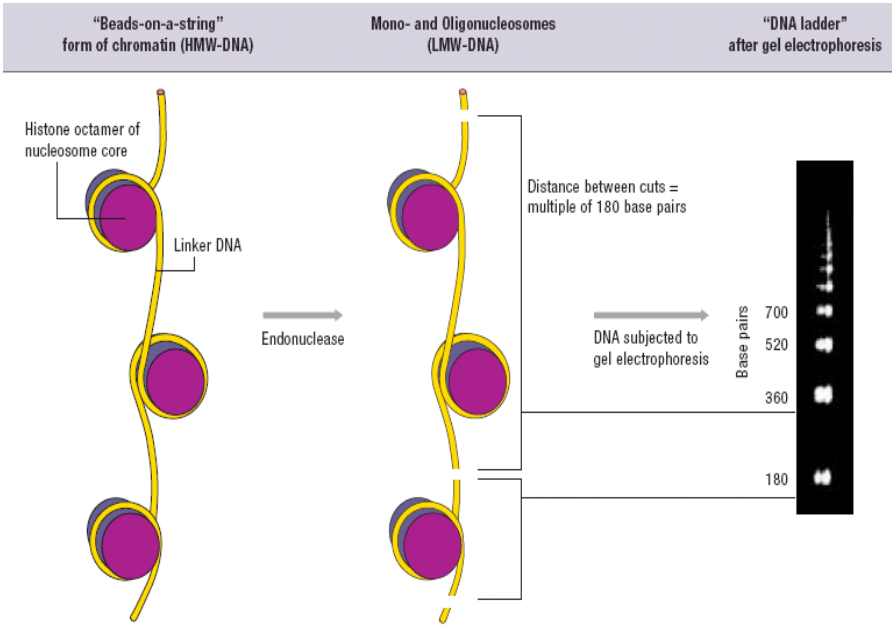


Figure 7 Analysis of DNA fragmentation by agarose gel electrophoresis. The fragmentations of DNA into different sizes of internucleosomes are represented as DNA ladders in agarose gel electrophoresis. However, apoptosis in some cell types can occur in the absence of cleavage and in the presence of fragmentation of DNA about 300 and/or 50 kbp (Brown *et al*., 1993).

2) Annexin V and Propidium Iodide

Annexin V, an anticoagulant with other biological effects, is a member of a family of proteins which exhibits Ca^{2+}dependent binding to negatively charged phospholipids, and it binds with highest affinity to phosphatidylserine (PS) (Andree *et al*., 1990) (Figure 8). Since PS translocation occurs early in apoptosis when cell membrane integrity is still intact, flow cytometric analysis using fluorescein isothiocyanate-(FITC) labeled annexin V is useful as a quantitative measurement of early apoptosis (Homburg *et al*., 1995; koopmen *et al*., 1994).

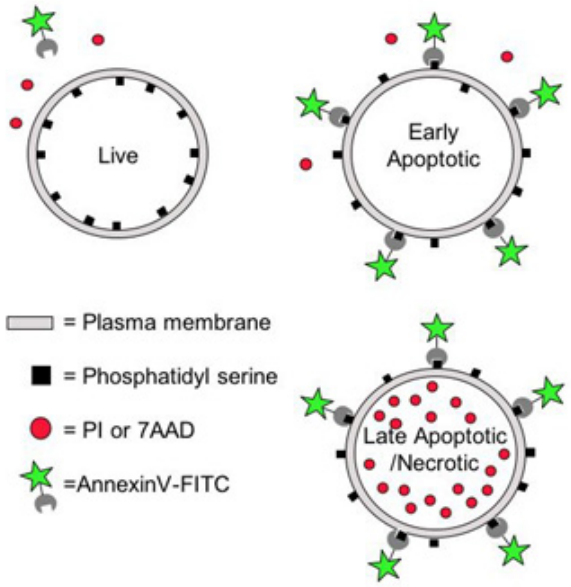


Figure 8 The detection of early apoptosis cells. Annexin V preferentially binds to early apoptotic cells whereas PI or 7AAD bind late apoptotic and necrotic cells (Homburg *et al*., 1995).

Flow cytometric determination of programmed cell death has also been used with the DNA intercalating dye PI and analyzed for cell cycle. When cells are permeabillized, treated with PI, and analyzed for DNA content, a sub-diploid (sub-G_0 /G_1) peak can be seen since the DNA in apoptotic cells stains particularly less intensely. Annexin V binding has been shown to reveal a much higher level of cells committed to apoptosis and that it occurs prior to any nuclear changes (Martin *et al*., 1996). This emphasizes the fact that PS externalization binding by annexin V is highly sensitive to detect early apoptotic cell. Unlike most of the apoptotic cells which retain their plasma membrane integrity, necrotic cells lose their plasma membrane integrity. A benefit of annexin V and PI method are that stained cells do not need to be fixed or permeabilized, thus PI staining enters only necrotic cells but is excluded

from apoptotic cells. Therefore, it can discriminate between necrosis and phases of apoptosis prior to secondary necrosis, one can test PI dye exclusion simultaneously with FTTC-labeled annexin V binding (Vermes *et al*., 1995). When annexin V binding is simultaneously occurring with PI uptake, necrosis may be the prevalent form of cell death unless cells in suspension are being collected and flow cytometry gates are set up too inclusively (too broad). Morphological analysis should resolve this issue. This assay can also be useful for measuring the kinetics of apoptosis (Martin *et al*., 1995).

2.3 Antioxidant

Antioxidants act to sequester free radicals and render them harmless. An antioxidant is an agent that prevents or inhibits oxidation. They are naturally occurring or synthetic substances that help protect cells from the damaging effects of oxygen free radicals (Davis *et al*., 1997). Antioxidants donate electrons to free radicals to convert them into harmless atoms and molecules. Several nutrients have antioxidant properties. These include vitamin E, manganese, glutathione, Co Q and vitamin C. These antioxidants all appear to be involved in the elimination of carbon-centered radicals and peroxyl radicals (Groff and Gropper, 2000).

2.3.1 Actions of hydrophilic and lipophilic antioxidants

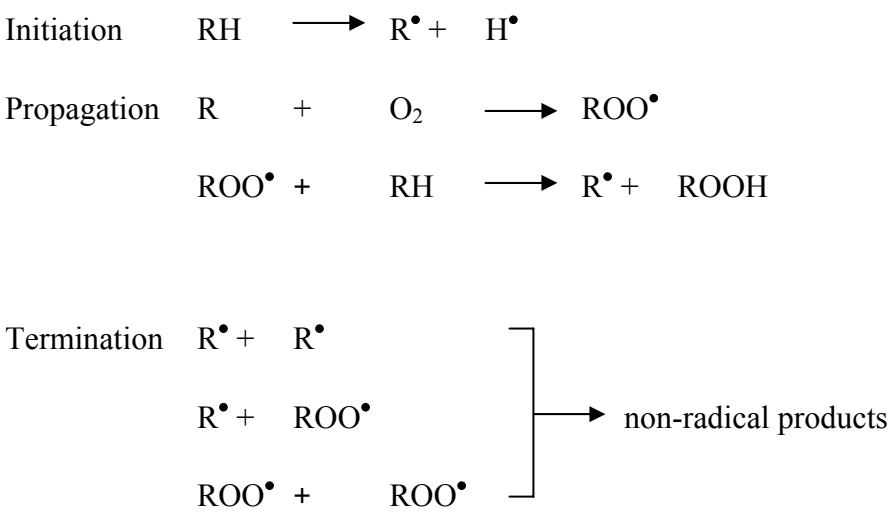
In biological systems, an antioxidant can be defined as any substance that, in low concentration compared with the oxidizable substrate, significantly delays or prevents oxidation of that substrate. The substrate, i.e. the oxidizable compound, is usually a lipid, but can be also a protein, DNA, or carbohydrate. In the case of lipid oxidation, the main mechanism of antioxidants is to act as radical chain-breakers.

Another mechanism is to act as preventive antioxidant oxygen scavenging or blocking the pro-oxidant effects by binding proteins that contain catalytic metal sites (Frankel and Meyer, 2000). The complexity of antioxidants needs to taken into account in free radical assays in testing for antioxidant activity. The complexity of a multicomponent oxidative biological material is overlooked compared to oxidation model systems that are models of lipids in their real environment. There is, moreover, no single test to evaluate the antioxidant activity of a compound. The antioxidant activity may vary widely depending on the environment of the lipid substrate. It has been shown that hydrophilic antioxidants are more effective in lipid systems, whereas lipophilic antioxidants work better in emulsions where more water is present (Frankel and Meyer, 2000). In lipophilic environment, hydrophilic antioxidants are oriented to oil-air interface, and give better protection against lipid oxidation than in a more hydrophilic environment, where hydrophilic antioxidants tend to be diluted and thus act poorly against lipid oxidation. Lipophilic antioxidants, in turn, are diluted in lipid environment and are not suitably oriented to the oil-air interface to inhibit the oxidation (Frankel *et al*., 1994). In testing antioxidants in a radical scavenging test, it should be remembered that this test evaluates only the radical scavenging activity of the compound, and not the other antioxidant mechanisms, such as metal chelation. In addition, the antioxidant action is more complex in real foods and biological systems where several mechanisms become effective (Halliwell and Gutteridge, 1989).

2.3.2 Antioxidant activity and mechanism

Lipid autoxidation involves a free radical chain reaction that is generally initiated by light, heat, radiation, metal ions etc (Shahidi and Naczk, 1995). The route

of autoxidation can be described in terms of initiation, propagation and termination reactions as illustrated as follows:



Oxygen and reactive oxygen species (ROS) are one of the major sources of primary catalysts that initiate oxidation *in vitro* and *in vivo* systems. Examples of oxygen centered free radical, known as ROS includes superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$) and $NO^{\bullet}$. Free radicals are responsible for aging and various chronic diseases since they attack the lipids of the cell membrane, modify protein and damage DNA (McCall and Frei, 1999). Many studies have shown that oxidative damage to cells and tissues are involved in chronic diseases development including cancers, aging, cataract, parkinsonism, stroke, myocardial infraction, and atherosclerotic cardiovascular disease (Cheeseman, 1993; Hodgson *et al*., 1996).

It is reported that antioxidants inhibit oxidation by acting at different stages in the oxidation reaction and they may have multiple mechanisms of action (Halliwell and Gutteridge, 1989; Dziezak, 1986):

1. Decreasing localized oxygen concentrations (oxygen scavenger)

2. Preventing chain initiation by scavenging initiating radicals (free radical inhibitor)

3. Binding catalysts such as metal ions to prevent initiating radical generation (metal inactivation)

4. Decomposing peroxides so that they cannot be reconverted to initiation radicals (peroxide decomposer)

5. Chain-breaking to prevent continued hydrogen abstraction by active radicals (chain breaker).

NO spontaneously reacts with triplet oxygen ($^3O_2$), decomposed and produce stable nitrite (NO_2^-) and nitrate (NO_3^-), which are indirect markers in evaluation of NO *in vitro*. By oxyhemoglobin, nitrite (NO_2^-) is oxidized to nitrate (NO_3^-), a major species in plasm (Jang and Murrell, 1998) Nitrite and nitrate produce carcinogenic nitrosamine by non-enzymatic N-nitrosation of primary and secondary amines. NO has been found to contribute to many diseases, such as various cancer, atherosclerosis, arthritis, cell apoptosis and necrosis. Specifically, NOs are related to the activities of cyclooxygenases, COX-1 and COX-2. In inflammation, macrophages are reported to produce increased amount of NO and $O_2^{\bullet-}$ generates $ONOO^-$, which enhance the activities of COXs and stimulates the eicosanoid productions (Ischiropoulos *et al*., 1992; Salvemini *et al*., 1993) Futhermore, $ONOO^-$ has been found to be a highly reactive oxidant and able to cause DNA damage (Vance *et al*., 1994).

Recently, several studies have been performed on NO formation. Flavonoids, such as apigenin, wogonin, luteolin, tectorigenin, and quercetin are reported to inhibit

NO production in the macrophage cell line RAW 264.7 (Sooliman and Mazzio, 1998; Kim *et al*., 1999). According to the study of Sadowska-Krowicka *et al*. (1998), genistein inhibited LPS-induced nitrite production in cultured macrophages and protected against LPS-induce necrosis despite its ability to cause apoptosis. Moreover, genistein, a know tyrosine kinase, is reported to suppress iNOS activity and/or iNOS gene expression (Sheu *et al*., 2001).

It is suggested that the reduction in production should result from the scavenging of NO radicals, direct inhibition of iNOS enzyme activity, and/or inhibition of iNOS gene expression (Sheu *et al.*, 2001; Park *et al.*, 2000). Therefore, suppression of NO level by phytochemicals may be a new and efficient approach for the treatment of inflammation and cancer.

2.3.2.1 DPPH free radical scavenging assay

Many antioxidant assays use accelerated oxidative conditions in lipid system by using high temperature and a high oxygen supply. The risk of degradation with such conditions during these tests is high for many antioxidants. In addition, this condition itself provokes lipid oxidation and such tests are not always representative of the natural lipid oxidation in food and human tissues (Bondet *et al*., 1997). Antioxidative activities are evaluated by various methods. The 2,2-diphenyl-l-picryl-hydrazyl radical ($DPPH^{\bullet}$) scavenging assay is widely used for its simplicity, ease, speed and sensitivity without the risk of thermal degradation of molecules tested, despite its biological irrelevance. The purple color $DPPH^{\bullet}$ is a relatively stable in alcoholic media and reacts with free radicals or other hydrogen donors, which result in a discolored DPPHH (Figure 9). The DPPH assay can be used to evaluate the

antioxidant capacity of antioxidants to donate hydrogen during a free radical attack (Liangli, 2002).

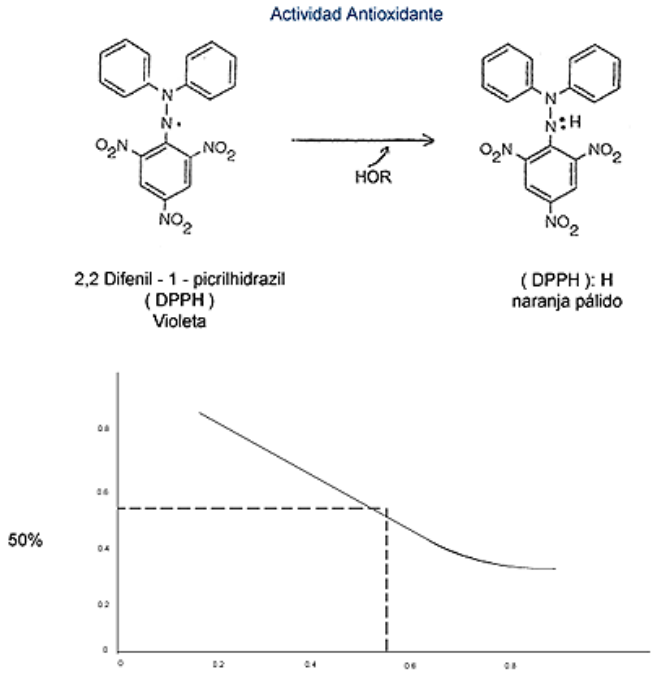


Figure 9 The 2,2-diphenyl-l-picryl-hydrazyl radical (DPPH$^{\bullet}$) scavenging assay. The purple color of DPPH$^{\bullet}$ is relatively stable in alcoholic media and reacts with free radicals or other hydrogen donors resulting in a discolored DPPHH (Liangli *et al*., 2002).

2.3.2.2 Principle of Ferric Reducing Antioxidant Power (FRAP)

Benzie and Strain (1996) have developed the FRAP method by considering the total antioxidant power as total reducing power since the antioxidants reduce the oxidants which can damage substrates. In FRAP, antioxidants reduce ferric tripyridyltriazine (Fe^{III}-TRTZ) complex to ferrous form, which has an intense blue

color at low pH of 3.6. The ferrous form can be monitored by measuring the change of absorbance at 593 nm. The absorbance value is directly related to the reducing power of antioxidants (Luengthanapol *et al*., 2003). Like ORAC, Trolox is used as the standard for comparing the reducing properties among samples. However, ascorbic acid can be used as the standard as well (Saha *et al*., 2004).

2.3.3 Antioxidant activity and seed coat color

Many studies have associated phenolic antioxidants with the color pigments of food plants. Comis (2000) stated that people who pay attention to the colors of foods they cook and serve are enhancing not only visual and gustatory pleasure, but nutritional effect as well. Tsuda (1993) screened various species of beans (*Phaseolus vulgaris* L.) for antioxidant activity, and several of them were markedly active, particularly the red and black types. The white beans revealed very weak activity. He stated that anthocyanins in bean seed coats were responsible for high activity of the colored types. Another study conducted by Amarowicz (1996) indicated that extracts obtained from five species of legumes with colored seed coat, pea, faba bean, lentil, everlasting pea, and broad bean, are characterized by high antioxidant activity. They also concluded that antioxidant substances in legumes are present mainly in the seed coat. Accumulation of flavonoid intermediates has been reported in unpigmented seed coats of legumes, barley (*Hordeum vulgare* L.) and *Arabidopsis thaliana* (L) Heynh. Flavones, flavonols and dihydroflavonols were reported to accumulate in the seed coats of white bean (*Phaseolus vulgaris*) and *Vicia faba* L. cv Blandine, whereas only proanthocyanidin was present in the dark-seeded *Vicia faba* cv. Alfred (Beninger and Hosfield, 1998; Beninger *et al*., 2000). Flavonoids found in an extract from the bean seed coat were strong antioxidants. A

genetic link was also found between bean color and flavonoids (Comis *et al*., 2000). Beninger and Hosfield (1999) stated that the normally colorless proanthocyanidin in beans undergoes secondary changes during seed maturation to form insoluble compounds with the cell wall and other phenolics in the seed coat, causing darkening. This darkening process was also proposed for proanthocyanidins in the brown testa of the caryopses of sorghum (Stafford *et al*., 1990). Seeds are exposed to oxidative damage due to oxygen, UV light, and other environmental factors. The seed coat contains numerous bioactive compounds, including polyphenols, which have antioxidant properties that protect the seeds against oxidative damage (Osawa *et al*., 1985). Troszyńska (2002) reported that extract from pea (*Pisum sativum* L.) seed coat exhibited pronounced antioxidant activity. Fractional separation of the extract indicated that it consists of various phenolic antioxidants such as tannins, flavonoids: flavone and flavonol glycosides and some phenolic acids (benzoic and cinnamic acids and cinnamic acid derivatives). They stated that the colored seed of pea is protected against oxidative damage by its seed coat antioxidant constituents. They concluded that phenolic substances occurring in the seed coat of colored pea cannot be neglected as a source of antioxidants. There are tannins in the seed coats of beans, with negligible amounts in the cotyledons (Deshpande *et al*., 1982). Bean varieties have different amounts of condensed tannins depending on the color of their seed coats. The white varieties contain lower concentrations of tannins than those with red, black or bronze seed coats (Elias *et al*., 1979). Kadam *et al*. (1982) reported that there are less tannins in mature bean seeds than in younger ones due to the polymerization of polyphenolic compounds to high-molecular weight insoluble polymers.

Phenolic compounds exist widely in plants. They are plant secondary metabolites, and they have an important role as defence compounds. Although the exact contribution of these secondary metabolites is still unclear, phenolic compounds are known to be important in the survival of a plant in its environment (Puupponen-Pimi *et al*., 2005). In addition to plants, phenolics exhibit several properties beneficial to humans. Several plant-derived medicines, which can prevent or cure diseases, are rich in phenolic compounds (Scalbert *et al*., 1993). In particular, phenolic compounds have been shown to exhibit protection against coronary heart disease and carcinogenesis (Hertog *et al*., 1995). They can be classified into the following subgroups: phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes, and complex phenolic polymers (Dewick *et al*., 2001).

Thin layer chromatography (TLC) has been applied in separating and identifying the phenolic acids in rapeseed meals (Fenton *et al*., 1980; Krygier *et al*., 1982). Usually TLC is used to control the purity of samples by gas chromatography. The TLC plates are coated with silica gel IB2-F or G-25 and fluorescent indicator UV-254. Fenton *et al*., 1980 used several solvent systems, namely, chloroform-formic acid and acetic acid (60:20:8:12), butanol-acetic acid-water (6:1:2) and butanol-acetic acid-water (4:1:5) for separating the polyphenolics from the unhydrolyzed extracts. Limitation on the use of TLC in analysis is the difficulties in quantification, as noted by Cai and Arntfield (2001). Quantification of the compounds requires some additional methods, such as purification with Sephadex and semi-preparative HPLC.

Anthocyanidins (flavan-3-en-3ols) are flavonoid groups, which commonly exist as glycosides and acylglycosides known as anthocyanins.

Anthocyanins are responsible for producing the blue and red colorations in fruits such as berries, cherries, and plums, vegetables such as red cabbage and radishes, and some grains, including sorghum (Herman 1976; Gous 1989). Data on anthocyanins in most cereals is limited, however, anthocyanin compounds such as apigeninidin-5-glucoside and luteolinidin-5-diglucoside (Nip and Burns, 1971) have been isolated from white and red sorghums; apigeninidin and luteolinidin in black sorghums (Gous 1989). Anthocyanin color is pH influenced: anthocyanins give off a red pigmentation at pH 3.5, and as the pH increases, anthocyanin coloring shifts to blue (Pierpoint *et al*., 1986). Anthocyanins also contribute some of the beneficial effects of consuming fruits and vegetables (Wang *et al*., 1997). Anthocyanins are vasoprotective (Lietti *et al*., 1976) and can decrease the onset of diabetes (Karaivanova *et al*., 1990). Anthocyanins also have anti-inflammatory (Lietti *et al*., 1976), anti-cancer, and chemo-protective properties (Karaivanova *et al*., 1990). Proanthocyanins, tannins, anthocyanins, and other flavonoids are partially absorbed in the human body (Pietta *et al*., 2000; Ross and Kassum, 2002). Tannins can be absorbed in the small intestine, but only as small oligomers (Deprez *et al*., 2000) (Figure 10). Most flavonoids are not absorbed directly, but are primarily degraded by large intestine microflora. Bacterial enzymes are able to cleave flavonoids and produce several phenolic acids which can be absorbed through the large intestine and enter circulation (Pietta *et al*., 1997 and Deprez *et al*., 2000). This information suggests that tannins and anthocyanins may be digestible and bioavailable to the human body as antioxidants.

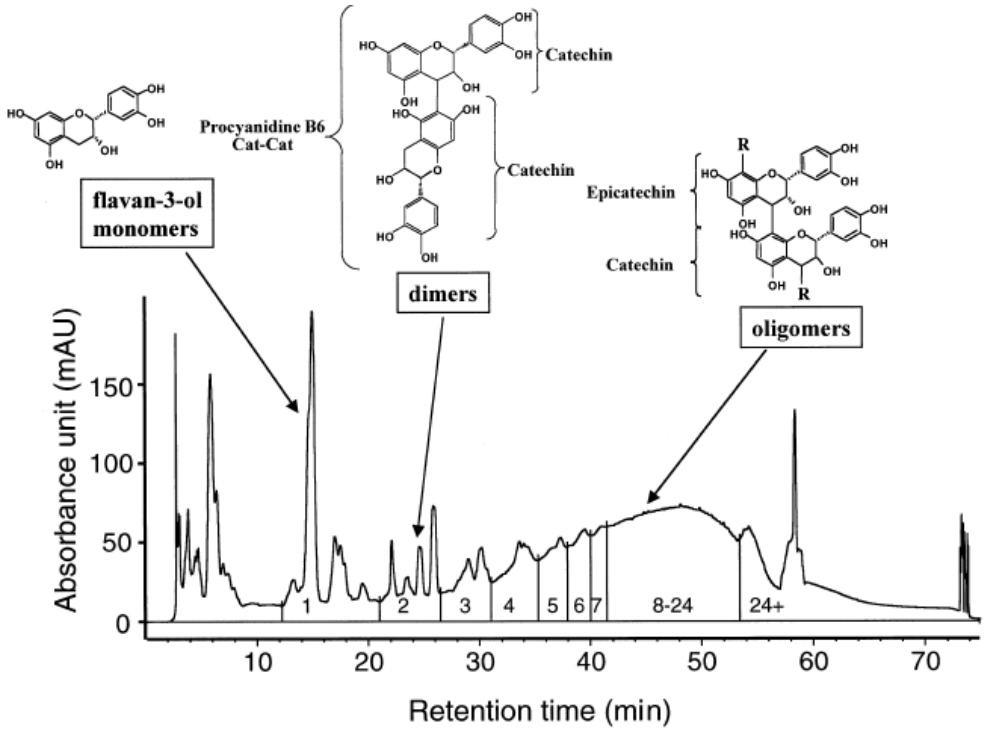


Figure 10 The separation of pycnogenol compounds by normal phase silica gel HPLC. The method used an acidic polar solvent system and UV detection as described by Cheynier and coworkers. Chemical structures of catechin and procyanidine B1 and B6 are shown as examples. (Deprez *et al*., 2000)

2.3.3.1 Seed coat of *Tamarindus indica* L.

Pant materials have long been used as traditional medicines for the treatment of a wide variety of ailments and diseases. Diets rich in vegetables and fruits can reduce cardiovascular disease (Howard and Kritchevsky, 1997; Hung *et al*., 2003; Mozaffarian *et al*., 2003; Katsube *et al*., 2004), notably by inhibiting LDL oxidation (Nicolle *et al*., 2004). Whole tamarind seed and kernels are rich in protein (13-27%), and the seed coat is rich in fibre (21%) and tannins (20%) (Table 1).

Table 1 Composition of tamarind seed, kernel and testa (%)

Constituent	Whole seed	Seed kernel (cotyledons)	Testa (seed coat)
Moisture	9.4-11.3	11.4-22.7	11.0
Protein	13.3-26.9	15.0-20.9	
Fat/oil	4.5-16.2	3.9-16.2	
Crude fibre	7.4-8.8	2.5-8.2	21.6
Carbohydrates	50.0-57.0	65.1-72.2	
Total Ash	1.60-4.2	2.4-4.2	7.4
Nitrogen-free extract	59.0		
Yield of TKP	50.0-60.0		
Calories/100g	340.3		
Total sugar	11.3-25.3		
Starch	33.1		
Tannin			20.2

Source: Modified from Anon, 1976; Morad *et al*., 1978; Ishola *et al*., 1990; Bhattacharyya *et al*., 1994.

The tamarind (*Tamarindus indica* L.) is a tree-type of plant which belongs to the Leguminosae family, Caesalpiniaceae sub family. It is indigenous to tropical Africa but has become naturalized in North and South America from Florida to Brazil, and is also cultivated in subtropica China, India, Pakistan, Indochina, Philippines, Java and Spain initially. The fruit shows a raddish-brown

color that turns black or black brown, becoming more aromatic and sour on ripening. *T. indica* L. pulp fruit is used for seasoning, as a food component and in juices. Its fruit is regarded as digestive, carminative, laxative, expectorant and blood tonic (Farnsworth and Bunyapraphatsara, 1992). Other parts of the plant present antioxidant (Tsuda *et al*., 1994), antihepatotoxic (Joyeux *et al*., 1995), anti-inflammatory (Rimbau *et al*., 1999), antimutagenic (Ramos *et al*., 2003) and antidiabetic activities (Maiti *et al*., 2004). The antiatherosclerosis potential of tamarind has not been previously investigated, and the seed coat is used to treat burns, wound healing and antidysenteric (Farnsworth and Bunyapraphatsara, 1992). With a particular astringent taste, tamarind seed coat contains high amount of polyphenolic compounds which exhibit strong antioxidative activity (Luengthanapol *et al*., 2003). The main active antioxidant isolated was chemically proved to be oligomeric proanthocyanidins, which function as a scavenger against peroxyl radicals, hydroxyl radicals and superoxide anions *in vitro*. It also acts as a protective agent against lipid peroxidation and oxidative damage of Ca^{2+}-ATPase in red blood cell membrane (Pumthong, 1999). The DPPH radical and ABTS cation radical scavenging activities were well proved with the ferric reducing antioxidant capacity of the extracts showed the highest hydroxyl radical scavenging activity of 56.6%. TAM exhibited good antioxidant activity (64.5-71.7%) against the linoleic acid emulsion system and the values were lower and higher than the synthetic antioxdiant, BHA and ascorbic acid, respectively (Siddhuraju, 2006).

Seed coat of *T.indica* significantly attenuated (as much as 68%) nitric oxide production induced by LPS and IFN-γ *in vitro* in a murine macrophage-like cell line, RAW 264.7 cells, and *in vitro* and *in vivo* in isolated peritoneal

macrophages. The preliminary safety studies in B6C3F1 mice demonstrated that the extract at 100-500 mg/kg can modulate the NO production without inducing any clinical signs of toxicity. Neither the development of a T-cell mediated sensitization responses to DNFB or HCA nor the dermal irritation to nonanoic acid nor DNFB is modulated by the *T. indica* treatment (Komutarin *et al*., 2004).

2.4 *In vivo* Anti-inflammation models

Pain is one of the classical signs of the inflammatory process in which sensitization of the nociceptors is the common denominator. This sensitization causes hyperalgesia or allodynia in humans, phenomena that involve pain perception (emotional component+nociceptive sensation).

The main mechanism involved in the hyperalgesic action of prostaglandins is traditionally considered to be due to a sensitizing effect on primary afferent nerves (Davies *et al*., 1984). The direct effects are mediated by the action of prostaglandins on EP/IP receptors and modulation of ion channels in primary afferents. The indirect effects are directed towards enhancing the sensitivity of sensory neurons to noxious agents such as heat and bradykinin and also assumed to contribute significantly to the PG-mediated peripheral sensitization (Figure 11).

Acetic acid causes algesia by liberating prostaglandins and sympathomimetic system mediators like PGE2 and PGF2a that excite pain nerve endings, and it is a sensitive method of screening both peripheral and central analgesic efficacy of agents (Collier *et al*., 1968; Shanmugas undaram and Venkataraman, 2005). The result of the writhing test alone does not ascertain whether the antinociceptive effects are central or peripheral. Thus, to clear the mode of the inhibitory effect of agents on the

nociceptive responses, the effect of the different extracts and fractions of the species under study on the tail flick test should be examined. It is known that the tail-flick response appears to be a spinal reflex, and is considered to be selective for centrally acting analgesic compounds (Ramabadran *et al*., 1989; Srinivasan *et al*., 2003). As noted, the reference narcotic analgesic drug, pethidine (50 mg/kg p.o.) exhibited significant antinociceptive effects in the tail flick test. The infusion, methanol extract and butanol and chloroform fractions of *Hypericum glandulosum* also produced a statistically significant but lesser in degree antinociceptive response to that of pethidine in the tail flick test. The results obtained on the writhing response and tail-flick test suggest that *Hypericum glandulosum* possesses both peripheral and central analgesic activity, although further studies are needed in order to know the mechanism behind the observed central antinociceptive action. On the other hand, the lack of influence of extracts and fractions of *Hypericum canariense* on the reaction time of mice submitted to the tail flick test (with the exception of the slight activity shown by the butanol fraction) is consistent with the interpretation that its analgesic property does not have a central origin, having an analgesic effect in the acetic acid writhing test that is mostly mediated via a peripheral mechanism by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonising the action of pain mediators at the target sites (Srinivasan *et al*., 2003).

As RNS, ROS and NO play such important roles in various health and diseases processes, and the wide range of therapeutic properties of flavonoids have long been known, the TAM extract that contains flavonoids among its major constituents, posses antioxidant and inhibitory activities on NO production *both in vitro* and *in vivo* should have some attenuating activities towards some

pathophysiological processes. The present studies further explored anti-inflamatory activity of TAM extract through iNOS and COX-2 expression, and anti-apoptotic effect on RAW 264.7 cells. In addition, *in vivo* anti-inflammation was evaluated by anti-nociceptive role of TAM extract using the tail flick and the acetic acid-induced writhing tests in a murine model.

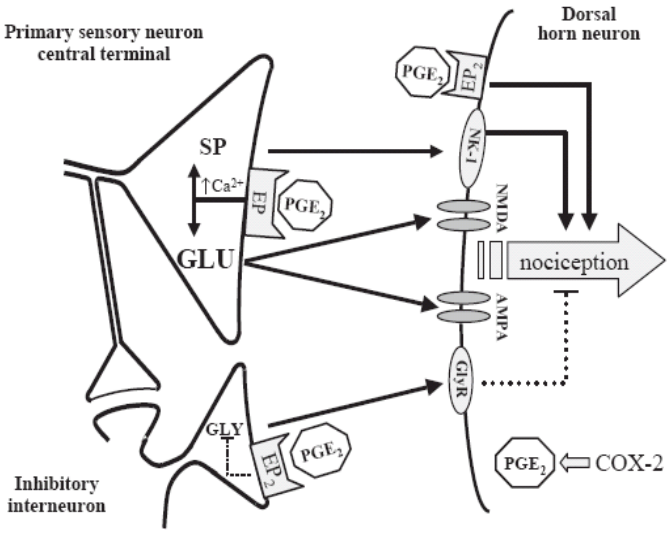


Figure 11 Molecular mechanisms of the sensitising action of PGE2 in dorsal horn. PGE2 exerts antihyperalgesic effects by acting on pre- and post-synaptic membranes of the primary afferent synapse. The activation of the presynaptic EP receptors leads to facilitated spinal release of glutamate and neuropeptides resulting in enhanced nociceptive processing. At the post-synaptic level, PGE2 can directly activate deep dorsal horn neurons via EP-2-like receptors located on dorsal horn neurons and block inhibitory glycinergic neurotransmission by activation of EP-2-like receptors in the inhibitory neurons. Activating and inhibiting pathways are depicted by solid and slashed lines, respectively. GLU = glutamate, GLY = glycine, SP = substance P (Chakraborty *et al*., 2004).

CHAPTER III

MATERIALS AND METHODS

The studies used *in vitro* model of mouse macrophage cell line RAW 264.7 gamma (NO-) cells activated with LPS and IFN-γ to induce NO production and inflammatory response, and etoposide to induce apoptosis. The inhibitory mechanism of NO production and the anti-inflammation by the extract was limited to the measurement of iNOS and COX-2 expression, respectively. Anti-apoptosis was assessed by DNA ladders formation in agarose gel and the annexin V-PI method. Anti-inflammation *in vivo* was assessed by two anti-nociceptive models, the acetic acid induced-writhing and tail flick tests. The overviews of the studies were summarized in Figure 12.

3.1 Materials

3.1.1 Experimental animals

Female ICR mice 6-8 week of age were obtained from Institutional Animal Care Building at Suranaree University of Technology, Nakhon Ratchasima. Mice were housed in stainless steel hanging cages with hardwood bedding and were provided tap water, and diet *ad libitum*. Mice were used to investigate the effects of the seed coat extract of TAM on *in vivo* anti-inflammatory activity. They were kept in a temperature-controlled room (25±2 ^{o}C) under a 12 hour light-dark cycle and 40-70% relative humidity.

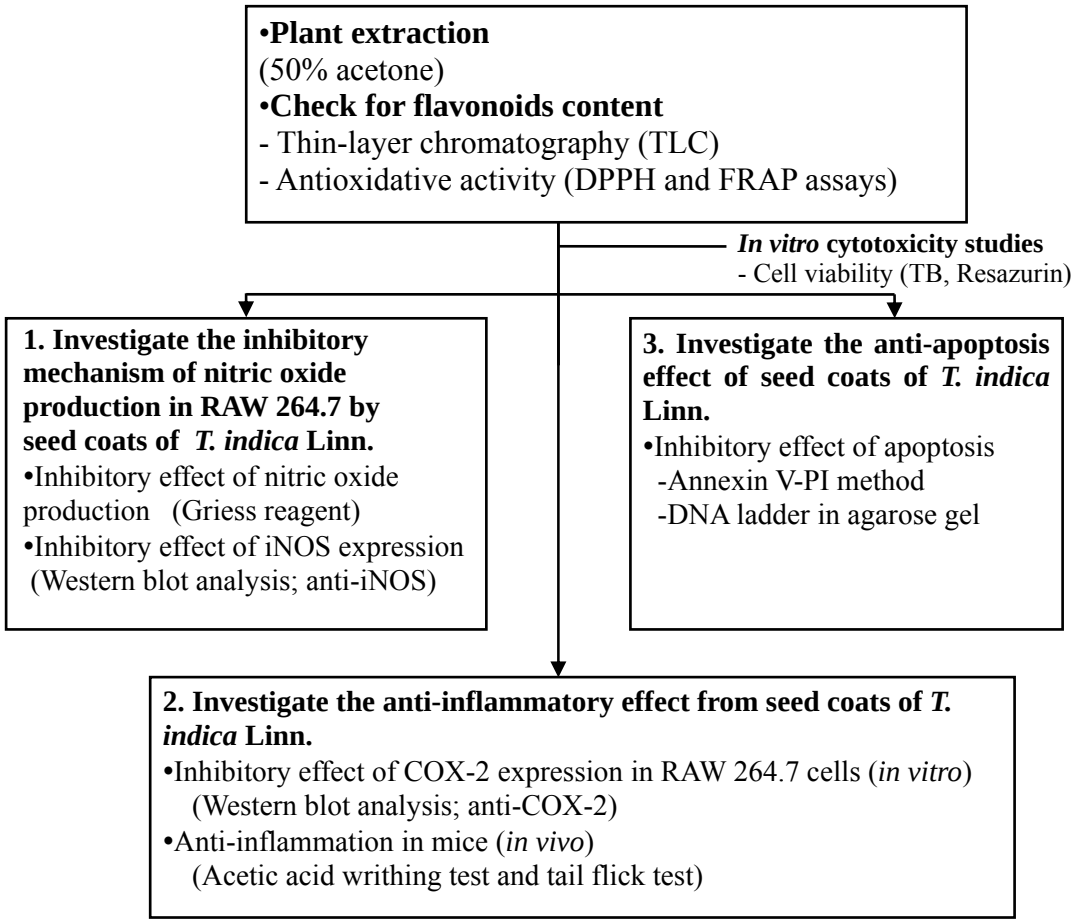


Figure 12 The overviews of the studies

3.1.2 Cell lines

The mouse macrophage cell line RAW 264.7 gamma NO (-) (ATCC, CRL-2278) were used to investigate the effects of the seed coat extract of TAM on *in vitro* cytotoxicity studies, inhibitory mechanism of NO production, anti-inflammation and anti-apoptosis. This cell line was commonly used in anti-inflammatory and anti-apoptosis studies because treatment with different stimuli including LPS, IFÑγ trigger the cell to express high levels of COX-2 and iNOS and eventually undergone apoptosis (Weisz *et al*., 1994).

3.1.2.1 Maintenance of RAW 264.7 cells

Cells were cultured and maintained in RPMI 1640 containing 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate adjusted to contain 1.5 g/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37^{o} C with 5% CO_2 in humidifies air. Exponentially growing cells were used for experiments when they reach about 80% confluence.

3.1.3 Chemicals, instruments, plastic and glasswares

The chemicals, materials and instruments employed in the present studies were summarized in Tables 2 and 3.

Table 2 List of chemicals used in the studies.

Name	Source
Acetic acid	Merck
Acrylamide	Sigma
Agarose	GIBCO
Ammonium persulfate	Merck, Darmstadt
Annexin V-FITC apoptosis detection kit II	BD Biosciences
Anti- iNOS (mouse monoclonal IgG1) (C-11)	Santa cruz
Anti-COX-2 (murine monoclonal IgG1) (Sc-1999)	Santa cruz
Anti-COX-2 (murine polyclonal) (160106)	Cayman
Boric acid (H_3BO)	Merck, Darmstadt

Table 2 (Continued)

Name	Source
Bromphenol blue	Sigma
Coomassie blue G250	Sigma
Dicolfenac	NOVARTIS
[3(4,5-dimethylthiazol-2-yl) ,5-diphenyltetrazolium bromide]-MTT	Wako company
Dimethyl sulfoxide (DMSO)	Wako company
DNA detection Kit	QIAGEN
DNA Ladder 100 bp	BIOLabs Inc.
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Sigma
ECL plus western blot detection reagent	Amersham
Ethidium bromide	Sigma
Ethyl alcohol	BDH
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Etoposide (20 mg/ml)	Fytosid
Fetal bovine serum (FBS)	Hyclone
Ferric-2,4,6-tripyridyl-s-triazine (Fe^{III}-TPTZ)	Sigma
Ferrous sulfate ($FeSO_4.7H_2O$)	Sigma
Folin & Ciocalteu's Phenol Reagent	Sigma
Hybond ECL Nitrocellulose membrane	Amersham
Hyperfilm ECL	Amersham
Gallic acid	Sigma

Table 2 (Continued)

Name	Source
Gentamicin	Gibco
Goat-anti-mouse-IgG-HRP conjugate (sc-2005, lot L1304)	Santa Cruz
Goat-anti-Rabbit IgG-HRP conjugate (10004301)	Cayman
Glycine	Sigma
N-2-hydroxethyl sulfuric acid (HEPES) buffer	Gibco
Interferon gramma (IFN-γ)	Sigma
Lipopolysaccharide (LPS)	Sigma
2-Mecaptoethanol (2-ME)	Sigma
Methyl alcohol	Sigma
3(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma
Non-idet P 40	Bio active
Penicillin and streptomycin solution	Sigma
Phosphate buffered saline (PBS)	Sigma
Phosphoric acid	Sigma
Proanthocyanidin (French paradox) from grape seed extract	Sigma
Rainbow coloured protein molecular weight marker	GE Healthcare
Resazurine	Sigma
RNase A	US Biology
RPMI 1640	Gibco
Sodium acetate	Aldrich

Table 2 (Continued)

Name	Source
Sodium azide	Sigma
Sodium bicarbonate	Sigma
Sodium carbonate	Sigma
Sodium dodecyl sulfate	Sigma
Sodium hydroxide	Carlo
Trisma base	Sigma
Triton X-100	Bio-Active
Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma
Trypan blue dye	Gibco
α-tubulin (mouse monoclonal IgG) (B-7, Sc 5286, lot J2505)	Santa cruz
Typsin	Gibco
Tween-20	Sigma
Vitamin C (ascorbic acid)	Fluka

Table 3 List of equipments, plastics and glasswares used in the studies.

Name	Source
6-well plate flat, bottom	Co-Star
24-well plate flat, bottom	Co-Star
96-well plate flat, round and U bottom	Co-Star

Table 3 (Continued)

Name	Source
Alundum extraction thimbles	Fisher Scientific
Blender	Molinex
Centrifuge machine	SORVALL
CO_2 incubator	SHEL LAB
Conical tubes	Nunc Co.
Coulter counter	Beckman
Electrophoresis system model # B1	BIORAD
Intelligent electrophoresis unit (model I My run)	Cosmo Bio
ELISA plate reader (model E6504)	BIORAD
FACScalibur cell analyzer (model FACSCalibur™ Flowcytometry)	Becton Dickinson
Glasswares	Pyrex Co.
Haemocytometer and cover slip	Fisher Scientific
Inverted microscope	Olympus optical
Laminar flow hood (model SG 600E)	Holten, Scientific
Light microscope	Nikon
Lyophilizer	Dura-dry
Microcentrifuge	SORVALL
Microscope slides	Fisher Scientific
Multichannel pipette	Gibco
Pasteur pipettes	Fisher Scientific

Petri dish	Nunc

Table 3 (Continued)

Name	Source
pH meter	Metrohm
Rotary evaporator with vacuum (model R205)	Buchi
Single frosted end slide	Fisher Scientific
Snap cap tubes	Nunc
Soxhlet extraction apparatus (model B-811)	Buchi
TLC plate Silica gel 60 F_{254} (Cat.OB 342214)	Merck
Water bath (model 122006180703)	SHEL LAB
Wallac Multilabel Counter (Model 1420)	Wallac
WEALTEC Dolphin-DOC ultraviolet analyzer	WEALTEC

3.2 Methods

3.2.1 Preparation of seed coat crude extract of TAM

After drying seeds from *Tamaridus indica* in a hot air oven at 140^{o}C for 2 hrs, the dried seed coats were separated from seeds by manual crushing and grounded into powder. The raw powder (Figure 13) was put into a thimble (medium porosity, 10-15 μm (Figure 14) and then into the soxhlet column. 50% acetone was placed into a round-bottomed flask, and connected to the soxhlet extraction apparatus. Several boiling chips were placed into a clean dry receiving flask. The extraction process was performed until the solvent had a clear color (appoximately 2-3 days). The reflux rate

was periodically checked and adjusted so that the heating rate was four to five exchanges per hour in the soxhlet thimble. When the extraction was complete, the thimble was removed and the sample was carefully transferred to a beaker. Any residual solvent was removed by filtering through cotton wool. The flask with the extract solution was placed on the rotary evaporator and the solvent was removed under vacuum, the extract was lyophilized in a lyophilizer at -50°C for two days. The powder of crude extracts was then kept in -20˚C freezers until use (Figure 15).

Figure 13 Raw powder of seed coat of *Tamarindus indica* L.

Figure 14 Soxhlet extraction apparatus.

Figure 15 Powder of seed coat extract from *Tamarindus indica* L. was evaporated from aqueous phase of 50% acetone extraction.

3.2.2 Flavonoid content and Antioxidant activity studies

3.2.2.1 The total phenolic content (TPC)

The total phenolic content of the phenolic extracts of TAM was determined according to Folin-Ciocalteu procedure with some modifications (Folin and Ciocalteau (1972). Since the powder of TAM extract is insoluble in water, all solutions were prepared in methanol/water (1:2). 0.02 mL of the phenolic extract or blank was pipetted into cuvettes, 1.58 mL of methanol/water (1:2) and 0.1 mL Folin-Ciocalteu reagent were added and mixed well. 5 min later, 0.3 mL sodium carbonate solution (7.5%) was added and shaked to mix. After incubation at room temperature for 1.5 h, the absorbance of each solution was determined at 765 nm with a Perkin-Elmer15 UV-Vis spectrophotometer, Norwalk, CT (Singleton and Rossi, 1965). Gallic acid was used as the standard compound. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of the 50 mg/mL standard gallic acid stock solution was added into each 100 mL volumetric flask, and then diluted to specific final volume with water to obtain phenol concentrations of 0, 50, 100, 150, 250 and 500 mg/g gallic acid, respectively.

3.2.2.2 Thin-layer chromatography system (TLC)

Each batch of TAM was checked for the content of flavonoids by TLC plate Silica gel 60 F_{254} using the solvents of toluene: acetone: formic acid at 6: 6: 1, respectively.

TLC experiments of the extract were performed as described previously (Pumthong, 1999). All TLC separations were performed on silica gel plates incorporated with a fluorescent indicator. First, mark the TLC plate with a pencil by lightly drawing a straight line parallel to the short dimension of the plate, about 1 cm from one end of the plate. Second, lightly made two small marks perpendicular to this

line to divide the line into thirds. This subdivided line would serve as a guide for placing the substance spots. Third, lightly drew a second line parallel to the first line and about 1 cm from the other end of the plate. Fourth, applied the extract or other reference compounds in methanol to the plate (at marviced position) as a small spot not larger than 1 mm in diameter. Then, developed the plate and allow the solvent front to rise to the second line prior to terminate the run. Visualization of the flavonoids were attained by spraying the sheets with DPPH.

3.2.2.3 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay measures the ability of the extracts to scavenge the violet colored of stable free radical DPPH. This assay was carried out as described by Saha (2004) with some modifications. Stock solution of TAM crude extract was prepared as 10 mg/mL in methanol. The solution was diluted to obtain different concentrations ranging from 500 to 3.9 μg/mL in methanol. Each concentration of extract was prepared in quadruplicates along with blanks and controls in a 96-wellplate. Then 5 μL of 10 mg/mL DPPH solution (in methanol) was added to each well. The plate was shaken gently and placed in the dark for 30 min at room temperature. The absorbance was then measured at 517 nm. Percentage of inhibition was calculated using the following formula:

$$\text{Percent inhibition} = \frac{\text{OD (DPPH)} - \text{OD (DPPH + sample)}}{\text{OD (DPPH)}} \text{ X } 100$$

3.2.2.4 Ferric Reducing /Antioxidant Power Assay (FRAP assay)

The FRAP assay, another method for assessing antioxidant power, is based on the reduction of ferric 2,4,6-tripyridyl-s-triazine [Fe(III)-TPTZ] to the ferrous 2,4,6-tripyridyl-s-triazine [Fe(II)-TPTZ] complex by a reductant at low pH which causes a colored complex to form. FRAP values are obtained by comparing the change absorbance at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

Prior to analysis, 1 mL extract was transferred into a 10 mL volumetric flask and diluted with the same solvent of extract. 0.1 mL diluted extract was transferred into test tubes and 3.0 mL of freshly prepared FRAP-reagent (25 mL of 300 mmol/L acetate buffer, pH 3.6 + 2.5 mL of 10 mmol/l TPTZ in 40 mmol/L HCl + 2.5 ml of 20 mmol/L $FeCl_3.6H_2O$) was added (Benzie and Strain, 1999). All measurements were done in triplicates. The standard curve was prepared using different concentrations (100–1000 μmol/L) of $FeSO_4 \cdot 7H_2O$. All solutions were prepared fresh and used on the day of preparation. In the FRAP assay, the antioxidant efficiency of the test compound was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were corrected for dilution and expressed in mol Fe^{II}/L, or as potency of vitamin C.

3.2.3 *In Vitro* cytotoxicity studies

Single cell suspensions of RAW 264.7 cells were used to evaluate the *in vitro* cytotoxicity of *T. indica* in order to find the range of optimum concentrations used for subsequent investigations. 100 μL of $5x10^6$ cells/mL were plated in a 96-well plate in the absence or presence of various concentrations of seed coat extract of *T. indica* either

for 24, 48 and 72 hours. Cell viability was evaluated by the trypan blue exclusion or Resazurin-based *in vitro* toxicology assay methods.

3.2.3.1 Trypan blue exclusion method

100 μL of the cell suspension were mixed with an equal volume of 0.4% (w/v) trypan blue. A pasteur pipet or pipetman were used to transfer a small amount of this diluted sample to one or both chambers of the hemocytometer and allow the chamber to fill by capillary action. The 10X object 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:

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

3.2.3.2 Resazurin-based *in vitro* toxicology assay

3.2.3.2.1 Preparation of TAM extract for use in cell cultures

50 mg powder of TAM extract was initially dissolved in 1 mL 100% DMSO to obtain 50 mg/mL TAM stock solution. Then the stock solution was further diluted with complete RPMI media to obtain desired concentrations.

A color of anthocyanins has been known to interfere with the detection of formazan product in the MTT assay (Wang and Mazza, 2002). Resazurin-based *in vitro* toxicology assay was selected to study the effect of TAM extract on cell viability in the present study.

The mouse macrophage cell line RAW 264.7 was cultured in RPMI media containing 10% heat-inactivate fetal bovine serum, 2mM glutamine, 100 U/ml penicillin and 100 µg/mL streptomycin. After reaching 80% confluence, cells were gently detached using a cell scraper. Cells were plated at a density of 6 x 10^5 cells/well in a 24 well plate. After 2 h incubation at 37^{o}C with 5% CO_2 in humidified air, different concentrations of TAM extract was added to obtain the final concentrations ranging from 0.01-100 µg/mL. Cultures were further incubated for another 24 h.

For the assessment of cell viability, resazurin dye solution, 10 times diluted from its original 10 mg/mL stock solution with complete RPMI, was added to each well of a 24 well plate. After incubation for additional 4 h at 37^{o}C and 5% CO_2, 200 µL of the supernatant with the bioreduced fluorescent resazurin intermediate (purple-red color) was transferred to a 96 well plate, and the absorbance was measured at 570 nm.

3.2.4 Nitrite studies

The anti-inflammatory property of TAM extracts was tested by measuring its effect on the pro-inflammatory mediator NO in activated RAW 264.7 murine macrophages. Measurement of nitrite accumulation in the culture medium was used to determine NO production. The culture medium was collected after 24 h incubation, and the nitrite concentration was measured by Griess reaction.

3.2.4.1 Measurement of nitrite

Nitrite concentration was determined using the Griess reagent as described by Padgett and Pruett (1992).

RAW 264.7 cells were plated at a density of 2 x 10^5 cells/well in a 96 well plate. Cells were grown for 2 h. to allow plate attachment prior adding TAM extract or other antioxidant controls. The final concentrations of TAM in the cultures were 1, 5 and 10 μg/mL, respectively. 500 μM vitamin C and 10 μg/mL GSE were used as other antioxidant controls. After 30 min incubation, RAW 264.7 cells were stimulated with 1 μg/mL LPS and 25 u/ml IFN-γ. The activated cells were further incubated for 24 h. Then 100 μL supernatants were collected to determine nitrite concentration and/or stored at -80^{o}C until use. 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. Nitrite in the samples was quantified by comparison with a standard curve. A nitrite multi points linear standard curve was determined using known concentrations of $NaNO_2$ ranging from 1.56 μM to 200 μM. Control cells were grown under identical conditions but were not exposed to the TAM extract or LPS /IFN-γ.

3.2.5 Evaluation of iNOS and COX-2 expression

The effect of the seed coat extract of *T.indica* on iNOS and COX-2 expression of activated RAW 264.7 cells were conducted using Western blots method.

1 mL of RAW 264.7 cells (2x10^6 cells) in RPMI 1640 containing 10% FBS and 1 μg/mL of LPS were plated in a 6 well plate in the presence or absence of various concentrations of the TAM extracts and incubated at 37^{o}C for 16-20 h. After incubation, cells were washed three times with PBS, placed in 150 of ice-cold lysis buffer (1 mL RIPA buffer, 10 μL 200 mM PMSF, 1 μL 2mM leupeptin, and 1 μL E-64)

for 15 min. Then the disrupted cells were transferred to microfuge tubes and centrifuged at 12,000 g and 4^{o}C for 30 min. The supernatant was collected, and the protein content of cell lysate was estimated by Lowry method (Maiti *et al*., 2004). The cell lysate was boiled for 5 min in 6X sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethnol, and 0.05 mg/mL of bromophenol blue) at a ratio of 6:1 (v/v). Proteins (30 µg per lane) were separated by electrophoresis in a 10%-SDS-polyacrylamide gel (150 min at 125 V) (Ban *et al*., 2004). The separated proteins were blotted onto nitrocellulose membranes (Pierce) (Figure 16). The membrane was blocked with 2% bovine serum albumin and 5% milk powder, 0.1% Tween 20 in PBS-buffer for 4 h, and incubated overnight with a 1:1000 dilution of the primary antibodies, anti-iNOS mouse monoclonal (Santa Cruz), or 1:2000 dilution of the primary antibodies anti-COX-2 mouse polyclonal (Cayman). After extensive washing with TPBS, the membranes were incubated with a 1:10,000 dilution of the secondary antibody goat-anti-mouse-HRP conjugate (Santa Cruz) or Goat-anti-Rabbit IgG-HRP conjugate (Cayman) for the iNOS and COX-2 expression, respectively. To control for equal loading of total protein in all lanes, blots were also stained with primary antibodies anti-tubulin mouse monoclonal (Santa cruz) at a dilution of 1:2000. Then the membranes were incubated with a 1:10,000 dilution of the secondary antibody goat-anti-mouse-HRP conjugate (Santa Cruz). Finally, blots were incubated for 5 minutes in ECL reagent (Pierce) and exposed to an CL-x Posure ^{TM} film about 5 minutes and 30 second for detecting iNOS and COX-2, respectively.

3.2.6 Evaluation of apoptosis

Assessment of cell apoptosis were evaluated by early and late apoptotic cell death by Annexin V-PE method, DNA fragmentation analysis was detected by

Figure 16 The separation of proteins by SDS-polyacrylamide gel electrophoresis. After separation, the separated protein were blotted onto nitrocellulose membranes.

visualization of DNA ladders in the agarose gel and fluorescence microscopy.

Confluent monolayer of RAW 264.7 cells were plated at a density of $1x10^6$ cells/well in a 6 well plate and grown for 2 h to allow plate attachment. The final concentrations of TAM extract were 1, 10 and 20 µg/mL, whereas the concentrations of antioxidant controls, GSE and vitamin C were 10 µg/mL and 500 µM, respectively. After 30 min incubation, 20 µg/mL etoposide was added for apoptosis induction. Then cells were further incubated for 24 h before evaluation of apoptosis.

3.2.6.1 Annexin V-PI method

The apoptotic cells were determined using the annexin V-FITC apoptosis detection kit II (BD Biosciences) as decribed by Michael *et al*. (2001) with slight modification. Briefly, cells were washed once with PBS and twice with binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and were resuspended in 100 µL binding buffer and 5 µL annexin V, 3 µL PI were added and incubated at room

temperature for 20 minutes in the dark. After incubation, cells were resuspended in 400 µL of binding buffer and analyzed by flow cytometry (Figure 17).

Figure 17 Becton Dickinson FACScalibur cell analyzer (model FACSCalibur™ Flowcytometry USA).

3.2.6.2 Visualization of DNA ladder formation

To separate linearized double-stranded DNA fragments, the agarose gel electrophoresis was applied. The migration of DNA in an electric field depends on the molecular size of the fragments. The size then can be estimated using DNA marker of known size. In this study DNA fragmentation was analyzed by agarose gel electrophoresis using the genomic DNA detection Kit (QIAGEN) (Kang *et al*., 2003) (Figure 18).

RAW 264.7 cells with $1x10^6$ cells/well were pre-incubated in complete RPMI medium in a 6 well plate at 37°C for 2 h Cells were then treated with TAM, GSE (1, 10, 20 µg/mL) or vitamin C (500 µM) for 24 h pior adding 10 µg/mL

Figure 18 The DNA fragmentation was visualized by electrophoresis on a 1.2% agarose gel and the gel was visualized with a WEALTEC Dolphin-DOC ultraviolet analyzer (WEALTEC, Corp., Sparks, Nevada, USA).

etoposide, the apoptotic inducer. 24 h later, the treated cells were washed twice with PBS and harvested by trypsinization. Cytosolic mononucleosomes and oligonucleosomes (180-bp nucleotides or multiples) were analyzed using DNA detection kit (QIAGEN) by following the procedures as described by the manufacturer's instructions (Appendix B). The DNA was loaded in 1.2% agarose gel containing 0.1 mg/mL ethidium bromide in 1X TBE buffer and electrophoresed at 80 volts for 90 min with 100 bp DNA markers (BIOLabs Inc.). The gel was visualized with a WEALTEC Dolphin-DOC ultraviolet analyzer (WEALTEC, Corp., Sparks, Nevada, USA).

3.2.7 *In vivo* anti-inflammatory activity

Antinociceptive activity was evaluated by using acetic acid-induced writhing test and tail flick test.

3.2.7.1 Acetic acid-induced writhing test

One hour after giving oral garvage (p.o.) of the vehicle, TAM extract or reference substance to groups of six mice, each mouse was given intraperitoneally 0.6% aqueous solution of acetic acid (0.1 mL/ 10g body weight). Immediately after the acetic acid injection, each animal was placed in a transparent observation cage and the number of writhes per mouse was counted for 45 min (Figure 19). The writhing activity consists of a contraction of the abdominal muscles together with a stretching of the hind limbs (Hern´andez-P´erez and Rabanal, 2002). The percentage of inhibition was calculated using the following ratio:

Percentage of protection = (control mean − treated mean)/control mean) X 100

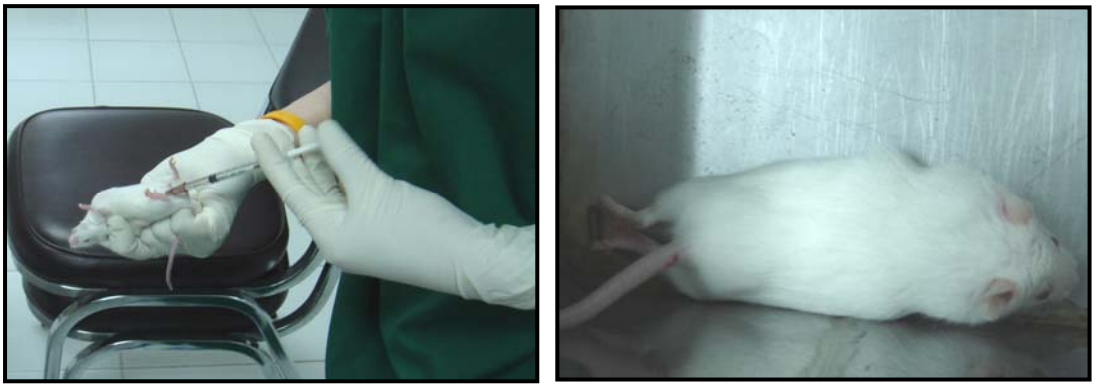


Figure 19 The evaluation of antinociceptive activity was evaluated by acetic acid induced-writhing test. The writhing responses of mice was caused by the intraperitoneal administration of acetic acid to induce abdominal constriction.

3.2.7.2 Tail flick test

The apparatus used in the tail flick test consisted of a circulating immersion water heater. The thermostat was adjusted so that a constant temperature of 51±1°C was maintained in the water bath. To begin treatment, the terminal 3 cm of each

mouse's tail was immersed in the water bath and the time in seconds taken to flick the tail was recorded. Only mice showing a pretreatment reaction time less than or equal to 3 s were selected for the study. Immediately after basal latency assessment, the plant extracts, reference substance or solvent were administered by the oral route to groups of six mice for 1 h prior to perform the tail flick test. Cut-off time was 10 s for tail flick measurements in order to minimise tissue injury (Hern´and ez-P´erez *et al*., 1995) (Figure 20).

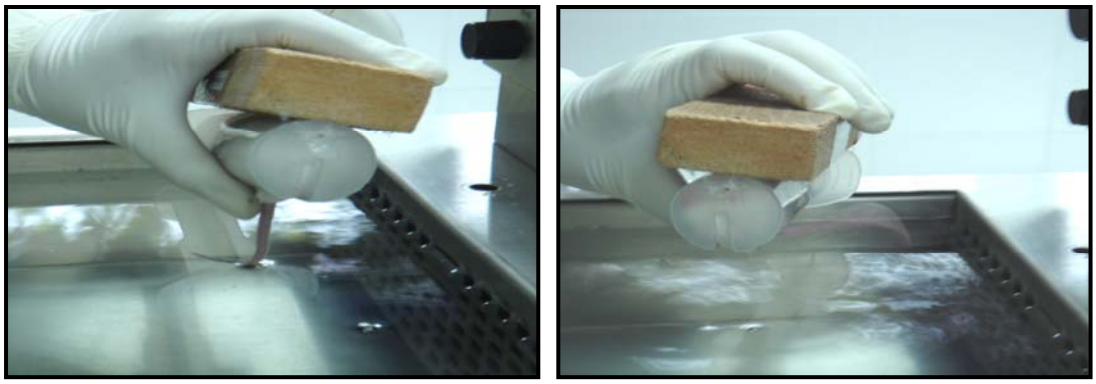


Figure 20 Tail-flick test.

3.3 Statistical analysis

Homogeneous data were evaluated by using a one-way analysis of variance (ANOVA), and differences between treatment groups and the vehicle control group was compared by using a Dunnett's two tails Test (Dunnett, 1995). Non-homogenous data was evaluated by using a non-parametric analysis of variance. Significance differences were compared between the experimental groups and the vehicle control groups by using the Wilcoxon rank test (Gross and Clark, 1975). In the results, the data is presented as the mean ±S.E. of mean.

CHAPTER IV

RESULTS

4.1 Flavonoids content and antioxidant activity studies

4.1.1 Thin-layer chromatography system (TLC)

In order to rapid screen and visualize quantitatively the free radical scavenging capacity (RSC) of TAM extract, each diluted sample was applied as a dot on a TLC plate and the RSC was detected by staining with DPPH solution. The appearance of yellow color in the spots shows a potential RSC of the dot samples (Soler-Rivas, 2000). The method is typically based on the inhibition of the accumulation of DPPH free radicals by the addition of antioxidants which scavenge the free radicals generating the changing color from purple of DPPH to yellow color or discolor of DPPHH, similar to the result of DPPH spectrophotometric assay.

Figure 21 showed the TLC chromatogram of *T.indica* seed coat extract (TAM) and grape seed extract (GSE). It indicated that the TAM extract, from origin to solvent front, could be separated by this system into six spots which each had different Rf values and displayed the discolor of DPPHH. This confirms that TAM extract contains at least six kinds of antioxidant compounds.

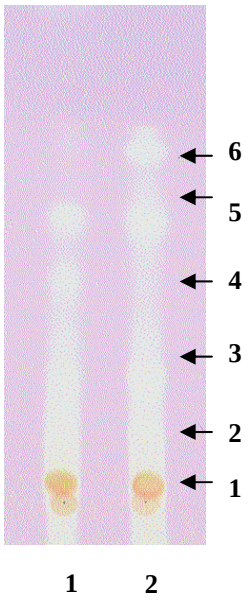


Figure 21 Thin layer chromatography (TLC) of TAM extract stained with a DPPH solution. The samples from left to right were extracts of GSE (lane 1) and TAM (lane 2) dissolved in ethanol and developed on toluene: acetone: formic acid at a ratio of 6:6:1, respectively.

4.1.2 The total phenolic compound contents (TPC)

Initial studies were conducted to investigate and determine the total phenolic compound contents (TPC) in TAM crude extract. Phenolic compounds occur in all vegetables and fruits as a diverse group of secondary metabolites which is a component of human diet, although the data for dietary intakes and metabolites is limited. They had been characterized by chemical reactivity and their quantitative complicated analysis. The amount of TPC in TAM was determined by using Folin-Ciocalteau's phenol reagent as modified from the method of Folin and Ciocalteau (1972).

The yield of TAM extract started from the weight of dried plant material till extracted by 50% acetone was 45.8%. The TPC of the extract was 178.6±3.36 mg/g GAE, which was higher than GSE which TPC contents was 133.2±2.39 mg/g GAE. The yield, TPC and IC_{50} of DPPH assay of TAM, GSE and vitamin C were summarized in Table 4 and Figure 22.

Table 4 The percentage of yield, amount of TPC and DPPH free radical scavenging activity of plant samples and standard (vitamin C).

Plant or standard	**Yield (%)** [a]	**Phenolic content (GAE (mg g^{-1}))**	**IC_{50} (µg/mL)** [b]
TAM	45.8	178.6 ± 3.36	13.2
GSE	-	133.2 ± 2.39	20.7
Vitamin C	-	-	38

Data represent means of three determinations ± SD (standard deviation)

[a] Percentage yields from the weight of dried plant material.

[b] DPPH free radical scavenging activity of TAM, GSE and standard vitamin C.

4.2 Antioxidant activity study

In order to obtain more complete information on the antioxidant activities of TAM extract, two different assays were employed including the DPPH and FRAP assays. The DPPH assay was employed to determine the ability of samples to capture free radicals. The FRAP assay was performed to determine the antioxidant power, is represented by the ability of reduction of ferric to ferrous at low pH causing the formation of colored ferrous-tripyridyltriazine complex.

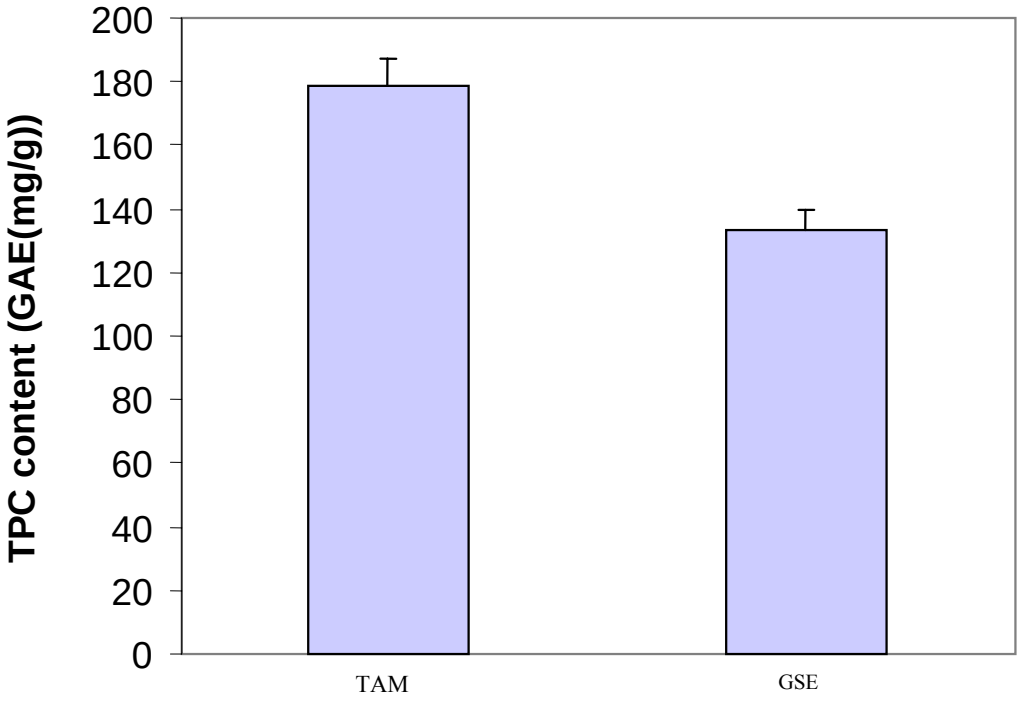


Figure 22 Comparison of TPC contents of TAM and GSE expressed as mg/g GAE. Each sample was measured in triplicates. All values are mean ± SD Values are significantly different from each other at $p \leq 0.01$

4.2.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical-scavenging activities of TAM along with the reference standards such as vitamin C was determined by the DPPH assay, and the results were shown in Table 4 and Figure 23. A lower value of IC_{50} indicates a higher antioxidant activity. The data showed that the extract of TAM possessed the higher DPPH radical scavenging activity than GSE and vitamin C, and their IC_{50} were significant differences ($p \leq 0.01$). The amount of IC_{50} in TAM was 13.2 µg/mL, whereas the IC_{50} of GSE and vitamin C were found 20.7 and 38 µg/mL, respectively.

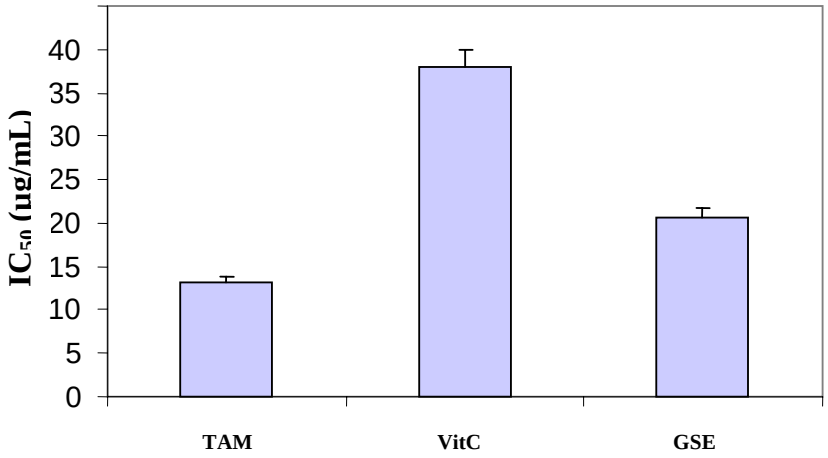


Figure 23 The free radical-scavenging activities of aqueous acetone extract of tamarind seed coat along with the reference standards such as vitamin C and GSE was determined by the DPPH assay.

4.2.2 Ferric Reducing/ Antioxidant Power (FRAP) assay

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability (Luengthanapol *et al*., 2003). Antioxidant potential of the TAM was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. The results of antioxidant capacities of TAM extract and vitamin C are given in Figure 24. The TAM extract was able to reduce ferric complex to ferrous form in a dose-dependent manner. The slope of reducing capability of TAM extract was 0.0083 whereas of vitamin C was 0.1437. Therefore, the potency of TAM was

approximately 1/17 time of vitamin C. Such potential reducing power activity might be attributed to the presence of dihydroxy type of benzene derivatives and (-) epicatechin presented in the TAM. However, vitamin C was found to possess more reducing antioxidant power than TAM.

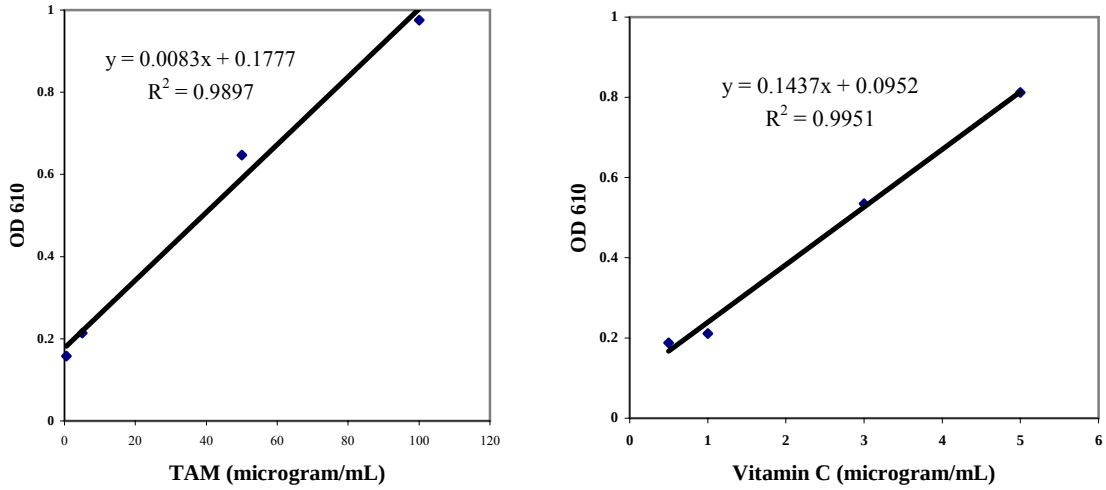


Figure 24 The total antioxidant activity by using Ferric Reducing/ Antioxidant Power Assay (FRAP) assay. The TAM was able to reduce ferric complex to ferrous form in a dose-dependent mode.

4.3 *In vitro* cytotoxicity studies

The TAM extract at the concentration of 0.01-100 μg/ml was added to the murine macrophage cell line (RAW 264.7). The mixtures were incubated at 37°C 5% CO_2 for 24 h, after which the viability of RAW cell was examined. The results showed no significant difference of percentage of viable cells of TAM treatment. TAM less than 10 μg/mL treated group did not induce any cytotoxic effect to RAW 264.7 cell compared to controls, as evaluated by trypan blue exclusion (TB) method and resazurin assay (Figure 25). However, at higher concentration, TAM was toxic to

cells as evidenced by % viability of cells was reduced from about 20 to 50% at 25 to 100 µg/ml TAM, as evaluated by resazurin assay.

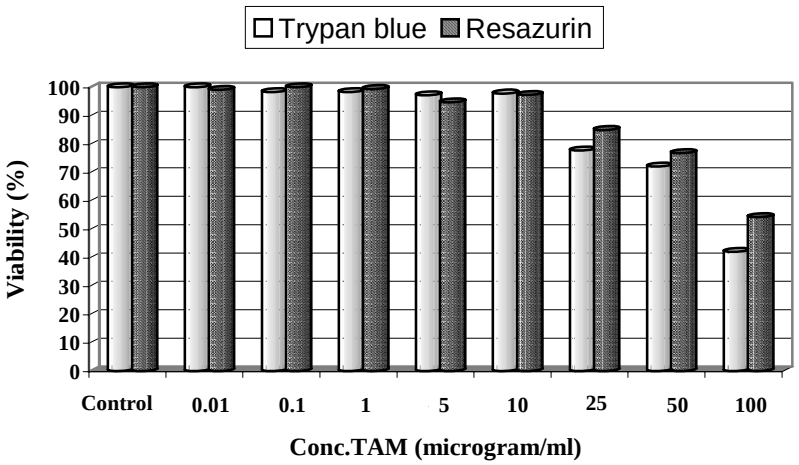


Figure 25 Cytotoxicity of TAM to RAW 264.7 cells by trypan blue and resazurin assays. TAM extract at concentrations ranging from 0.01-10 µg/mL did not induce any cytotoxicity to RAW 264.7 cells. (error bars are not visible due to small value)

4.4 Nitrite studies

Anti-inflammatory properties of the extract and reference standard were tested by measuring their effects on the pro-inflammatory mediators nitric oxide (NO) in activated murine macrophages cell line (RAW264.7). Measurement of nitrite accumulation in the culture medium was used to determine NO production. The culture medium was collected after 24 h incubation, and the nitrite concentration was measured by Griess reaction, a general index for measuring nitric oxide formation (Padgett and Pruett,1992).

4.4.1 Griess reagent

To determine the best conditions for inducing NO production by murine macrophages, RAW 264.7 cells were pre-treated with RPMI complete media with various concentrations of LPS or IFN-γ alone as shown in Figures 26 and 27. LPS or IFN- γ alone stimulated the highest NO production by RAW 264.7 at a concentration of 5 μg/mL and 50 u/mL, respectively. As 5 μg/mL LPS and 50 u/mL IFN- γ induced the amount of NO only slightly higher than at 1 μg/mL LPS and 25 u/mL IFN- γ, we selected 1 μg/mL LPS and 25 u/mL IFN- γ for subsequent studies. The combination of 1 μg/mL LPS and 25 u/mL IFN- γ could provide additive effect since the amounts of NO released was enhanced compared to the amount released by each stimulant separately (Figure 28). Therefore, the combination of 1 μg/ml and 25 u/mL IFN-γ were used to induce NO production by RAW 264.7 cells in subsequent studies.

Time course study of RAW 264.7 cells activated with LPS/IFN- γ at different incubation time points (0, 2, 4, 8, 10, 12, 14, 16, 18, 20, 24, 32, 36 and 48 h) revealed that NO production, measured as nitrite, increased dramatically from the basal level of ~6 to ≥ 40 μM within 24 h. NO production increased 3 folds after a 10 h stimulation of LPS/IFN-γ and gradually increased to over 10 folds at a 48 h (Figure 29).

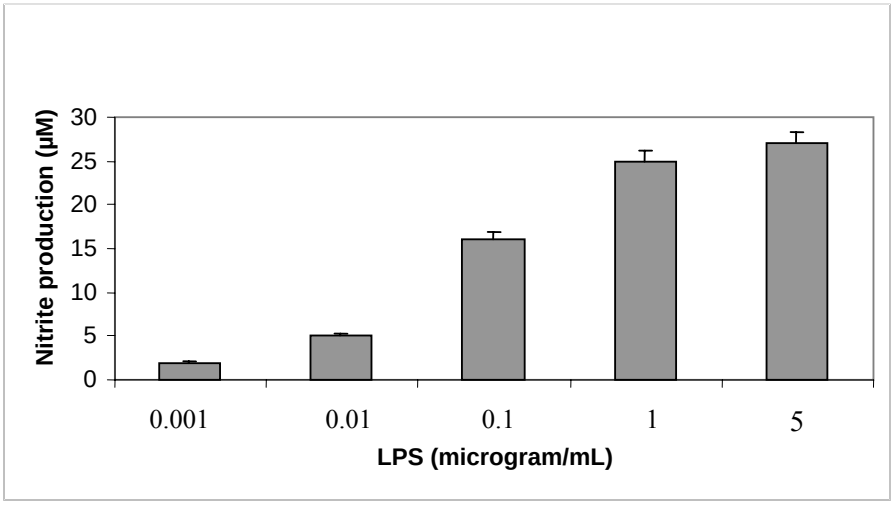


Figure 26 Concentration-dependent production of NO by RAW 264.7 cells stimulated with LPS (0.001-5 µg/mL) for 24 h, after which the NO released was measured as nitrite using the Griess reagent. The columns represent the means ± SE of three independent experiments, each was done in quadruplicates.

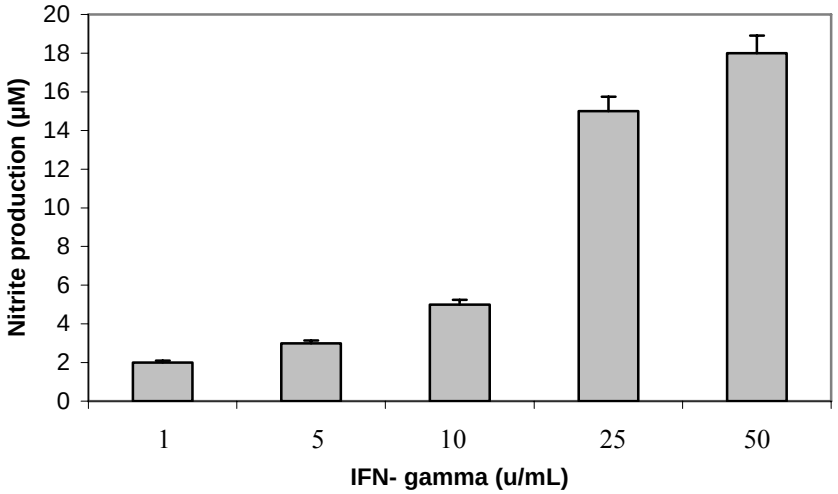


Figure 27 Concentration-dependent production of NO by RAW 264.7 cells stimulated with IFN-γ (1-50 u/mL) for 24 h, after which the NO released was measured as nitrite using the Griess reagent. The columns represent the means ± SE of three independent experiments, each was done in quadruplicates.

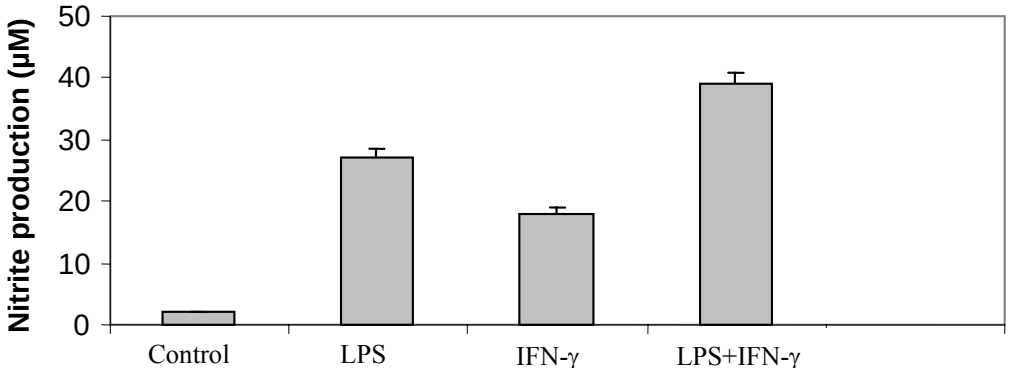


Figure 28 Concentration-dependent production of NO by RAW 264.7 cells stimulated with 1 μg/mL LPS, 25 u/mL IFN-γ alone and LPS / IFN-γ for 24 h, after which the NO released was measured as nitrite using the Griess reagent. The columns represent the means ± SE of three independent experiments, each was done in quadruplicates.

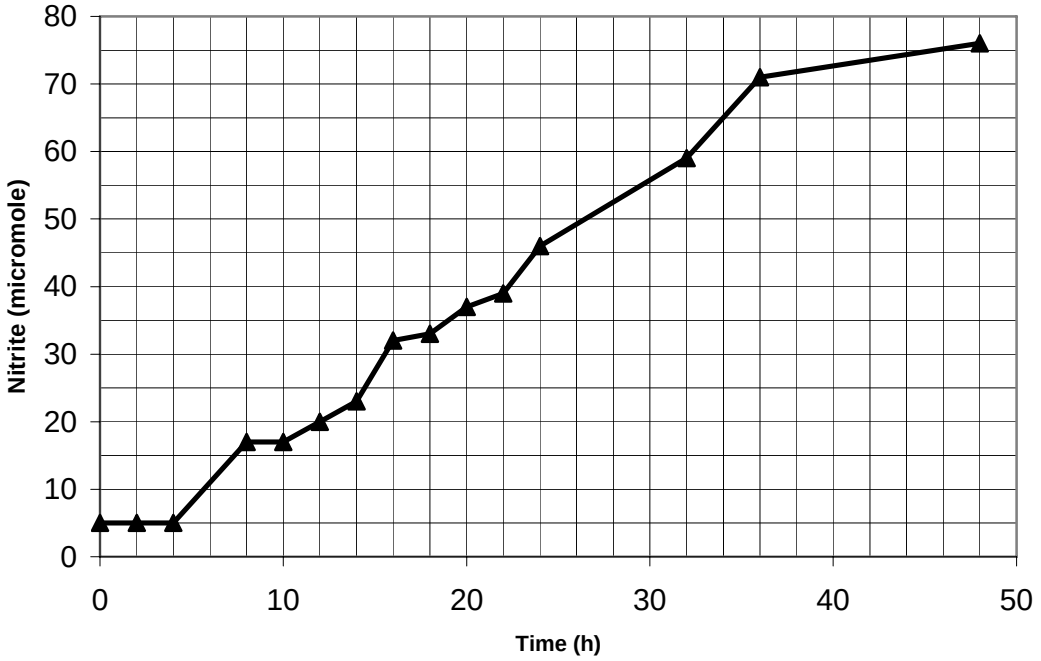


Figure 29 Time course of NO expression in RAW 264.7 cells. Cells were incubated with 1 μg/mL LPS plus 25 u/mL IFN-γ for the indicated time periods. The data is a representative of four independent experiments.

To determine the effects of oligomeric proanthocyanidin (OPCs) and other phenolic compounds on NO production, different concentrations of TAM (1-10 μg/mL) and other phenolic compounds, GSE (10 μg/mL) and vitamin C (500 μM) were incubated with the LPS/IFN-γ-activated RAW 264.7 macrophages.

TAM extract induced a dose-dependent inhibition of NO production at concentration range that had no effect on cell viability. The TAM extract induced a high suppression about 60% of NO production by LPS and IFN-γ activated RAW 264.7 cells at concentration of 10 μg/mL, whereas 10 μg/mL GSE and 500 μM

vitamin C induced the inhibition of NO production by 33 and 42%, respectively. (Figures 30 and 31).

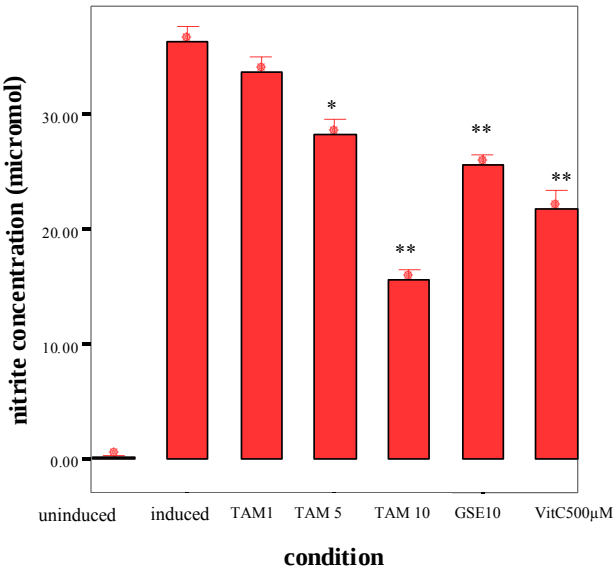


Figure 30 Effect of TAM extract on LPS plus IFN-γ activated induced nitrite production in RAW 264.7 cells. The cells were treated with difference concentrations of TAM (µg/mL) for 24 h. Production of nitrite was determined by Griess reaction, as described in materials and methods. All data represent the mean±SD of three experimental determinations. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences from the control group.

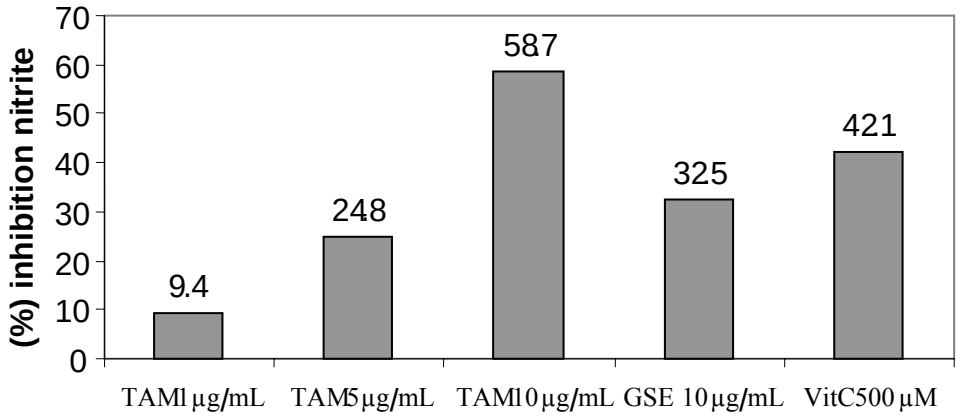


Figure 31 The inhibitory effect of TAM on NO production. The TAM extract induced a high suppression (≈60%) of NO production by LPS and IFN-γ activated RAW 264.7 cells at concentrations that had no effect on cell viability.

4.5 Evaluation of iNOS and COX-2 protein expression

In order to elucidate the mechanism by which TAM inhibits NO production in LPS and IFN-γ activated macrophages, the iNOS protein expression was investigated. iNOS protein was sufficiently expressed after stimulation with 1 μg/mL LPS and 25 u/mL IFN-γ for 24 h, and the presence of TAM at 1 and 10 μg/mL markedly decreased iNOS expression in a concentration-dependent manner. The suppression of iNOS was about 68% at 10 μg/mL TAM extract, whereas 10 μg/mL GSE and 500 μM vitamin C displayed about 40.7% and 32.3% suppression by densitometry, respectively. (Figure 32) There was no significant effect on the level of tubulin synthesis in the same samples.

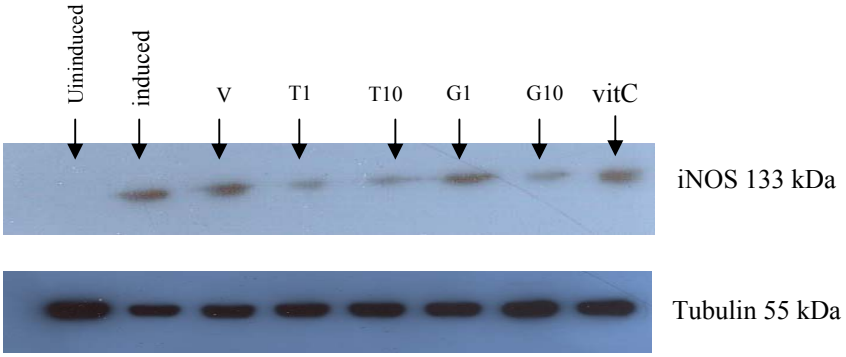


Figure 32 Western blot analysis of iNOS expression in RAW 264.7cells exposed to TAM extract in the presence of LPS/IFN-γ. RAW 264.7 cells were activated with 1 μg/mL LPS plus 25 u/mL IFN-γ for 24 h, with and without TAM extract (T1=1, T10=10 μg/mL) compared with control vitamin C (500 μM) and GSE (10 μg/mL). Equal amounts of total protein (30 μg/lane) were loaded.

Western blot analysis of COX-2 expression in Figure 33 showed that TAM extract produced a dose dependent suppression of COX-2 protein. Compared to VH control, COX-2 protein was sufficiently expressed after stimulation with 1 μg/mL LPS and 25 u/mL IFN-γ for 24 h, and the presence of TAM at 1 and 10 μg/mL markedly decreased COX-2 expression in a concentration-dependent manner. The suppression of COX-2 was about 59.6% at 10 μg/mL TAM extract, whereas 10 μg/mL GSE and 500 μM vitamin C displayed about 45.2% and 20.2% suppression, respectively. (Figure 33)

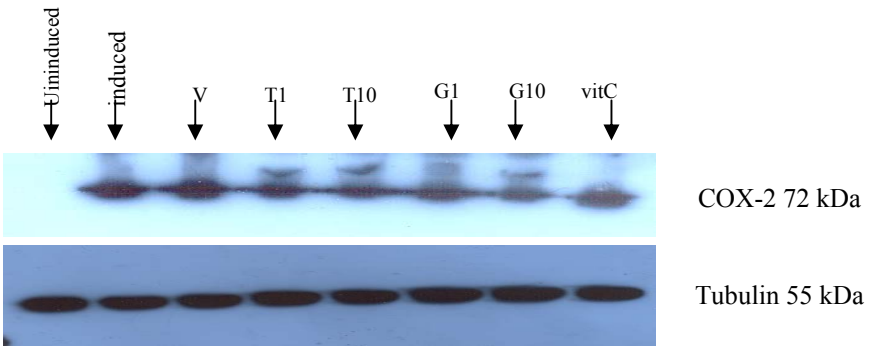


Figure 33 Western blot analysis of COX-2 expression in RAW 264.7 cells exposed to TAM extract in the presence of LPS/IFN-γ. RAW 264.7 cells were activated with 1 μg/mL LPS plus 25 u/mL IFN-γ for 24 h, with and without TAM extract (T1=1, T10=10 μg/mL) compared with control vitamin C (500 μM) and GSE (10 μg/mL). Equal amounts of total protein (30 μg/lane) were load.

4.6 Evaluation of apoptosis

4.6.1 Annexin V-PI method

The anti-apoptotic effect of TAM extract was determined by using annexin V-FITC and propidium iodide (PI) method. Annexin V is a Ca^{2+}-dependent binding protein with high affinity for phosphatidylserine (PS) which exposed in the membrane of early apoptotic cells. Viable cells with intact membranes exclude annexin V, whereas the membranes of dead and damaged cells are permeable to annexin V. Cells that stain positive for Annexin V-PE are undergoing early apoptosis. Cells that stain positive for both Annexin V-PE and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for Annexin V-PE but positive for PI are necrotic cells.

RAW 264.7 cells were treated with various concentrations of TAM extract (1, 10 and 20 μg/mL), GSE (1, 10 and 20 μg/mL) and vitamin C (500 μM) for 24 h before adding the apoptosis inducer, etoposide. The presence of TAM and GSE in the culture inhibited the etoposide-induced apoptosis in RAW 264.7 cells. Representative results of two anti-apoptotic studies were shown in Figure 34-37. TAM extract induced stronger anti-apoptotic effect on induced RAW 264.7 cells compared to GSE and vitamin C. The percentage of apoptotic RAW 264.7 cells decreased from 16.1% in control cells to 6.1% after treatment with 10 μg/mL of TAM, whereas the apoptotic cells were decreased to only 12.2 and 14.8% after exposure to 10 μg/mL GSE and 500 μM vitamin C, respectively. Overall, the percentage of apoptotic RAW 264.7 cells was decreased after TAM, GSE and vitamin C treatment.

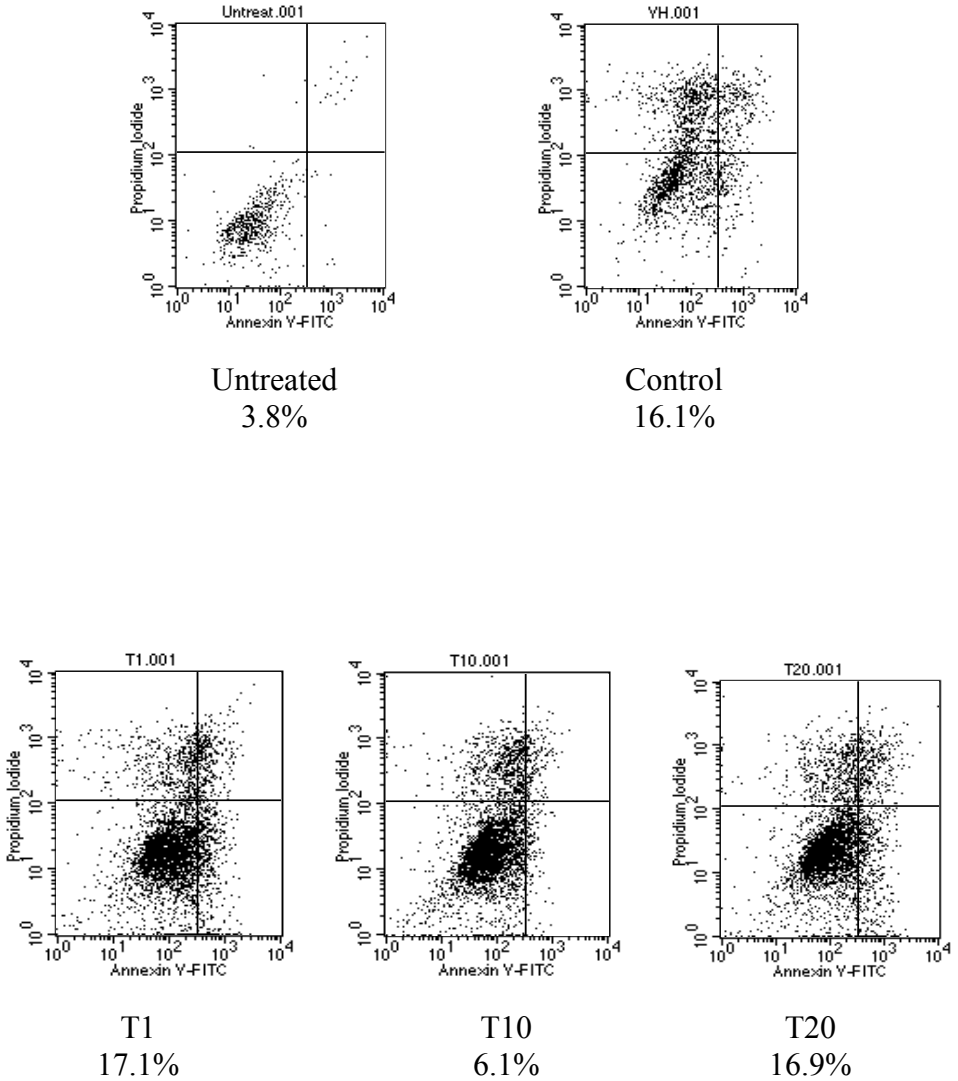
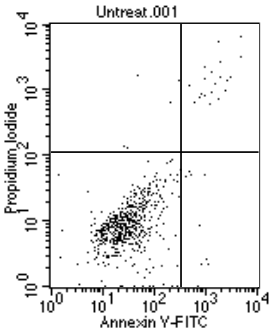
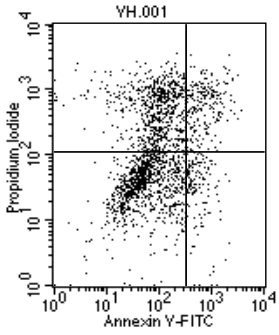


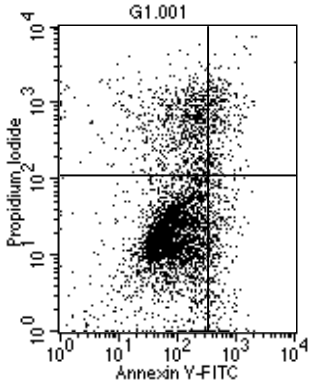
Figure 34 Anti-apoptotic effect of TAM extract on activated RAW 264.7 cells. Cells were treated with VH control (0.08% DMSO) or various concentrations of TAM extract for 24 h before adding 20 μg/mL etoposide. Data indicates the percentage of annexin V-FITC and PI stained cells.



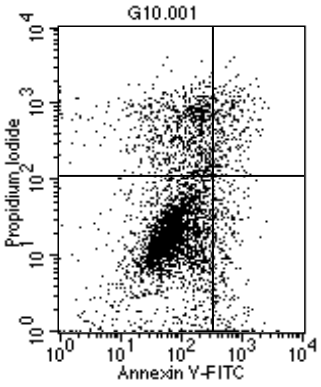
Untreated
3.8%



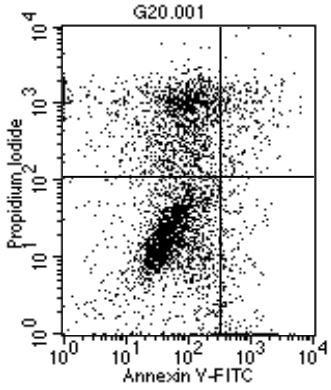
Control
16.1%



G1
12.2%

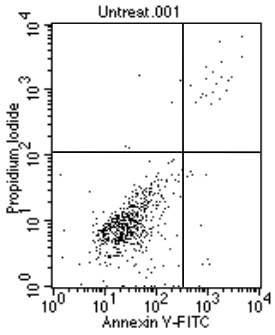


G10
12.2%

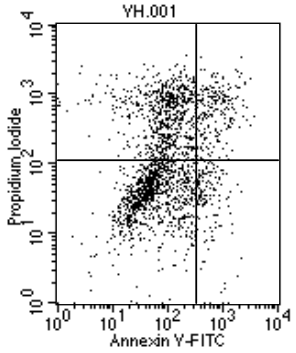


G20
14.8%

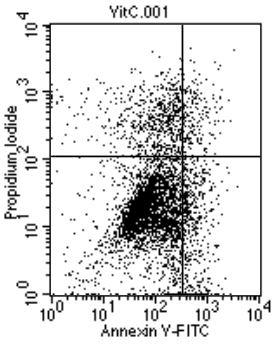
Figure 35 Anti-apoptotic effect of GSE on activated RAW 264.7 cells. Cells were treated with VH control (0.08% DMSO) or various concentrations of GSE for 24 h before adding 20 μg/mL etoposide. Data indicates the percentage of annexin V-FITC and PI stained cells.



Untreated
3.8%



Control
16.1%



Vitamin C 500 μM
14.8%

Figure 36 Anti-apoptotic effect of vitamin C on activated RAW 264.7 cells. RAW cells were treated with VH control (0.08% DMSO) for 24 h before adding 20 μg/mL etoposide. Data indicates the percentage of annexin V-FITC and PI stained cells.

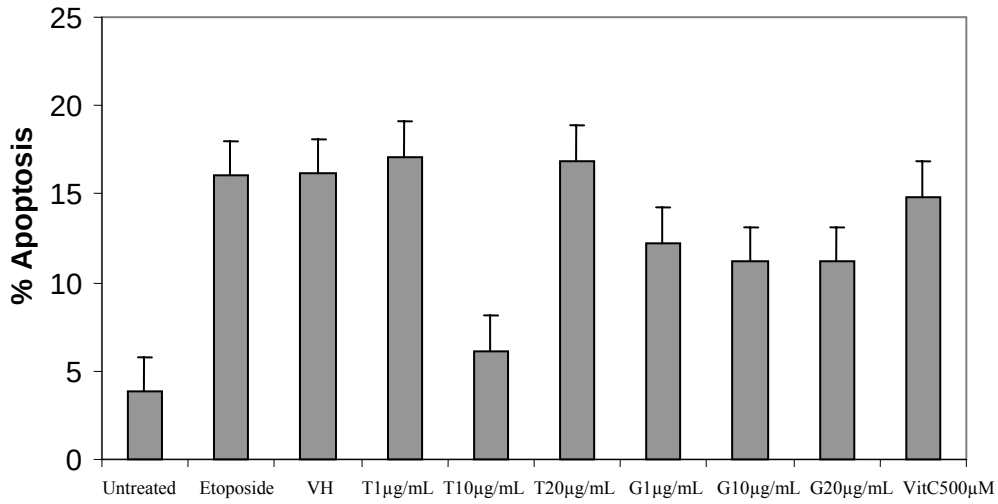


Figure 37 Effect of TAM, GSE and vitamin C on percent cell death of RAW 264.7 cells. Cells were treated with 0.08% DMSO or various concentrations of TAM extract (T1 = 1, T10 = 10, T20 = 20 µg/mL) and GSE (G1 = 1, G10 = 10, G20 = 20 µg/mL) and 500 µM vitamin C prior exposing 20 µg/mL etoposide for another 24 h. Cells were harvested and stained with annexin V-FITC and PI according to the manufacturers' instructions. Ten thousand events were collected and analyzed for each sample, by flow cytometer (Becton Dickinson; FACScalibur cell analyzer (model FACSCalibur^{TM}).

4.6.2 Visualization of DNA ladder formation

After preexposure of RAW 264.7 cells with TAM, GSE and vitamin C for 24 h and subsequently treated with etoposide for 24 h, the DNA fragmentation of RAW 264.7 cells was evaluated on 1.2% agarose gel eletrophoresis by using DNA minikit method (QIAGEN) and stained with ethidium bromide. Analysis of the agarose gel electrophoresis pattern revealed a ladder, which was absent in cells not subjected to the anti-cancer drug, etoposide. As clearly observed in Figure 38, TAM,

GSE and vitamin C decreased the fragmented DNA in all etoposide treated cells. Also, the decrease of DNA ladder in TAM and GSE treated cells was dose dependent. Therefore, the DNA ladder analysis confirmed the reduction of apoptosis by TAM, and provided the support evidence observed in flow cytometry studies.

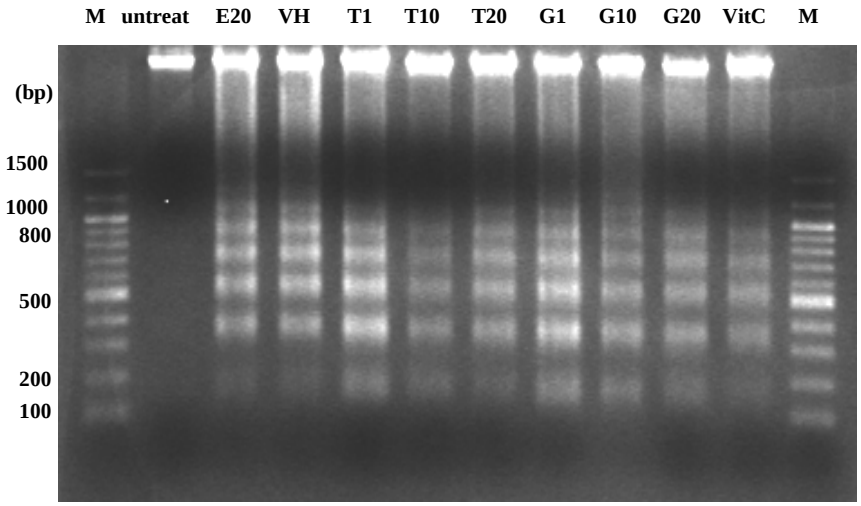


Figure 38 The reduction of DNA ladder formation by TAM, GSE and vitamin C. DNA fragmentation of RAW 264.7 cells preexposured to various concentrations of TAM extract and GSE and vitamin C for 24 h and subsequently treated with 20 µg/mL etoposide for another 24 h. DNA ladder was analyzed by DNA mini kit method using 1.2% agarose gel electrophoresis and staining with ethidium bromide. Lane M = 100 base-pair ladder marker, E20 = culture treated with 20 µg/mL etoposide, VH = 0.08% DMSO treated cells, T1-T20 = 1, 10 and 20 µg/mL of TAM extract treated cells G1-G20 = 1, 10 and 20 µg/mL GSE treated cells, vitC = 500 µM vitamin C treated cells.

4.7 *In vivo* anti-inflammatory activity

4.7.1. Antinociceptive activity

Antinociceptive activity was evaluated by acetic acid induced writhing and tail flick tests. The effect of TAM extract on acetic acid-induced writhing responses in mice was shown in Table 5. It was found that all extracts of this plant at the doses assayed caused a significant inhibition on the writhing responses induced by 0.6% acetic acid when compared with control, with values ranging from 14.8 to 44% of inhibition, Nevertheless, none of the screened extracts reached the values obtained for dicolfenac at 25 mg/kg, p.o. (67.8%), the non-steroidal anti-inflammatory chosen as a reference drug. The data concerning the antinociceptive effect of the TAM extracts and fractions on the tail flick test in mice are summarised in Table 5. Pretreatment of mice with 400 mg/kg TAM extracts, p.o. showed significant analgesic activity in the tail flick test responses to the nociceptive stimuli ($p < 0.05$).

The results of dose–response effects of TAM extract on acetic acid-induced writhing in mice were presented in Table 5. TAM at doses of 200 and 400 mg/kg significantly inhibited the writhing response of mice caused by the intraperitoneal administration of acetic acid. The maximal inhibition of the nociceptive response was 44% for TAM at the dose of 400 mg/kg, which is only slightly less than that of dicolfenac (67.8%) at the dose of 25 mg/kg. TAM exerts its pain-relieving effect in a dose-dependent manner. The dose of 400 mg/kg TAM and 25 mg/kg diclofenae significantly induced a protection against abdominal constriction ($p < 0.01$) (Figure 39). From these results, it seems that the extract possesses both peripheral and central analgesic activities in mice.

Table 5 Analgesic effects of TAM extract and diclofenae on acetic acid-induced writhing and tail flick test.

Treatment groups	Dose (mg/kg, p.o.)	Tail flick[a] (second)	Writhing times[a] (times/45 min) (mean ± S.E.M.)	Writhing inhibition (%)
VH Control	8% DMSO	4.4±3.11	34.8±4.21	-
Diclofenae	25	7.8±2.21***	11.2±3.27***	67.8±4.49
TAM	100	5.2±4.56	29.6±2.77	14.8±3.13
TAM	200	5.1±4.24	24.4±2.52**	29.8±5.23
TAM	400	6.4±3.55**	19.5±3.05**	44.0±5.93

[a] Number of mice per group was six.

** Statistical significance: $p < 0.05$ (vs. control group)

*** Statistical significance: $p < 0.01$ (vs. control group)

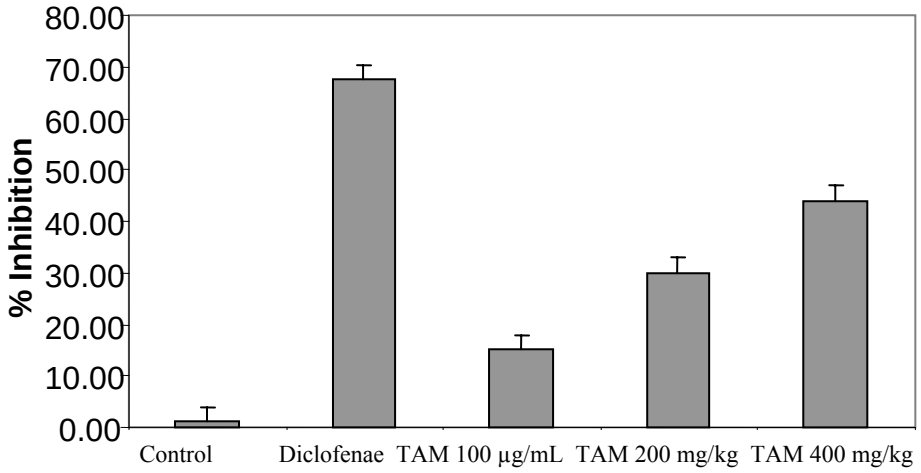


Figure 39 Inhibition of the nociceptive response of TAM (100, 200 and 400 mg/kg) on acetic acid-induced writhing responses in mice. Control = 8% DMSO treated mice, diclofenae = 25 mg/kg treated mice. Numbers of mice per treatment group was six.

CHAPTER V

DISCUSSION

Since TAM seed coat contains 4-16% of oil, it is essential to remove the fat prior isolating the phenolics compounds from the ground and homogenizated seeds (Shahidi, 1995) The defatting can be performed with methanol/ammonium/water (Naczk and Shahidi, 1989) or with carbon tetrachloride (Li and Rassi, 2002). The extraction with hexane in a Soxhlet apparatus is the most common procedure (Naczk *et al*., 1992a; Wanasundara *et al*., 1996). Hulls may also be removed after extraction (Krygier *et al*., 1982; Kozlowska *et al*., 1983a; Kozlowska *et al*., 1983b). Defatted rape seed meal is usually extracted with organic solvents which most commonly used are aqueous methanol, ethanol, or acetone. When extracting TAM tannins, 50% aqueous acetone is the most common extraction solvent (Vuorela, 2005). Acetone was used in the present study as solvent for extraction of crude material from *T. indica* as it is miscible with water, is volatile, and has low toxicity to bioassays used (Eloff *et al*., 1998). Another advantage of acetone is the ease of removal from fractions collected within a relatively short period.

Positive DPPH test suggests that the TAM extract is a free radical scavenger. The high content of polyphenolic compounds in *T. indica* seed coat extracts (TAM) registered its higher DPPH radical scavenging activity than vitamin C and GSE. Whilst in the DPPH model, the antioxidant efficiency of phenolic compounds is mainly dependent on the number of hydroxyl groups capable of donating hydrogen

to the radical. In 1995 Brand-Williams found that the scavenging activity of seed coat extracts would be related to substitution of hydroxyl groups in the aromatic rings of phenolics thus contributing to their hydrogen donating ability. Pumthong (1999) demonstrated the antioxidant activity of the *T. indica* seed coat extract composed of flavonoids including tannins, polyphenols, anthocyanidins, and olygomeric proanthocyanidins, these results agree with recently reported antioxidant property of the tamarind seed, which was attributed to the oligomeric flavonoids: procyanidin B_2 (II), procyanidin trimer (IV), procyanidin tetramer (V), procyanidin pentamer (VI) and procyanidin hexamer (VII) (Sudjaroen *et al*., 2005) (Figure 40).

Moreover, the activities in all the investigated samples are directly proportional to the concentration of total phenolics content (TPC). Elias *et al*. (1979) quantitated amounts of phenolic compounds in tamarind seeds by analytical HPLC and reported that tamarind seeds contained 6.54 g/kg total phenolics which mainly represented by oligomeric procyanidin tetramer, hexamer, trimer and pentamer with lower amounts of procyanidin B_2 and (-)-epicatechin. The amounts of oligomeric procyanidins detected in seeds were 29.3 g/kg, in pericarp were 18.6 g/kg. A basic evaluation of the polymeric tannins using (-)-epicatechin as external standard revealed approximate values of 325 mg/kg in the seeds (Table 6). Apart from these, Hagerman *et al*. (1998) has reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals and that the effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups. Through oxidative phenolic coupling reactions, phenolic dimers and oligomers are formed. Each dimer or oligomer may retain its original number of reactive hydroxyl groups. This will enhance antioxidant

and or antiradical activity, if it is still soluble. Given that the tamarind seeds especially, contained substantial amounts of these oligomeric proanthocyanidins, their antioxidant and antiscarvenging activities are highly warranted.

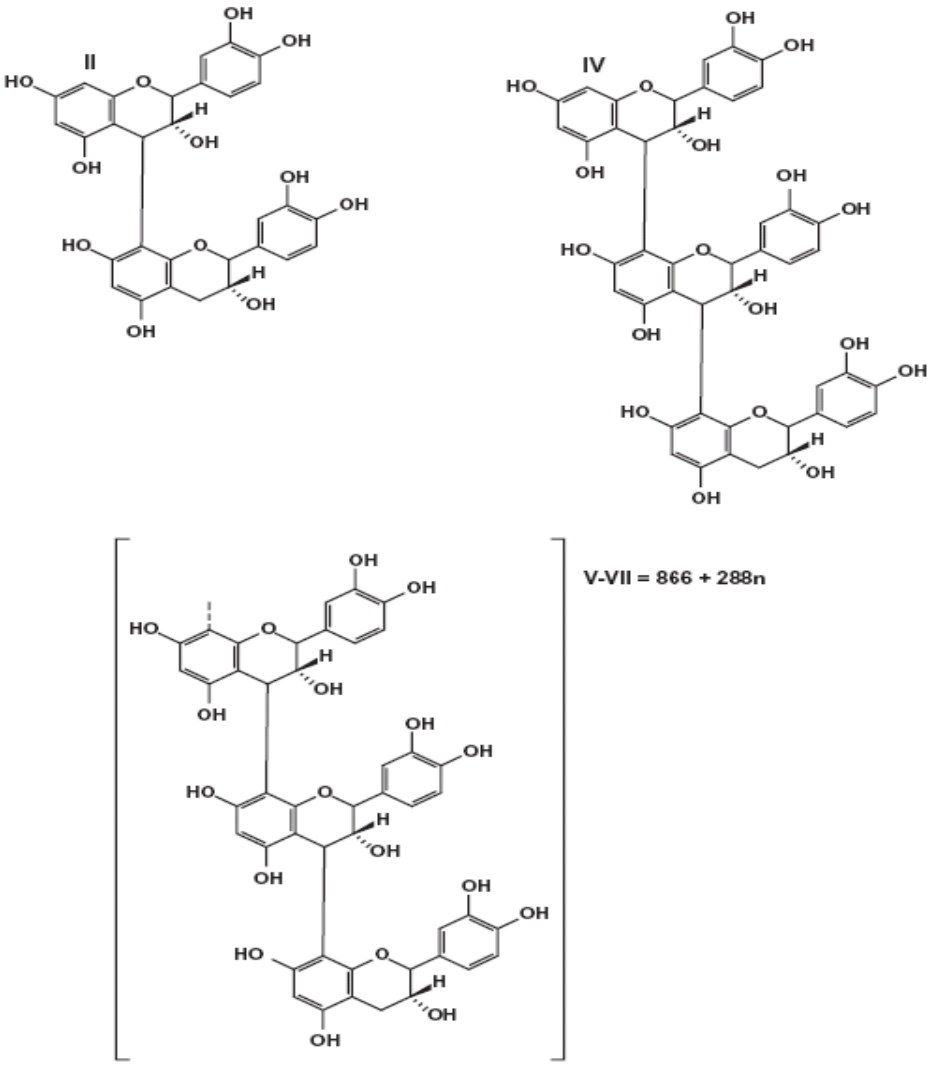


Figure 40 The structures of the oligomeric flavonoids are procyanidin B_2 (II), procyanidin trimer (IV), procyanidin tetramer (V), procyanidin pentamer (VI) and procyanidin hexamer (VII). (Sudjaroen *et al*., 2005)

Table 6 Content of phenolic compounds (mg/kg) in seeds of tamarind fruits (Gu *et al*., 2003).

Compound	Tamarind seeds (mg/kg)
Procyanidin B_2	359
(-)-Epicatechin	325
Procyanidin trimer	1180
Procyanidin tetramer	1975
Procyanidin pentamer	1149
Procyanidinhexamer	1559
Polymeric tannins	20,290
Polymeric tannins	2340
Total (g)	**6.537**
Polymeric tannins were not included in the total.	

FRAP assay is a simple and reliable test depends upon the reduction of ferric 2,4,6-tripyridyl-s-triazine [Fe(III)-TPTZ] to the ferrous 2,4,6-tripyridyl-s-triazine [Fe(II)-TPTZ] complex by a reductant at low pH (Benzie and Strain, 1999). The potential reducing power activity of TAM extract in the FRAP assay might be attributed to the presence of dihydroxy type of benzene derivatives and (-)-epicatechin presented in the seed coat. However, vitamin C was found to have more reducing antioxidant power than TAM. Nevertheless, the recent *in vivo* investigation has revealed that the consumption of dark chocolate, which contains phenolics, in particular (-)-epicatechin, proved to be potential antioxidants through ferric-reducing antioxidant power assay (*in vitro*) and increased the total antioxidant capacity of blood plasma (Serafini *et al*., 2003). This study suggests that the phenolic substances from TAM are potent antioxidant sources. Nonetheless, evaluating the occurrence of

tannin-protein interactions in the seed coat samples by testing both *in vitro* protein digestibilities together with the assessment of antioxidant properties *in vivo* might be a fruitful approach for advocating TAM as nutraceuticals.

The highly unstable gas, nitric oxide (NO), appears to be a major macrophage mediator of tumor cell killing (Manthey *et al*., 1994). However, high level of NO production may induce host cell death and inflammatory tissue damage (Mineo *et al*., 1997). Murine macrophage cell line RAW 264.7 has previously been found to produce NO in response to LPS and the combination of LPS and IFN-γ. Stimulation of macrophages to produce NO requires two signals. The first, IFN-γ, primes them to respond to the second triggering signal of LPS. Although the triggering signal cannot precede the priming signal, the two can be provided simultaneously (Scuro *et al*., 2004). Therefore, both INF-γ and LPS were used in the present study to activate RAW 264.7 cells for 24 h to induce NO production. Direct measurement of NO is difficult, both because of the small amounts present and the lability of NO in the presence of oxygen. NO is currently measured by spectroscopic methods, including chemiluminescene (Foster *et al*., 2003 and Gladwin *et al*., 2002), ultraviolet (UV) visible spectroscopic (Kelm *et al*., 1997), and electron paramagnetic resonance (EPR) (Kuppusamy *et al*., 1996). The UV visible spectroscopic method for NO determination is based on the Griess reagent which is a mixture of sulfanilamide, HCl, and N-(1-napthyl)-ethylenediamine (NED). The spectrum of the product of this reaction shows a band at 548 nm. The absorbance of this peak is proportional to the nitrite concentration which is propotional to the amount of NO production. A second UV visible spectroscopic method is based on the reaction of oxyhemoglobin (HbO_2) with NO to form methemoglobin (metHb) and (NO^-_3) and is used to determine rates

of NO production. The reaction is also accompanied by significant changes in the absorption spectrum of HbO_2. The spectral changes form the basis of the hemoglobin NO assay, a well-established spectrophotometric technique that allows the quantification of NO in solution (Gow *et al*., 1999). The present study used the Griess reaction to determine NO production since the method is simple, inexpensive, and sensitive enough to detect the induced form of NO production.

In early studies, TAM could modulate the NO production in B6C3F1 mice at 100-500 mg/kg, the dose range that does not induce any clinical signs of toxicity. Neither the development of a T-cell mediated sensitization responses to DNFB or HCA nor the dermal irritation to nonanoic acid or DNFB was modulated by the *T. indica* treatment. Moreover, TAM was found to inhibit NO production in macrophages both *in vivo* and *in vitro* (Komutarin *et al*., 2004). The present study confirmed the modulation of NO production by TAM in activated RAW 264.7 cells at the lower range of concentrations.

The present study also revealed that TAM extract suppressed NO production of LPS and IFN-γ activated RAW 264.7 via down modulation of iNOS expression in a dose dependent manner. In addition, TAM extract also induced dose related decrease of COX-2 expression. The effect on COX-2 was less pronounced than iNOS. The suppression of TAM at 10 μg/mL, as analyzed in the same blotted membrane, was about 59.6% and 68% for COX-2 and iNOS, respectively. The mechanism of inhibition of iNOS and COX-2 expression are not clear. The modulation either at the transcriptional and/or post-transcriptional levels could also involve the inhibition of gene expression. Flavonoids, such as quercetin, apigenin, and tea catechins, mediated anti-inflammatory activity via the inhibition of COX-2, and iNOS. For example, tea

catechins inhibit the production of inflammatory mediators by down-regulating COX-2, and reduced NO by inhibiting the expression of iNOS (Higdon and Frei, 2003). The inhibition of transcription factors, including NF-κB and activator protein-1 have been reported as the mechanism of iNOS suppression and the anti-inflammatory effects of glucocorticoids (Bourke and Moynagh, 1999). Whether TAM mediated suppression of iNOS and COX-2 expressions are related to NF-κB inhibition need to be further investigated.

Other studies have demonstrated that various flavonoids such as rutin, quercetin, luteolin, hesperidin and biflavonoids produced significant antinociceptive and/or anti-inflammatory activities (Bittar *et al*., 2000; Calixto *et al*., 2000; Ramesh *et al*., 1998). The present study demonstrated that the acetone extract from TAM which contains flavonoids among its major constituents seems to possess both peripheral and central analgesic activities in mice, since the extract was able to inhibit acetic acid-induced writhing episodes and exhibit antinociception in the tail flick test. Acetic acid causes algesia by liberating endogenous substances and many others that excite pain nerve endings. The writhing test is a sensitive method of screening both peripheral and central analgesic efficacy of agents (Collier *et al*., 1968). Oral administration of TAM significantly reduced the number of mouse abdominal constriction following acetic acid, indicating analgesic activity for both species at the doses assayed. The results of this writhing test alone did not ascertain whether the antinociceptive effects are central or peripheral. Thus, to clear the mode of the inhibitory effect of both species on the nociceptive response, the effect of the TAM extract on the tail flick test was examined. It is known that the tail-flick response appears to be a spinal reflex, and is considered to be selective for centrally acting analgesic compounds

(Ramabadran *et al*., 1989; Srinivasan *et al*., 2003). As noted, the reference narcotic analgesic drug, votalren (25 mg/kg p.o.) exhibited significant antinociceptive effects in the tail flick test. The infusion, acetone extract of TAM also produced a statistically significant but less in degree antinociceptive response than dicolfenac in the tail flick test. The results obtained on the writhing response and tail-flick test suggest that TAM possesses both peripheral and central analgesic activity, although further studies are needed in order to know the mechanism behind the observed central antinociceptive action. On the other hand, the lack of influence of TAM in low dose (100 mg/kg) on the reaction time of mice submitted to the tail flick test is consistent with the interpretation that its analgesic property does not have a central origin. An analgesic effect in the acetic acid induced writhing test is mostly mediated via a peripheral mechanism by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonising the action of pain mediators at the target sites (Srinivasan *et al*., 2003).

The discovery and characterization of the COX-2 enzyme early in the 1990s led to many studies on selective inhibitors of this isoform. COX-2 is considered to play an important role in the nociceptive transmission in mechanical allodynia (DeLeo *et al*., 2000), chemically induced pain (Yamamoto and Nozaki-Taguchi, 1996), hyperalgesia (Hay and Belleroche, 1997), and thermally induced acute pain (Yamamoto and Nozaki-Taguchi, 2002). Tegeder *et al*. (2001) have reported that, even with a sufficient concentration of celecoxib in the spinal cord to inhibit COX-2, systemically, administered celecoxib has no effect on formalin-induced flinching behavior or prostaglandin-E_2 increase. It has been demonstrated that endotoxin-induced hyperalgesia can be prevented by indomethacin and DFU by inhibition of

COX-1 and/or COX-2, respectively (Tunctan *et al*., 2006). Thus, the mode of the inhibitory effect of TAM extract on the nociceptive responses might involve with inhibition of COX-2 expression. Whether TAM extract can also affect COX-1 expression need to be further investigated.

Apoptosis, or programmed cell death, is believed to be an intrinsic death program that cells activate; thus, they actively contribute to their own deaths. The nucleus undergoes a relatively characteristic metamorphosis during apoptosis, first chromatin condensation, and then nuclear condensation (Pelfrey *et al*., 1995). RAW 264.7 cells treated with etoposide drug undergo the classical morphological changes associated with apoptosis. DNA fragmentation and the characteristic DNA laddering were observed in these cells. Analysis of the agarose gel electrophoretic pattern revealed a ladder, which was absent in cells that were not subjected to etoposide, a commonly used apoptosis inducing agent (Shaulian *et al*., 1996). The degree of DNA fragment was lowered at 10 µg/mL TAM extract. When 10 µg/mL of GSE was added to the culture medium, the degree of apoptosis was lowered, as shown by the DNA fragmentation and the annexin V-PI method in the flow cytometry. The mechanism of such effect may be in two ways: one is metal chelation because of the properties of the phenol-hydroxyl group; the other may be the reduction property of double bonds in aromatic rings (Dive *et al*., 1997). Notably, the reduction of apoptosis was not observed at high concentration (20 µg/mL) of both TAM and GSE. This could be explained by the cytotoxic effect of both compounds at this concentration. Hence, if Tamarind seed coat phenolics are used as food additives or phytochemicals, the safety and toxicology of phenolics have to be distinguished in detail and extensively studies.

CHAPTER VI

CONCLUSION

The polyphenol was isolated from seed coat of *Tamaridus indica* Linn., and several biological activities were investigated by: (1) two different antioxidant activity assays; (2) inhibitory activity of NO formation by activated RAW 264.7 macrophage cell line; (3) iNOS expression as the inhibitory mechanism (4) anti-inflammatory activity *in vitro* and *in vivo* models; and finally (5) anti-apoptosis of the RAW 264.7 macrophage cell line.

For the rapid screening of RSC by TLC, It indicates that the crude extract could be specially separated by this system into six spots from origin to solvent front that each had different Rf values. The yield of the TAM extracts was 45.8% and the total phenolic content was 178±3.8 mg/g GAE which was higher than GSE whose content was found 133.2±2.39 mg/g GAE. The free radical-scavenging activities of aqueous acetone extract of TAM was compared with other well know antioxidant compound such as vitamin C and GSE. The present study showed that the extract of TAM registered the higher DPPH radical scavenging activity than vitamin C and GSE. The amount of IC_{50} in the crude TAM extract was 13 µg/mL, much lower than the IC_{50} of GSE and vitamin C which were 20.7 µg/mL and 38.7 µg/mL, respectively.

The TAM extract was able to reduce ferric complex (Fe^{III}-TRTZ) to ferrous form in a dose-dependent mode. The slope of reducing capability of TAM extract was 0.0083 whereas of vitamin C was 0.1437. Therefore, the potency of TAM was

approximately 1/17 time of vitamin C. This study suggests that the phenolic substances from seed coats of T. indica are potent antioxidant sources.

TAM at the concentrations equals or less than 10 μg/ml did not induce any cytotoxicity to RAW 264.7 cells, as evaluated by TB and resazurin assay. The resazurin method was chosen to substitute for MTT assay as the color of anthocyanins interfere with the detection of formazan product in the MTT assay.

Anti-inflammatory properties of the extract and standard were tested by measuring their effects on the pro-inflammatory mediators nitric oxide (NO) in activated macrophages RAW264.7. The TAM extract induced a high suppression (≈60%) of NO production by LPS and IFN-γ activated RAW 264.7 cells at concentration of 10 μg/mL that had no effect on cell viability. The anti-inflammatory property of TAM was also supported by the in vitro inhibition of COX-2 protein expression in activated RAW 264.7 cells.

In order to elucidate the mechanism by which TAM inhibits nitrite production, its effect on iNOS protein expression was investigated. TAM shows a dose-dependent inhibition of iNOS protein expressions *in vitro*.

Antinociceptive activity was evaluated by acetic acid induced writhing test and tail flick test, TAM extracts more than 400 mg/kg; p.o. showed significant analgesic activity in the tail flick test in responses to the nociceptive stimuli ($p < 0.05$). From these results it seems that the extract possesses both peripheral and central analgesic activities in mice. TAM at the doses of 200 and 400 mg/kg significantly inhibited the writhing response of mice caused by the intraperitoneal administration of acetic acid. The maximal inhibition of the nociceptive response was 44% for TAM at the dose of 400 mg/kg, which was slightly less than 67.8% of diclofenae at the dose of

25 mg/kg. TAM also exerts its pain-relieving effect in a dose-dependent manner.

The percentage of apoptotic RAW 264.7 cells decreased from 16.1% in control cells to 6.1 % after treatment with 10 µg/mL of TAM. Similarly, the apoptotic cell was also decreased from 16.1% in control cells to 11.2% after treatment with 10 µg/mL GSE. The fragmented DNA was clearly observed in treated cells in a dose dependent manner, whereas the DNA content of uninduced apoptotic cells did not provide any ladders.

Tamarind is an important source of food in tropical regions, but at present, the waste products of the canning industry such as the pericarp and seeds are discarded in Thailand. Seeds which are waste products appear to have real potential as safe and low-cost sources of chemopreventive natural products and is worthwhile to be developed as effective and inexpensive natural antioxidants in nutraceutical.

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APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

1. Folin-Ciocalteau Micro Method

- **Gallic Acid Stock Solution**

Gallic acid	0.500	gm
Ethanol	10	mL

Dilute to volume with distilled water (store at $4^{o}C$)

- **Sodium Carbonate Solution**

Anhydrous sodium carbonate	200	gm
Water	800	mL

Bring to a boil. After cooling, add a few crystals of sodium carbonate, and after 24 h, filter and add distilled water to 1 L.

(Store at Room temperature)

2. DPPH assay

- Stock DPPH Solution (1 mM)

DPPH	0.0985	gm
Methanol	250	mL
(Filtrate, keep in freezer)		

- Working DPPH (0.2 mM)

Stock DPPH Solution (1 mM)	10	mL
Methanol	40	mL

3. FRAP assay

- Acetate buffer (300mM, pH 3.6)

Sodium acetate.$3H_2O$	3.1	gm
Glacial acetic acid	16	mL
Distilled water to 1 litre		
(Store at 4^oC)		

- Dilute HCl: 40 mM

Conc. HCl (1 M)	1.46	mL
Distilled water to 1 liter		
(Store at Room temperature)		

- TPTZ (10 mM)

TPTZ	0.062	gm
HCl (40 mM)	20	mL
(Freshly prepared)		

- Ferric chloring (20 mM)

$FeCl_3.6H_2O$	0.108	gm
Distilled water	20	mL

(Freshly prepared)

- Working FRAP reagent

Acetate buffer (300mM, pH 3.6)	200	mL
TPTZ (10 mM)	20	mL
Ferric chloring (20 mM)	20	mL
Distilled water	24	mL

(Freshly prepared)

4. Nitrite assay

- Griess reagent

Phosphoric acid	3.5	mL
Sulfanilamide	1	gm
N-(1-napthyl)-ethylenediamine (NED)	0.1	gm

Adjust volume with MQ water to 100 mL

(Filter sterile, store at 4^{o}C)

5. Western blot

- RIPA buffer

PBS 1X	100	mL
NP-40	1	mL
SDS	0.1	gm

(Store at 4^{o}C)

- Lysis buffer

RIPA buffer	1	mL

PMSF (200 mM)	10	μL
Leupeptin (2 mM)	1	μL
E-64 (1 mM)	1	μL
(Freshly prepared)		

- 6X Sample Buffer

Tris-base	0.59	gm
distilled water	8.5	mL
SDS	1.5	gm
2ME	0.6	mL
Glycerol	7.5	mL
Bromophenol blue	7.5	mg
(Store at 4^{o}C)		

- 10% SDS-polyacrylamide gel

SDS	10	gm
distilled water	100	mL
(Store at Room temperature)		

- 10% AP solution

Ammonium persulfate	0.1	gm
distilled water	1	mL
(Freshly prepared)		

- 30% acrylamide

Acrylamide	30	gm
bis- acrylamide	0.8	gm
distilled water	100	mL

(Filtrate, store at 4^{o}C)

- 1.5M Tris Cl, pH 8.8

  Tris-base 18.165 gm

  distilled water 80 mL

  Adjust pH 8.8 by HCl then adjust volume to 100 ml

  (Filtrate, store at 4^{o}C)

- 0.5 M TrisCl, pH 6.8

  Tris-base 6 gm

  distilled water 80 mL

  Adjust pH 6.8 by HCl, then adjust volume to 100 mL

  (Filtrate, store at 4^{o}C)

- Running buffer (10 X)

  Tris-base 30 gm

  Glycine 14.4 gm

  SDS 10 gm

  distilled water to 1 liter

  (Filtrate, store at 4^{o}C)

- Running buffer (1X)

  Running buffer (10 X) 100 mL

  distilled water 900 mL

  (Filtrate, store at 4^{o}C)

- Blotting buffer (1X)

  Tris-base 3.03 gm

  Glycine 14.4 gm

Methanol	200	mL
distilled water to 1 liter		
(Filtrate, store at 4^{o}C)		

- TPBS 0.1% Tween 20

PBS 1X	1,000	mL
Tween 20	1	mL
(Filtrate, store at 4^{o}C)		

- 5% smilk milk

Skim milk	0.75	gm
TPBS 0.1% Tween 20	15	mL
(Freshly prepared)		

- Coomassie stain solution

Coomassie blue	0.05	gm
Methanol	80	mL
Glacial acetic acid	14	mL
distilled water to 100 mL		
(Store at room temperature)		

- Destaining solution

Methanol	5	mL
Glacial acetic acid	7	mL
distilled water to 100 mL		
(Store at room temperature)		

- 10% Resolving gel (12.05 mL for 2 gel)

30% acrylamide	3.984	mL

1.5 M Tris/SDS pH 8.8	2.988	mL
distilled water	4.98	mL
10% AP	84	µL
TEMED	15	µL
(Freshly prepared)		

- Staking gel (4.99 mL for 2 gel)

30% acrylamide	0.65	mL
0.5 M Tris/SDS pH 6.8	1.25	mL
distilled water	3.05	mL
10% AP	34	µL
TEMED	5	µL
(Freshly prepared)		

- Reagent A (Lowry method)

Na_2CO_3	5.3	gm
NaOH (0.1N)	250	mL
(Store at 4^oC)		

- Reagent B (Lowry method)

$CuSO_4.5H_2O$	0.05	gm
Sodium citrate	0.1	gm
distilled water	10	mL
(Store at 4^oC)		

- Reagent C (Lowry method)

Folin	1	mL
distilled water	1	mL

(Freshly prepared)

- Reagent D (Lowry method)

Reagent B (Lowry method)	1	mL
Reagent A (Lowry method)	1	mL

(Freshly prepared)

- Primary antibodies iNOS or COX-2 (1:500)

Primary antibodies (iNOS or COX-2) (200µg/ mL)	20	µL
Skim milk	0.1	gm
TPBS	10	mL

(Freshly prepared)

- Secondary antibody HRP-conjugated rabbit-anti-mouse-IgG (1:10,000)

Secondary antibody HRP (200 µg/ 0.5 mL)	1	µL
Skim milk	0.1	gm
TPBS	10	mL

(Freshly prepared)

- Tubulin antibody (1:1,000)

Tubulin antibody (200 µg/ mL)	10	µL
Skim milk	0.1	gm
TPBS	10	mL

(Freshly prepared)

6. DNA fragmentation

- 1.2% agarose gel

Agarose gel	1.2	gm

distilled water	100	mL

(Freshly prepared)

- TBE buffer 10X solution

Boric acid	55	gm
Tris-base	109	gm
EDTA	9.3	gm
distilled water	800	mL

Dissolve and adjust volume to 1 liter, store the 10X solution in glass bottles at room temperature and discard any batches that develop a precipitate.

- TBE buffer 1 X solution

TBE buffer 10 X solution	100	mL
distilled water	900	mL

(Filtrate, store at Room temperature)

- RNaseA 100 mg/mL

RNaseA	50	mg
DDW.	500	µL

(Aliquot, store at 4^{o}C)

- DNA ladder 100 bp (keep in -20^{o}C) (DNA detection kit (QIAGEN))

Stock solution= 500 µg/mL = 0.5 µg/µL = 500 ng/µL

Working solution = 50 ng/µL

Stock 5 µL + 45 µL AE

- DNA mini kit (QIAGEN)

AW1 + ethanol 25 mL

AW2 + ethanol 30 mL

Keep in RT

- Ethidium bromide staining (10 mg/mL, FW=394.3)

distilled water	100	mL
Ethidium Bromide	1	g

Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.

7. Acetic acid-induced writhing method

- 0.6% acetic acid

Acetic acid	0.6	mL
distilled water	100	mL

(Filtrate, store at room temperature)

APPENDIX B

REAGENTS FOR CELL CULTURE

1. Reagents for cell culture

- **FBS/FCS (inactivated, Hyclone)**

  Slowly thaw the frozen FBS in a breaker filled with water.

  Put in water bath at 37^{o}C till completely thaw.

  Heat inactivate (56 ^{o}C, 20 min), gentle mix every 5-10 min

  Aliquot 50 ml each tube

  (Store at -20^{o}C)

- **RPMI 1640 1X**

RPMI-1640 1X with L-glutamine, with phenol red	1	pack
$NaHCO_3$	2	gm

  Adjust volume to 1,000 mL with MQ.water

  Adjust pH to 0.2-0.3 below desired (7.4)

  (Filter sterile, store at 4^{o}C)

- **RPMI 1640 1X (complete media**

RPMI-1640 1X	200	mL
FBS (inactivated, Hyclone)	25	mL
HEPES buffer 1 M	3.75	mL
Penicillin+Streptomycin	150	µL

  (Store at 4^{o}C)

- **PBS 10X**

KH_2PO_4	0.288	gm
NaCl	18	gm
$Na_2HPO_4.7H_2O$	1.5	gm
MQ.water	200	mL

(Autoclave 121^oC, 15 min, store at 4^oC)

- **PBS 1X**

PBS 10X	20	mL
MQ.water	180	mL

(Store at 4^oC)

- **Hepes buffer (1M)**

Hepes	23.83	gm
MQ.water	100	mL

(Store at 4^oC)

- **Penicillin/Streptomycin (100X)**

Penicillin	0.6	gm
Streptomycin	1.34	gm
PBS 1X	100	mL

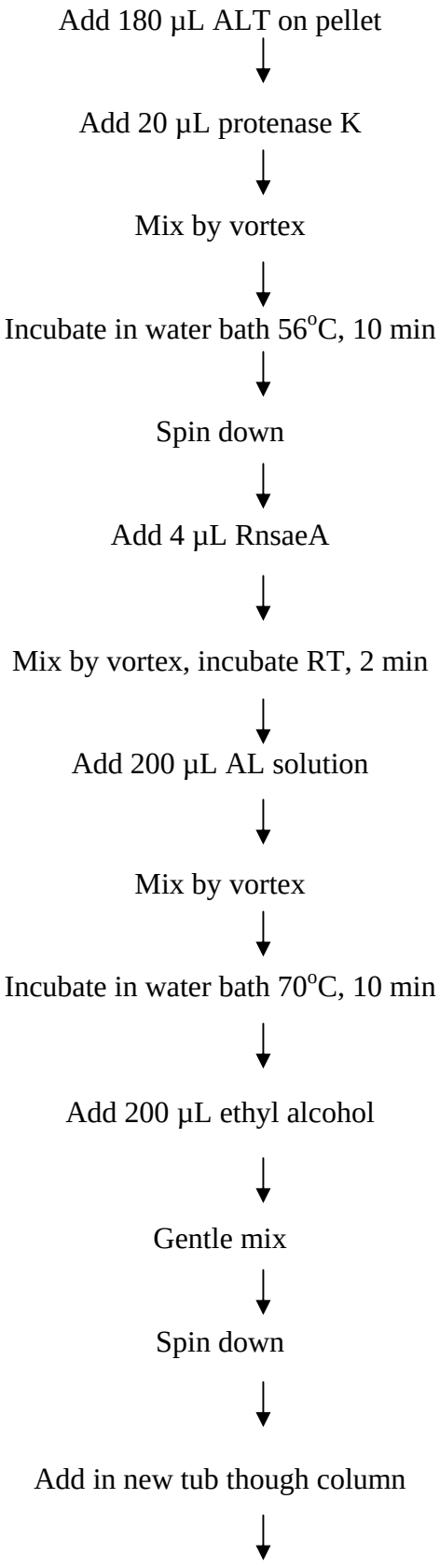
(Aliquot, store at -20^oC)

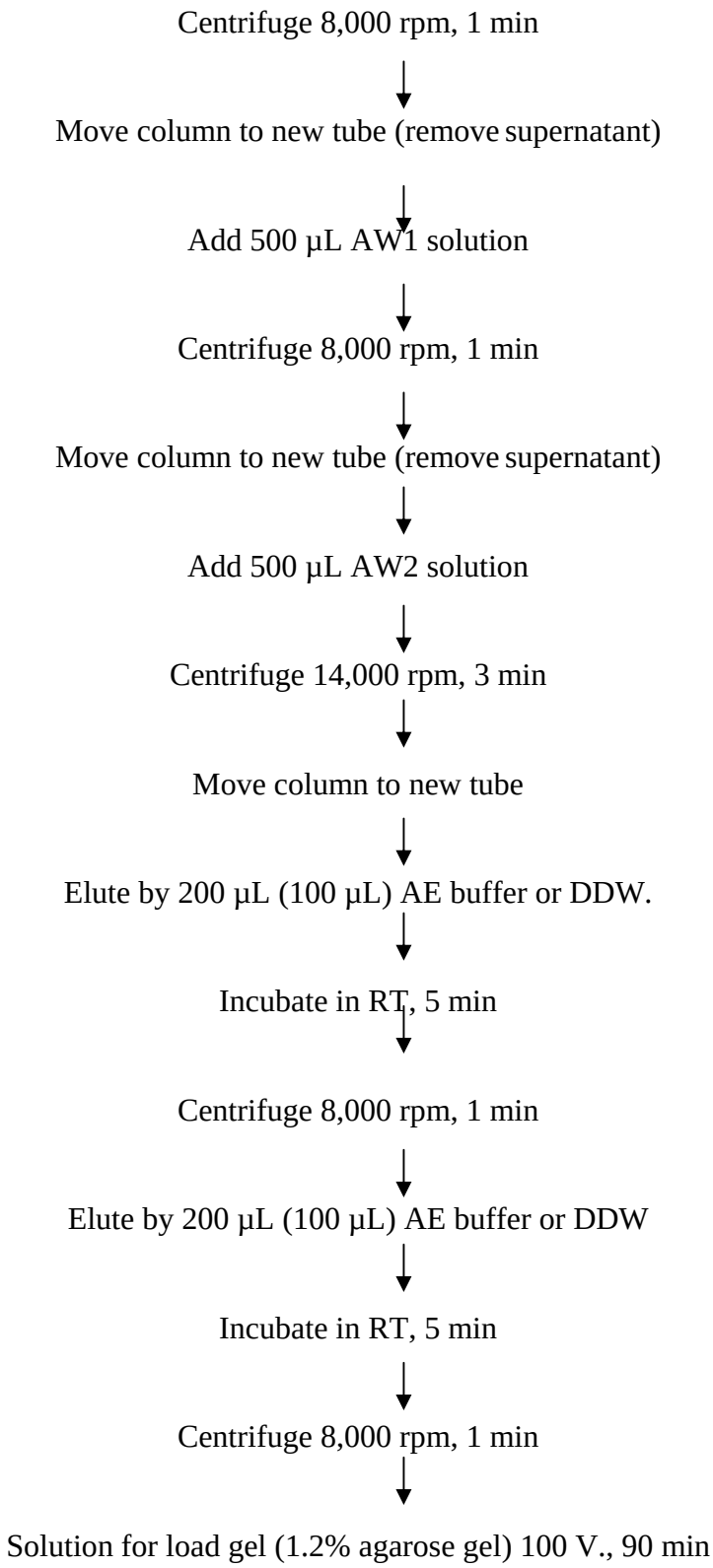
- **Trysin/EDTA**

Trysin	0.25	gm
EDTA	0.04	gm
PBS 1X	100	mL

(Aliquot, store at -20^oC)

2. DNA mini kit manufacturer's instructions




Centrifuge 8,000 rpm, 1 min
Move column to new tube (remove supernatant)
Add 500 µL AW1 solution
Centrifuge 8,000 rpm, 1 min
Move column to new tube (remove supernatant)
Add 500 µL AW2 solution
Centrifuge 14,000 rpm, 3 min
Move column to new tube
Elute by 200 µL (100 µL) AE buffer or DDW.
Incubate in RT, 5 min
Centrifuge 8,000 rpm, 1 min
Elute by 200 µL (100 µL) AE buffer or DDW
Incubate in RT, 5 min
Centrifuge 8,000 rpm, 1 min
Solution for load gel (1.2% agarose gel) 100 V., 90 min

APPENDIX C

DETERMINATION PROTIEN/ DNA CONCENTRATIONS

1. Protein (Lowry method)

- Prepare a series of dilutions of 5 mg/mL bovine serum albumin in the same buffer containing the unknowns, to give concentrations of 3.9 to 500 micrograms/mL (0.0039 to 5 mg/mL).
- Add 2 µL each dilution of standard, protein-containing unknown, or buffer (for the reference) to 100 µL reagent A in separate test tubes and mix.
- Incubate the tubes 10 min in room temperature.
- Add 100 µL reagent B to each tube, mix, incubate 10 min at room temperature.
- Rapidly add 20 µL reagents C to each tube, mix, incubate 30 min in room temperature.
- Measure absorbance at 620 nm by ELISA plate reader.

2. DNA content method

- Fill two cuvettes with TE buffer. Read and record the A_{260} of the sample cuvette against the blank. Repeat at 280 nm.

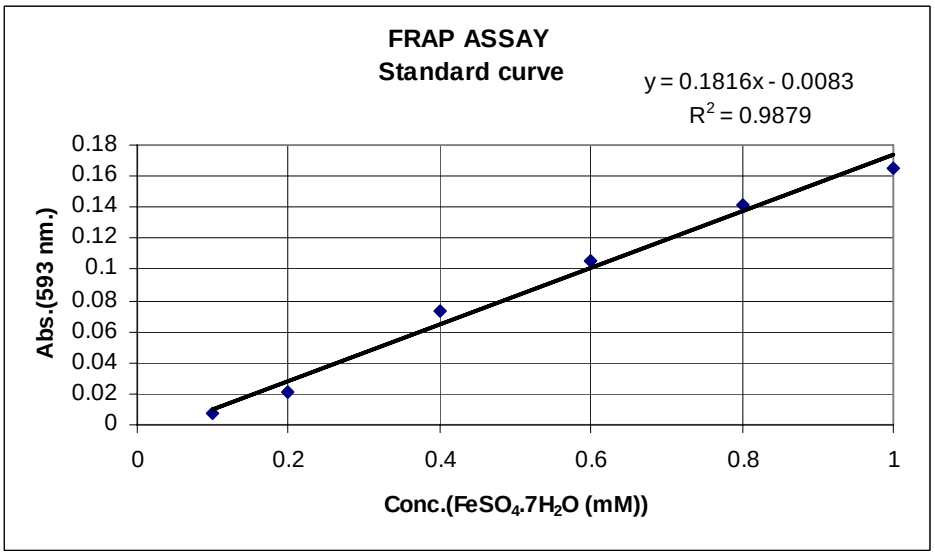
- Dilute the DNA in 400 mL of TE such that the A_{260} is ideally between 0.1 to 1.0, mix well.
- Empty and clean the sample cuvette and add the diluted DNA.
- Record absorbance of DNA sample at both 260 and 280 nm. Correct the readings as necessary using the blank values determined in step 1.
- The absorbance at 260 nm allows calculation of the concentration of DNA in the sample. An O.D. of 1 corresponds to approximately 50 µg/mL for double-stranded DNA, 40 µg/mL for single-stranded DNA, and 32 to 34 for typical oligonucleotides. The $^{A_{260}}/_{A_{280}}$ ratio can provide a very rough estimate of the purity of the nucleic acid. Relatively pure preparations of DNA has $^{A_{260}}/_{A_{280}}$ values of 1.8 and 2.0, respectively. Phenol contamination will result in significantly lower $^{A_{260}}/_{A_{280}}$ ratios. Such contamination makes accurate quantitation of DNA impossible. Note however that the A_{260}/A_{280} ratio can not be used to determine whether there is significant protein contamination in a nucleic acid preparation. Protein absorbs at 280 nm too little to make this estimation at all reliable.

APPENDIX D

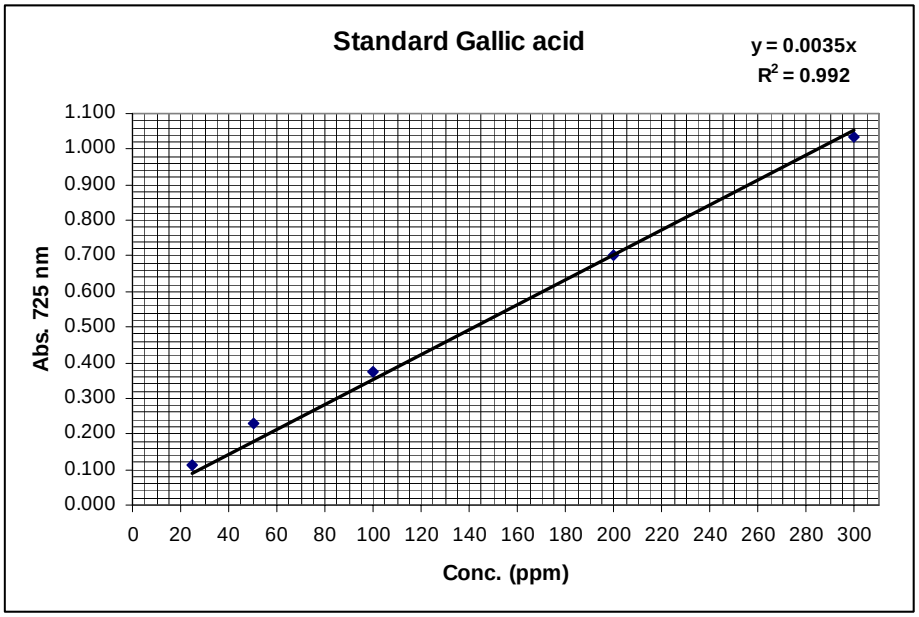
STANDARD CURVES

1. Standard curve

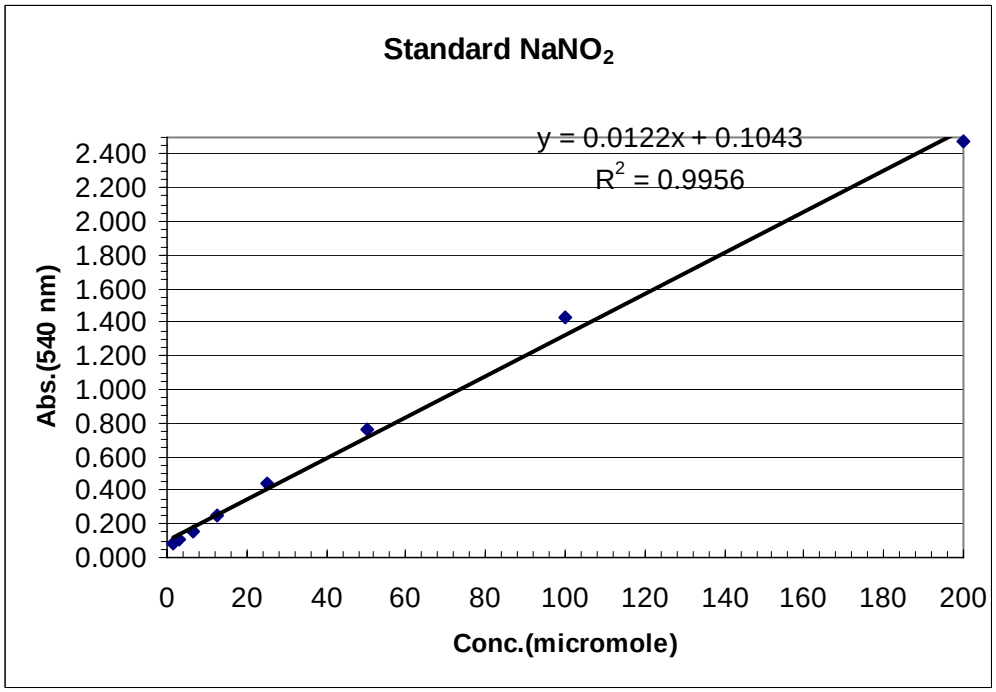
- FRAP assay



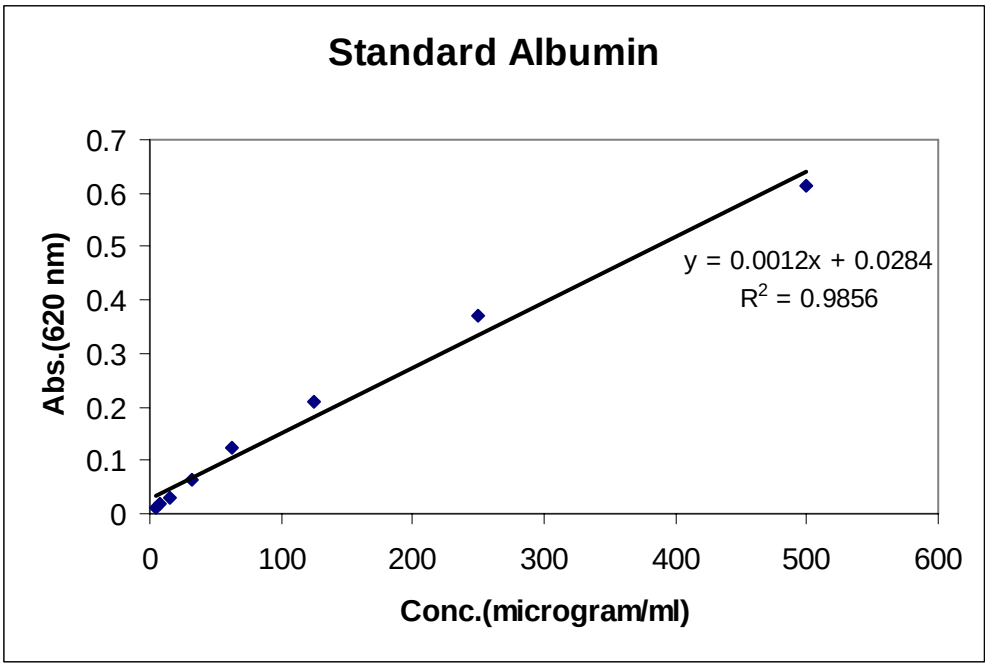
- Total phenolics by Folin-Ciocalteu assay


Standard Gallic acid
y = 0.0035x
R2 = 0.992
Abs. 725 nm
1.100
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Conc. (ppm)

- Nitrite assay



- Lowry method



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