BIOLOGICAL PROPERTIES AND TOXICITY OF WAN KHAN MAK (*AGLAONEMA SIMPLEX* BL.) FRUIT EXTRACT

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ฤทธิ์ทางชีวภาพและความเป็นพิษของสารสกัดจากผลว่านขันหมาก (*Aglaonema simplex* Bl.)

นางสาวรัตนา เกียรติทรงชัย

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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ว่านขันหมาก (*Aglaonema simplex* Bl.) เป็นพืชในวงศ์ Araceae ที่ถูกกล่าวขานว่าเป็นพืชมหัศจรรย์ มีสรรพคุณทางเภสัชวิทยาหลายด้าน แต่ปัจจุบันยังไม่มีรายงานวิจัยเกี่ยวกับฤทธิ์ทางด้านชีวภาพ หรือศึกษาความเป็นพิษของว่านขันหมาก การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อหาสารพฤกษเคมี ฤทธิ์ต้านออกซิเดชัน ต้านภูมิแพ้ และต้านการอักเสบ และการทดสอบความเป็นพิษแบบเฉียบพลันโดยการป้อน และการฉีดเข้าหลอดเลือดดำ รวมถึงศึกษาฤทธิ์ก่อกลายพันธุ์ของสารสกัดแอลกอฮอล์หยาบจากผลของว่านขันหมาก จากการสกัดผลอบแห้งของว่านขันหมากด้วย 95% เอทิลแอลกอฮอล์ได้ปริมาณสุทธิสารสกัดหยาบ ร้อยละ 12.65 ของน้ำหนักสารสกัดแห้ง โดยมีปริมาณสารต่อกรัมสารสกัดแห้งดังนี้ สารประกอบฟีนอลิกเทียบเท่าน้ำหนักกรดแกลลิค 56.73±0.37 มิลลิกรัม มีสารประกอบฟลาโวนอยด์เทียบเท่าน้ำหนักคาเทชิน 5.03±0.03 มิลลิกรัม มีสารโปรแอนโทไซยานิดินเทียบเท่าน้ำหนักคาเทชิน 7.02±0.12 มิลลิกรัม ฤทธิ์ต้านอนุมูลอิสระของสารสกัดเมื่อประเมินโดยวิธี DPPH จะมีค่า IC_{50} เท่ากับ 399.77±15.33 ไมโครกรัมต่อมิลลิลิตร และค่าของ FRAP ซึ่งเทียบเท่าน้ำหนักวิตามินซี 44.07±0.59 ไมโครกรัมต่อกรัมสารสกัดแห้ง นอกจากนี้สารสกัดสามารถลดการสร้างอนุมูลอิสระ ภายในเซลล์แมคโครฟาจ RAW264.7 ที่ถูกชักนำให้เกิดสภาวะเครียดออกซิเดชันโดย *tert*-Butyl hydroperoxide (*t*BuOOH) เมื่อติดตามโดยใช้ DCFH-DA probe ที่ให้สารเรืองแสง ส่วนการทดสอบพิษแบบเฉียบพลันโดยการป้อนและการฉีดเข้าหลอดเลือดหนูเม้าส์ ICR พบว่าสารสกัดไม่ก่อให้เกิดพิษหรือเกิดการตายในสัตว์ทดลอง การทดสอบฤทธิ์ก่อกลายพันธุ์โดยประเมินจากความผิดปกติของโครโมโซมของเซลล์ไขกระดูกหนูแรท Wistar พบว่าสารสกัดไม่ก่อให้เกิดความเสียหายต่อโครโมโซมและไม่แตกต่างจากกลุ่มควบคุม นอกจากนี้ระดับความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ (0.125-0.5 mg/ml) ของสารสกัดมีฤทธิ์ยับยั้ง β-hexosaminidase ที่หลั่งพร้อมสารก่อภูมิแพ้ของเซลล์ RBL-2H3 และลดการสร้างไนตริกออกไซด์ของเซลล์ RAW 264.7 เมื่อถูกกระตุ้นด้วย LPS และ IFN-γ ทั้งนี้ ปริมาณการยับยั้งแปรผันโดยตรงกับความเข้มข้นของสารสกัดที่เพิ่มขึ้น นอกจากนั้นความเข้มข้นเดียวกันของสารสกัดสามารถลดระดับการแสดงออกของเอมไซม์ iNOS และ COX-2 ผลโดยรวมจากการศึกษาครั้งนี้ชี้ชัดว่าสารสกัดจากผลของว่านขันหมากเป็นแหล่งของสารต้านอนุมูลอิสระธรรมชาติซึ่งมีราคาถูก ปลอดภัย

และมีฤทธิ์ทางเภสัชวิทยาที่มีศักยภาพต่อการพัฒนาให้เป็นผลิตภัณฑ์ธรรมชาติเพื่อป้องกันโรคหรือเป็นผลิตภัณฑ์อาหารเสริมต่อไปในอนาคต

สาขาวิชาชีววิทยา
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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RATANA KIATSONGCHAI : BIOLOGICAL PROPERTIES AND TOXICITY OF WAN KHAN MAK (*AGLAONEMA SIMPLEX* BL.) FRUIT EXTRACT. THESIS ADVISOR : ASST. PROF. BENJAMART CHITSOMBOON, Ph.D. 156 PP.

WAN KHAN MAK/ ANTIOXIDANT/ TOXICITY/ ANTI-ALLERGY/ ANTI-INFLAMMATORY

Wan Khan Mak (WKM) is the plant in Araceae family that has scientific name *Aglaonema simplex* Bl. Though Wan Khan Mak has been referred as a miracle plant possessing several ethno-pharmacologic properties, neither research in its claimed biological activities nor its toxicity have been explored. The present study aimed to investigate the *in vivo* toxicity, the phytochemicals and reported the antioxidant, anti-allergic and anti-inflammatory activities of 95% ethanol crude extract of dried fruits of WKM. The results showed that the yield of WKM crude extract was 12.65% of the dry weight and the contents per gram dry extract of total phenolics, flavonoids and proanthocyanidins were 56.73±0.37 mg GAE, 5.03±0.03 mg CE and 7.02±0.12 mg CE, respectively. For antioxidant studies, the IC_{50} of DPPH scavenging activity of the extract was 399.77±15.33 μg/ml and the FRAP value was 44.07±0.59 μg AEAC/g dry extract. WKM extract could effectively attenuate intracellular reactive oxygen species generation in *t*BuOOH-induced oxidative stress in macrophage RAW264.7 cells as monitored by using DCFH-DA fluorescent probe. *In vivo* investigation of acute oral toxicity and systemic toxicity revealed that no clinical signs of toxicity or effects on survival were observed in all WKM-treated ICR mice. Likewise, *in vivo* mutagenic effect evaluated by chromosomal aberration in bone marrow cells from WKM-treated

Wistar rats also revealed no chromosomal damages that was different from the vehicle control. At non-cytotoxic concentration (0.125-0.5 mg/ml) WKM dose dependently suppressed antigen-induced degranulation of RBL-2H3 cells. In addition, WKM also strongly suppress NO production in concomitant with iNOS and COX-2 suppression in LPS plus IFN-γ-activated RAW264.7 cells, in a dose-related manner. Overall, the present data suggest that WKM extract has a potential as a safe and effective source of natural antioxidants with many pharmaceutical properties and is worthwhile to be developed as natural chemopreventive or nutraceuticals products in the future.

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LIST OF ABBREVIATIONS

Ab	Antibody
Abs	Absorbance
Anti-DNP	monoclonal anti-body of Dinitrophenal
ATCC	American Type Culture Collection
Bp	Basepair
BSA-DNP	bovine serum abumin Dinitrophenal
COX-2	cyclooxygenase-2
DCFH-DA	2',7'-dichlorofluorescin-diacetate
DMSO	Dimethylsulfoxide
DPPH$^{\bullet}$	2, 2-diphenyl-1-picrylhydrazyl radical
ECL	Enhanced chemiluminescence
EMEM	Eagle minimum essential media
FBS	Fatal bovine serum
FRAP	Ferric reducing antioxidant power
h	hour(s)
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

LIST OF ABBREVIATIONS (Continued)

kDa	Kilodalton
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
$^1O_2$	Singlet oxygen
$O_2^{\bullet -}$	Superoxide anion
$OCl^{\bullet}$	Hypochloride
O.D.	Optical density
$OH^{\bullet}$	Hydroxyl radical
$OONO^-$	Peroxynitrite
OPCs	Oligomeric proanthocyanidins
PBS	Phosphate buffer saline
PI	Propidium Iodide
PIPES	piperazine-N,N'-bis (2-ethanesulfonic acid)
RNS	Reactive nitrogen species

LIST OF ABBREVIATIONS (Continued)

ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute number 1640
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Trypan blue exclusion
TPC	total phenolic content
Trolox	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
VH	Vehicle

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Despite a bloom of research in Thai herbs in recent years, there is a few research in Wan, the general name for sedges or orchid, compared with other herbs. Traditional Thai medicine textbooks; for example, Medical Relief by Phraya Pitprasartvej in 1909, Rural Medicine by Phraya Pongsa Visutatibodi, MD. in 1921, Wan Category by Luang Prapatsanpakorn in 1933 and Wan and Their Characteristics by Leun Kanhakanjana, have mentioned that most, if not all, of Wan are herbs, which possess many medicinal and superstitious effects, including poisonous-sting insect and disease cure, drug detoxification, general healing, wards off snakes, protecting against enemies, scaring away the devil, and amulet of good luck and commerce. Wan has been categorized into 34 families, 512 genera and over 1,700 species. There are many interesting species of Wan and one of those is Wan Khan Mak. Thai-traditional medicine has cited Wan Khan Mak as a miracle plant. Wan Khan Mak has been referred to possess many ethno-medical properties such as anti-aging, firming the skin, strengthens teeth, grey hairs prevention and energy booster (Botanical Garden, 2005; Apinya sanyasee, 2005).

Folk wisdom has long recognized plants for their medicinal and protective properties. Only recently, though, has science established that plants could

also play more comprehensive roles in the human diet. Many phytochemicals that provide plants for their color, flavor, smell and texture, may help to prevent some diseases such as cancer, heart disease, inflammation and various chronic diseases. Example of some well-known phytochemicals possessing pharmaceutical properties are curcumin in curcuma, gingerol in ginger, capsaicin in chilli pepper, catechin in tea leaves, diallyl sulphide in garlic, indole-3-carbinol in cabbage, sulphoraphane in broccoli, lycopene in tomatoes, resveratrol in grapes, quercetin in onion and potato and flavonoids in colorful fruits such as oligomeric proanthocyanidin (OPC) in grape seeds (Surh, 2003).

Nitric oxide (NO) is a short-lived free radical and intercellular messenger that plays important roles in a variety of physiological processes and biological functions such as smooth muscle relaxation, penile erection, platelet inhibition, neurotransmission, immune responses, inflammation, vascular homeostasis and macrophage-mediated cytotoxicity (Alessandro *et al*., 1994; Kang *et al*., 2002). Over production of NO has been associated with oxidative stress and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune disease and chronic inflammation, mutation, allergy and apoptosis (Park *et al*., 2000; Morgan, 2000; Sandoval *et al*., 1997; Kolb and Kolb-Bachofen, 1992). Production of NO by macrophages is enhanced upon activation by bacterial endotoxins and cytokines mainly via an increase of the intracellular content of the inducible isoform of nitric oxide synthase (i-NOS) (Alessandro *et al*., 1994). The over production of NO induced by lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ) in mouse macrophage cell line RAW264.7 can be simply measured by Griess reagent (Park *et al*., 2000; Nathan and Xie, 1994). Many phytochemicals, especially,

flavonoids can inhibit the over production of NO production induced by i-NOS such as quercetin, epigenin, wogonin, luteolin, tectorignin, galangin, morin, epigallocatechin gallate, ginkgo biloba extract, silymarin, hesperidin, grape seed extract, pine bark extract and tamarind seed pericarp extract (Kim *et al*., 1999; Raso *et al*., 2001; Kobuchi *et al*., 1997; Sakata *et al*., 2003; Chan *et al*., 1997; Kang *et al*., 2002; Fine *et al*., 2000; Komutarin *et al*., 2004). Particularly, OPCs extracts from French maritime pine bark or Pygnogenol® have been reported for their antioxidants activities, inhibition of NO production and many pharmacological properties such as antibacterial, anti-inflammatory, anti-viral, anti-carcinogenic, anti-allergic, immune stimulating, anti-platelet aggregation, decreased capillary permeability and fragility (Fine, 2000; Ray *et al*., 1999; Dhanalakshmi *et al*., 2003; Shafiee *et al*., 2003).

In the present day, allergic disease is a serious problem worldwide, especially in the developed countries. Substances that cause allergic reaction are called allergens which are generally found in everyday-life environment; for example, pollens, cosmetics, food, dust mites, mold spores and animal dander. Second exposure to the allergen leads to the cross-linking of bound IgE, triggering the release of many pharmacological active mediators such as histamine, serotonin, prostaglandin, leukotrienes and cytokines from mast cells and basophils. These mediators cause smooth muscle contraction, increased vascular permeability and vasodilation (Goldsby *et al*., 2002). When Rat basophilic leukemia (RBL-2H3) cell line was stimulated by DNP-BSA antigen, it released histamine or some indicator in the supernatant which could be measured by colorimetric method (Goda *et al*., 1999; Tewtrakul and Subhadhirasakul, 2007).

Nowadays, in certain places where Wan Khan Mak has earned its reputation as a miracle plant, its fruits have been sold expensively ranging from 200-400 baths each. Part of the reason might be the plant is rarely found, and it might not bear fruits every year if grown under inappropriate weather and conditions (Apinya sanyasee, 2005). Nevertheless, at present, there is still no research and studies or any scientific evidences to support Wan Khan Mak's claimed ethno-medical properties.

Up till the last few years, most people are not well realized the pharmacological values of Wan Khan Mak. It has been planted only for decoration or as plant amulet to dispel bad luck, or attract good luck and fortune. In contrary, recently, people are increasingly aware of Wan Khan Mak's therapeutic values. Spreading from words of mouth, those who consumed Wan Khan Mak stated that it promoted good health, freshen the feeling, anti-body aching and energize the body. Furthermore, some severe allergic patients claimed that their symptoms were distinctively better after consumption of Wan Khan Mak within a few days. One patient also indicated that the efficacy of this plant is better than many anti-allergic medicines prescribed by his doctor. However, this patient has some concern about its long-term toxicity (personal interview).

This study aimed to explore the general toxicity and some biological properties of Wan Khan Mak in addition to determine its basic chemical composition. The toxicity studies were be evaluated *in vivo* by using acute oral and systemic toxicity tests, and the mammalian bone marrow chromosome aberration test. Certain *in vitro* biological activities of the extract including antioxidant activity, the inhibition of NO production and the anti-allergic reaction of the extract were be investigated.

1.2 Research objectives

The objectives of the study were:

1. To determine chemical composition of Wan Khan Mak: Proximate analysis, polyphenolics, flavonoids and proanthocyanidins contents.
2. To investigate acute oral and systemic toxicities of Wan Khan Mak in ICR mice.
3. To investigate mutagenic activity of Wan Khan Mak in Wistar rat.
4. To examine *in vitro* antioxidant activity of Wan Khan Mak by using DPPH, FRAP and 2´, 7´-dichlorofluorescin-diacetate (DCFH-DA) assays.
5. To investigate *in vitro* anti-allergic effect of Wan Khan Mak: Inhibition of histamine released in activated RBL-2H3 cells.
6. To investigate the effect of Wan Khan Mak on NO production induced by LPS and IFN-γ activated RAW264.7 cells.

1.3 Research hypotheses

1. The ethanolic fruit extract of Wan Khan Mak contains phytochemicals and has biological activities toward antioxidation.
2. The ethanolic fruit extract of Wan Khan Mak shows no-toxicity effect toward acute toxicity, systemic toxicity and mutagenic toxicity.
3. The ethanolic fruit extract of Wan Khan Mak can inhibit of histamine released in activated RBL-2H3 cells.
4. The ethanolic fruit extract of Wan Khan Mak can inhibit NO production by LPS and IFN-γ-activated RAW264.7 cells.

1.4 Scope and limitations of the study

This research only focused on the studies of ethanolic fruit extract of Wan Khan Mak (WKM). The chemical composition of the extract was limited to proximate analysis, total phenolic and proanthocyanidin contents. The acute toxicity tests were limited to a single oral gavage and intravenous injection of WKM extract to ICR mice. The toxicity from long term use was evaluated by the mutagenic effect of the extract by both *in vitro* and *in vivo* assays. The *in vitro* mutagenic assay was limited to the Ames test, and *in vivo* study was only limited to the study of chromosome aberration of Wistar rats exposed to the extract by oral gavage. The antioxidant activities of the extract were evaluated by three different methods: DPPH, FRAP, and DCFH-DA assays. The anti-inflammatory activity of the extract was evaluated *in vitro* using the suppression of NO production, iNOS and COX-2 expression in LPS and IFN-γ-activated RAW264.7 model. The anti-allergic activity of the extract was investigated *in vitro* using the suppression of β-hexosaminidase released by DNP-activated RBL-2H3 cells.

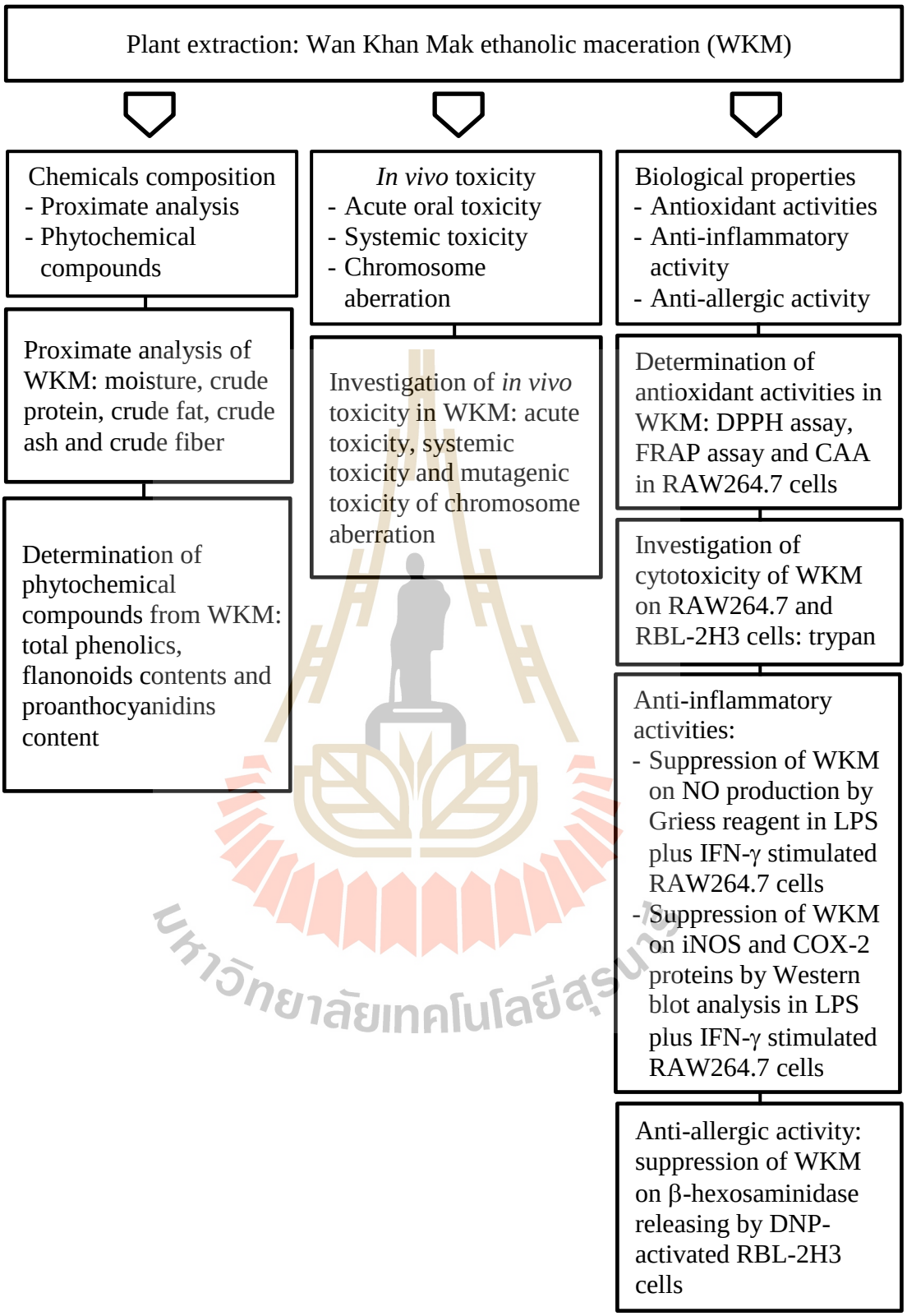

Figure 1.1 Scope and limitations of the study.

WKM = Wan Khan Mak

1.5 Expected results

1. Basic data for the toxicity of *Aglaonema simplex* Bl.'s fruits is a direct benefit to the consumer.
2. Provide information of toxicological and biological effects of *Aglaonema simplex* Bl.'s fruits.
3. Scientific evidences to proof some claimed pharmacological properties of *Aglaonema simplex* Bl.'s fruit in Thai traditional medicine that no research has been conducted before.
4. Strengthen the knowledge and the general practices in Thai traditional medicine.

CHAPTER II

LITERATURE REVIEWS

2.1 *Agloanema simplex* Bl.

Wan Khan Mak or the local people in Chanthaburi province called "Prome Teen Soung" (Figure 2.1) is the plant in Araceae family that has scientific name *Aglaonema simplex* Bl. (Tem Samittanan, 2001). It is in the same family with Dump Cane (*Homalomena rubescens* Kunth.) in Thai called "Soaw noi pra pang" and same genus with Chinese Evergreen (*Aglaonema modestum*) in Thai called "Kheew Muen Phee". The other members of this genus are *A. commutatum, A. brevispathum, A. costatum, A. crispum, A. nebulosum, A. nitidum, A. pictum, A. rotundum, A. siamense, A. tenuipes, A. brevispathum* and so on (Absolute astronomy, 2009). The greenish plant, about 1 meter high, is shoot from the underground bulb and the dark green shiny leave, 2 inches wide, are ovate shape and acuminate tip. The white or yellowish flower is borne in terminal raceme, at the leaf axil and produces a cluster of bright red-orange fruits. The young fruits of Wan Khan Mak are light green and turned to red-orange when it ripens. It is widely distributed in every region in Thailand especially on rocky foothill slopes and likes to grow in mixed evergreen-deciduous forests under shady areas which are usually moist. The method of taking Wan Khan Mak, based on Thai traditional medicinal book, is to swallow whole fresh ripe fruits or to chew dried fruits (Apinya Sanyasee, 2005).

Figure 2.1 Wan Khan Mak (*Aglaonema simplex* Bl.)

2.2 Traditional used and phytochemical properties of *Agloanema simplex* Bl.

Though Wan Khan Mak has been referred as a miracle plant possessing several ethnopharmacologic properties, neither research nor studies have been reported on chemical composition, general toxicity and any biological effects of Wan Khan Mak in both domestic and international database. However, there are a few studies of other member of *Aglaonema* related species on international database such as ultrastructure of lycopene containing chromoplasts in fruits of *Aglaonema commutatum* Schott (*Araceae*) (Knoth, 1981). Anti-hyperglycemic effects of N-containing sugars from *Aglaonema treuii* in diabetic mice compared with other plants

(Nojima, 1998) and the inhibitory activity of *Aglaonema modestum* Schott ex Engl. on nitric oxide synthase (iNOS) in lipopolysaccharide-activated macrophages compared to other Chinese medicinal plants (Ryu *et al*., 2001). Furthermore, there have been some reports of phytochemicals isolated from genera of *Aglaonema* such as a survey of polyhydroxyalkaloids in species of 52 genera of Araceae revealed that the presence of 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine and α-homonojirimycin from *Aglaonemateae* can suggest some correlation among other plants in the same family such as *Nephthytis* Schott, *Anchomanes* Schott and *Pseudohydrosme* Engl. (Kite *et al*., 1997). The isolation of Homoazasugars: α-homonojirimycin and homonojirimycin isomers including the new chemical, α-4-di-epi-homonojirimycin or α-homoallonojirimycin that are a glycosidase inhibitors from *Aglaonema treubii* Engl. has also been reported (Martin *et al*., 1999). Notably, glycosidase and glycosyltransferase inhibitors have attracted considerable interest for their potential roles in curing various diseases such as cancer, diabetes and virus infection including AIDS (Winchester *et al*., 1992; Asano *et al*., 2000; Jacob, 1995). In the recent year, there has been study of genetic relationships in 9 species of *Aglaonema* by amplified fragment length polymorphism (AFLP) marker (Chen *et al*., 2004)

Folk wisdom has long recognized plants for their medicinal and protective properties. Only recently, though, has science established that plants could also play more comprehensive roles in the human diet. Many phytochemicals that provide plants for their color, flavor, smell and texture, may help to prevent some diseases such as cancer, heart disease, inflammation and various chronic diseases. Example of some well-known phytochemicals possessing pharmaceutical properties are curcumin in curcuma, gingerol in ginger, capsaicin in chilli pepper, catechin in tea leaves,

diallyl sulphide in garlic, indole-3-carbinol in cabbage, sulphoraphane in broccoli, lycopene in tomatoes, resveratrol in grapes, quercetin in onion and potato and flavonoids in colorful fruits such as oligomeric proanthocyanidin (OPC) in grape seeds (Surh, 2003).

2.3 Phytochemicals composition

Fruits and vegetables, which are among the perishable commodities, are important ingredients in the human dietaries. Due to their high nutritive value, they make significant nutritional contribution to human well-being. The perishable fruits and vegetables are available as seasonal surpluses during certain parts of the year in different regions and are wasted in large quantities due to absence of facilities and know-low for proper handling, distribution, marketing and storage. Furthermore, massive amounts of the perishable fruits and vegetables, produced during a particular season result in a glut in the market and become scarce during other seasons. Neither can they all be consumed in fresh condition nor sold at economically viable prices (Aberoumand and Deokule, 2008; Abuye *et al*., 2003).

Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits, vegetables, and medicinal plants (Hertog, Hollman, Katan, and Kromhout, 1993; Parr and Bolwell, 2000; Barros, Dueñas, Carvalho, Ferreira, and Santos-Buelga, 2012). The beneficial effects derived from plant phenols have been attributed to their antioxidant properties (Heim, Tagliaferro, and Bobilya, 2002). They are also considered to possess anti-atherogenic, anti-allergenic, anti-inflammatory, antimicrobial, anti-thrombotic, vasodilatory, and cardioprotective effects. Basic structures of phenolic compounds possess an aromatic

ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (Balasundram, Sundram, and Samman, 2006). Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. More than 8,000 phenolic phytochemicals have been identified (Croft, 1998). Phenolic compounds are plant secondary metabolites that are frequently attached to sugars (glycosides). Occasionally, phenolic compounds also occur in plants as aglycones. Thus, these phenolics can be classified into non-soluble compounds (condensed tannin, lignins, and cell-wall bound hydroxycinnamic acids) and soluble compounds (phenolic acids, phenylpropanoids, flavonoid, and quinones) (Bravo, 1998). Phenolic compounds can be divided into several classes based upon their chemical structures. Though such structural diversity results in the range of phenolic compounds that occur in nature, the three most important groups of dietary phenolics are phenolic acid, tannins, and flavonoids (King and Young, 1999). Phenolic acids consist of two subgroups: the hydroxybenzoic and hydroxycinnamic acids. The two major dietary hydroxybenzoic acids are ellagic (Figure 2.2A) and gallic acid (Figure 2.2B), which usually occur as hydrolysable tannins and are found mainly in berries and nuts (Maas and Galletta, 1991). Tannins, the relatively high molecular weight compounds, are divided into 2 groups. Condensed tannins are the first group, which are polymers of catechins or epicatechins. And the second group is hydrolyzable tannins which are polymers of gallic acid (Chang, Collins, Bailey, and Coffey, 1994; King and Young, 1999). Flavonoids are the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Baxter, Harborne, and Moss, eds., 1999). Flavonoids are low molecular

weight compounds, consisting of fifteen carbon atoms which are arranged in a C6-C3-C6 configuration. Essentially, the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge usually in the form of heterocyclic ring, C (Bohm, 1998; Marken and Beecher, 2000) (Figure 2.3). Flavonoids can be further subdivided into 6 major subclasses, based on the connection of an aromatic ring to the heterocyclic ring, as well as the oxidation state and functional groups of the heterocyclic ring. In addition, within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns (Beecher, 2003). The six major subclasses of flavonoids include flavones, flavonols, flavanones, catechins or flavanols, isoflavones, and anthocyanidins (Ross and Kasum, 2002). Chemical structures and major food sources of flavonoid subclasses are shown in Figure 2.4.

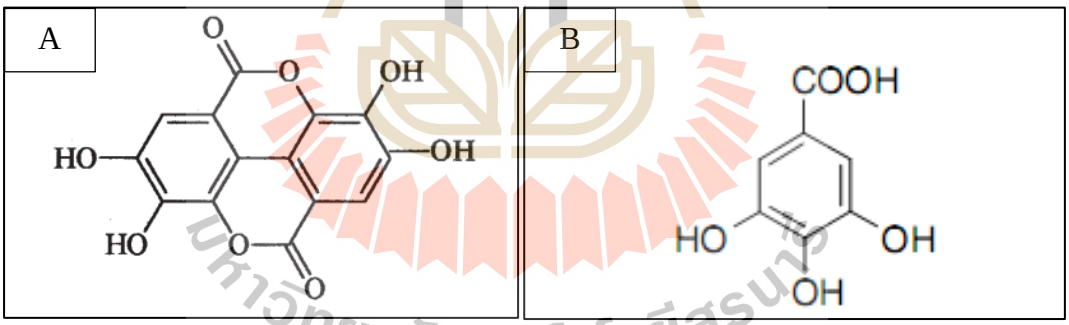

Figure 2.2 Structure of (A) ellagic acid (King and Young, 1999), and (B) gallic acid (Balasundram *et al*., 2006).

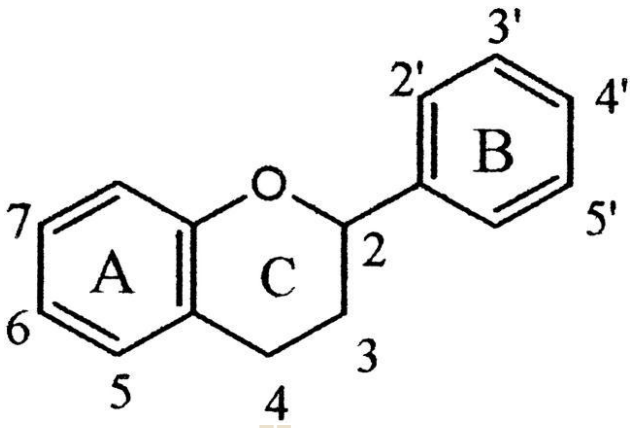

Figure 2.3 Generic structure of a flavonoid molecule (Balasundram *et al*., 2006).

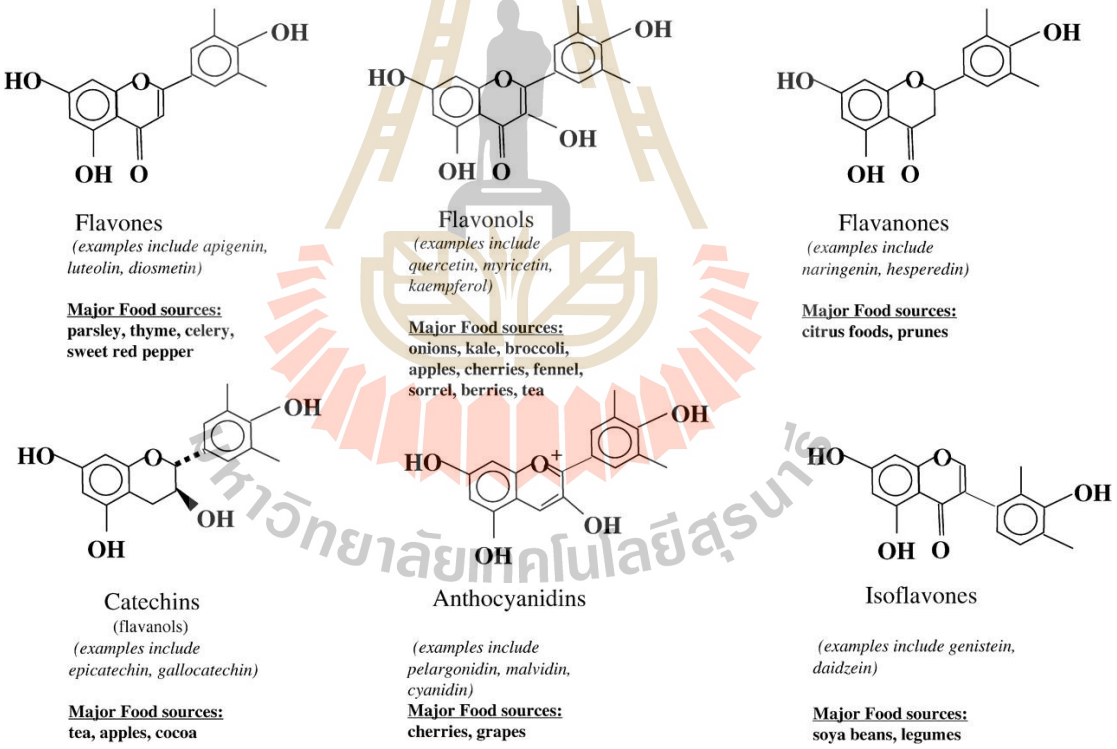

Figure 2.4 Chemical structures and major food sources of flavonoid subclasses (Ross and Kasum, 2002).

2.4 Free radicals and antioxidants

In recent years, there has been considerable interest in free radicals and related species. They are mainly derived from oxygen (reactive oxygen species/ROSs), including not only the oxygen radicals (superoxide anion $O_2^{\cdot -}$, hydroxyl radicals $^{\cdot}OH$ etc.) but also some non-radical derivatives. In addition, they are also derived from nitrogen (reactive nitrogen species/RNSs) such as nitric oxide (NO) and RNSs, which play also important roles in biology similar to ROS (Hallwell, 2006; Fang, Seki, and Maeda, 2009). Free radicals are very small highly reactive molecules due to the presence of unpaired valence shell electrons. They are very short lived, with half-lifes in milli-, micro- or nanoseconds. The well-regulated levels of ROS and RNS are produced to maintain cellular homeostasis in the normal healthy tissue and they play important roles as signaling molecules that regulate cell growth and reduction-oxidation (redox) status (Devasagayam *et al*., 2004). Free radicals are normally generated in our body by various endogenous systems. They are generated from the autoxidation and consequent inactivation of small molecules such as reduced thiols and flavins, and from the activity of certain oxidases, lipoxygenases, cyclooxygenases, peroxidases, and dehydrogenases. (Machlin and Bendich, 1987; Nicholis and Budd, 2000). Other sources of free radicals also include redox cycling of xenobiotics, exposure to environmental (atmospheric pollutants and cigarette smoking etc.) or physicochemical agents (ultraviolet rays and radiation etc.), or toxic chemicals, overnutrition, or pathophysiological state (Machlin and Bendich, 1987; Cadenas and Davies, 2000; Devasagayam and Kamat, 2002) (Figure 2.5). However, during time of environmental stress, free radicals can adversely alter lipids, proteins, and DNA, resulting in significant damage to cell structure and functions. Membrane lipids

present in subcellular organelles are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations (Devasagayam, Boloor, and Ramasarma, 2003). Oxidation of proteins by ROSs/RNSs can result in loss of enzyme activity (Stadtman, 1992, 2001). Free radical damage to DNA can result in mutagenesis and carcinogenesis. The hydroxyl radical is known to react with all components of the DNA molecule. Hydroxyl radicals can cause damage to purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999).

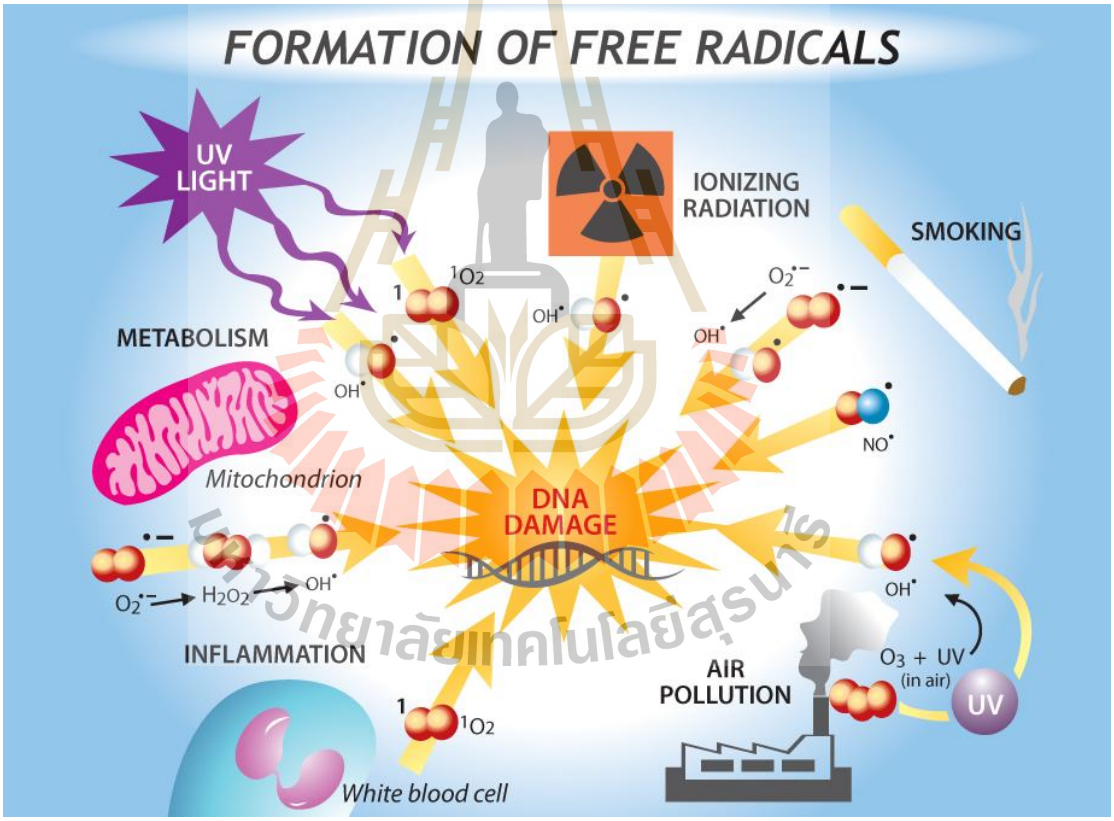

Figure 2.5 Sources of free radicals (casparcg.org, http://casparcg.org/wp-content/uploads/2015/07/free-radical.jpg., 2014).

Oxidative stress or overproduction of ROSs and RNSs have been implicated in aging and a number of human diseases such as atherosclerosis, malaria, rheumatoid arthritis, neurodegenerative diseases, ischemia-reperfusion (I/R) injury, cardiovascular diseases, viral pathogenesis, hypertension, formation of drug resistant mutant, drug-induced tissue injury, and inflammation (Aruoma, 1998; Fang *et al*., 2009). Antioxidants are known as substances or nutrients that neutralize free radicals in various stages (Sies, 1997). Nature has endowed each cell with adequate protective mechanisms against any harmful effects of free radicals. As shown in Figure 2.6, these defense systems include intracellular superoxide dismutase (SOD), glutathione peroxidase, and glutathione reductase. Other non-enzymatic antioxidants include essential nutrient such as vitamin E and C. Vitamin E acts as a chainbreaking antioxidant which prevents the propagation of free radical reactions in all cell membranes in our body. Vitamin E is also considered as the standard antioxidant to which other compounds with antioxidant activities are compared. Ascorbic acid or vitamin C is also part of the normal protecting mechanism (Buettner, 1993; Mahadik and Mukherjee, 1996; Kagan *et al*., 2002). Other non-enzymatic antioxidants also include carotenoids, flavonoids, and related polyphenols such as α-lipoic acid, glutathione. Carotenoids, such as beta-carotene, lycopene, lutein and other carotenoids, function as important antioxidants to quench O_2 and $ROO^{\cdot}$ (Stahl and Sies, 2003). Flavonoids, mainly present as coloring pigments in plants also function as potent antioxidants at various levels. Antioxidant can act at the levels of prevention, interception, and repair. Preventive antioxidants attempt to stop the formation of ROS like intracellular SOD. Interception of free radicals is mainly mediated by radical scavengers such as vitamin C and E, glutathaione, other thiol compounds, carotenoids,

flavonoids etc. Repair enzymes are involved at the repair and reconstitution level of antioxidants (Devasagayam *et al*., 2004). Natural products from dietary components such as Indian spices, Thai spices, traditional Chinese spices as well as medicinal plants are known to possess antioxidant activity. They have important roles in bioprospecting of new medicines from medicinal plants, which are also rich sources of antioxidants. Current estimate indicates that about 80% of people in developing countries still rely on traditional medicine from medicinal plants and animals for various diseases prevention and treatment (Devasagayam *et al*., 2004).

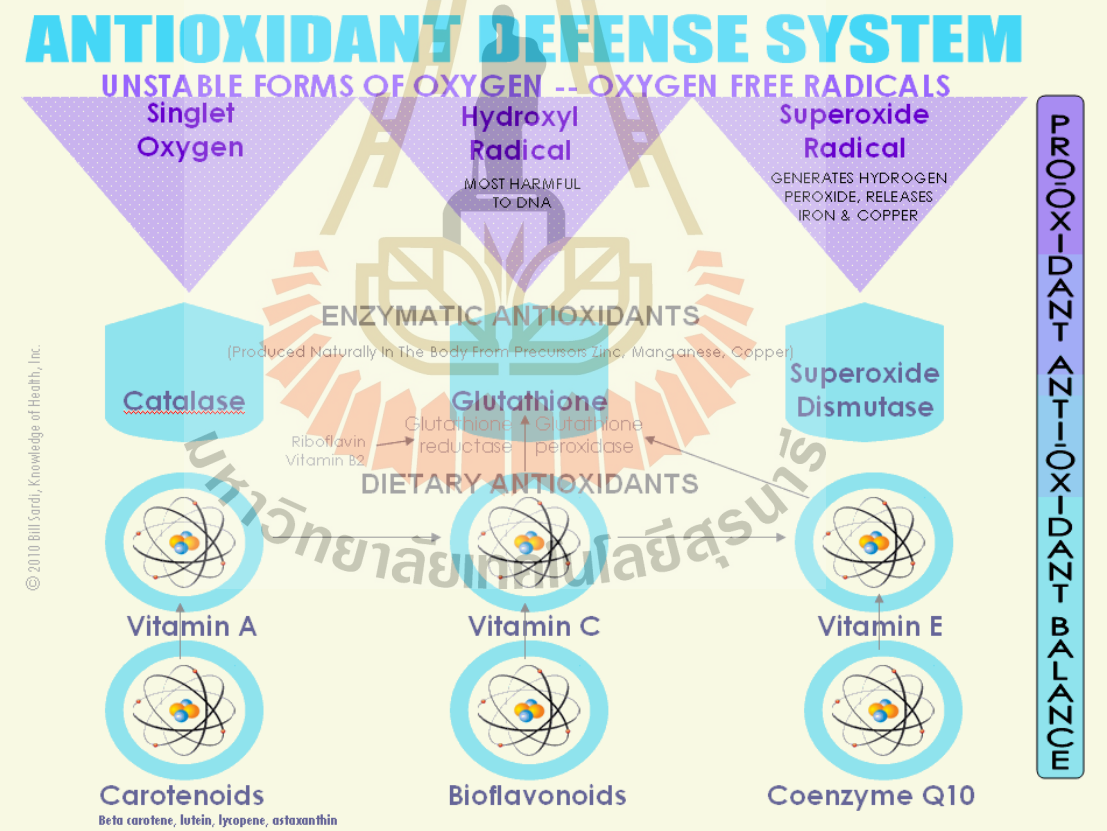

Figure 2.6 Antioxidant defense system (Lewrockwell, http://www.lewrockwell. com/ sardi/antioxidant-defense-big.gif, 2010).

2.5 Toxicity test

Acute oral toxicity data are used to satisfy hazard classification and labelling requirements, for risk assessment for human health and the environment, and when estimating the toxicity of mixtures. The provision of either a point estimate of the LD_{50} value or range estimate of the LD_{50} generally meets the acute oral toxicity data requirements for classification for all regulatory authorities in the areas of industrial chemicals, consumer products and for many pesticide applications **(**OECD guideline 423).

Acute systemic toxicity testing is the estimation of the human hazard potential of a substance by determining its systemic toxicity in a test system (currently animals) following an acute exposure. Its assessment has traditionally been based on the median lethal dose (LD_{50}) value - an estimate of the dose of a test substance that kills 50% of the test animals. For a substance to have systemic toxic effects it must be absorbed by the body and distributed by the circulation to sites in the body where it exerts toxic effects. The liver may transform a circulating drug or chemical to another form (biotransformation), and this new metabolite may be the one causing the observed toxicity (USP<88>).

The purpose of the in vitro chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells (Evans, 1976; Ishidate and Sofuni, 1985; Galloway *et al*., 1987). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this guideline is not designed to measure

numerical aberrations and is not routinely used for that purpose. Chromosome aberrations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

CP generates alkylating metabolites following biological activation, resulting in formation of mutant cells (Vainio *et al*., 1992). The CYP450 system consists of over 50 related proteins that catalyze the oxidation of many structurally unrelated compounds of endogenous and exogenous origin. They play an important role in the activation and/or inactivation of many drugs. CYP450 activity displays high interindividual variability because of induction or inhibition by drugs and environmental, dietary factors, and genetic factors (Shimada *et al*., 1994). CP is activated and inactivated by different CYP450 enzymes. At least 6 different CYP450s play a role in CP metabolism. Among them, CYP2B6 is the major enzyme responsible for the bioactivation of CP (Lang *et al*., 2001). Anti-genotoxic agents especially those present in natural substances act through different cellular pathways involving endogenous sequestration of mutagens by various enzymes (Heddle *et al*., 1999; Flora, 1998). Preventing the formation of carcinogens from precursors, blocking the metabolic activation of carcinogens by increasing the activation of detoxification enzymes might inhibit initiation of cancer (Dhuley *et al*., 1993). The previous study demonstrated that alkaloids and flavonoids have chemopreventive effects against most of the carcinogens (Surh *et al*., 1998).

The in vitro chromosome aberration test may employ cultures of cell lines or primary cell cultures. The cells used are selected on the basis of growth ability in

culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations. At the present time, the available data suggest that it is important to consider the p53 status, genetic (karyotype) stability, DNA repair capacity and origin (rodent versus human) of the cells chosen for testing (Pfuhler *et al*., 2011). These characteristics may be considered relevant for demonstration of chemical safety in human population.

Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system is a co-factorsupplemented post-mitochondrial fraction prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (Ames, McCann and Yamasaki 1975; Maron and Ames 1983; Natarajan, Tates, van Buul, Meijers and de Vogel, 1976; Matsuoka, Hayashi and Ishidate, 1979) or a combination of phenobarbitone and β-naphthoflavone (Elliot *et al*., 1992; Matsushima, Sawamura, Hara and Sugimura, 1976; Galloway, 1994) . The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (Elliot et. al., 1992; Matsushima, Sawamura, Hara and Sugimura, 1976). The S9 fraction typically is used at concentrations ranging from 1-10% (v/v) in the final test medium. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of chemical being tested.

2.6 Inflammation

Nitric oxide (NO) is a short-lived free radical and intercellular messenger that plays an important role in a variety of physiological processes and biological function

such as smooth muscle relaxation, penile erection, platelet inhibition, neurotransmission, immune responses, inflammation, vascular homeostasis and macrophage-mediated cytotoxicity (Alessandro *et al*., 1994; Kang *et al*., 2002). Over production of NO has been associated with oxidative stress and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune disease and chronic inflammation, mutation, allergy and apoptosis (Park *et al*., 2000; Morgan, 2000; Sandoval *et al*., 1997; Kolb and Kolb-Bachofen, 1992). Production of NO by macrophages is enhanced upon activation by bacterial endotoxins and cytokines mainly via an increase of the intracellular content of the inducible isoform of nitric oxide synthase (i-NOS) (Alessandro *et al*., 1994). The over production of NO induced by lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ) in mouse macrophage cell line RAW264.7 can be simply measured by Griess reagent (Komutarin *et al*., 2004; Park *et al*., 2000; Nathan and Xie, 1994). Many phytochemicals, especially, flavonoids can inhibit the induced NO production by i-NOS such as quercetin, epigenin, wogonin, luteolin, tectorignin, galangin, morin, epigallocatechin gallate, ginkgo biloba extract, silymarin, hesperidin, grape seed extract, pine bark extract and tamarind seed pericarp extract. (Kim *et al*., 1999; Raso *et al*., 2001; Kobuchi *et al*., 1997; Sakata *et al*., 2003; Chan *et al*., 1997; Kang *et al*., 2002; Fine *et al*., 2000; Komutarin *et al*., 2004). Particularly, OPCs extract from French maritime pine bark or Pygnogenol® have been reported for their antioxidant activities, inhibition of NO production and many pharmacological properties such as antibacterial, anti-inflammatory, anti-viral, anti-carcinogenic, anti-allergic, immune stimulating, anti-platelet aggregation, decreased capillary permeability and fragility. (Fine, 2000; Ray *et al*., 1999; Dhanalakshmi *et al*., 2003; Shafiee *et al*., 2003)

Inflammation is the reaction of vascularized tissue to local injury. The causes of inflammation are many and varied but most commonly result from an immune response to infectious microorganisms (Sommer, 2005) as shown in Figure 2.7. Inflammation can be classified into acute and chronic. Though the temporal profiles differ, both acute and chronic inflammation share common mechanisms with different time scales and amplitudes of the biological response (Cheng *et al*., 2010). Acute inflammation is the early or immediate response to vascular changes where the widespread effects of inflammatory mediators produce pain, heat, and swelling, usually of short duration (Sommer, 2005).

Subacute or chronic phase is characterized by the development of specific humoral and cellular immune responses to the pathogen present at the site of tissue injury (Faghali and Wright, 1997). Chronic inflammation is self-perpetuating and may be present for weeks, months, or even years. It may develop as the result of a recurrent or progressive acute inflammatory process or from low-grade, smoldering responses that fail to evoke an acute attack (Sommer, 2005). During both the acute and chronic inflammatory state, a variety of soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction. In addition, a variety of these soluble mediators regulate resident cells and the newly recruited inflammatory cells and some of these mediators contribute to the systemic responses associated with the inflammatory state.

The soluble factors that mediate these responses are divided into four main categories: (1) inflammatory lipid metabolites and the numerous derivatives from arachidonic acid such as prostaglandins and leukotrienes, (2) three cascades of soluble proteinase/substrates such as clotting and complement which generate numerous

proinflammatory peptides, (3) NO, a potent endogenous vasodilator, (4) a group of cell-derived polypeptides, known as cytokines, which to a large extent orchestrate the inflammatory response (Faghali and Wright, 1997). Thus, many inflammatory conditions are associated with many and varied factors including production of large amounts of NO (Liu and Hotchkiss, 1995), proinflammatory cytokines (TNF-α and IL-6) (Faghali and Wright, 1997), inducible enzymes (iNOS and COX-2) (Dannhardt and Kiefer, 2001; Calixto, Otuki, and Santos, 2003), and numerous of genes are expressed in association with the inflammatory process by activating several types of transcription factors (NF-κB, C/EBP, and AP-1) (Koj, 1996).

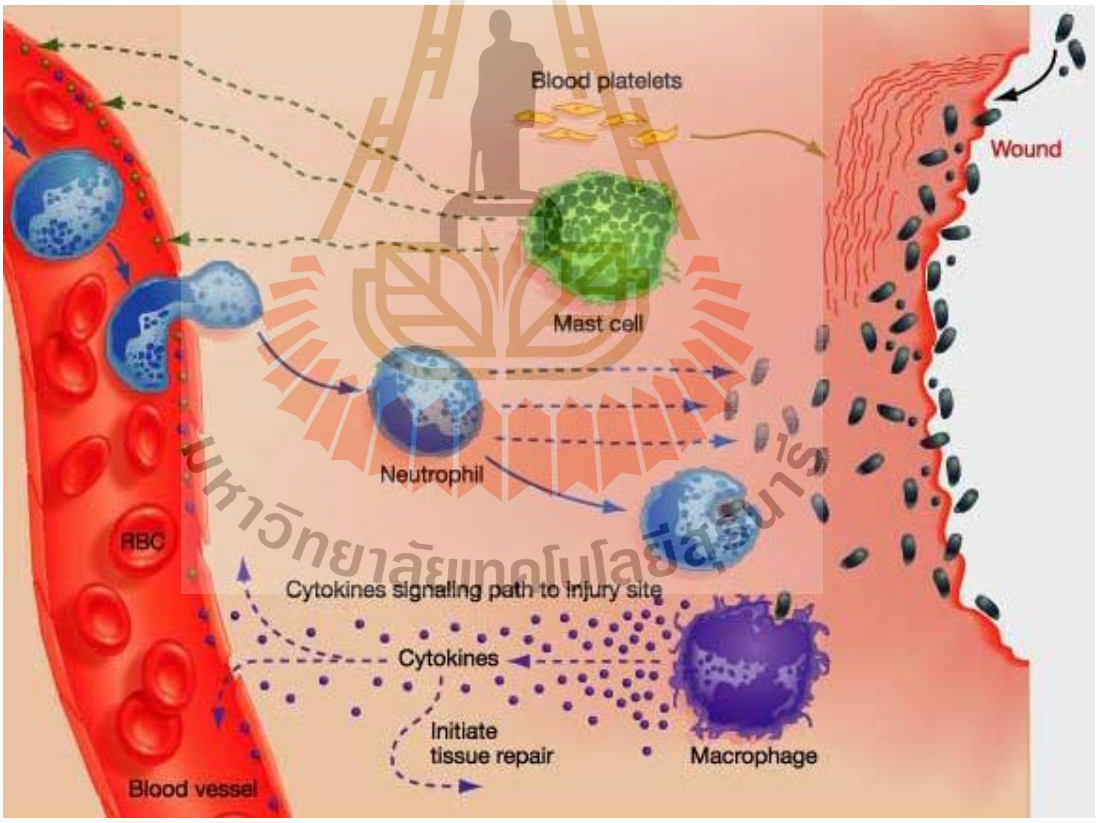

Figure 2.7 Inflammatory response against pathogens (Inflammation relief guide, http://www.inflammationreliefguide.com/wp-content/uploads/2011/10/Inflammatory-Response2.jpg., 2011).

NO is an important bioregulatory molecule regulating numerous physiological functions such as blood pressure, neural signal transmission, and platelet functions, as well as playing critical roles in the anti-pathogen and tumoricidal response of the immune system. Low concentrations of NO are sufficient, in most cases, to affect these functions (Liu and Hotchkiss, 1995; Wink *et al.*, 1998). However, during infection and inflammation, in vivo formation of NO is increased suggesting that NO concentrations are well above those found in normal physiological functions in infection of tissues. Increased NO levels may bring about some adverse effects of NO such as formation of carcinogenic N-nitroso compounds, deamination of DNA bases and mutagenesis, oxidation of DNA bases and tissue injury caused by potent oxidative agents (Liu and Hotchkiss, 1995). It is likely that the release of NO via iNOS has also been implicated as a deleterious agent in various pathophysiological conditions and tissue damages (Moncada, Palmer, and Higgs, 1991; Wink *et al.*, 1998). Figure 2.8 shows physiological and pathological roles of NO. The molecular NO is synthesized from the guanidino group of L-arginine by three isoforms of the enzyme NO synthase (NOS). Each isoform has been described and cloned: endothelial cell NOS (ecNOS or type 3), brain NOS (bNOS, nNOS, or type 1), and inducible macrophage-type NOS (iNOS or type 2) (Cuzzocrea, Riley, Caputi, and Salvemini, 2001). Historically, NOS have been classified into two distinct categories, constitutive (nNOS and ecNOS) and inducible (iNOS). Each isoform is the product of a distinct gene (Wink *et al.*, 1998). As shown in Figure 2.9, biosynthesis of NO, generally, nNOS and ecNOS are present continuously in neurons and endothelial cells, respectively, and require elevation in intracellular Ca2+ and attendant activation of calmodulin (Ca2+/calmodulin dependent) to produce NO. These isoforms are

regulated primarily by calcium influx and generate low levels of NO for brief periods of time in response to receptor or physiological stimulation. Therefore, when NO is formed by vascular endothelial, it diffuses into the vascular smooth muscle cells adjacent to the endothelium where it binds to and activates guanylyl cyclase. This enzyme catalyzes the dephosphorylation of GTP to cGMP, which serves as a second messenger for many important cellular functions, particularly for signaling smooth muscle relaxation (Moncada *et al*., 1991; Wink *et al*., 1998). Another enzyme, iNOS needs to be induced by cytokines primarily by cells of the myeloid lineage, macrophages, enthothelial cells, and a number of other cells and can generate locally high concentration of NO for prolonged periods of time (Wink *et al*., 1998). iNOS is expressed typically in response to immunological stimuli, bacteria lipopolysaccharide (LPS), and a variety of proinflammatory cytokines, and produces nanomoles, rather than picomoles of NO (Cuzzocrea *et al*., 2001). iNOS is a cytosolic Ca2+-independent enzyme. It requires tetrahydrobiopterin as well as other cofactors, and its induction is suppressed by glucocorticoids (Moncada *et al*., 1991). Induction of iNOS can also be suppressed by numerous agents, including thrombin, macrophage deactivation factor, transforming growth factor-β, platelet-derived growth factor, interleukin (IL)-4, IL-8, IL-10, and IL-13 (Cuzzocrea *et al*., 2001). Macrophages are capable of producing wide ranges of NO concentrations depending on the source of stimulus such as LPS, proinflammatory cytokines, or LPS plus proinflammatory cytokines. Interestingly, macrophages are stimulated by LPS plus IFN-γ. The amount of NO generation is higher when compared to stimulation with LPS alone or LPS plus TNF-α or IL-1β (Cheng *et al*., 2010).

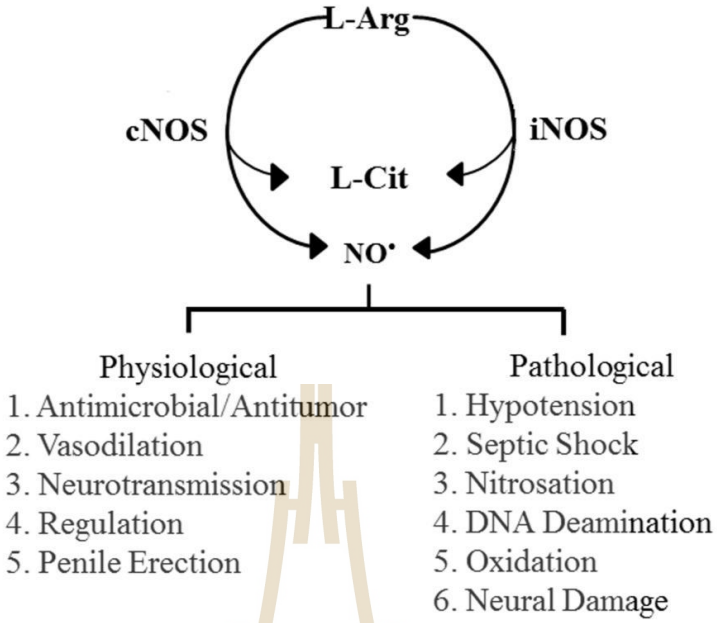

Figure 2.8 Physiological and pathological roles of NO (Liu and Hotchkiss, 1995).

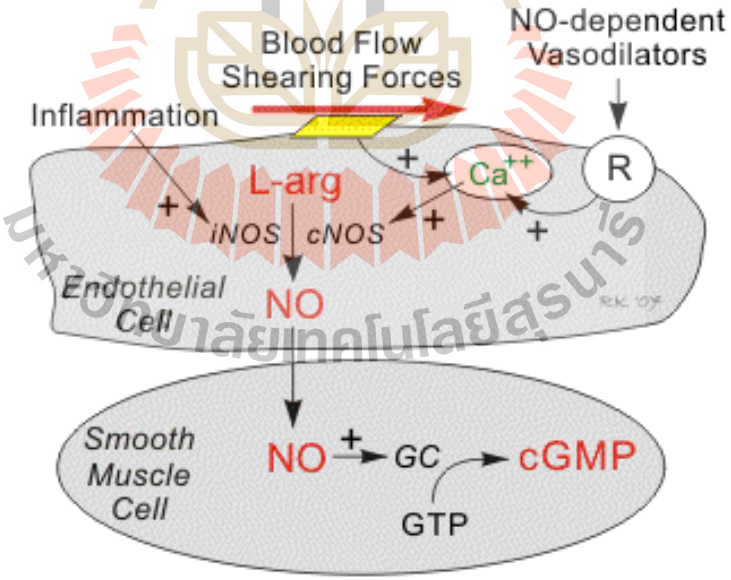

Figure 2.9 Biosynthesis of NO (Cardiovascular physiology concepts, http:// www.cvphysiology.com/Blood%20Flow/BF011%20%20nitric%20oxide.gif., 2008).

The biological effects of NO can be divided into two types, direct and indirect. The direct effects of NO generally occur at low concentrations, whereas indirect effects occur at much higher concentration. Direct effects are those chemical reactions in which NO reacts directly with given biological targets. Low levels of NO can react directly with heme-containing proteins such as guanylate cyclase, oxyhemoglobin, and cytochrome P450, and thereby may account for the neuromodulatory effects of nNOS and the vasodilatory effects of ecNOS. In contrast, the indirect effects require the reaction of NO with oxygen (O_2) or superoxide (O_2^-) to generate RNS which subsequently react with the biological targets and change their downstream functions. These reactions require high local concentrations of NO of which iNOS may be the sole biological source (Wink *et al*., 1998; Thomas *et al*., 2008; Cheng *et al*., 2010). Figure 2.10 shows the overview of the chemistry of direct and indirect reactions of NO.

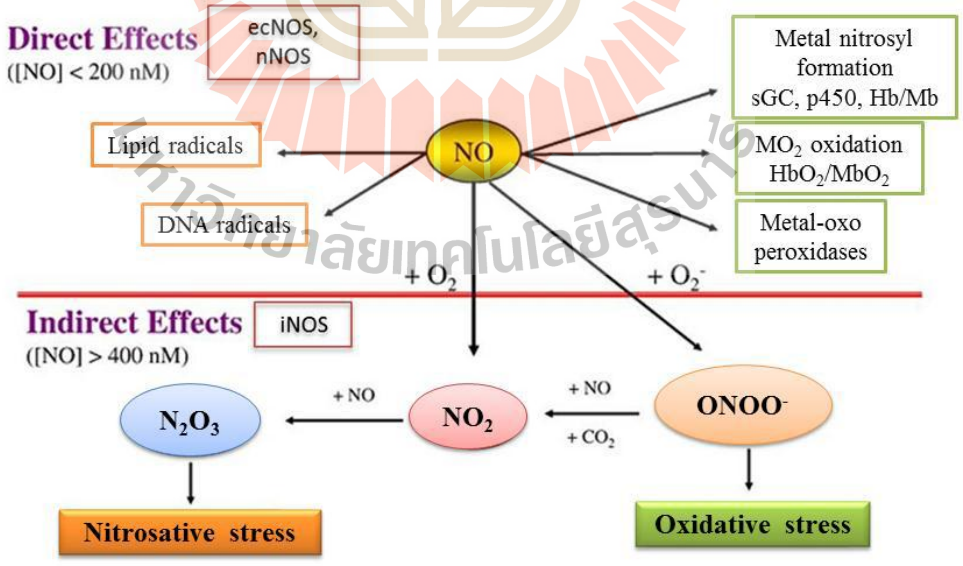

Figure 2.10 The chemistry of direct and indirect reactions of NO (adapted from Thomas *et al*., 2008).

Cyclooxygenase (COX) or prostaglandin H2 synthase (PGHS), first purified in 1976 and cloned in 1988, is the key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid (AA) (DeWitt and Smith, 1988; Merlie, Fagan, Mudd, and Needleman, 1988; Yokoyama, Takai, and Tanabe, 1988). Two closely related forms of COX known as COX-1 and COX-2 have been identified. Both isoforms are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Both isoenzymes transform AA to PGs, but they differ in their distribution and their physiological roles. Thus, rather than classifying PG biosynthesis into physiological and pathological, it may be better to classify based on COX isoforms: either constitutive or induced. COX-1 activity is constitutive and is present in nearly all cell types at a constant level, whereas COX-2 activity is normally absent from cells, and when induced, the protein level can increase or decrease in a matter of hours after a single stimulus (Vane, Bakhle, and Botting, 1998).

It is now known that under basal conditions the constitutive enzyme COX-1 is expressed in nearly all tissues including the kidney, colon, stomach, liver, spleen, heart, lung, and brain (Vane *et al*., 1998; Dannhardt and Kiefer, 2001). In contrast, COX-2 expression is largely undetectable unless it is induced by inflammatory stimuli in cells such as macrophages, endothelial cells, and synoviocytes. Such stimuli are proinflammatory cytokines (IL-1α/β, IL-2, IFN-γ, and TNF-α), endotoxin, mitogens, oncogenes (phorbolic ester), growth factors (fibroblast growth factor, FGF; platelet derived growth factor, PDGF; epidermal growth factor, EGF), hormones (luteinizing hormone, LH) and disorders of water-electrolyte homeostasis. The anti-inflammatory cytokines, IL-4, IL-10, and IL-13, and glucocorticoids can decrease induction of

COX-2 (Dannhardt and Kiefer, 2001; Wang and Dubois, 2010). In addition, COX-2 expression is regulated at both transcriptional and post-transcriptional levels. It is well established that the COX-2 transcription can be regulated by various transcription factors such as NF-κB, C/EBP, CREB, NFAT, AP-1, and PPAR (Wang and Dubois, 2010).

Induction of COX-2 by several stimuli associated with cell activation and inflammation assured the relationship of this particular isoform to inflammatory disease in general. Apart from its involvement in inflammatory process, COX-2 seems to play a role in angiogenesis, colon cancer, and Alzheimer's disease, based on the fact that it is expressed during these diseases. The discovery of the COX-2 has made possible the design of new drugs that can reduce inflammation without removing the protective PGs in the stomach and kidney made by COX-1 (Vane *et al*., 1998). Therfore, the selective COX-2 inhibitors have offered a new perspective for anti-inflammatory treatment.

2.7 Hypersensitivity

In the present day, allergic disease is a serious problem worldwide, especially in the developed countries. Substances that cause allergic reaction are called allergens which are generally found in everyday-life environment; for example, pollen, cosmetics, food, dust mites, mold spores and animal danders. Second exposure to the allergen leads to the cross-linking of bound IgE, triggering the release of many pharmacological active mediators such as histamine, serotonin, prostaglandin, leukotrienes and cytokines from mast cells and basophils. These mediators cause smooth muscle contraction, increased vascular permeability and vasodilation

(Goldsby *et al*., 2002). When Rat basophilic leukemia (RBL-2H3) cell line was stimulated by DNP-BSA antigen, it released histamine which could be measured by HPLC (Suzuki *et al*., 2005; Yamamoto *et al*., 2004; Nakatani, 2002).

Phenolics are important secondary metabolites in plants and are widely distributed in various vegetables and fruits. In addition to carbohydrates and fruit acids, phenols represent the third most abundant constituent in grapes (Shi, Yu, Pohorly, and Kakuda, 2003). Many commonly known natural phenolic compounds include flavonoids, lignans, stilbenes and phenolic acids. The mono-and polyphenolic substances in grapes can be categorised into phenolic acids and related compounds (e.g. caffeic acid, ferulic acid, vanillic acid and gallic acid), and flavonoids (e.g. colourless flavan-3-ols, coloured flavanones and anthocyanins), respectively (Shi *et al*., 2003).

Mast cells are granule-containing secretory cells in the immune system. They are widely distributed in the connective tissues throughout the body and mostly reside under the skin, respiratory and gastrointestinal tracts for the direct interaction with environmental stimuli (Theoharides *et al*., 2010). Mast cells are the important effector cells playing a pivotal role in both immediate and late-phase reactions of type I hypersensitivity. Activation of mast cells can be initiated by allergen-induced crosslinking of IgE bound by the high-affinity IgE receptors (FceRI) on the cells (Abramson, Licht, and Pecht, 2006; Abramson and Pecht, 2007). Cross-linked FceRI then initiates a complex network of signalling events that lead to phosphorylation of tyrosine kinases and mobilisation of Ca^{2+} into cytosol (Pecht and Corcia, 1987). Within seconds to minutes of activation, mast cells degranulate to release various preformed mediators, including β-hexosaminidase, histamine and serotonin, that

ultimately contribute to acute inflammation and chronic allergic disorders such as seasonal rhinitis, food allergy and asthma (Abramson *et al*., 2006) (Figure 2.11). RBL-2H3 is a rat basophilic leukaemia cell line, a cultured tumour analogue of mast cells, sharing various similar characteristics with mucosal mast cells (Seldin *et al*., 1985). It has been considered an excellent model system for the study of mast cell secretion (Aketani, Teshima, Umezawa, and Sawada, 2001) and screening for agents with anti-allergic potential (Aketani *et al*., 2001; Huang *et al*., 2008).

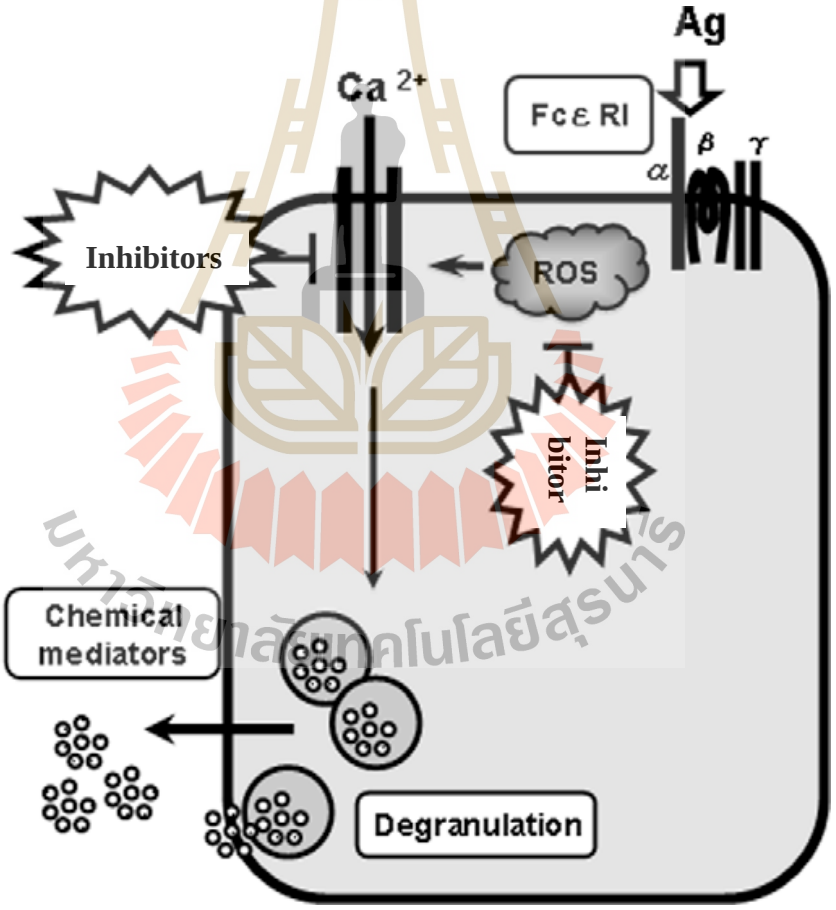

Figure 2.11 The degranulation by antigen in RBL-2H3 cells (adapted from Itoh *et al*., 2009).

Although a bloom of research in Thai herbs in recent years, there were fewer researches in Wan, especially Wan Khan Mak (*Aglaonema simplex* Bl.). Wan Khan Mak is the plant in Araceae family that the Thai-traditional medicine has cited as a miracle plant. Nevertheless, at present, the studies of its toxicity and the scientific evidences to support Wan Khan Mak's claimed ethno-medical properties are still very limited. This study aims to explore the general toxicity and some biological properties of Wan Khan Mak in addition to determine its basic chemical composition. The toxicity studies will be evaluated *in vivo* by using acute oral and systemic toxicity tests, and the mammalian bone marrow chromosome aberration test. Certain *in vitro* biological activities of the extract including antioxidant activity, the inhibition of NO production and the anti-allergic reaction of the extract were investigated.

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. The inhibitory mechanism of NO production and anti-inflammation by the extract was limited to the measurement of iNOS and COX-2 expression, respectively. Rat basophilic leukemic 2H3 cell line was triggered by cross linked of specific IgE antibody and its allergen to induce hypersensitivity response. The inhibitory mechanism of degranulation by the extract was limited to measurement of beta-hexosaminidase. Toxicities *in vivo* were evaluated by three methods, the acute toxicity test, the systemic toxicity test and the chromosome aberration test. The proximate analysis such as crude protein and crude fat were determined by Kjeldahl method and Soxhlet extraction method, respectively. The moisture and ash were determined using the weight difference method. Fiber content was estimated from the loss in weight of the crucible and its content on ignition. Carbohydrate (NFE; nitrogen free extract) was determined when the sum of the percentages of moisture, ash, crude protein, crude fat (EE; ether extract) and crude fiber were subtracted from 100. The phytochemical composition of the extract, total phenolic conpounds, total flavonoid contents and proanthocyanidin contents, were evaluated by Folin-Ciocalteu's method, aluminium chloride colorimetric method and vanillin assay, respectively.

3.1 Materials

3.1.1 Chemicals and reagents

Cyclophosphamide monohydrate, colchicine, ascorbic acid, 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), penicillin G, streptomycin sulfate, resveratrol, 2',7'-dichlorofluorescin-diacetate (DCFH-DA), and tertbutyl hydroperoxide (tBuOOH), were ordered from Sigma-Aldrich (St. Louis, MO). KARYOMAX®Giemsa Stain, RPMI medium 1640 and Hank's balanced salt solution (HBSS) were obtained from Gibco Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). Quercetin dihydrate was obtained from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Finally, 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Sigma-Aldrich Chemine Gmbh (Steinheim, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue solution were purchased from Invitrogen Molecular Probes (Eugene, OR). penicillin G, streptomycin sulfate, N-(1-Naphthyl) ethylenediamine dihydrochloride, sodium nitrite, and LPS (Escherichia coli O111:B4), were purchased from Sigma-Aldrich (St. Louis, MO). Mouse interferon gamma (mIFN-γ) and ECL western blotting substrate were purchased from Piearce Protein Research Products (Rockford, IL). Anti-iNOS mouse monoclonal, anti-tubulin mouse monoclonal and secondary antibody goat-anti-mouse-HRP conjugate were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. anti-COX-2 mouse polyclonal was ordered from Cayman Chemical, Ann Arbor, MI. nitrocellulose membrane was purchased from Amersham, Pittsburgh, PA.

3.1.2 Experimental animals

Wistar rats and ICR mice, from the National Laboratory of Animal Center, Salaya, Nakorn Pathom, Thailand, were used for mutagenic study and *in vivo* toxicity of Wan Khan Mak, respectively. All animal procedures and their care were conducted under the protocol approved by animal care and usage committee of Suranaree University of Technology (SUT), and The Thailand Institute of Scientific and Technological Research (TISTR).

3.1.2.1 ICR mice

ICR mice, from the National Laboratory of Animal Center, Salaya, Nakorn Pathom, Thailand were acclimatized for a week before use and housed in stainless steel cages with food and tap water *ad libitum* (Figure 3.1). They were kept in a temperature-controlled room at 25±2°C under a 12 hour light-dark cycle.

Figure 3.1 ICR mice (*Mus Musculus*).

3.1.2.2 Wistar rat

Wistar rats, 5-7 weeks old, from the National Laboratory of Animal Center, Salaya, Nakorn Pathom, Thailand were housed in stainless steel cages with food and tap water ad libitum. They were kept in a temperature-controlled room at 25±2°C under a 12 hour light-dark cycle.

3.1.3 Cell lines

The mouse macrophage cell line (RAW264.7) gamma $NO^{(-)}$ (ATCC, CRL-2278) was used for cellular antioxidant activity (CAA) assay and inhibitory mechanism of NO production. Meanwhile the rat basophilic leukemia (RBL-2H3) cell line (ATCC, CRL-2256) was used for β-hexosaminidase release assay.

3.1.3.1 Maintenances of RAW264.7 cells

Cells were cultured at 37°C, 5% CO_2 in humidifies air and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (Figure 3.2). Exponentially growing cells were used for the experiments when they reached approximately 80% confluence.

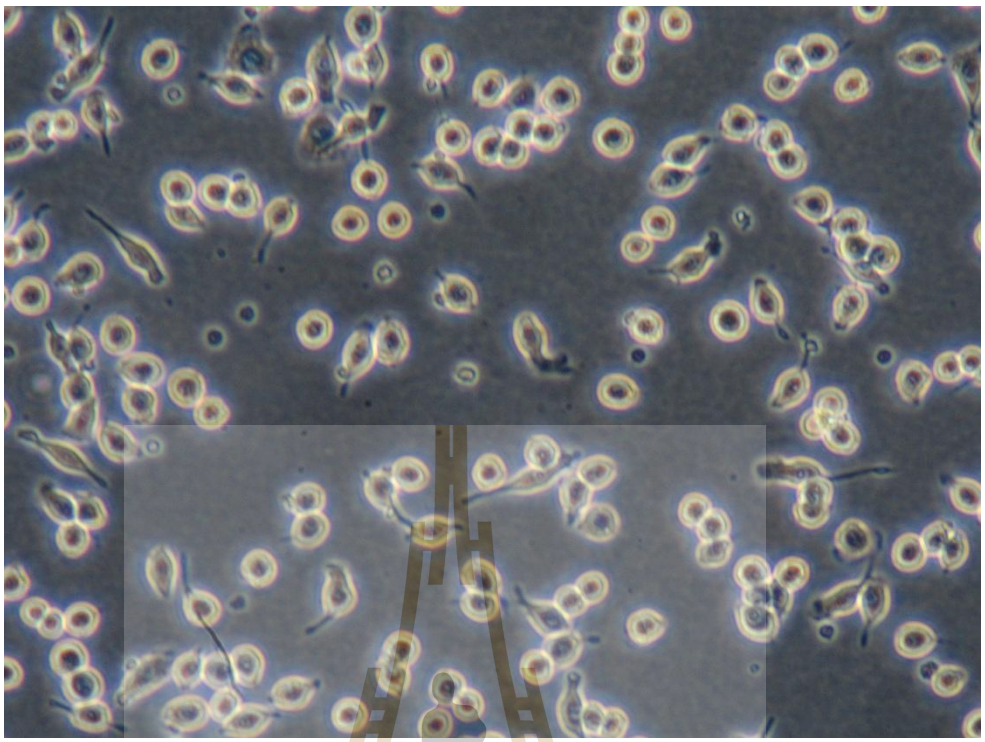

Figure 3.2 Characteristics of RAW264.7 cell line in RPMI1640 media viewed under light microscope.

3.1.3.2 Maintenances of RBL-2H3 cells

Cells were cultured at 37°C, 5% CO_2 in humidifies air and maintained in MEM supplemented with 15% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. Exponentially growing cells were used for the experiments when they reached approximately 80% confluence (Figure 3.3).

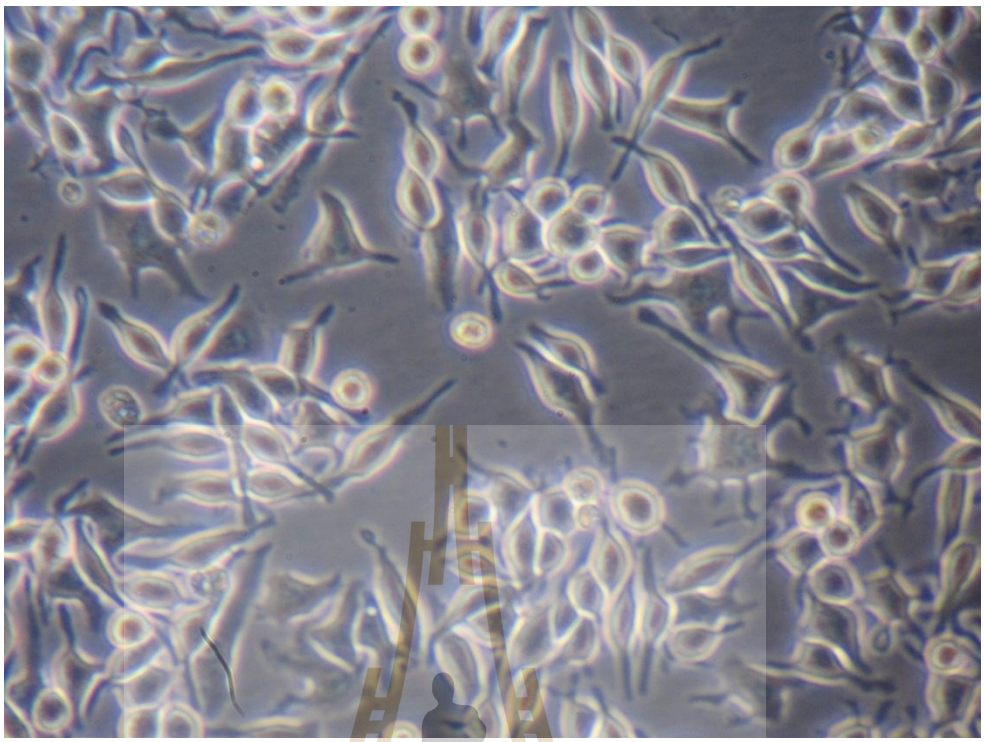

Figure 3.3 Characteristics of RBL-2H3 cell line in MEM media viewed under light microscope.

3.1.4 Plant materials

The fruits of Wan Khan Mak were collected from Amphur Pukthongchai, Nakhon Ratchasima province, Thailand (Figure 3.4). The plant was identified and authenticated by the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 186333) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Figure 3.4 *Agloanema simplex* Bl. (WKM) in Amphur Pukthongchai, Nakhon Ratchasima province, Thailand.

3.2 Methods

3.2.1 Plant extract preparation

The fruits were dried at 40°C in a hot-air oven (Memmert GmbH & Co. KG, Schwabach, Germany) then ground to powder and extracted by maceration in 95% ethanol for 48 hours at room temperature. After centrifugation, the extract was

concentrated by rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and dried by lyophilization (Freeze-Zone 12 plus, Labconco Corporation, Missouri).

3.2.2 Determination of chemical constituents

The chemical constituents of dried fruits of Wan Khan Mak, including moisture, crudes proteins, fats, ash and fibers were determined according to the standard methods of AOAC (1997). The moisture and ash were determined using the weight difference method (Figure 3.5 and 3.8) and fiber content estimated from the loss in weight of the crucible and its content on ignition (Figure 3.9). The Kjeldahl method (Figure 3.6) and Soxhlet extraction method (Figure 3.7) were used for determination of crude protein and crude fat, respectively.

Figure 3.5 Weight difference method for moisture content.

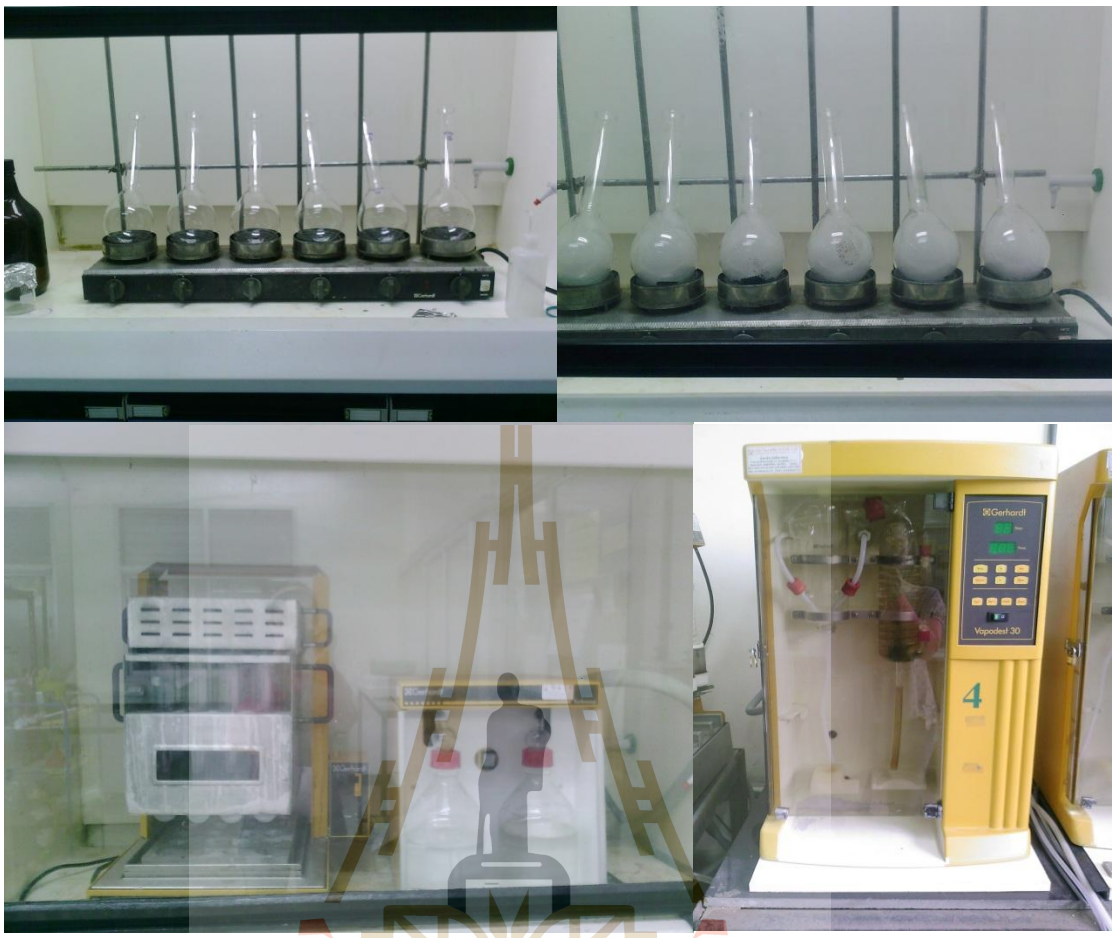

Figure 3.6 Kjeldahl method for determined crude protein.

Figure 3.7 Soxhlet extraction method for determined crude fat.

Figure 3.8 Weight difference method for crude ash.

Figure 3.9 Fiber content was estimated from the loss in weight of the crucible and its content on ignition.

3.2.3 Determination of phytochemical compounds

3.2.3.1 Total phenolic content (TPC)

The amount of total phenolic in the extracts was performed using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Briefly, 100 µl of the extracts or different concentration of gallic acid ranging from 0.05-0.3 mg/ml were taken in a test

tube, then 2 ml of 2% sodium carbonate (Na_2CO_3) was added and mixed thoroughly. The tubes were vortexed and after 2 min 100 µl Folin-Ciocalteu reagent diluted 2 fold with water was added, vortexed immediately and allowed the mixure to stand at room temperature for 30 min. The absorbance of extracts was measured spectrophotometrically at 750 nm. Gallic acid was used to prepare the standard curve. The concentration of phenolic compounds of the extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

3.2.3.2 Total flavonoids content (TFC)

The total flavonoid in the crude extracts was measured using the Aluminum Chloride Colorimetric Method (Liu *et al*., 2002). 250 µl of plant extract or positive control (Catechin at ranging from 0.05-0.5 µg/ml), 1.25 ml of DI water and 75 µl of 5% sodium nitrite were added to a glass test tube and vortexed. After 6 mins, 150 µl of 10% aluminum chloride was added, vortexed and the mixture was allowed to stand for 5 min, then 500 µl sodium hydroxide (1M) and 275 µl of distilled water were added and vortexed. The absorbance of extracts was measured spectrophotometrically at 510 nm. Catechin was used to prepare the standard curve. The flavonoid content of the extracts was expressed as milligrams of catechin equivalent (CE) per gram of dry extract.

3.2.3.3 Proanthocyanidins content

The vanillin assay was one of the most commonly used for PA (proanthocyanidin) estimation (Price *et al*., 1978). It is widely employed as a method for quantitative determination of condensed tannin (proanthocyanidins) in plant

materials, such as fruits (Goldstein and Swain, 1965), sorghum grain (Burns, 1971), and forage legumes (Broadhurst and Jones, 1978). When a monomeric or polymeric flavan-3-ol reacts with vanillin under acidic conditions, a red color develops with an absorption maximum at 500 nm.

A modified vanillin assay (Sun *et al*., 1998) was adopted for quantification of the total proanthocyanidin content of the ethanolic plant extract. An aliquot 125 µl of extract or Catechin (0.02-0.2 µg/ml) was added to a solution of 4% vanillin-methanol (750 µl) and 4% HCl-methanol (375 µl) in a glass test tube. The red coloration was read spectrophotometrically at 500 nm. Catechin was used as reference standard.

3.2.4 Determination of antioxidant properties

3.2.4.1 DPPH assay

The antioxidant activity of the extract was measured with respect to hydrogen donating or radical scavenging ability using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The various concentrations of the extract (0.2 ml) in ethanol were pipetted into a glass test tube and ethanol solution of DPPH (3 ml) was added to each tube. The tube was shaken gently and left to stand in the dark at room temperature for 30 min. the absorption was measured at 517 nm by a spectrophotometer (Katsube *et al*., 2004). Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. Inhibition of free-radical DPPH in percentage was calculated as the following equation:

$$\text{Percent Inhibition (I \%)} = \frac{(A_{\text{blank}} - A_{\text{sample}}) \times 100}{A_{\text{blank}}}$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Trolox was used as a standard reference. IC_{50} value, the concentration of sample required for 50% scavenging of the DPPH free radical, was determined from the curve of percent scavenging plotted against the concentration. Each determination was carried out in quadruplicates.

3.2.4.2 FRAP assay

The total antioxidant potential of the extract was determined using a ferric reducing ability of plasma (FRAP) assay (Benzie and Stain, 1996). The principle of this method is based on the ability of the antioxidants to reduce the colorless form ferric (Fe^{+3}) to its ferrous (Fe^{+2}), blue colored form. The freshly prepared FRAP reagent contains 10 mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl plus 20 mM ferric chloride ($FeCl_3$) and 0.3 M acetate buffer; pH 3.6 at the raio 1:1:10, respectively. Then various concentrations of the extract (0.1 ml) were mixed with the FRAP reagent (3 ml). The absorbance was measured at 593 nm after standing for 30 min. The standard curve was prepared using different concentrations of ascorbic acid standard solution (0.002-0.04 µg/ml). All determinations were performed in triplicate.

3.2.4.3 Cellular antioxidant activity (CAA) assay

Antioxidant activity was evaluated using the DCFH-DA assay as described by Dufour *et al*. (2007). Briefly, RAW264.7 cells were plated in 96 well

black clear-bottom plates at 1×10^6 cells/ml and incubated for 24 h at 37°C and 5% CO_2. Cells were incubated either with various concentrations of Wan Khan Mak (125, 250 and 500 µg/ml), or quercetin. After 24 h, cell were washed with Hank's balance salt solution (HBSS) and incubated for 30 min with HBSS containing 20 µM DCFH-DA. Cells were then washed again with HBSS 3 times and treated with 500 µM *tert*-butylhydroperoxide (*t*-BHP) in HBSS. Fluorescence was measured after 3 h on the automated 96-well plate reader using an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

3.2.5 *In vivo* toxicity

3.2.5.1 Acute oral toxicity test

Acute oral toxicity of Wan Khan Mak was performed according to the Organization for Economic Cooperation Development (OECD) guideline 423 (2001). Briefly, 5 male and 5 female ICR mice were used for each dose group. The extract (2,000 and 5,000 mg/kg body weight) and vehicle control (5% TWEEN80) was single administered to mice by oral gavage. The animals were observed for general signs of toxicity at least once during the first 30 minutes with special attention given during the first 4 hours and thereafter at least once daily for a total of 14 days.

3.2.5.2 Acute systemic toxicity test

The acute systemic toxicity of the extract was evaluated using the protocol for safety assessment of biologic products described by the U.S. Pharmacopeia (USP <88>). A group of 5 male ICR mice were injected intravenously

(i.v.) with Wan Khan Mak extract (625 mg/kg body weight in 0.9% NaCl). After that, animals were observed for general signs of toxicity over 72 h compared to the vehicle control group (0.9% NaCl) and thereafter at least once daily for a total of 14 days. The animals were performed gross anatomical dissection at the day 14.

3.2.5.3 *In vivo* mutagenicity test

In vivo mutagenetic effect of Wan Khan Mak was performed using the mammalian bone marrow chromosome aberration test as described in the OECD Guideline 475 (1997). Wistar rats were treated via oral gavage with a single dose of 5,000 mg/kg body weight of the WKM extract. The vehicle and positive control groups received 5% TWEEN80 and 50 mg/kg body weight cyclophosphamide, respectively. Cyclophosphamide (CP) was freshly prepared by dissolving 12 mg CP in 1 ml of sterile distilled water at a dose of 50 mg/kg body weight which used as standard mutagen. After 24 h of administration, mitotic arrest was initiated at 90 min. prior to sacrifice the animals by injection of colchicine (3.5 mg/kg body weight, i.p.) (Jha and Singh, 1995; Kirkland *et al*., 2007). Bone marrow cells were collected from femurs by flushing with 3 ml HBSS medium (pre-warm at 37°C) by a hypodermic syringe, and washed with 0.075 M KCl (pre-warm at 37°C) (WHO, 1985; US EPA, 1996; Preston *et al*., 1987). Then, cells were vortexed and centrifuged at 1,000 rpm for 10 min. After discarding the supernatant, the pellet was fixed in 5ml of cold freshly prepared fixative (acetic acid/methanol 1:3) and change for 3 times. For the last time, the pellet was stored in the fixative at least 1 h or overnight before slide preparation. Finally, the slide was prepared by May-Grunwald-

Giemsa technique (Figure 3.10) and the cells with structural aberrations were counted under a light microscope (Nesslany *et al*., 2004; Charles *et al*., 2005).

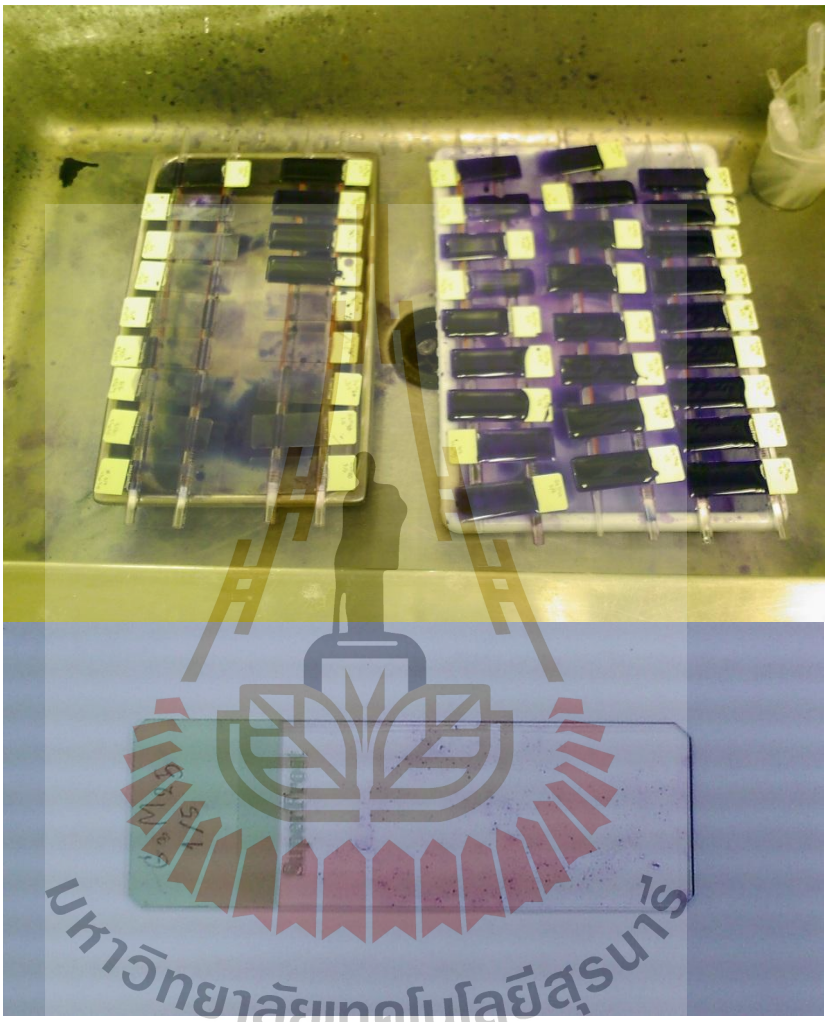

Figure 3.10 Slides were prepared by May-Grunwald-Giemsa technique.

3.2.6 Dose range-finding studies

In vitro cytotoxicity of Wan Khan Mak on RAW264.7 and RBL-2H3 was conducted in order to find the optimum dose-range for subsequent studies. Both cells were incubated in the absence or presence of various concentrations of the fruit

extract for 24 h. Cell viability was determined by MTT and Trypan blue exclusion methods.

3.2.6.1 MTT method

MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tratrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria. Then, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring yielding purple MTT formazan crystals (dark purple colure) which are insoluble in aqueous solutions. The crystal formazan product can be dissolved in acidified isopropanol or DMSO and measured spectrophotometrically. An increase in cell number results in increasing amount of formazan product which in turn, is proportional to the absorbance at 540 nm. As reduction of MTT can only occur in metabolically active cells, the level of MTT formazan formed could be a measurement of cell viability (Martin and Clynes, 1993; Byun *et al*., 2008).

3.2.6.1.1 Cytotoxic effect of WKM extract towards RAW264.7

Cells RAW264.7 (100 µl) were seeded in a 96-well plate at the concentration of 1×10^5 cells/well. Cells were incubated at 37°C, 5% CO_2 for overnight. WKM were dissolved in DMSO to make stock solution and diluted to various concentrations in media from 125 to 500 µg/ml (the final concentration of DMSO was 0.1%). After overnight incubation, the media was removed from the culture and were replaced with the extract or the vehicle control and further incubated for 24 h. Cell viability was determined with MTT assay.

After incubation with WKM extract (125 - 500 µg/ml.) for 24 h, the supernatant was removed and 100 µl of 0.5 mg/ml MTT in PBS was added to each well and then cells were incubated for 4 h. The media was removed and formazan product was dissolved by 100 µl DMSO. The inhibition of cell growth induced by WKM was detected by measuring the optical density (OD) at 540 nm by a microplate reader.

The percentage of cell viability was calculated according to the following equation:

$$\text{Percentage of the viable cell} = \frac{\text{average OD of test group}}{\text{average OD of control group}} \times 100$$

3.2.6.1.2 Cytotoxic effect of WKM extract towards RBL-2H3

Cells RBL-2H3 (100 µl) were seeded in a 96-well plate at the concentration of 4×10^4 cells/well. Cells were incubated at 37°C, 5% CO_2 for overnight. WKM were dissolved in DMSO to make stock solution and diluted to various concentrations in media from 125 to 500 µg/ml (the final concentration of DMSO was 0.1%). After overnight incubation, the media was removed from the culture and were replaced with the extract or the vehicle control and further incubated for 24 h. Cell viability was determined with MTT assay.

After incubation with WKM extract (125 - 500 µg/ml) for 24 h, the supernatant was removed and 100 µl of 0.5 mg/ml MTT in PBS was added to each well and then cells were incubated for 4h. The media was removed and formazan product was dissolved by 100 µl DMSO. The inhibition of cell growth induced by

WKM was detected by measuring the optical density (OD) at 540 nm by a microplate reader.

The percentage of cell viability was calculated according to the following equation:

$$\text{Percentage of the viable cell} = \frac{\text{average OD of test group}}{\text{average OD of control group}} \times 100$$

3.2.6.2 Trypan blue exclusion method

Trypan blue is one of the most recommended dyes for assessing cell viability by dye exclusion method because it is inexpensive, dependable and efficient. The trypan blue exclusion test is used to determine the number of viable cells based on the principle that intact plasma membranes in live cells exclude specific dyes, but dead cell do not. In this study, a cell suspension is simply mixed with dye and then visually examined under a microscope to determine whether cells take up or exclude dye. Dead cells absorb TB into the cytoplasm because of loss of membrane integrity, whereas live cells remain unstained. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Thus, the relative number of dead and live cells is obtained by counting the number of stained (dead) and unstained (live) cells using a haemocytometer viewed under an optical microscopy.

3.2.6.2.1 Cytotoxic effect of WKM extract towards RAW264.7

Cells RAW264.7 (100 µl) were seeded in a 96-well plate at the concentration of 1×10^5 cells/well. Cells were incubated at 37°C, 5% CO_2 for

overnight. WKM were dissolved in DMSO to make stock solution and diluted to various concentrations in media from 125 to 500 µg/ml (the final concentration of DMSO was 0.1%). After overnight incubation, the media was removed from the culture and were replaced with the extract or the vehicle control and further incubated for 24 h. Cell viability was determined with Trypan blue exclusion method.

After incubation to WKM for 24 h the supernatant was removed, cells in each well were trypsinized by adding 20 µl of 0.25% trypsin·EDTA in PBS and incubated at 37°C for 10 min. Then, the trypsin activity was neutralized by resuspended with 100 µl complete media with 10% FBS. The plate was placed on ice, 50 µl of cell suspension was pipetted to a micro centrifuge tube and an equal volume of 0.4% (w/v) trypan blue was added to the tube and mixed thoroughly. The micropipette was used to transfer a small amount of this mixer to a chamber of the haemocytometer. The 10X objective lens was used to focus on the grid lines of the chamber. The viable cells (bright light) and dead cells (stained 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 equation:

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

3.2.6.2.2 Cytotoxic effect of WKM extract towards RBL-2H3

Cells RBL-2H3 (100 µl) were seeded in a 96-well plate at the concentration of 4×10^4 cells/well. Cells were incubated at 37°C, 5% CO_2 for

overnight. WKM were dissolved in DMSO to make stock solution and diluted to various concentrations in media from 125 to 500 µg/ml (the final concentration of DMSO was 0.1%). After overnight incubation, the media was removed from the culture and were replaced with the extract or the vehicle control and further incubated for 24 h. Cell viability was determined with Trypan blue exclusion method.

After incubation to WKM for 24 h, the supernatant was removed, cells in each well were trypsinized by adding 20 µl of 0.25% trypsin·EDTA in PBS and incubated at 37°C for 10 min. Then, the trypsin activity was neutralized by resuspended with 100 µl complete media with 15% FBS. The plate was placed on ice, 50 µl of cell suspension was pipetted to a micro centrifuge tube and an equal volume of 0.4% (w/v) trypan blue was added to the tube and mixed thoroughly. The micropipette was used to transfer a small amount of this mixer to a chamber of the haemocytometer. The 10X objective lens was used to focus on the grid lines of the chamber. The viable cells (bright light) and dead cells (stained 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 equation:

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

3.2.7 Anti-allergy method

Rat basophilic leukemic 2H3 cells were triggered by crosslinking specific surface membrane inbound IgE multivalent allergen to induce hypersensitivity

response and hence the basophilic cell degranulation release of histamine, β-hexosaminidase and other inflammatory mediators. The inhibitory mechanism of degranulation by the extract was evaluated by measurement of β-hexosaminidase, concomitant released with histamine (Schwartz, Lewis, Seldin, and Austen, 1981).

Inhibitory effect of Wan Khan Mak Extract on the degranulation of β-hexosaminidase from activated RBL-2H3 cell line was investigated by *in vitro* method. RBL-2H3 cells were seeded overnight into a 96 well plate at a concentration of 5×10^4 cells/well and then passively incubated with mouse anti-dinitrophenyl monoclonal antibody (anti-DNP IgE) for overnight (Fischer *et al*., 1995; Saito *et al*., 2004). Cells were then washed with PIPES buffer consisting of 10 mM PIPES, 140 mM NaCl, 5 mM glucose, and pH 7.4, and incubated with vehicle control (0.1% DMSO) or various concentrations of test extract (WKM at 125, 250 and 500 µg/ml) or positive control (10 µM Quercetin) for 12 h. After incubation, cells were washed with PIPES buffer then sensitized with dinitrophenyl bovine serum albumin (DNP-BSA) for 30 min at 37°C. The amount of β-hexosaminidase in the supernatant was analyzed by colorimetric method (Goda *et al*., 1999; Tewtrakul and Subhadhirasakul, 2007).

3.2.8 Anti-inflammation method

The anti-inflammatory property of Wan Khan Mak ethanolic extracts was tested by measuring its effect on the pro-inflammatory mediator NO and COX-2 expression in RAW 264.7 murine macrophages activated with LPS and IFN-γ.

3.2.8.1 Nitrite assay

RAW264.7 cells were plated at a concentration of 1×10^5 cells/well (100 µl) in a 96 well flat bottom plate for 24 h at 37°C, in an atmosphere of 5% CO_2 plus air. Cells were incubated with or without various concentrations of 100 µl Wan Khan Mak extract or other antioxidant control (10 µM Quercetin). four hours later, cells were stimulated with 1 µg/ml LPS and 25 unit/ml IFN- γ (50µl) and further incubated for another 24 h. The NO production was determined by measuring nitrite concentration using the Griess reagent (Green *et al*., 1982). Briefly, following incubation, 100 µl of culture supernatant from each well was transferred to another 96-well plate. Then an equal volume of Griess reagent (1% (w/v) sulfanilamide, 0.1% (w/v) napthylethylelenediamine dihydrochloride (NED) and 3.5% (v/v) phosphoric acid in DI water) was added. The nitrite concentrations were measured spectrophotometrically at 540 nm using $NaNO_2$ as standard (Page *et al*., 1996).

3.2.8.2 Expression of nitric oxide synthase (iNOS) and COX-2

The effect of the WKM extract on iNOS and COX-2 expression of activated RAW 264.7 cells were conducted using Western blots method.

RAW264.7 cells were plated at a concentration of 2×10^6 cells/well in a 6 well plate for 24 h at 37°C, in an atmosphere of 5% CO_2 plus air. Cells were incubated with or without various concentrations of 100 µl WKM extract or standard control (10 µM quercetin). Four hours later, cells were stimulated with 1 µg/ml LPS and 25 unit/ml IFN- γ and further incubated for another 24 hours. After incubation, cells were washed three times with PBS, placed in 150 µl of ice-cold lysis buffer (1 mL RIPA buffer, 10 µL 200 mM PMSF, 1 µL 2mM leupeptin, and 1 µL E-

64) for 20 min. Then the disrupted cells were transferred to microcentrifuge tubes and centrifuged at 12,000 g and 4°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 (15 μg per lane) were separated by electrophoresis in a 7.5%-SDS-polyacrylamide gel (120 min at 125 V) (Ban *et al*., 2004). The separated proteins were blotted onto nitrocellulose membranes (Amersham, Pittsburgh,PA). The membrane was blocked with 5% bovine serum albumin in 0.1% Tween 20 and PBS buffer (TPBS) for 4 h, and incubated 2h. with a 1:1000 dilution of the primary antibodies, anti-iNOS mouse monoclonal (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or 1:2000 dilution of the primary antibodies anti-COX-2 mouse polyclonal (Cayman Chemical, Ann Arbor, MI). After extensive washing with TPBS, the membranes were incubated 1h. 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 Protien Research Product) and exposed to the film about 5 minutes for detecting iNOS and COX-2.

3.3 Location of research

This study was performed at the Center for Scientific and Technological Equipment (CSTE), Suranaree University of Technology, and The Thailand Institute of Scientific and Technological Research (TISTR).

3.4 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 Turkey's HSD test. 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). P values of less than 0.05 were considered as statistically significant.

CHAPTER IV

RESULTS

4.1 Phytochemicals profile

4.1.1 Percent yield of the WKM extraction

The percentage yield of crude ethanolic WKM fruit extract started from the weight of dried grounded plant material till extracted by 95% absolute ethanol was 12.65 ± 1.27%.

4.1.2 Proximate composition of dried fruit of Wan Khan Mak

Proximate analysis of the dried ground fruits of Wan Khan Mak demonstrated the presence of moisture in the dry matter 11.96 ± 1.39%, crude protein (CP) 71.44 ± 2.25%, crude fat (EE) 0.9 ± 0.08%, crude ash 4.72 ± 0.12%, crude fiber (CF) 0.11 ± 0.002% and available carbohydrate (NFE; Nitrogen Free Extract) 10.88 ± 1.17%. (Figure 4.1)

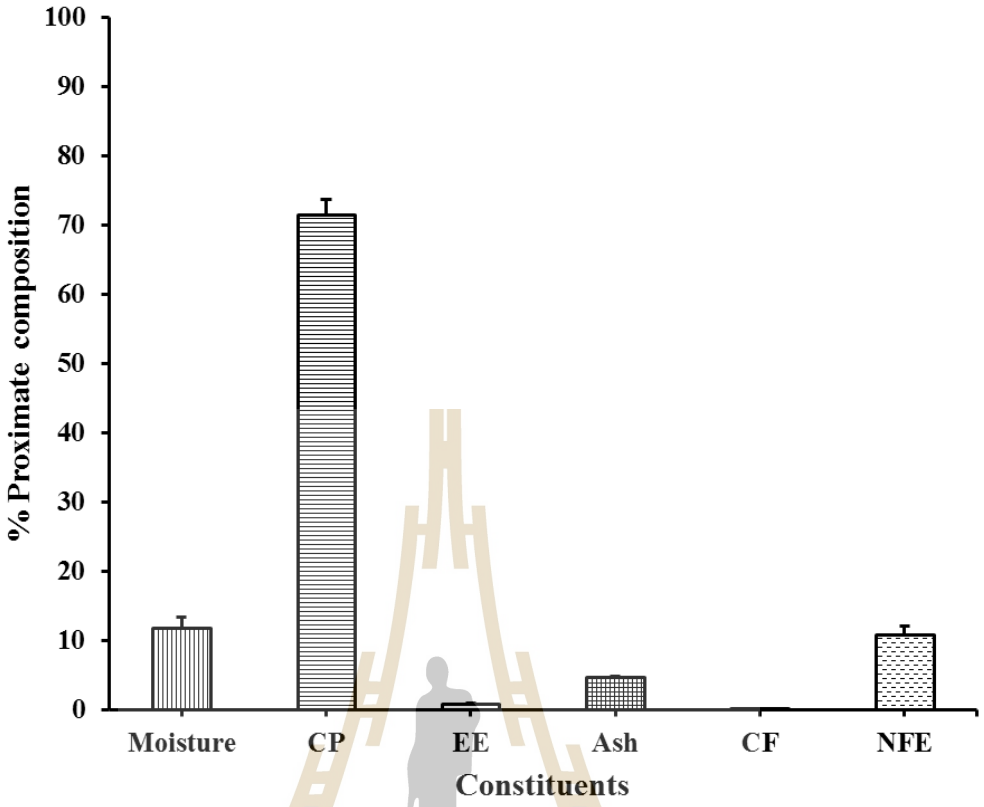

Figure 4.1 Proximate composition of dried ground fruits of Wan Khan Mak. CP= crude protein, EE= crude fat, CF= crude fiber and NFE= available carbohydrate.

4.1.3 Total phenolic content (TPC)

Wan Khan Mak fruits were used to investigate the good sources of antioxidant properties. Initial studies were conducted to investigate and determine the total phenolic compound contents (TPC) in WKM ethanolic 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 WKM was determined by using Folin- Ciocalteau's phenol reagent as modified from the method of Folin and Ciocalteau (1972).

The total phenolic content (TPC) was analyzed by fitting the calibration curve of gallic acid (R^2 = 0.997). The total phenolic of extract was 56.75 ± 0.37 mg GAE/g dry extract (shown in Table 4.1). The content of total phenolic was expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g dry extract).

4.1.4 Total flavonoids content (TFC)

The total flavonoids content (TFC) was analyzed by fitting the calibration curve of catechin (R^2 = 0.985). The total flavonoids content of the extract was 5.03 ± 0.03 mg CE/g dry extract (shown in Table 4.1). The content of total flavonoids was expressed as mg catechin equivalent per gram of dry extract (mg CE/g dry extract).

4.1.5 Proanthocyanidins content (PA)

The proanthocyanidins content was analyzed by fitting the calibration curve of catechin (R^2 = 0.951). The proanthocyanidins content of the extract was 7.02 ± 0.12 mg CE/g dry extract (shown in Table 4.1). The content of proanthocyanidins was expressed as mg catechin equivalent per gram of dry extract (mg CE/g dry extract).

Table 4.1 Total phenolics, total flavonoids and proanthocyanidins contents of Wan Khan Mak fruit extract.

Phytochemical component	Wan Khan Mak extract
Total phenolics (mg GAE/g dry extract)	56.75 ± 0.37
Total flavonoids (mg CE/g dry extract)	5.03 ± 0.03
Proanthocyanidins (mg CE/g dry extract)	7.02 ± 0.12

Data were mean ± SD., (n = 3).

4.2 Antioxidant properties of extract from WKM fruits

In order to obtain more information on the antioxidant activities of WKM extract, two different assays of DPPH and FRAP were used in the study. 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. The assay is based on the ability of sample to reduce ferric to ferrous at low pH causing the formation of colored ferrous-tripyridyltriazine complex.

4.2.1 DPPH radical scavenging activity

The DPPH assay was widely and one of the most extensively used methods to evaluate *in vitro* antioxidant activity in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as ethanolic extract of plants. DPPH radical is scavenged by antioxidants through the donation of a hydrogen, forming the reduced DPPH-H. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The DPPH radical scavenging activity (%) and IC_{50} values of the WKM extracts in comparison with other commonly used antioxidants are shown in Table 4.2. The percentages of $DPPH^{\cdot}$ inhibition by all compounds were gradually increased in dose dependent manner. However, the scavenging capacity of the extract was much lower than other positive controls. The IC_{50} of the extract was about 100-200 fold higher than the antioxidant controls. In this study, the scavenging ability of quercetin and catechin were a comparable and were higher than Trolox. The result suggested that the WKM extract exhibited the capability to scavenge DPPH free radicals. Similar to other antioxidant

controls, the percentage of DPPH inhibition by WKM extract was gradually increase in a dose-dependent manner. The percentages inhibition of the scavenging activity against DPPH radicals in WKM extract at the concentrations of 78, 156, 313 and 625 µg/ml were 20.46 ± 0.11%, 38.08 ± 0.22%, 54.86 ± 0.25% and 76.19 ± 1.09%, respectively. The IC_{50} of WKM extract was 399.77 ± 15.33 µg/ml. The percentages inhibition of the scavenging activity against DPPH radicals in quercetin at the concentrations of 0.5, 1.0, 2.0 and 3.0 µg/ml were 13.33 ± 0.32%, 27.16 ± 0.58%, 53.96 ± 0.70% and 77.00 ± 0.85%, respectively. The IC_{50} of quercetin was 2.002 ± 0.022 µg/ml. The percentages inhibition of the scavenging activity against DPPH radicals in catechin at the concentrations of 0.5, 1.0, 2.0 and 3.5 µg/ml were 23.27 ± 0.13%, 41.26 ± 0.20%, 62.14 ± 0.49% and 77.30 ± 0.20%, respectively. The IC_{50} of catechin was 2.144 ± 0.010 µg/ml. The percentages inhibition of the scavenging activity against DPPH radicals in Trolox at the concentrations of 1.5, 3.0, 4.5 and 8.0 µg/ml were 15.50 ± 0.31%, 35.12 ± 0.25%, 53.93 ± 0.87% and 86.82 ± 0.06%, respectively. The IC_{50} of Trolox was 4.462 ± 0.015 µg/ml. The Trolox equivalent free radical scavenging activity was 1.116 g /100 g WKM.

Table 4.2 DPPH free radical scavenging activity of WKM extract and standard antioxidants, quercetin, catechin and trolox.

Sample	Conc. (μg/ml)	%Inhibition	IC_{50} (μg/ml)
WKM	78	20.46 ± 0.11	399.77 ± 15.33
	156	38.08 ± 0.22	
	313	54.86 ± 0.25	
	625	76.19 ± 1.09	
Quercetin	0.5	13.33 ± 0.32	2.002 ± 0.022
	1.0	27.16 ± 0.58	
	2.0	53.96 ± 0.70	
	3.0	77.00 ± 0.85	
Catechin	0.5	23.27 ± 0.13	2.144 ± 0.010
	1.0	41.26 ± 0.20	
	2.0	62.14 ± 0.49	
	3.5	77.30 ± 0.20	
Trolox	1.5	15.50 ± 0.31	4.462 ± 0.015
	3.0	35.12 ± 0.25	
	4.5	53.93 ± 0.87	
	8.0	86.82 ± 0.06	

Data were mean ± SD., (n = 3).

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 WKM was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. the reducing antioxidant power of the extract in FRAP assay was expressed as µg of ascorbic acid equivalent antioxidant capacity (AEAC) per gram dry weight of the extract and was shown in Table 4.3. Ferric reducing ability power (FRAP) values of WKM extracts was 44.07 ± 0.51 (µg AEAC /g dry wt.).

Table 4.3 Ferric reducing ability power (FRAP) values of WKM extracts.

Sample	**FRAP (µg AEAC /g dry wt.)**
WKM extract	44.07 ± 0.51

Data were mean ± SD., (n = 3).

4.2.3 Cellular antioxidant activity (CAA)

Cellular antioxidant activity (CAA) assay is based on the ability of compounds to prevent the formation of DCF by *Tert*-butylhydroperoxide (*t*-BuOOH)-generated peroxyl radicals in RAW264.7 cells. The increment of DCF fluorescence emission following ROSs-mediated oxidation of DCFH was followed for 180 min. The Figure 4.3 showed the effect of WKM extract on DCF-emission intensity at 180 min. time point. The result suggested that standard antioxidants (positive control)

quercetin (10 μM), and WKM extract (0.125, 0.25 and 0.5 mg/ml) could significantly scavenge ROSs induction when compared to the vehicle control (VH) (p<0.05). Also, WKM decreased DCF-fluorescence emission in a dose-dependent manner. Notably, the highest concentration of WKM (0.5 μg/ml) was decreased more ROSs than the antioxidant positive control quercetin (10 μM) in DCF formation following *t*-BuOOH-mediated oxidation of DCFH. The percentage inhibition of ROS formation exhibited by WKM extract and quercetin to scavenge was shown in Figure 4.2. WKM extract at the concentrations of 0.125, 0.25 and 0.5 mg/ml produced a dose-dependent inhibition of ROS production by 21.24 ± 0.88%, 40.64 ± 0.86% and 64.31 ± 1.59%, respectively. Whereas the inhibition of 10μM quercetin was 47.84 ± 0.50%.

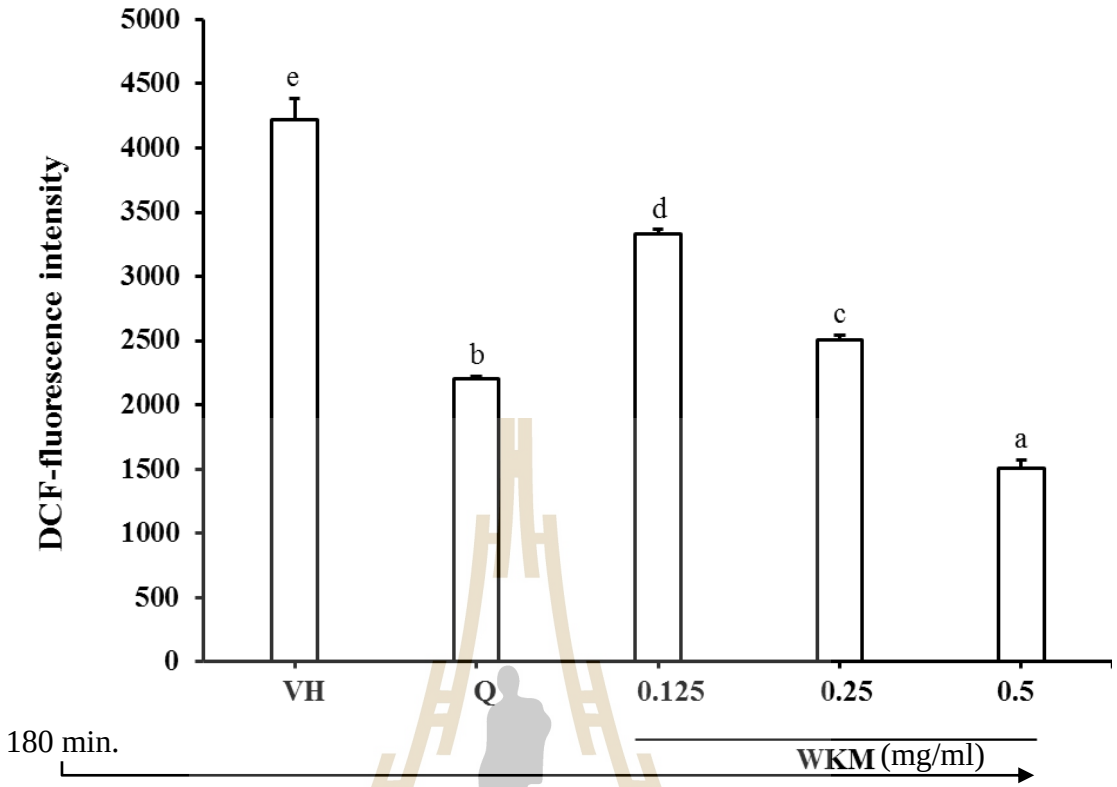

Figure 4.2 The cellular radical scavenging activity of WKM extract in tBuOOH-activated RAW264.7 cells. Intracellular ROSs level generated in cells was measured by the 2',7'-dichlorofluorescin-diacetate (DCFH-DA) assay after addition of the *t*-BuOOH for 180 min. RAW264.7 cells were pretreated with vehicle solution, quercetin or WKM extract for 24 h. *t*-BuOOH was added in the culture after incubation with DCFH-DA for 30 min.VH = vehicle control, Q = 10μM quercetin, WKM = Wan Khan Mak extract 0.125, 0.25 and 0.5 mg/ml. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance)

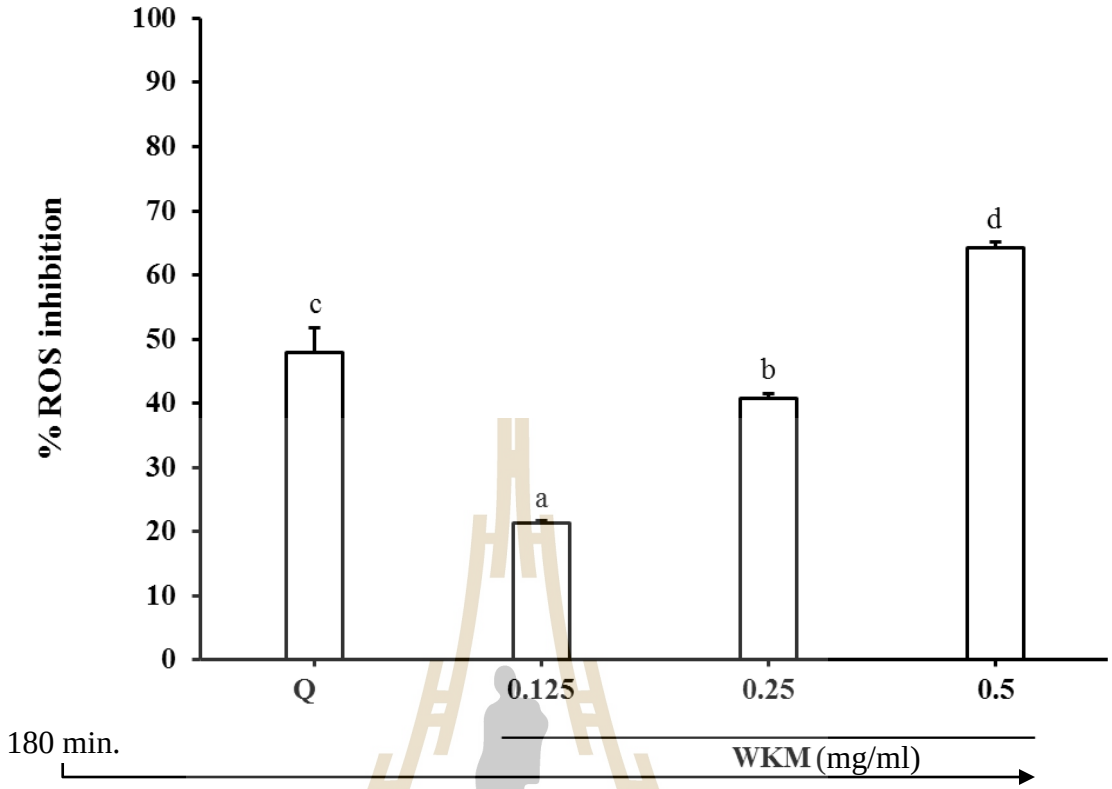

Figure 4.3 The percentage inhibition of ROS production by WKM extract and quercetin in the DCFH-DA assay. Q = 10µM quercetin, WKM = Wan Khan Mak extract 0.125, 0.25 and 0.5 mg/ml. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance)

4.3 *In vivo* toxicity

4.3.1 Acute toxicity

Acute toxicity tests can provide preliminary information on the toxic nature of a material for which no other toxicology information is available. In most acute toxicity tests, each test animal is administered a single (relatively high) dose of the test substance, observed for 1 or 2 weeks for signs of treatment-related effects. Some acute toxicity tests (such as the "classical" LD_{50} test) are designed to determine the

mean lethal dose of the test substance. The median lethal dose (or LD_{50}) is defined as the dose of a test substance that is lethal for 50% of the animals in a dose group. LD_{50} values have been used to compare relative acute hazards of industrial chemicals, especially when no other toxicology data are available for the chemicals. However, many important observations of toxicity are not represented by LD_{50} values or by slopes of dose-response curves for lethality. For example, information about morbidity and pathogenesis may have more toxicological significance than mortality, and these endpoints also should be evaluated in short term toxicity tests (Food and Drug Administration [FDA], 1988). The observation including: skin and fur, eyes, mucous membrane, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behavioural patterns. Furthermore, the attention direct to observations are salivation, lethargy, sleep, diarrhea, coma, tremors and convulsions (OECD guideline 423, 2001). The present study used the protocol of OECD guideline 423 (2001) to conduct the *in vivo* toxicity test of WKM. This protocol used fewer animals, and are thus much more cost efficient than the conventional test designed to determine LD_{50s} (Food and Drug Administration [FDA], 1988). In this study ICR mice were single administered with WKM extract 2000 and 5000 mg/kg body weight or VH control (TWEEN80) by oral gavage and the animals were observed for signs of toxicity for 14 days. Table 4.4 showed the effect of WKM extract on body weight of mice in oral acute toxicity test. The result showed that WKM did not alter the body weight in both treated groups. No toxic symptoms or death were observed in any animals and all of them lived up to 14 days.

Table 4.4 Body weight of mice in oral acute toxicity test after treated with WKM fruits extract.

Groups	**Control**		**WKM extract**			
	(5% TWEEN80)		**2000 mg/kg**		**5000 mg/kg**	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Day 0	34.6±1.67	27.6±2.19	34.8±1.79	28.0±1.00	34.2±2.17	28.2±1.64
Day 7	39.4±3.21	31.6±1.52	37.8±2.39	30.8±1.48	40.2±2.59	32.6±1.95
Day 14	40.0±3.08	31.2±1.79	38.4±1.95	32.2±1.29	40.8±2.86	33.2±0.82

Values are mean ± S.D., n = 5, *P-values < 0.05

4.3.2 Systemic toxicity test

In vivo systemic tests evaluate the impairment or activation of a system rather than the impairment of individual cells or organs. Systemic toxicity tests (USP<88>) evaluate a test article's potential to induce a systemic response after exposure to the test article. Categories are based on duration of exposure, dose and route. A systemic toxicity testing is the most commonly performed, and includes a single exposure with a 72-hour observation period. This test is considered negative if none of the animals injected with the test article show a significantly greater biological reaction than the animals treated with the control article. If two or more mice die, or show signs of toxicity, or if three or more mice lose more than 2g of body weight, the test article is considered unsafe. This study evaluated the potential toxic effects of a single-dose systemic injection of WKM extract in male ICR mice. The extract was prepared in saline solution (0.9% NaCl) for 625 mg/kg body weight and

was injected intravenously in groups of five mice. The animals were observed for signs of toxicity or other biological reactivity for seventy-two hours after administration. Table 4.5 display the body weight and the weight of vital organs of mice in systemic toxicity test after treated with WKM fruits extract. No toxic symptoms or death were observed in any of the animals and they lived up to 14 days. A gross necropsy at the end of the experiment revealed no apparent changes in any organ. There were no changes either in the corporal weight or the weight of the principle organs and all treated animals exhibited a gain in body weight comparable to controls.

Table 4.5 The body weight and vital organs weight of mice in systemic toxicity test after treated with WKM fruits extract.

Groups	Control (0.9% NaCl)	625 mg/kg BW of WKM in 0.9% NaCl
body weight (g)	43.0 ± 2.45	42.4 ± 2.70
liver (g)	2.87 ± 0.24	2.75 ± 0.12
liver/BW (%)	6.25 ± 0.42	6.29 ± 0.31
kidney (g)	0.99 ± 0.07	0.95 ± 0.12
kidney/BW (%)	2.21 ± 0.10	2.16 ± 0.19
heart (g)	0.31 ± 0.06	0.29 ± 0.02
heart/BW (%)	0.65 ± 0.13	0.66 ± 0.08
lung (g)	0.33 ± 0.01	0.28 ± 0.04
lung/BW (%)	0.69 ± 0.10	0.64 ± 0.11
spleen (g)	0.15 ± 0.03	0.13 ± 0.01
spleen/BW (%)	0.33 ± 0.07	0.30 ± 0.02

Values are mean ± S.D., n = 5., *P-values < 0.05

4.3.3 Chromosome aberrations in rat bone marrow cells

The results of chromosomal analysis in Wistar rat bone marrow cells at metaphase stage when oral administration the vehicle control (0.9% NaCl), WKM extract (5,000 mg/kg body weight) or CP (cyclophosphamide; 50 mg/kg body weight) were summarized in Table 4.6. The number of mitotic cells (Figure 4.4) was determined by counting 2000 cells for each animal. The mitotic index (M.I.) represents the fraction of cell proliferation activity and changes in the mitotic index reflect the cytotoxic effect of the test compound (WHO, 1985). In these study, the mitotic index in the vehicle control group were 13.20 ± 0.99 (male) and 9.60 ± 1.06 (female), while in WKM- and CP-treated groups, the values were 12.00 ± 0.92 (male), 9.10 ± 1.35 (female), and 3.30 ± 0.24 (male), 3.00 ± 0.22 (female), respectively. The mitotic index of WKM extract group was decreased but did not show any statistically significant difference from the negative control group. On the other hand, the values in both WKM extract and negative control groups were showed statistically significant differences from CP-treated group.

The percentage of chromosome aberration with break (Figure 4.5 and Table 4.6) in vehicle control group were 0.12 ± 0.01 (male) and 0.11 ± 0.02 (female), in WKM-treated group (5,000 mg/kg body weight) were 0.06 ± 0.01 (male) and 0.07 ± 0.01 (female), in CP-treated group were 3.90 ± 0.59 (male) and 3.88 ± 0.50 (female). Though, exposure to WKM showed the trend of decreasing percentage of chromosome aberration with break from VH control by 50% in male and about 64% in female, forever, the values were not statistically significant. As, expected, the mutagen positive control CP increased the percentage of chromosome aberration with break by 32.5 fold in male and by 35.2 fold in female, when compared to the vehicle

control group. In addition, CP exposure also induced aberrant cells with chromosome exchange and chromosome multiple aberration, whereas, none of these two types of chromosome aberrant were observed in both VH control and WKM-exposed groups.

In concordance with the result of chromosome aberration, the percentage of total cells aberration in vehicle control and WKM-treated groups (5,000 mg/kg body weight) was not statistically different, though, the percentage of cell aberration was appeared to reduce by 50% in male and about 64% in female upon exposure to WKM. CP-exposed group also significantly increased total cells with aberration by about 30 and 32 fold in male and female, respectively when compared to vehicle control and WKM-treated groups.

In summary, the M.I. of WKM extract group did not exhibit any statistically significant difference from the vehicle control group, while the mutagenic positive control CP markedly decreased M.I. significantly when compared to both vehicle control and WKM-treated groups. This means that WKM extract at the given dose has no cytotoxic effects on bone marrow cells. The major change of chromosome that induced by WKM extract was break which was the same as in vehicle control group while The major change of chromosome in CP-treated group were break, exchange and multiple aberrations. There was no significant difference in chromosome damage per cell between vehicle control group and WKM-treated group. This means that WKM extract at the given dose had no chromosome damage to the rat bone marrow cells.

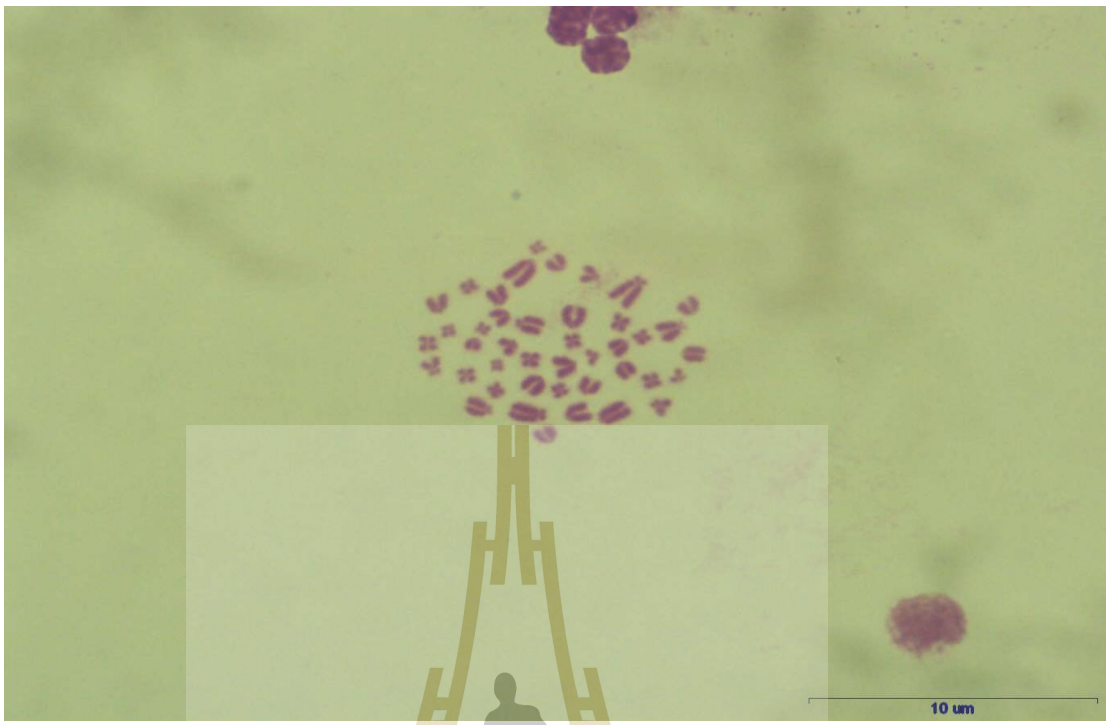

Figure 4.4 The well spread normal metaphase chromosome of bone marrow cells from Wistar rat. The photograph was taken from digital camera under magnification at 100x objective.

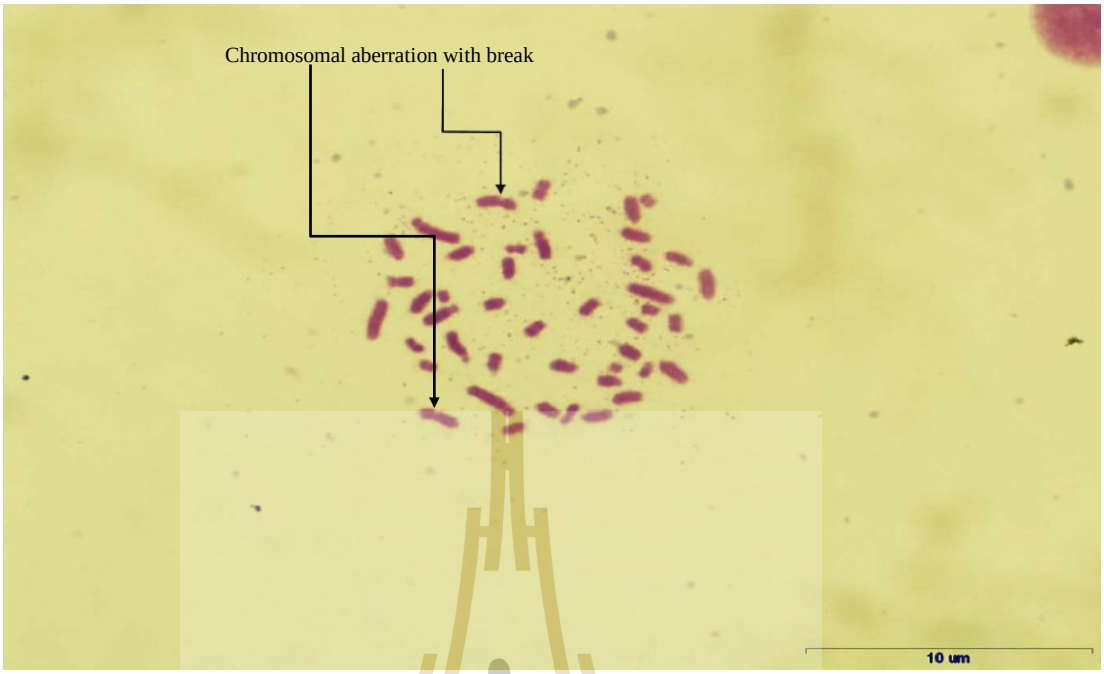

Figure 4.5 Examples of the chromosomal aberrations with break which was found in rats treated with vehicle control, WKM extract or cyclophosphamide; 50 mg/kg BW. The photograph was taken from digital camera under magnification at 100x objective.

Table 4.6 Chromosome aberration rates in Wistar rat bone marrow cells treated with 5,000 mg/kg WKM fruits extract.

Treatment (mg/kg BW)	Sex	M.I.# ± SEM (%)	Cell of Chromosome Aberration (%)			Chromosome Damage per Cell
			Break	Exchange	Multiple	
0.9% NaCl	**male**	13.20 ± 0.99	0.12 ± 0.01	0	0	0.01 ± 0.00
	female	9.60 ± 1.06	0.11 ± 0.02	0	0	0.01 ± 0.00
WKM (5000 mg/kg BW)	**male**	12.00 ± 0.92	0.06 ± 0.01	0	0	0.01 ± 0.00
	female	9.10 ± 1.35	0.07 ± 0.01	0	0	0.01 ± 0.00
CP (50mg/kg BW)	**male**	3.30 ± 0.24*	3.90 ± 0.59*	2.56 ± 0.48*	4.26 ± 1.13*	1.88 ± 0.31*
	female	3.00 ± 0.22*	3.88 ± 0.50*	2.55 ± 0.46*	4.23 ± 1.24*	1.53 ± 0.17*

Values are mean ± SEM, n = 5, #mitotic index, *p-values < 0.05

4.4 Anti-allergic activity

4.4.1 Dose range-finding study

In vitro cytotoxicity effect of WKM extract on rat basophilic leukemia (RBL-2H3) was investigated using the MTT colorimetric assay and trypan blue exclusion method. The optimum concentration range of WKM that had no direct cytotoxic effect on RBL-2H3 cells applicable model was conducted in RBL-2H3 cells was obtained and was used for further studies.

4.4.1.1 MTT method

RBL-2H3 cells were incubated for 24 h with VH control, various concentrations of WKM (0.5, 0.25 and 0.125 mg/ml), or 10 µM quercetin. Then, the cell viability of RBL-2H3 cells was determined by MTT assay. The result (Figure 4.6) suggested that WKM at the concentration of 0.125, 0.25 and 0.5 mg/ml had no cytotoxic effect towards RBL-2H3 cells as evidenced by no significant difference in cell viability was observed in WKM exposed cultures when compared to vehicle control cells (Cell viability was about 90%). The antioxidant positive control quercetin at 10 µM was also not cytotoxic to RBL-2H3 cells.

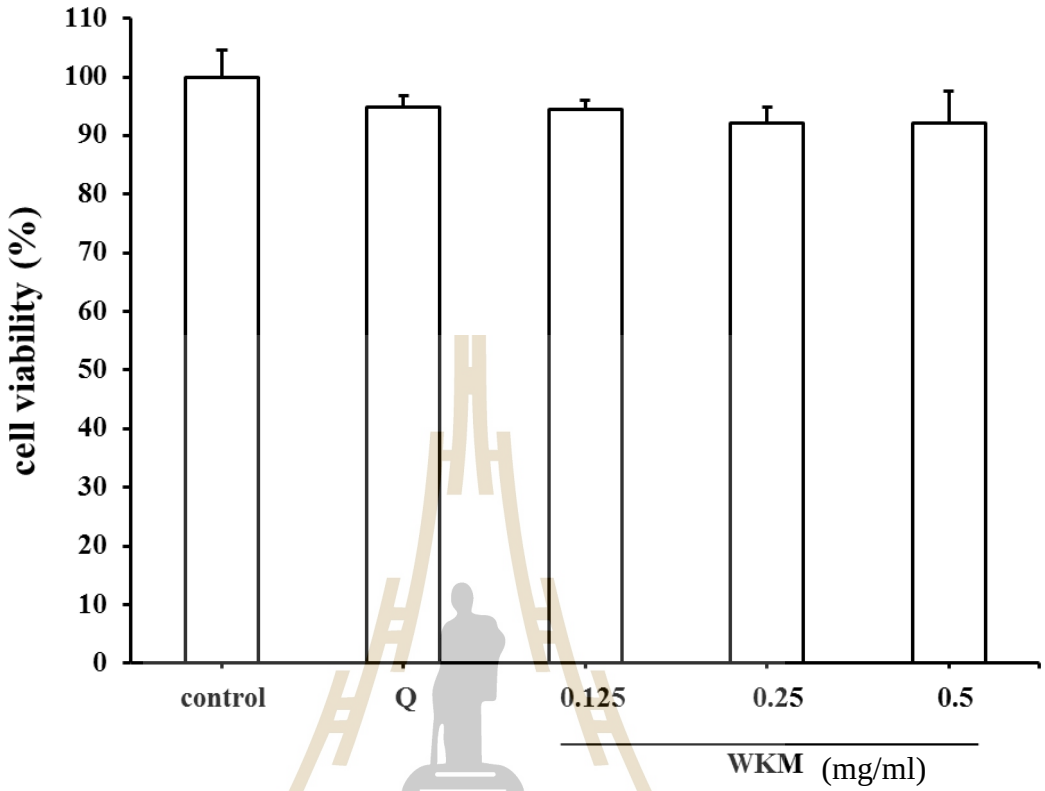

Figure 4.6 The toxicity effect of WKM extract and quercetin on RBL-2H3 cells evaluated by the MTT assay.

4.4.1.2 Trypan blue exclusion method

The viability of RBL-2H3 cells exposed to WKM extract was also confirmed by trypan blue exclusion method (Figure 4.7). The result was in concordance with MTT assay, the percentage of rat basophilic leukemia cells viability when treated with various concentration of the extract (0.125, 0.25 and 0.5 mg/ml) were 87.11 ± 5.78, 88.81 ± 2.31 and 90.94 ± 1.65%, respectively and 10 µM quercetin was 86.33 ± 1.21, which were not significantly different from controls ($p < 0.05$).

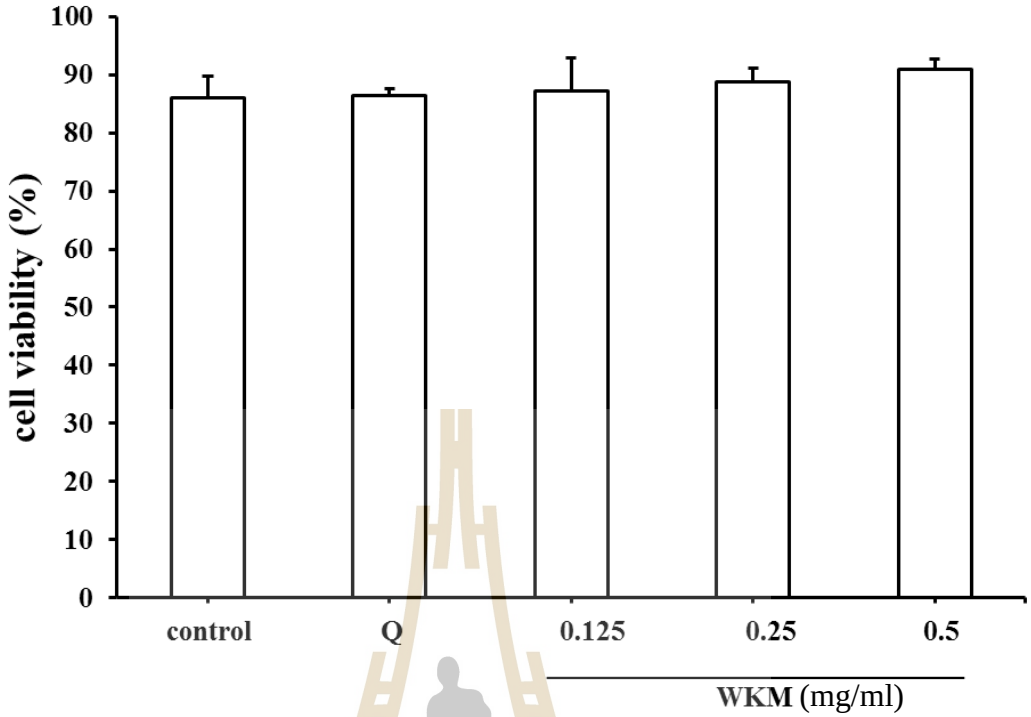

Figure 4.7 The toxicity effect of WKM extract and quercetin on RBL-2H3 cells as determined by Trypan blue exclusion method.

4.4.2 β-Hexosaminidase assay

During immediate-type allergy (type I hypersensitivity), histamine is released from activated mast cells via a degranulation process by IgE-allergenic stimulation. β-Hexosaminidase is located in the secretory granules of mast cells where histamines are stored, and is released along with histamine when mast cells are immunologically activated (Schwartz *et al*., 1981; Marquardt and Wasserman, 1983)**.** Thus, β-hexosaminidase activity can be used as degranulation marker of allergic response of the mast cells and has been widely used in biochemical studies of allergies as a screening method for anti-allergic agents (Fischer *et al*., 1995; Choi *et al*., 1996). Several therapeutic agents that inhibit the release of pharmacologically active chemical mediators from mast cells or basophils are now in clinical use

(Marone *et al*., 2002). Moreover, many kinds of natural products have been reported to show anti-allergic effects (Choo *et al*., 2003). To investigate the anti-allergic activity of the selected plant, the rat basophil leukemia cell line, RBL-2H3 was used. RBL-2H3 cells were used for *in vitro* assay to assess the anti-allergic activity of WKM extracts. Inhibitory effects of the WKM extract on Ag-stimulated degranulation from RBL-2H3 cells was investigated by stimulation of IgE-sensitized RBL-2H3 cells with DNP-BSA in the absence or the presence of the WKM extract, and 10 μM quercetin was used as positive control. Results in Figure 4.8 showed that WKM fruit extract could decamp modulate Ag-stimulated degranulation of RBL-2H3 cells. The percentage of β-hexosaminidase release was decreased by WKM in dose-related manner compared to the vehicle control, the percentage of β-hexosaminidase release at all concentration of WKM were significantly lower than vehicle control ($p < 0.05$). WKM at the concentration of 0.125, 0.25 and 0.5 mg/ml significantly decreased the degranulation by 15.62, 33.70 and 60.25%, respectively (Figure 4.8). Notably, the maximum inhibitory effect on degranulation exerted by 0.5 mg/ml of extract was as effective as 10 μM of quercetin, the positive control in the study.

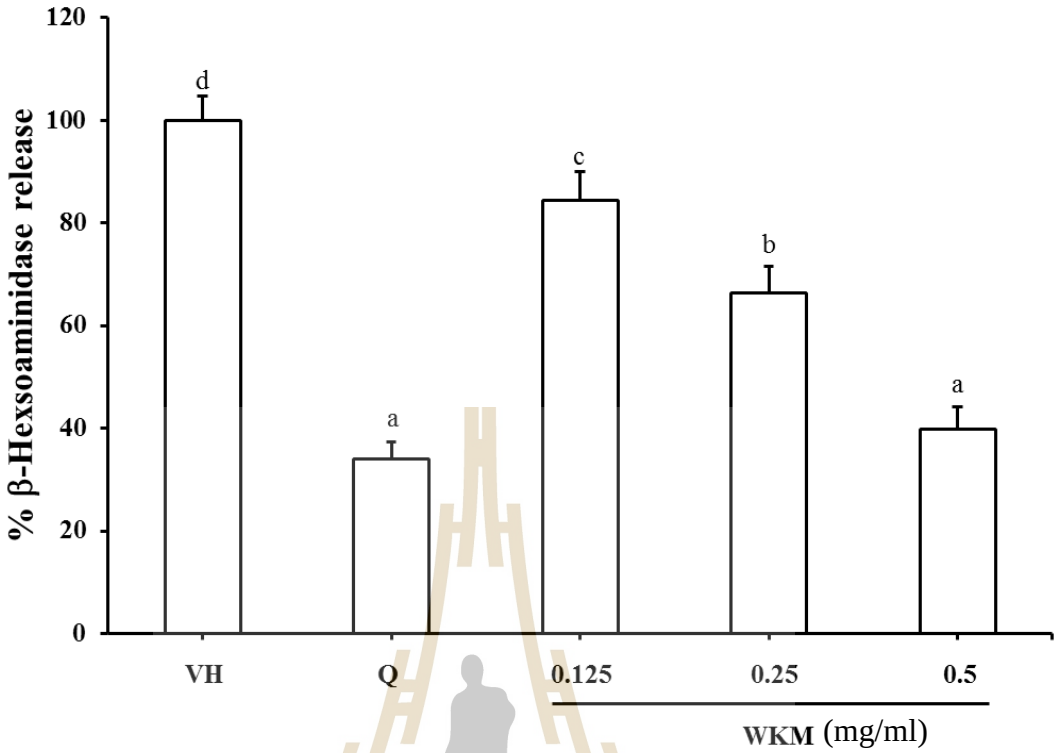

Figure 4.8 The release of β-hexosaminidase by Ag-stimulated degranulation from rat basophilic leukemia RBL-2H3 cells. Values are mean ± SD (n = 3) of the percentage release of β-hexosaminidase. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance).

4.5 anti-inflammatory activities

4.5.1 Dose range-finding study

In vitro cytotoxic effect of WKM extract on mouse macrophage cell line (RAW264.7) was investigated using the MTT colorimetric assay and trypan blue exclusion method. The dose range of WKM that was not cytotoxic towards RAW246.7 cells in this preliminary study was chosen for further use in subsequent studies.

4.5.1.1 MTT method

The viability of RAW264.7 cells exposed to WKM extract was determined by MTT assay (Figure 4.9). Cells were incubated for 24 h with or without various concentrations of WKM (0.125, 0.25 and 0.5 mg/ml). The percentage of mouse macrophage cells viability when treated with various concentration of the extract (0.5, 0.25 and 0.125 mg/ml) were 102.40 ± 11.22, 110.18 ± 11.70 and 116.20 ± 10.83%, respectively, and 10 µM quercetin was 110.08 ± 11.54%. Therefore, the results indicated that neither WKM extract (up to 0.5 mg/ml) nor 10 µM quercetin was cytotoxic towards RAW264.7 cells.

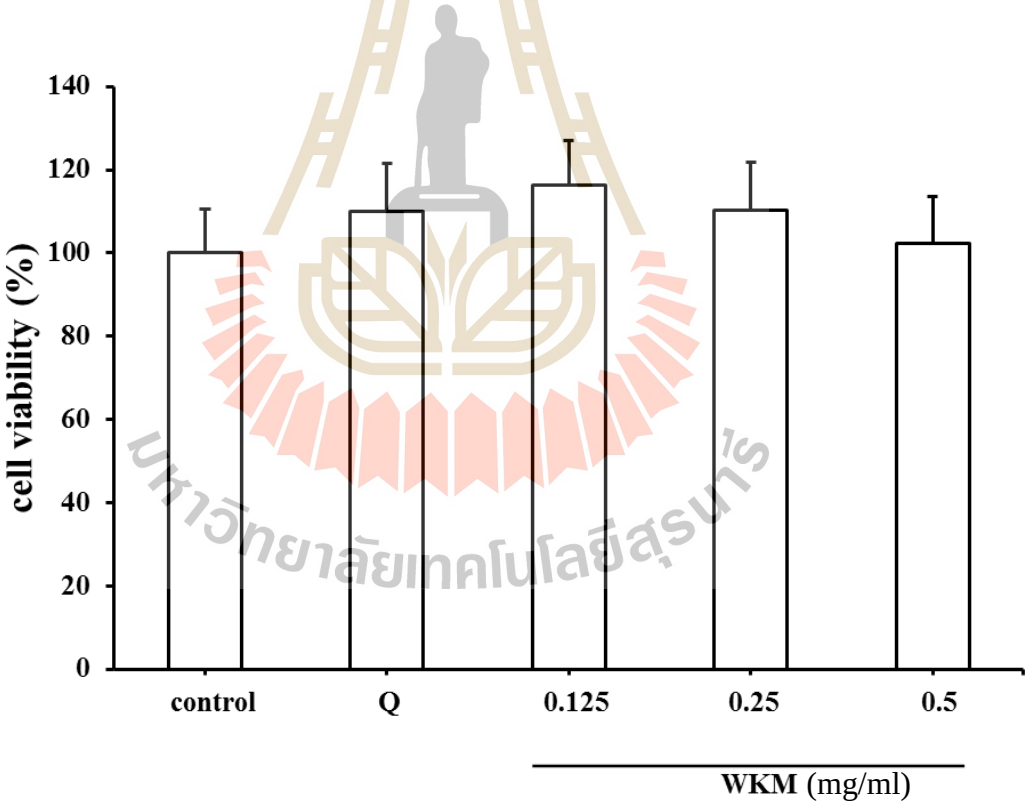

Figure 4.9 The toxic effect of WKM extract and quercetin on cell viability of RAW264.7 cells as evaluated by MTT assay.

4.5.1.2 Trypan blue exclusion method

The viability of RAW264.7 cells exposed to WKM extract was also confirmed by trypan blue exclusion method (Figure 4.10). Cells were incubated for 24 h with or without various concentrations of WKM (0.125, 0.25 and 0.5 mg/ml). In agreement with MTT assay, the percentage of viability of RAW264.7 cells exposed to WKM at the concentrations of 0.125, 0.25 and 0.5 mg/ml were 93.18 ± 7.17, 94.57 ± 13.14 and 96.64 ± 14.53%, respectively, which were not significantly different from the vehicle control ($p > 0.05$). At 10 µM quercetin was 94.92 ± 18.67%, which was also not cytotoxic towards RAW264.7 cells. The result indicated that no significant difference of percentage of viable cells in WKM and quercetin treatment ($p > 0.05$).

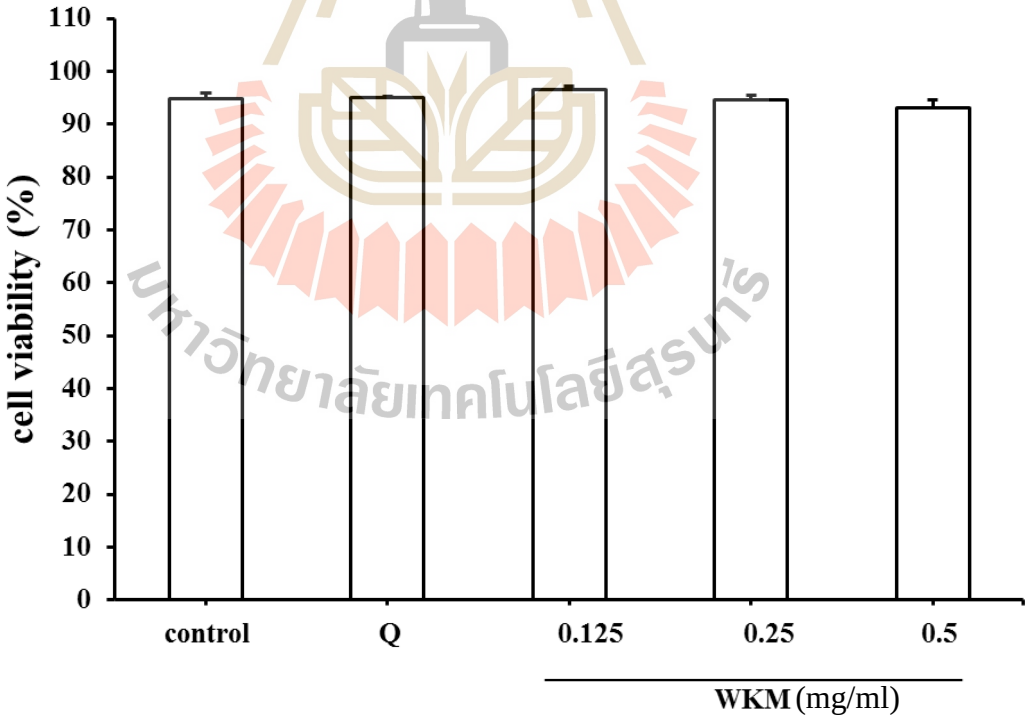

Figure 4.10 Cytotoxic effect of WKM extract and quercetin on cell viability of RAW264.7 cells as determined by Trypan blue exclusion method.

4.5.2 Expression of Nitrite oxide production

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

RAW264.7 cells were pretreated with antioxidant, quercetin or WKM for 4 h, then, stimulated for 24 h with LPS plus IFN-γ, and measured for NO production using the Griess assay. As shown in Figure 4.11, Naïve RAW264.7 cells (NA) secreted basal levels of NO, while the production of NO was increased to 31.06 ± 0.77 μM in LPS plus IFN-γ activated RAW264.7 cells. Preincubation with the antioxidant control, 10 μM quercetin, decreased the nitrite level to 6.69 ± 0.09 μM, while, pretreated with WKM at concentrations of 0.125, 0.25 and 0.5 mg/ml decreased the nitrite level to 24.96 ± 0.22, 16.10 ± 0.12 and 4.70 ± 0.04 μM, respectively.

These results clearly demonstrated that WKM extract induced a dose-dependent suppression of NO production by LPS plus IFN-γ activated RAW264.7 cells at the concentration range that had no effect on cell viability (Figure 4.9 and 4.10). The percent of suppression of NO production by WKM at concentrations of 0.125, 0.25 and 0.5 mg/ml was 18.83 ± 0.70, 47.66 ± 0.40 and 84.71 ± 0.15%, respectively, at 10 μM, quercetin induced the suppression of NO production by 78.26 ± 0.29% (Figures 4.12).

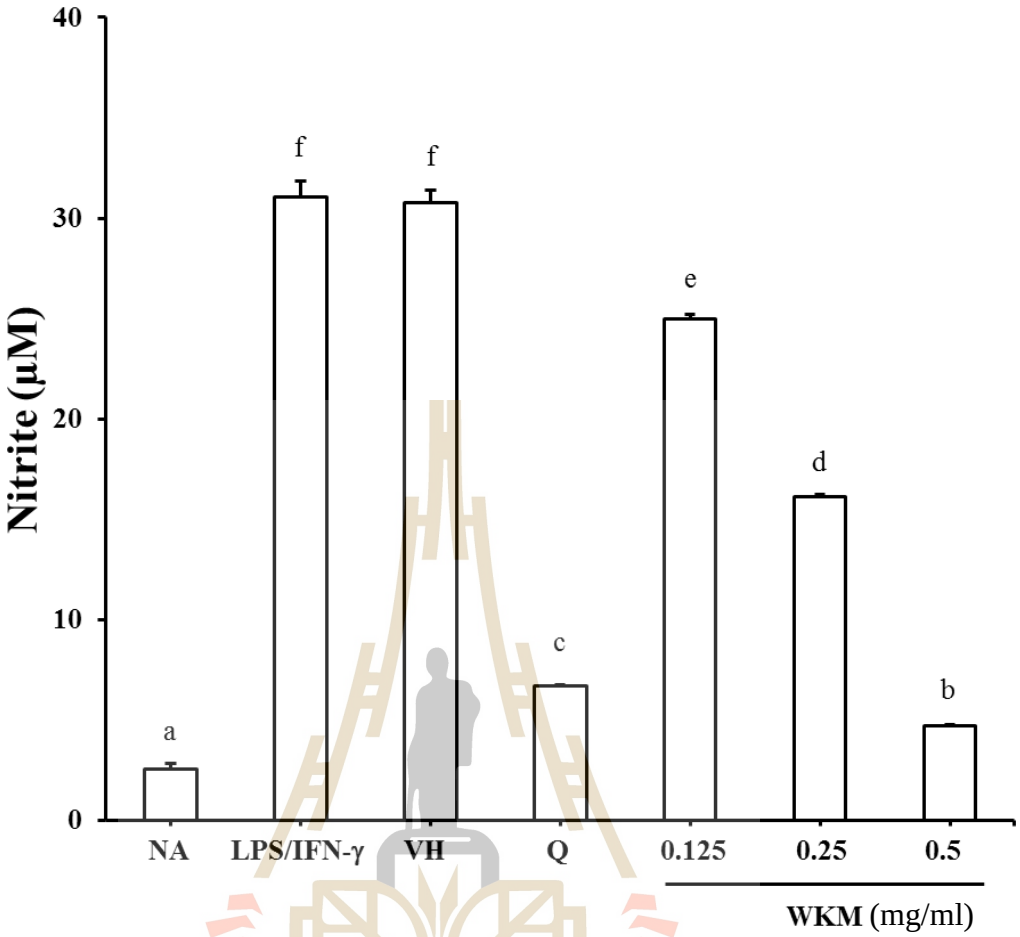

Figure 4.11 Effect of WKM extract on nitrite oxide production by LPS plus IFN-γ activated RAW 264.7 cells. The cells were pretreated with different concentrations of WKM (0.125-0.5 mg/mL) for 4 h then stimulated with LPS (1 µg/mL) plus IFN-γ (25 U/mL) for 24 h. Production of nitrite was determined by Griess reaction, as described in materials and methods. All data represent the mean±SD (n=4) of representative of three independent experiments with a similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.

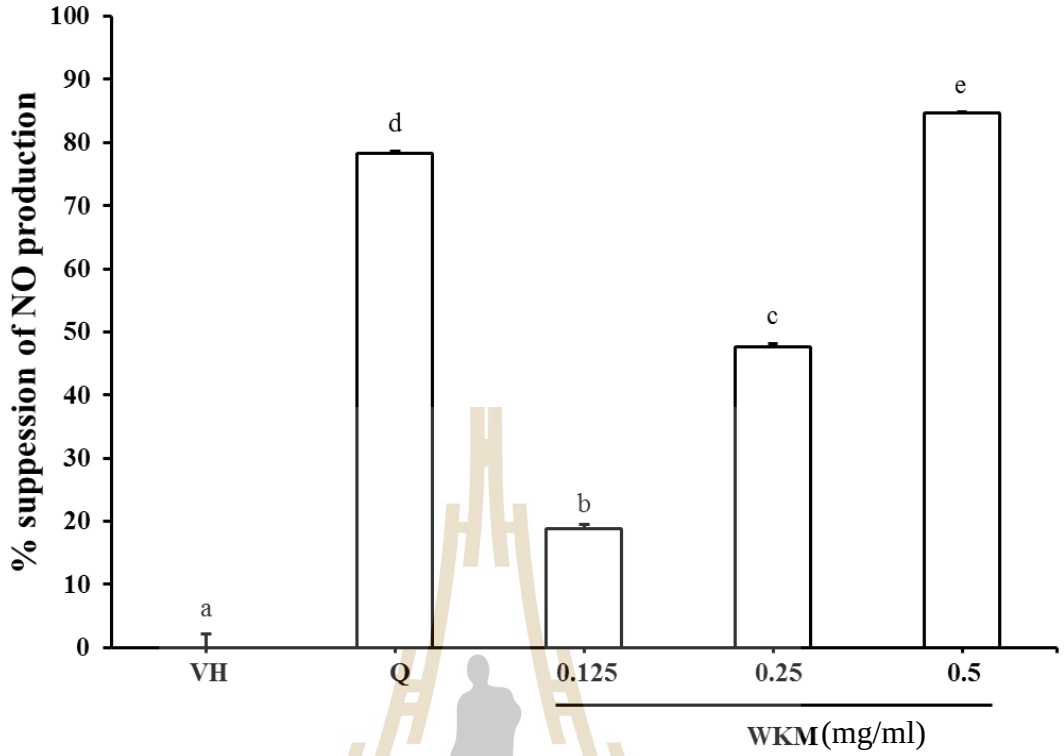

Figure 4.12 The suppression effect of WKM on NO production by LPS and IFN-γ activated RAW 264.7 cells at the concentrations that had no effect on cell viability. All data represent the mean±SD (n=4) of representative of three independent experiments with a similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.

4.5.3 Suppression of iNOS and COX-2 protien expressions in LPS plus IFN-γ-activated RAW264.7 cells

In order to elucidate the mechanism by which WKM inhibits NO production in LPS and IFN-γ activated macrophages, the iNOS protein expression was investigated by Western blotting analysis. RAW264.7 cells were pretreated with antioxidants, quercetin (10 μM), or WKM at concentrations of 0.125, 0.25 and 0.5 mg/ml for 4 h prior subsequently activated with LPS (1 μg/mL) plus IFN-γ (25 U/mL)

for 24 h. Total proteins were extracted and analyzed by Western blotting for the expressions of iNOS. LPS plus IFN-γ-induced increases in iNOS expression compared to unstimulated naïve culture (Figure 4.13). As expected, anti-oxidant controls (10 μM quercetin) decreased LPS plus IFN-γ-induced iNOS protein level. Compared to the corresponding controls, WKM extract produced a dose-dependent suppression of iNOS level in LPS plus IFN-*γ* activated RAW264.7 cells (Figure 4.13) suggesting the suppression of NO production by WKM was mediated by decreasing the expression of iNOS. As determined by densitometry, the suppression of iNOS was 58.96% at 10 μM quercetin, whereas the suppression by 0.125, 0.25 and 0.5 mg/ml WKM were about 12.86%, 90.26% and 97.54%, respectively. There was no significant effect on the level of tubulin synthesis in the same samples.

Western blot analysis of COX-2 expression by densitometry showed that WKM extract produced a dose dependent suppression of COX-2 protein in LPS and IFN-γ-activated RAW264.7 cells (Figure 4.14). When compared to the unstimulated naïve control, the suppression of COX-2 expression by WKM at the concentrations of 0.125, 0.25 and 0.5 mg/ml were 3.86%, 47.58% and 98.21%, respectively, whereas the suppression by positive controls (10 mM quercetin) was 87.38%. There was no significant effect of WKM or quercetin on the level of house-keeping gene tubulin was observed in the same samples. Therefore, the anti-inflammatory activity of the extract was suppressed by the strong suppression of NO and COX-2 expression in the LPS and IFN-γ-activated RAW264.7 cells.

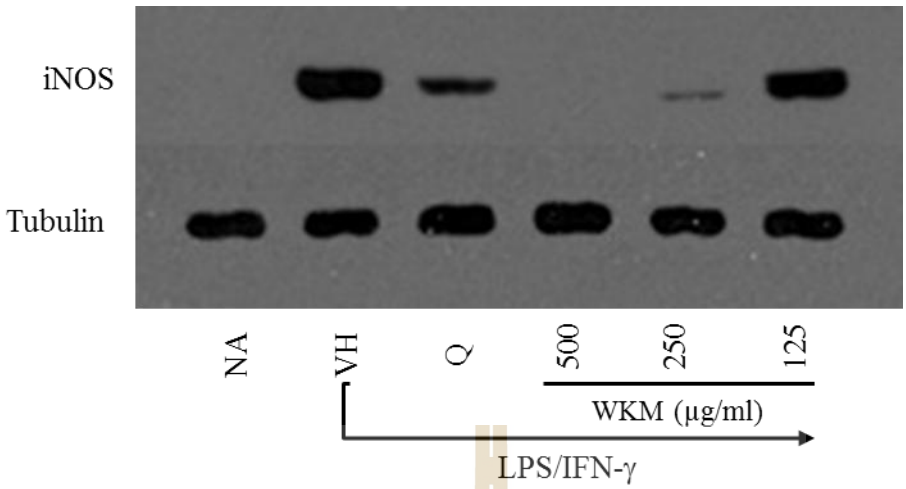

Figure 4.13 Effect of WKM on LPS plus IFN-γ-induced iNOS protein levels in RAW26.7 cells. The relative expression of proteins was quantified densitometrically using ImageJ software and normalized according to tubulin reference bands. Data are a representative of at least two independent experiments. NA = naïve, VH = vehicle, Q = 10 µM quercetin, WKM extract at 0.5, 0.25 and 0.125 mg/ml, respectively.

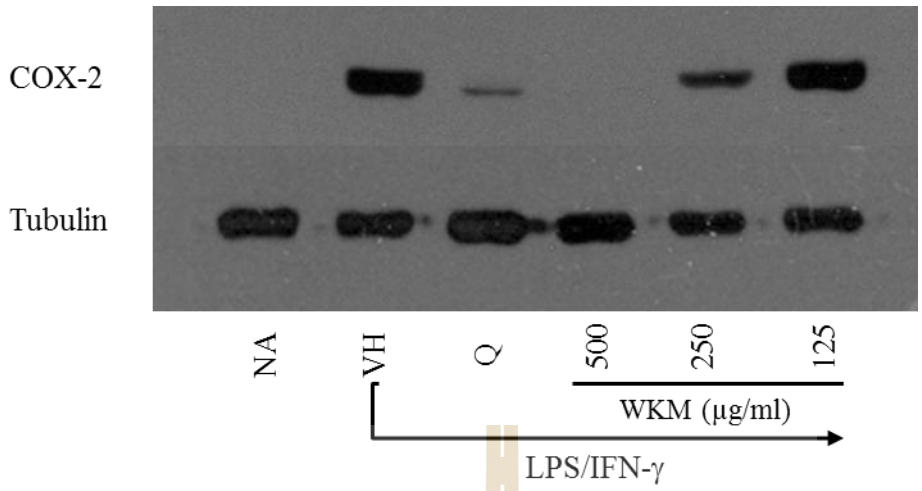

Figure 4.14 Effect of WKM on LPS plus IFN-γ-induced COX-2 protein levels in RAW26.7 cells. The relative expression of proteins was quantified densitometrically using ImageJ software and normalized according to tubulin reference bands. Data are a representative of at least two independent experiments. NA = naïve, VH = vehicle, Q = 10 µM quercetin, WKM extract at 0.5, 0.25 and 0.125 mg/ml, respectively.

CHAPTER V

DISCUSSIONS

Although a bloom of research in Thai herbs in recent years, there were fewer researches in Wan, especially Wan Khan Mak (*Aglaonema simplex* Bl.). Wan Khan Mak is the plant in Araceae family that the Thai-traditional medicine has cited as a miracle plant. Nevertheless, at present, the studies of its chemical composition, general toxicity and the scientific evidences to support Wan Khan Mak's claimed ethno-medical properties are still very limited in both domestic and international database.

Folk wisdom has long recognized plants for their medicinal and protective properties. Only recently, though, has science established that plants could also play more comprehensive roles in the human diet. Many phytochemicals that provide plants for their color, flavor, smell and texture, may help to prevent some diseases such as cancer, heart disease, inflammation and various chronic diseases. Example of some well-known phytochemicals possessing pharmaceutical properties are curcumin in curcuma, gingerol in ginger, capsaicin in chilli pepper, catechin in tea leaves, diallyl sulphide in garlic, indole-3-carbinol in cabbage, sulphoraphane in broccoli, lycopene in tomatoes, resveratrol in grapes, quercetin in onion and potato and flavonoids in colorful fruits such as oligomeric proanthocyanidin (OPC) in grape seeds (Surh, 2003).

It is well known that the major components of plant extracts that act as antioxidants are phytochemicals such as phenolic compounds and flavonoids. The presented study showed that phenolics, flavonoids and proanthocyanidins were found in WKM fruits extracts when assessed with Folin-Ciocalteu, aluminum trichloride and vanillin assay, respectively. WKM extract also had antioxidant activities when evaluated with DPPH and FRAP assays. Similarly, the present study also suggested that flavonoids and phenolics could exhibit antioxidant activities in the extracts from WKM.

Several methods have been performed and developed to measure the total antioxidant activity of natural medicinal plants *in vitro*. due to the complex composition of phytochemicals and oxidative process, the antioxidant capacity of medicinal plant extracts should not be investigated by using only one single method (Bohm, Schlesier, Harwat, and Bitsch, 2001). The use of at least two methods should be employed in order to evaluate the total antioxidant activity. The secondary metabolites in plants, vitamin C, Trolox, catechin and quercetin were used as positive antioxidant controls in the present study as they are the most frequently used antioxidant standards for food samples. DPPH assay is one of the most extensively used methods to evaluate antioxidant activity. The present study revealed that the IC_{50} from DPPH radical scavenging capacity of WKM was 399.77 ± 15.33 µg/ml and had ferric reducing power (FRAP value) as 44.07 ± 0.51 µg AEAC/g dry weight. Though DPPH and FRAP assays are based on a single electron transfer reaction (Bunea *et al*., 2011), their characteristics, sensitivities, mechanisms of the reaction, and endpoints are totally different. For instance, DPPH method is based on the free radical scavenging activity while FRAP measures the reducing power of sample to reduce

Fe^{+3} to Fe^{+2} (Benzie and Strain, 1999). FRAP assays also evaluate the chain-breaking antioxidant potential (Ghiselli *et al*., 1995).

Though both DPPH and FRAP assays are frequently used methods, they have some drawbacks for antioxidant activity measurement. In DPPH assay, other compounds may have high absorbance or interference at the same wavelength at 515 nm. In addition, DPPH radical is absent in living organisms. The drawbacks of the FRAP method are that the compounds with low redox potential (lower than 0.77) still can reduce the Fe^{+3} even though they do not behave as antioxidants *in vivo*. Interfering compounds may also absorb at the same wavelength, and the assay is also performed at a non-physiological pH (Pérez-Jiménez *et al*., 2008). Therefore, the present study also determined the scavenging activity in the cell-based assay using intracellular fluorescent probe DCFH-DA. For evaluation of cellular antioxidant activity, the macrophage is usually the cell of choice in studying ROSs-mediated cellular events since they could generate high amount of ROSs following stimulation. The present study demonstrated that antioxidant standard, 10 μM quercetin, exerted a strong inhibition of ROSs generation in murine macrophage RAW264.7 cells induced by *t*BuOOH over a period of 180 min. In addition to extracellular antioxidant capacity, WKM also possessed intracellular antioxidant activity in scavenging ROSs and decreasing the oxidation of DCFH. Similarly, the crude water and ethanol extracts of various plants leaves and fruits containing phenolic compounds had been shown previously to act as antioxidants in the cell-base assay using DCFH-DA probe (Aiyegoro and Okoh, 2009, 2010; Shi, Xu, Hu, Na, and Wang, 2011; Shukla, Mehta, Mehta, and Bajpai, 2012; Serra, Duarte, Bronze, and Duarte, 2011). In addition, polyphenols are known to support bioactive activities in medicinal plants. Therefore,

the antioxidant activity of WKM might be attributed to the presence of phenolic and flavonoid in ethanol crude extracts of WKM. In summary, the extract from WKM fruits contained phenolic, flavonoid and proanthocyanidin compounds and could display antioxidant properties by both chemical and cell-base antioxidant assays. Notably, though WKM displayed lower DPPH radical scavenging activity than the antioxidant positive controls, quercetin, catechin and Trolox (Table 4.2), the extract at 0.25-0.5 mg/ml could exhibit a high potential to reduce ROS-induced oxidative stress in RAW264.7 cells, as compared to 10 µM quercetin (Figure 4.3). This could be explained by differences by modes of antioxidant action in both assays. The DPPH method is based on reduction of stable DPPH nitrogen radicals in presence of antioxidants (Huang *et al*., 2005). In DCFH-DA cell-base assay, DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent dichlorofluoroscein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (Yen, Chiang, Wu, and Yeh, 2003). Therefore, the observed antioxidant potential of the WKM extract in DPPH and DCFH-DA assays might be mediated by a different mechanism and/or different antioxidant constituents in extract. In fact, many important classes of antioxidants including flavonoids, one of main chemical constituents in WKM extract, contain a huge number of natural antioxidant compounds with a diversity of their modes of action (Kumar and Pandey, 2013). Depending on what specific phytochemical constituents present in the extract are providing the antioxidant activity, their discrete chemical structures, positions, numbers, and types of substitutions of features can influence their redox properties

and hence their antioxidant potentials. The structure antioxidant activity relationships of flavonoids have been studied, for example, multiple hydroxyl groups of flavan nucleus of flavonoid favor antioxidant and chelating activity, methoxy group has unfavorable steric effects and increase lipophilicity, a double bond and carbonyl group in the herterocycle of the nuclear structure offer a more stable radical and hence increase the activity (Heim, Tagliaferro, and Bobilya, 2002). Phenolics may have hydroxyl or methoxyl groups; while flavones possess hydroxyl, keto, and free carboxylic groups; and flavonoids have flavan nucleus with different types of substitutions. Typically, all of these compounds can provide different reducing properties.

From the foregoing discussions, it is clear that not a single method can give a comprehensive prediction of antioxidant efficacy. Therefore, more than one method is recommended and there should be greater caution in extrapolating the extracellular data to intracellular-based assay (Aruoma, 2003).

In acute oral toxicity study, ICR mice were single administered with WKM extract 2000 and 5000 mg/kg body weight or VH control (TWEEN80) by oral gavage. The result showed that WKM did not alter the body weight gain in both treated groups. No toxic symptoms or death were observed in any animals and all of them lived up to 14 days.

The *in vivo* systemic test was a method to determine the potential toxic effects of a single-dose extract. The extract was prepared in saline solution (0.9% NaCl) for 625 mg/kg body weight and was injected intravenously in groups of five mice. Result displays the body weight and the relative weight of vital organs of mice in systemic toxicity test after treated with WKM fruits extract. No toxic symptoms or

death were observed in any of the animals and they lived up to 14 days. A gross necropsy at the end of the experiment revealed no apparent changes in any organ. There were no changes either in the corporal weight or the weight of the principle organs and all treated animals exhibited a gain in body weight comparable to controls.

Based on the present studies by acute oral toxicity, the concentrations of 5000 mg WKM extract /kg body weight were chosen for the chromosome aberration study. WKM extract at this dose were freshly prepared in the form of suspension in 0.9% NaCl and mixed well before oral administration. Cyclophosphamide (CP), a standard mutagen, was freshly prepared and was single injected intraperitoneally to rats at a dose of 50 mg/kg body weight in the positive control group. The animals were sacrificed under ether anesthesia 24 h after administration.

In this study, metaphase cells with one or more chromosome aberrations were scored from 50 well-spread metaphases per animal (250 metaphases per sex per treatment group, 5 male and 5 female) at random. The types of aberration (break, exchange and multiple aberrations) were scored and recorded strictly in accordance with the method of Ito and Ito (2001). The severity of chromosome aberrations included the aberrations with break, exchange and multiple aberrations, but not aberrations with gap (Ito and Ito, 2001). The result in the present study indicated that WKM extract exhibited no evidence of mutagenic potential at the highest concentration of 5000 mg WKM/kg body weight, as evaluated by chromosome aberration with break, exchange and multiple aberrations. The level of chromosome break and exchange of WKM-treated group was in the base line and was comparable to the negative control group.

In the last decades, the relationship between food, nutrition and cancer, and the knowledge that cancer may be a preventable disease have been extensively explore with an increased interest in studying the mutagenic or antimutagenic potential of some dietary constituents (Azevedo *et al*., 2003). Considerable emphasis has been laid down on the use of dietary constituents to prevent the mutagen-induced mutation and/or chromosomal damages due to their relative non-toxic effects. Whether the non-toxic and non-mutagenic dose of Wan Khan Mak will also possess the anti-mutagenic potential should be further investigate. The positive mutagen CP, a chemotherapeutic drug, is an indirect mutagen, damages chromosomes through generate of free radicals and alkylating DNA thereby producing mutation (Povirk and Shuker, 1994). CP was often used as positive control in genotoxic test, both in laboratory animals or in cell cultures in the presence of liver S-9 fraction. The types of chromosomal aberrations induced by CP as a positive control were reported to be chromosome break, chromatid break, chromatid exchange, chromosomal exchange and ring chromosome (IARC, 1981). However, the present study only focused on the acute toxicity, further investigation in the long term toxic effects study of WKM extract are needed.

In the present day, allergic disease is a serious problem worldwide, especially in the developed countries. Substances that cause allergic reaction are called allergens which are generally found in everyday-life environment; for example, pollen, cosmetics, food, dust mites, mold spores and animal danders. Second exposure to the allergen leads to the cross-linking of bound IgE, triggering the release of many pharmacological active mediators such as histamine, serotonin, prostaglandin, leukotrienes and cytokines from mast cells and basophils. These mediators cause

smooth muscle contraction, increased vascular permeability and vasodilation (Goldsby *et al*., 2002). When Rat basophilic leukemia (RBL-2H3) cell line was stimulated by DNP-BSA antigen, it released histamine which could be measured by HPLC (Suzuki *et al*., 2005; Yamamoto *et al*., 2004; Nakatani, 2002).

Most people are not well realized the pharmacological values of Wan Khan Mak up till resent years. Spreading from words of mouth, those who consumed Wan Khan Mak stated that it promoted good health, freshen the feeling, anti-body aching and energize the body. Furthermore, some severe allergic patients claimed that their symptoms were distinctively better after consumption of Wan Khan Mak within a few days. One patient also indicated that the efficacy of this plant is better than many anti-allergic medicines prescribed by his doctor. However, this patient has some concern about its long-term toxicity (personal interview). Nowadays, in certain places where Wan Khan Mak has earned its reputation as a miracle plant, its fruits have been sold expensively ranging from 200-400 baths each. The reason might be the plant is rarely found (Apinya sanyasee, 2005). Nevertheless, at present, there is still no research and studies or any scientific evidences to support Wan Khan Mak's claimed anti-allergy property.

The release of mediators by degranulation is one of the key phenomena observed during allergic inflammation (Theoharides *et al*., 2010). In the present study, the anti-allergic effect of WKM extract was investigated using the antigen-induced degranulation in IgE-sensitised RBL-2H3 cells. RBL-2H3 cells is known to be an analog of rat mucosal mast cell with function similar to both primary mast cells and normal basophils. RBL-2H3 cells are a popular model for studying of IgE-mediated degranulation and is considered as a common screening test for the immediate phase

of type I anti-allergic reaction (Cheong *et al*., 1998; Han *et al*., 2012). Among four allergic disease types, the type I (or immediate type) hypersensitivity is mediated by allergen cross-linking of FcεRI-bound IgE on surface of mast cells. Activated mast cells degranulate to release various mediators, including histamine, β-hexosaminidase and other proinflammatory cytokines such as IL-4 and TNF-α (Abramson *et al*., 2006). Therefore, reduction or inhibition of mast cell-mediated inflammatory responses is crucial to avoid immediate hypersensitivity responses in atopic individuals. Although applications of WKM, as anti-inflammatory activities have been reported (Ryu *et al*., 2001), the anti-allergic effects of WKM still remain elusive. In the current study, we found the inhibitory effects of WKM on the degration of IgE-sensitised basophilic cells. The granule-associated β-hexosaminidase is an exoglycosidase and is released in parallel with other chemical mediators (e.g. histamine and serotonin) upon antigen crosslinking of FcεRI. Therefore, the release of β-hexosaminidase has been commonly regarded as useful indicators to evaluate the activation of mast cells in various acute allergic and inflammatory responses (Abramson *et al*., 2006; Pecht and Corcia, 1987). This assay is a convenient tool for studying signal transduction pathways leading to exocytosis of mast cells as well as screening potential drugs for the blockade of mast cell degranulation (Granberg, Fowler, and Jacobsson, 2001). Cheong *et al*. (1998) reported the possible relationships of structure to the inhibitory activities of flavonoids based on the results study with more than 20 compounds using a similar assay method by measuring the release determination of β-hexosaminidase from Ag-activated RBL-2H3cells. The anti-allergic effect of quercetin was shown by high suppression of β-hexosaminidase

releases in RBL-2H3 cells (Yun, Kang, Park, and Nam, 2010). Mastuda (2002) suggested that the hydroxyl groups of quercetin play a significant role in exerting the anti-allergic activity.

Measurement of mitochondrial metabolic rate using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a popular method used to indirectly evaluate cell viability. However, the metabolic activity may be changed or variation of results can be obtained by different conditions or chemical treatments including pH, green tea polyphenol (Wang, Henning, and Heber, 2010; Plumb, Milroy, and Kaye, 1989; Hsu *et al*., 2003) and color interference from media or samples. Maioli *et al*. (2009) reported that certain drugs/compounds cause alterations in mitochondrial activity in the MTT assay. In the present study, the color interference from sample and media in the MTT assay was minimized by subtracting the sample with blank sample (extract plus media without cells). In addition, the MTT was prepared in colorless PBS and the colored extract and media was removed before MTT addition.

In results clearly showed that WKM reduced IgE-mediated β-hexosaminidase release in a dose-dependent manner (15.62, 33.70 and 60.25% inhibition at 125, 250 and 500 µg/ml, respectively). A significant inhibition on release of β-hexosaminidase was observed with pretreatment of WKM (125-500 µg/ml) for the length of 12 h. This indicates that WKM is capable of inhibiting the degranulation of RBL-2H3 cells. However, further research in animal model should be concluded to find out the *in vivo* anti-allergic activity of the extract.

Nitric oxide (NO) is a short-lived free radical and intercellular messenger that plays an important role in a variety of physiological processes and biological

functions such as smooth muscle relaxation, penile erection, platelet inhibition, neurotransmission, immune responses, inflammation, vascular homeostasis and macrophage-mediated cytotoxicity (Alessandro *et al*., 1994; Kang *et al*., 2002). Production of NO by macrophages is enhanced upon activation by bacterial endotoxins and cytokines mainly via an increase of the intracellular content of the inducible isoform of nitric oxide synthase (iNOS) (Alessandro *et al*., 1994). The over production of NO induced by lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ) in mouse macrophage cell line RAW264.7 can be simply measured by Griess reagent (Komutarin *et al*., 2004; Park *et al*., 2000; Nathan and Xie, 1994). Particularly, OPCs extract from French maritime pine bark or Pygnogenol® have been reported for their antioxidant activities, inhibition of NO production and many pharmacological properties such as antibacterial, anti-inflammatory, anti-viral, anti-carcinogenic, anti-allergic, immune stimulating, anti-platelet aggregation, and decreased capillary permeability and fragility (Fine, 2000; Ray *et al*., 1999; Dhanalakshmi *et al*., 2003; Shafiee *et al*., 2003).

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.

The present study revealed that WKM extract suppressed NO production by LPS and IFN-γ activated RAW 264.7 cells via down modulation of iNOS expression in a dose dependent manner. In addition, WKM extract also induced dose related decrease of COX-2 expression. The effect on COX-2 was less pronounced than iNOS. The suppression of WKM at 250 μg/ml, as analyzed in the same blotted membrane, was about 47.58% and 90.26% for COX-2 and iNOS, respectively. The *in vitro* anti-

inflammatory activity of WKM suggested by suppression of NO and COX-2 expression might be related to its antioxidant property. Several polyphenolic compounds from various agricultural food sources (e.g. pecans, millets, green tea and grapeseed) with antioxidation, also possess anti-inflammatory, anti-allergic and anti-cancer activities (Chandrasekara and Shahidi, 2011; de la Rosa *et al*., 2011; Forester and Waterhouse, 2009). For example, flavonoids, gallic acid, curcumin and quercetin (Yun, Kang, Park, and Nam, 2010; Sakata *et al*., 2003). Therefore, quercetin was used as the positive control in both anti-allergy and anti-inflammatory activities test.

The mechanism of inhibition of iNOS and COX-2 expression are still unclear. 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 WKM mediated suppression of iNOS and COX-2 expressions are related to NF-κB inhibition need to be further investigated.

CHAPTER VI

CONCLUSIONS

Proximate analysis of the dried ground fruits of Wan Khan Mak (WKM) demonstrated that crude protein (CP) was the main compound of the plant next were moisture, available carbohydrate (NFE; Nitrogen Free Extract), crude ash, crude fiber and finally, crude fat (EE). The yield of WKM crude extract was 12.65% of the dry weight. The total phenolic compound, total flavonoid and proanthocyanidins contents were determined by Folin-Ciocalteu, aluminium tricholride colorimetric and vanillin assays, respectively. The present study indicated that WKM fruit extract contained phenolic, flavonoid and proanthocyanidin compounds and displayed antioxidant properties by both chemical antioxidant (DPPH and FRAP) and intracellular-base assay using DCFH-DA probe. Acute oral and systemic toxicity studies revealed that WKM did not alter body weight gain or induced any signs or symptoms of toxicity and all of the animals lived up to 14 days. A gross necropsy at the end of the experiment (14 days) of the systemic toxicity revealed no apparent changes either in the corporal weight or the weights of the principle organs. The chromosomal aberration study showed that WKM extract at 5000 mg/kg body weight has no cytotoxic effects or caused no chromosome damages to the rat bone marrow cells. Inhibitory effects of the WKM extract on Ag-stimulated degranulation from RBL-2H3 cells was investigated by stimulation of IgE-sensitized RBL-2H3 cells. WKM at the highest concentration 0.5 mg/ml significantly decreased the degranulation up to

60.25%. Anti-inflammatory properties of the extract was evaluated by measuring the effects on the pro-inflammatory mediator nitric oxide (NO) in LPS plus IFN-γ activated murine macrophages cell line (RAW264.7). The results clearly demonstrated that WKM extract induced a dose-dependent suppression of NO production by LPS plus IFN-γ activated RAW264.7 cells at the concentration range that had no effect on cell viability. The anti-inflammatory activity of WKM extract was further confirmed by a concomitantly down regulation of iNOS and COX-2, in a dose dependent manner.

This study provides information of toxicological and biological effects of the plant that are direct benefits to the consumers. Overall, the present results reveal that WKM extract is relatively safe for comsumers and has a potential anti-allergic and anti-inflammatory activities, and it is worthwhile to be developed as natural chemopreventive products or nutraceuticals in the future. However, further investigation in long term toxicity study and the mechanisms involving anti-allergic and anti-inflammatory activities of the WKM extract are still required.

REFERENCES

REFERENCES

Aberoumand, A., and Deokule, S. S. (2008). Comparison of compounds of some edible plants of Iran and India, Pakistan. **Journal Nutrition**. 7(4): 582-585.

Abramson, J., and Pecht, I. (2007). Regulation of the mast cell response to the type I Fce receptor. **Immunological Reviews**. 217: 231-254.

Abramson, J., Licht, A., and Pecht, I. (2006). Selective inhibition of the FceRI-induced de novo synthesis of mediators by an inhibitory receptor. **EMBO Journal**. 25(2): 323-334.

Absolute astronomy (2009). **Aglaonema.** [Online]. Available: http://www.absoluteastronomy.com/topics/Aglaonema.

Abuye, C., Urga, K., Knapp, H., Selmar, D., Omwega, A., Imungi, J., and Winterhalter, P. (2003). A survey of wild, green, leafy vegetables and their potential in combating micronutrient deficiencies in rural populations. **East African Medical Journal**. 80: 247-252.

Aiyegoro, O. A., and Okoh, A. I. (2009). Phytochemical screening and polyphenolic antioxidant activity of aqueous crude leaf extract of *Helichrysum pedunculatum*. **International Journal of Molecular Sciences**. 10: 4990-5001.

Alessandro, W., Shinobu, O., Cicatiello, L., and Esumi, H. (1994). Dual mechanism for the control of inducible-type NO synthase gene expression in macrophages during activation by interferon-γ and bacterial lipopolysaccharide. **Journal of Biological Chemistry.** 269(11): 8324-8333.

Aletor, V., and Adeogun, O. (1995). Chemical analysis of the fruit of Vitex doniana (Verbenaceae). **Food Chemistry**. 53: 375-379.

Ames, B. N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. **Mutation Research.** 31: 347-364.

AOAC. (1990). **Official methods of analysis, 14th edition, Association of Official Analytical Chemists, Washington DC**. Arlington, Virginia, USA.

AOAC. (1995). **Official Methods of Analysis of AOAC International.** 16th edition. AOAC International Suite 500, Maryland USA.

Apinya Sanyasee (2005). **Wan Khan Mak the Miracle Plant.** [Online]. Available: http://www.mahamdo.com/job/apinya/index.asp.

Aruoma, O. I. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. **Journal of the American Oil Chemists' Society.** 75: 199-212.

Aruoma, O. I. (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. **Mutatiom Research**. 523-524: 9-20.

Asano, N., Nash, R. J., Molyneux, R. J., and Fleet, G. W. J. (2000). Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. **Tetrahedron:Asymmetry**. 11(8): 1645-1680.

Azevedo, L., Gomes, J. C., Stringheta, P. C., Gontijo, A. M., Padovani, C. R., Ribeiro, L. R., and Salvadori, D. M. (2003). Black bean (*Phaseolus vulgaris* L.) as a protective agent against DNA damage in mice. **Food and Chemical Toxicology**. 41: 1671-1676.

Balasundram, N., Sundram, K., and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses. **Food Chemistry**. 99: 191-203.

Barros, L., Dueñas, M., Carvalho, A.M., Ferreira, I.C.F.R., and Santos-Buelga, C. (2012). Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. **Food and Chemical Toxicology.** 50: 1576-1582.

Baxter, H., Harborne, J. B., and Moss, G. P. (eds.). (1999). **Phytochemical dictionay: handbook of bioactive compounds from plants (2nd ed.)**. London: Taylor & Francis.

Beecher, G. R. (2003). Overview of dietary flavonoids: nomenclature occurrence and intake. **Journal of Nutrition**. 133: 3248S-3254S.

Benzie, I. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. **Analytical Biochemistry.** 239: 70-76.

Bohm, B. A. (1998). **Introduction to flavonoids**. Amsterdam: Harwood Academic Publishers.

Botanical garden of Udomsuksa School (2005). **Wan the Decoration Plant and Good Name.** [Online]. Available: http://www.udomsuksa.ac.th/Latphrao/botanical/botanic24.asp.

Bourke, E., and Moynagh, P.N. (1999). Antiinflammatory effects of glucocorticoids in brain cells, independent of NF-kappa B. **Journal of Immunology**. 163: 2113-2119.

Bradley, J. R. (2008). TNF-mediated inflammatory disease. **Journal of Pathology**. 214: 149-160.

Bralley, E. E., Hargrove, J. L., Greenspan, P., and Hartle, D. K. (2007). Topical anti-inflammatory activities of Vitis rotundifolia (muscadine grape) extracts in the tetradecanoylphorbol acetate model of ear inflammation. **Journal Medicinal Food**. 10(4): 636-642.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. **Nutrition Reviews**. 56(11): 317-333.

Broadhurst, R. B., and Jones, W. T. (1978). Analysis of condensed tannins using acidified vanillin. **Journal of the Science of Food and Agriculture**. 29: 788-794.

Buettner, G. R. (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. **Archives of Biochemistry and Biophysics**. 300(2): 535-543.

Bunea, A., Rugina, O. D., Pintea, A. M., Sconta, Z., Bunea, C. I., and Socaciu, C. (2011). Comparative polyphenolic content and antioxidant activities of some wild and cultivated blueberries from Romania. **Notulae Botanicae Horti Agrobotanici Cluj-Napoca**. 39(2): 70-76.

Burns, R. E. (1971). Method for estimation of tannin in sorghum grain. **Agronomy Journal** 63: 511-512

Butler L. G., Price M. L., and Brotherton J. E. (1982). Vanillin assay for proanthocyanidins (condensed tannins): modification of the solvent for estimation of the degree of polymerization. **Journal of Agricultural Food Chemistry**. 30(6): 1087-1089

Byun, J. W., Choo, S. H., Kim, H. H., kim, Y. J., Hwang, Y. J., and Kim, D. Y. (2008). Evaluation of board sperm viability by MTT reduction assay in Beltsville thawing solution extender. **Asian-Australasian Journal of Animal Sciences**. 21(4): 494-498.

Cadenas, E., and Davies, K. J. A. (2000). Mitochondrial free radical generation, oxidative stress, and aging. **Free Radical Biology and Medicine**. 29: 222-230.

Calixto, J. B., Otuki, M. F., and Santos, A. R. S. (2003). Anti-inflammatory compounds of plant origin. Part I. action on arachidonic acid pathway, nitric oxide and nuclear factor κB (NF-κB). **Planta Medica**. 69: 973-983.

Cardiovascular physiology concepts. (2008). **Nitric oxide** [Online]. Available: http://www.cvphysiology.com/Blood%20Flow/BF011%20%20nitric%20oxide.gif.

Casparcg. (2014). **Antioxidants** [Online]. Available: http://casparcg.org/wp-content/uploads/2015/07/free-radical.jpg.

Chan, M. M., Fong, D., Ho, C., and Huang, H. (1997). Inhibition of Inducible Nitric Oxide Synthase Gene Expression and Enzyme Activity by Epigallocatechin Gallate, a Natural Product from Green Tea. **Biochemical Pharmacology** 54: 1281-1286.

Chandrasekara, A., and Shahidi, F. (2011). Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. **Journal of Agricultural and Food Chemistry**. 59(1): 428-436.

Chang, M-J., Collins, J. L., Bailey, J. W., and Coffey, D. L. (1994). Cowpeas tannins

related to cultivar, maturity, dehulling and heating. **Journal of Food Science**. 59: 1034-1036.

Charles, G. D., Spencer, P. J., Schisler, M. R., Cifone, M., Budinsky, R. A., and Gollapudi, B. B. (2005). Mode of mutagenic action for the biocide bioban CS-1246 in mouse lymphoma cells and implications for its *in vivo* mutagenic potential. **Toxicological Sciences.** 84: 73-80.

Chen, J., Devanand, P. S., Norman, D. J., Henny, R. J., and Chao, C. C. (2004). Genetic Relationships of Aglaonema Species and Cultivars Inferred from AFLP Markers. **Annals of Botany (Lond).** 93(2): 157-166.

Cheng, R., Glynn, S., Flores-Santana, W., Switzer, C., Ridnour, L., and Wink, D. A. (2010). Nitric oxide and redox inflammation in cancer. **Advances in Molecular Toxicology**. 4: 157-182.

Cheong, H., Choi, E. J., Yoo, G. S., Kim, K. M., Ryu, S. Y., and Ho, C. (1998). Desacetylmatricarin, an anti-allergic component from *Taraxacum platycarpum*. **Planta Medica.** 64(6): 577-578.

Choo, M. K., Park, E. K., Han, M. J., and Kim, D. H. (2003) Antiallergic activity of ginseng and its ginsenoside. **Planta Medica**. 69: 518-522.

Croft, K. D. (1998). The chemistry and biological effects of flavonoids and phenolic acids. **Annals New York Academy of Sciences**. 854: 435-442.

Cuzzocrea, S., Riley, D. P., Caputi, A. P., and Salvemini, D. (2001). Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. **Pharmacological Reviews**. 53(1): 135-159.

Dannhardt, G., and Kiefer, W. (2001). Cyclooxygenase inhibitors-current status and future prospects. **European Journal of Medicinal Chemistry**. 36: 109-126.

de la Rosa, L. A., Alvarez-Parrilla, E., and Shahidi, F. (2011). Phenolic compounds and antioxidant activity of kernels and shells of Mexican pecan (Carya illinoinensis). **Journal of Agricultural and Food Chemistry**. 59(1): 152-162.

Devasagayam, T., and Kamat, J. (2002). Biological significance of singlet oxygen. Indian **Journal of Experimental Biology**. 40: 680-692.

Devasagayam, T., Boloor, K., and Ramasarma, T. (2003). Methods for estimating lipid peroxidation: an analysis of meritis and demeritis. **Indian Journal of Biochemistry and Biophysics**. 40: 300-308.

Devasagayam, T., Tilak, J., Boloor, K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. (2004). Free radicals and antioxidants in human health: current status and future prospects. **Journal of the Association of Physicians of India**. 52: 794-804.

DeWitt, D. L., and Smith, W. L. (1988). Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. **Proceedings of the National Academy of Sciences of the United States of America**. 85: 1412-1416.

Dhanalakshmi, S., Agarwal, R., and Agarwal, C. (2003). Inhibition of NF-κB pathway in grape seed extract-induced apoptotic death of human prostate carcinoma DU145 cells. **International Journal of Oncology.** 23(3): 721-727.

Dhuley, J. N., Raman, P. H., Mujumdar, A. M., and Naik, S. R. (1993). Inhibition of lipid peroxidation by piperine during experimental inflammation in rats. **Indian Journal of Experimental Biology**. 31: 443-445.

Duchler, M., and Stepnik, M. (2008). Cytotoxic effects of a combination of three natural compounds to leukemia cells *in vitro*. **Cancer Therapy.** 16: 733-740.

Dufour, D., Pichette, A., Mshvildadze, V., Bradette-Hebert, M. E., Lavoie S., Longtin, A., Laprise, C., and Legault, J. (2007). Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Ledum groenlandicum* Retzius. **Journal of Ethnopharmacology.** 111: 22-28.

Elliot, B. M., Combes, R. D., Elcombe, C. R., Gatehouse, D. G., Gibson, G. G., Mackay, J. M., and Wolf, R.C. (1992). Report of UK environmental mutagen society working party. Alternatives to Aroclor 1254-induced S9 in *in vitro* genotoxicity assays. **Mutagenesis**. 7: 175-177.

Evans, H. J. (1976). **Cytological methods for detecting chemical mutagens. In: chemical mutagens, principles and methods for their detection, Vol. 4**, Hollaender, A. (ed) Plenum Press, New York and London, pp. 1-29.

Fang, J., Seki, T., and Maeda, H. (2009). Therapeutic strategies by modulating oxygen stress in cancer and inflammation. **Advanced Drug Delivery Reviews.** 61: 290- 302.

Feghali, C. A., and Wright, T. M. (1997). Cytokines in acute and chronic inflammation. **Frontiers in Bioscience**. 2: d12- d26.

Feghali, C. A., Bost, K. L., Boulware, D. W., and Levy, L. S. (1992). Mechanisms of pathogenesis in scleroderma. I. Over production of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. **Journal of Rheumatology.** 19(8): 1207-1211.

Fine, A. M. (2000). Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. **Alternative Medicine Review.** 5(2): 144-151.

Fischer, M. J. E., Paulussen, J. J. C., Horbach, D. A., Roelofsen, E. P. W., Miltenburg, J. C., Mol, N. J., and Janssen, L. H. M. (1995). Inhibition of mediator release in RBL-2H3 cells by some H1-antagonist derived anti-allergic drugs: Relation to lipophilicity and membrane effects. **Inflammation Research.** 44: 92-97.

Flora, S. D. (1998). Mechanism of inhibitors of mutagenensis and carcinogenesis. **Mutation Research**. 402: 151-158.

Foster, G. D., Wyatt, H. R., Hill, J. O., McGuckin, B. G., Brill, C., Mohammed, B. S., Szapary, P. O., Rader, D. J., Edman, J. S., and Klein, S. (2003). A randomized trial of a low-carbohydrate diet for obesity. **The New England Journal of Medicine**. 348: 2082-2090.

Galloway, S. M., Aardema, M. J., Ishidate, M. Jr., Ivett, J. L., Kirkland, D. J., Morita, T. M., and Sofuni, T. P. (1994). Report from working group on *in vitro* tests for chromosomal aberrations. **Mutation Research.** 312: 241-261.

Galloway, S. M., Armstrong, M. J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A. D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, G. H., Resnick, M. A., Anderson, G., and Zeiger, E. (1987). Chromosome aberration and sister chromatid exchanges in Chinese hamster ovary cells: Evaluation of 108 chemicals. **Environmental and Molecular Mutagenesis.** 10 (suppl. 10), 1-35.

Ghiselli A., Serafi ni M., Maiani G., Azzini E., and Ferro-Luzzi A. (1995). A fluorescence-based method for measuring total plasma antioxidant capability. **Free Radical Biology and Medicine** 18: 29-36.

Giri, A., Khynriam, D., and Prasad, S. B. (1998). Vitamin C mediated protection on cisplatin induced mutagenicity in mice. **Mutation Research.** 421: 139-148.

Gladwin, M. T., Wang, X., Reiter, C. D., Yang, B. K., Vivas, E. X., Bonaventura, C., and Schechter, A. N. (2002). S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O2/NO-linked allosteric function. **The Journal of Biological Chemistry**. 277: 27818-27828.

Goda, Y., Hoshino, K., Akiyama, H., Ishikawa, T., Abe, Y., Nakamura, T., Otsuka, H., Takeda, Y., Tanimura, A., and Toyoda, M. (1999). Constituents in watercress: Inhibitors of histamine release from RBL-2H3 cells induced by antigen stimulation. **Biological and Pharmaceutical Bulletin**. 22(12): 1319-1326.

Goldsby, R. A., Kindt, T. J., Osborne, B. A., and Kuby, J. (2002). **Immunology, 5th ed**. W.H. Freeman and Company, New York, pp. 361-370.

Goldstein J.L., and Swain T. (1965) The inhibition of enzymes by tannins. **Phytochemistry** 4: 185-192

Gow, A. J., Luchsinger, B. P., Pawloski, J. R., Singel, D. J., and Stamler, J. S. (1999). The oxyhemoglobin reaction of nitric oxide. **Proceedings of the National Academy of Sciences**. 96: 9027-9032.

Granberg,M., Fowler, C. J., and Jacobsson, S. O. (2001). Effects of the cannabimimetic fatty acid derivatives 2-arachidonoylglycerol, anandamide, palmitoylethanolamide and methanandamide upon IgE-dependent antigen-induced b-hexosaminidase, serotonin and TNF alpha release from rat RBL-2H3 basophilic leukaemic cells. **Naunyn-Schmiedebergs Archives of Pharmacology**. 364(1): 66-73.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbauum, S. R. (1982). Analysis of nitrate, nitrite and (^{15}N) nitrate in biological fluids. **Analytical Biochemistry.** 126: 131-138.

Halliwell, B., and Gutteridge, J. M. C. (1999). **Free radicals in biology and medicine** (3rd ed.). Oxford: Clarendon Press.

Han, Y. S., Kim, M. S., and Hwang, J. K. (2012). Macelignan Inhibits Histamine Release and Inflammatory Mediator Production in Activated Rat Basophilic Leukemia Mast Cells. **Inflammation**. 35(5): 1723-1731.

Heddle, J. A., Moody, J. A., Thompson, L. U., Torous, D. K., and Trentin, G. (1999). New approaches to antimutagenesis. **Journal of Environmental Pathology, Toxicology and Oncology**. 18: 95-101.

Heim, K. E., Tagliaferro, A. R., and Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. **Journal of Nutritional Biochemistry**. 13: 572-584.

Hertog, M. G., Hollman, P. C., Katan, M. B., and Kromhout, D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. **Nutrition and Cancer**. 20: 21-29.

Higdona, J. V., and Freia, B. (2003). Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. **Critical Reviews in Food Science and Nutrition**. 43(1): 89-143.

Hsu, S., Bollag, W. B., Lewis, J., Huang, Q., and Singh, B. (2003). Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. **Journal of Pharmacology and Experimental Therapeutics**. 306: 29-34.

Huang D., Ou B., and Prior R.L. (2005). The chemistry behind antioxidant capacity assays-Reviews. **Journal of Agricultural and Food Chemistry**. 53: 1841-1856.

Huang, F., Yamaki, K., Tong, X., Fu, L., Zhang, R., and Cai, Y. (2008). Inhibition of the antigen-induced activation of RBL-2H3 cells by sinomenine. International. **Immunopharmacology.** 8(3): 502-507.

Inflammation relief guide. (2011). **Inflammation.** [Online]. Available: http://www.inflammationreliefguide.com/wp-content/uploads/2011/10/Inflammatory-Response2.jpg.

Ishidate, M. Jr., and Sofuni, T. (1985). **The *in vitro* chromosomal aberration test using chinese hamster lung (CHL) fibroblast cells in culture. In: Progress in Mutation Research**. Oxford: Elsevier Science Publishers.

Isong, E., and Idiong, U. (1997). Nutrient content of the edible leaves of seven wild plants from Nigerian. **Plant Foods for Human Nutrition**. 51: 79-84.

Ito, Y., and Ito, M. (2001). Suppressive effect of (-)-Epigallocatechin gallate on aflatoxin B1-induced chromosome aberrations in rat bone marrow cells. **Journal of Health Science**. 47: 248-57.

Itoh, T., Ninomiya, M., Yasuda, M., Koshikawa, K., Deyashiki, Y., Nozawa, Y., and Koketsu, M. (2009). Inhibitory effects of flavonoids isolated from *Fragaria ananassa* Duch on IgE-mediated degranulation on rat basophilic leukemia RBL-2H3. **Bioorganic and Medicinal Chemistry.** 17: 5374-5379.

Jacob, G. S. (1995). Glycosylation inhibitors in biology and medicine. **Current Opinion in Structural Biology.** 5(5): 605-611.

Jha, A. M., and Singh, A. C. (1995). Clastogenicity of lanthanides: induction of chromosomal aberration in bone marrow cells of mice *in vivo*. **Mutation Research.** 341: 193-197.

Kagan, V. E., Kisin, E. R., Kawai, K., Serinkan, B. F., Osipov, A. N., Serbinova, E. A., Wolinsky, I., and Shvedova, A. A. (2002). Toward mechanism-based antioxidant interventions. **Annals of the New York Academy of Sciences**. 959: 188-198.

Kang, J. S., Jeon, Y. J., Kim, H. M., Han, S. H., and Yang, K. (2002). Inhibition of inducible nitric-oxide synthase expression by silymarin in lipopolysaccharide-stimulated macrophages. **Journal of Pharmacology and Experimental Therapeutics**. 302: 138-144.

Katsube, T., Tabata, H., Ohta, Y., Yamasaki, Y., Anuurad, E., Shiwaku, K., and Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin–Ciocalteu assay. **Journal of Agricultural and Food Chemistry.** 52: 2391-2396.

Kelm, S., and Schauer, R. (1997). Sialic acids in molecular and cellular interactions. **International Review of Cytology**. 175: 137-240.

Kim, H. K., Cheon, B. S., Kim, Y. S., Kim, S. Y., and Kim, H. P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure–activity relationships. **Biochemical Pharmacology.** 58: 759-765.

King, A., and Young, G. (1999). Characteristics and occurrence of phenolic phytochemicals. **Journal of the American Dietetic Association**. 99: 213-218.

Kirkland, D., Whitwell, J., Deyo, J., and Serex, T. (2007). Failure of antimony trioxide to induce micronuclei or chromosomal aberrations in rat bone-marrow after sub-chronic oral dosing. **Mutation Research.** 627: 119-128.

Kite, G. C., SHARP, H. J., HILL, P. S., and BOYCE, P. C. (1997). Polyhydroxyalkaloids in the Aroid tribes Nephthytideae and Aglaonemateae: Phytochemical support for an intertribal relationship. **Biochemical Systematics and Ecology.** 25(8): 757-766.

Kobuchi, H., Kroy-Lefaxi, M. T., Christen, Y., and Packer, L. (1997). Ginkgo biloba extract (egb 761): inhibitory effect on nitric oxide production in the macrophage cell line raw 264.7. **Biochemical Pharmacology.** 53: 897-903.

Koj, A. (1996). Initiation of acute phase response and synthesis of cytokines. **Biochimica et Biophysica Acta.** 1317: 84-94.

Kolb, H., and Kolb-bachofen, V. (1992). Nitric oxide: A pathogenic factor in autoimmunity. **Immunology Today**. 13: 157-160.

Komutarin, S., Butterworth, A. L., Keil, D., Chitsomboon, B., Suttajit, M., and Meade, B J. (2004). Extraxt of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages *in vitro* and *in vivo*. **Food and Chemical Toxicology.** 42(4): 649-658.

Kumar, S., and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. **Scientific World Journal**. 2013: Article ID 162750.

Kuo, P. L., Hsu, Y. L., Lin, T. C., Lin, L. T., and Lin, C. C. (2004). Induction of apoptosis in human breast adenocarcinoma MCF-7 cells by prodelphinidin B-2, 3, 30-di-Ogallate from Myrica rubra via Fas-mediated pathway. **Journal of Pharmacy and Pharmacology**. 56(11): 1399-1406.

Kuppusamy, P., Chzhan, M., Wang, P., and Zweier, J. L. (1996). Three-dimensional gated EPR imaging of the beating heart: Time-resolved measurements of free radical distribution during the cardiac contractile cycle. **Magnetic Resonance in Medicine**. 35: 323-328.

Lang, T., Klein, K., and Fischer, J., Nüssler, A. K., Neuhaus, P., Hofmann, U., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2001). Extensive genetic polymorphism in the human liver. **Pharmacogenetics**. 11: 399-415.

Lewrockwell. (2010). **Antioxidants.** [Online]. Available: http://www.lewrockwell.com/sardi/antioxidant-defense-big.gif.

Li, W. G., Zhang, X. Y., Wu, Y. J., and Tian, X. (2001). Anti-inflammatory effect and mechanism of proanthocyanidins from grapeseeds. **Acta Pharmacologica Sinica** 22(12): 1117-1120.

Liu, R. H., and Hotchkiss, J. H. (1995). Potential genotoxicity of chronically elevated nitric oxide: a review. **Mutation Research**. 339: 73-89.

Maas, J. L., and Galletta, G. J. (1991). Ellagic acid, an anticarcinogen in fruits, especially in strawberries: a review. **HortScience.** 26(1): 10-14.

Machlin, L. J., and Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. **Journal of the Federation of American Societies for Experimental Biology**. 1: 441-445.

Mahadik, S. P., and Mukherjee, S. (1996). Free radical pathology and antioxidant defense in schizophrenia: a review. **Schizophrenia Research**. 19: 1-17.

Maioli, E., Torricelli, C., and Pacini, A. (2009). Critical Appraisal of the MTT Assay in the Presence of Rottlerin and Uncouplers. **Biological Procedures**. 11: 227-240.

Manthey, J. A., McCoy, D. L., and Crowley, D. E. (1994). Stimulation of rhizosphere iron reduction and uptake in response to iron deficiency in citrus rootstocks. **Plant Physiology and Biochemistry**. 32: 211-215.

Maron, D. M., and Ames, B. N. (1983). Revised methods for the *Salmonella* mutagenicity test. **Mutation Research.** 113: 173-215.

Marone, G., Galli, S.J., and Kitamura, Y. (2002). Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. **Trends in Immunology** 23: 425-427.

Martin, A., and Claynes, M. (1993). Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. **Cytotechnology.** 11: 49-58.

Martin, O. R., Compain, P., Kizu, H., and Asano, N. (1999). Revised structure of a homonojirimycin isomer from Aglaonema treubii: First example of a naturally occurring α-homoallonojirimycin. **Bioorganic & Medicinal Chemistry Letters**. 9(21): 3171-3174.

Mastuda, H., Morikawa, T., Kazuho U, Hiromi, M., and Masayuki, Y. (2002). Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF-α and IL-4 production from RBL-2H3 cells. **Bioorganic and Medicinal Chemistry.** 10: 3123-3128.

Matsuoka, A., Hayashi, M., and Ishidate, M. Jr. (1979). Chromosomal aberration tests on 29 chemicals combined with S9 mix *in vitro*. **Mutation Research.** 66: 277-290.

Matsushima, T., Sawamura, M., Hara, K., and Sugimura, T. (1976). A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation systems.

In: de Serres, F. J., Fouts, J. R., Bend, J. R. and Philpot, R. M. (eds) ***In Vitro* Metabolic Activation in Mutagenesis Testing**. Elsevier, North-Holland, pp. 85-88.

Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988). Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). **Journal of Biological Chemistry**. 263: 3550-3553.

Mineo, M., Nakamura, N., Kitajima, K., Ueda, M., Tsutsmishita, Y., Futakis, S., and Takaishi, Y. (1997). Benzoquinones inhibit the expression of inducible nitric oxide synthase gene. **Biochemical Biophysical Research Communication**. 239: 367-371.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. **Pharmacological Reviews**. 43(2): 109-142.

Morgan, L. (2000). Nitric oxide: A challenge to chiropractic. **Journal of Canadian Chiropractic Association**. 44(1): 40-48.

Nakatani, K., Atsumi, M., Arakawa, T., Oosawa, K., Shimura, S., Nakahata, N., and Ohizumi, Y. (2002). Inhibitions of histamine release and prostaglandin E2 synthesis by mangosteen, a Thai medicinal plant. **Biological and Pharmaceutical Bulletin.** 25(9): 1137-1141.

Natarajan, A. T., Tates, A. D, van Buul, P. P. W., Meijers, M., and de Vogel, N. (1976). Cytogenetic effects of mutagens/carcinogens after activation in a microsomal system *in vitro*, I. induction of chromosome aberrations and sister chromatid exchanges by diethylnitrosamine (DEN) and dimethylnitrosamine

(DMN) in CHO cells in the presence of rat-liver microsomes. **Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis**. 37: 83-90.

Nathan, C., and Xie, Q. (1994). Regulation of biosynthesis of nitric oxide. **Journal of Biological Chemistry.** 269(19): 13725-13728.

Nesslany, F., Brugier, S., Mouriès, M. A., Curieux, F., and Marzin, D. (2004). *In vitro* and *in vivo* chromosomal aberrations induced by megazol. **Mutation Research.** 560: 147-158.

Nicholis, D. G., and Budd, S. L. (2000). Mitochondria and neuronal survival. **Physiological Reviews.** 80(1): 315-360.

Nojima, H., Kimura, I., Chen, F. J., Sugihara, Y., Haruno, M., Kato, A., and Asano, N. (1998). Antihyperglycemic effects of N-containing sugars from *Xanthocercis zambesiaca*, *Morus bombycis*, *Aglaonema treubii*, and *Castanospermum australe* in streptozotocin-diabetic mice. **Journal of Natural Products.** 61(3):397-400.

OECD—**Guideline for the Testing of Chemicals, No. 423**: Acute Oral toxicity-acute toxic class method, 17th December 2001.

OECD—**Guideline for the Testing of Chemicals, No. 475**: Mammalian bone marrow chromosome aberration Test, 21st July 1997.

Padgett, E. L., and Pruett, S. B. (1992). Evaluation of nitrite production by human monocyte-derived macrophages. **Biochem Biophys. Res. Commun.** 186: 775-779.

Page, C., Sanceau, J., Drapier, J. C., and Wietzerbin, J. (1996). Differential expression

of inducible NO synthase in two murine macrophage cell lines. **Immunology**. 89:274-280.

Park, Y. C., Rimbach, G., Saliou, C., Valacchi, G., and Packer, L. (2000). Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-K secretion, and NF-UB-dependent gene expression in RAW 264.7 macrophages. **FEBS Letters**. 465: 93-97.

Parr, A. J., and Bolwell, G. P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. **Journal of the Science of Food and Agriculture**. 80: 985-1012.

Pecht, I., and Corcia, A. (1987). Stimulus-secretion coupling mechanisms in mast cells. **Biophysical Chemistry**. 26(2-3): 291-301.

Pérez-Jiménez, J., Arranz, S., Tabernero, M., Díaz-Rubio, M. E., Serrano, J., Goñi, I., and Saura-Calixto, F. (2008). Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: extraction, measurement and expression of results. **Food Research International**. 41: 274-285.

Plumb, J. A., Milroy, R., and Kaye, S. B. (1989). Effects of the pH dependence of 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide-formazan absorption on chemo-sensitivity determined by a novel tetrazolium-based assay. **Cancer Research**. 49: 4435-4440.

Porter, L., Hrhtich, T., and Chan, C. R. (1986). Acid butanol proanthocyanidin assay. **Phytochemistry.** 25: 223-230.

Povirk, L. F., and Shuker, D. E. (1994). DNA damage and mutagenesis induced by nitrogen mustards. **Mutation Research**. 318: 205-226.

Price, M. L., van Scoyoc, S., and Butler, L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. **Journal of Agricultural and Food Chemistry**. 26: 1214-1218.

Raso, G. M., Meli, R., Carlo, G. D., Pacilio, M., and Carlo, R. D. (2001). Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. **Life Science**. 68: 921-931.

Ray, S. D., Kumar, M. A., and Bagchi, D. (1999). A Novel Proanthocyanidin IH636 Grape Seed Extract Increases *in vivo* Bcl-X**L** Expression and Prevents Acetaminophen-Induced Programmed and Unprogrammed Cell Death in Mouse Liver. **Archives of Biochemistry and Biophysics.** 369(1): 42-58.

Ross, J. A., and Kasum, C. M. (2002). Dietary flavonoids: bioavailability, metabolic effects, and safety. **Annual Reviews of Nutrition**. 22: 19-34.

Ryu, J., Aus, H., Lee, H., Feng, L., Qun, W., Han, Y., and Han, B. (2001). Inhibitory activity of Chinese medicinal plants on nitric oxide synthesis in lipopolysaccharide-activated macrophages. **The Journal of Applied Pharmacology.** 9: 183-187.

Saito, S., Maruyama, Y., Kamiyama, S., Nakahata, N., and Ohizumi, Y. (2004). *Ephedrae herba* in *Mao-Bushi-Saishin-To* Inhibits IgE-Mediated Histamine Release and Increases cAMP Content in RBL-2H3 Cells. **Journal of Pharmacological Sciences.** 95: 41-46.

Sakata, K., Hirose, Y., Qiao, Z., Tanaka, T., and Mori, H. (2003). Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. **Cancer Letters.** 199: 139-145.

Sandoval, M., Zhang, X., Liu, X., Mannick, E. E., Clark, D. A., and Miller, M. J. S. (1997). Peroxynitrite-induced apoptosis in T84 and RAW264.7 cells: Attenuation by L-ascorbic acid. **Free Radical Biology and Medicine.** 22(3): 489-495.

Saura-Calixto, F. (2008). Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: extraction, measurement and expression of results. **Food Research International**. 41: 274-285.

Schwartz, L. B., Lewis, R. A., Seldin, D., and Austen, K. F. (1981). Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. **The Jounal of Immunology**. 126(4): 1290-1294.

Scuro, L. S., Simioni, P. U., Gabriel, D. L., Saviani, E. E., Modolo, L.V., Tamashiro, W. M. S. C., and Salgado, I. (2004). Suppression of nitric oxide production in mouse macrophages by soybean flavonoids accumulated in response to nitroprusside and fungal elicitation. **BMC Biochemistry**. 5(5): 1-8.

Seldin, D. C., Adelman, S., Austen, K. F., Stevens, R. L., Hein, A., and Caulfield, J. P. (1985). Homology of the rat basophilic leukaemia cell and the rat mucosal mast cell. **Proceedings of the National Academy of Sciences of the United States of America.** 82(11): 3871-3875.

Sena, L., VanderJagt, D., Rivera, C., Tsin, A., Muhammadu, I., Mahamadou, O. ,Milson, M., Pastosyn, A., and Glew, R. (1998). Nutritional profile of some edible plants from Mexico, **Plant Foods for Human Nutrition** 52: 17-30.

Serra, A. T., Duarte, R. O., Bronze, M. R., and Duarte, C. M. M. (2011). Identification of bioactive response in traditional cherries from Portugal. **Food Chemistry.** 125: 318-325

Shafiee, M., Carbonneau, M. A., Urban, N., Descomps, B., and Lagar, C. L. (2003). Grape and grape seed extract capacities at protecting LDL against oxidation generated by Cu^{2+}, AAPH or SIN-1 and at decreasing superoxide THP-1 cell production. A comparison to other extracts or compounds. **Free Radical Research.** 37(5): 573-584.

Sharma, N., Trikha, P., and Athar, M., and Raisuddin, S. (2001). Inhibition of benzo(a)pyrene-and cyclophosphamide-induced mutagenicity by *Cinnamomum cassia*. **Mutation Research**. 480-481: 179-188.

Shi, J., Yu, J., Pohorly, J. E., and Kakuda, Y. (2003). Polyphenolics in grapeseedsbiochemistry and functionality. **Journal of Medicinal Food**. 6(4): 291-299.

Shi, Y-X., Xu, Y-K., Hu, H-B., Na, Z., and Wang, W-H. (2011). Preliminary assessment of antioxidant activity of young edible leaves of seven *Ficus* species in the ethnic diet in Xishuangbanna, Southwest China. **Food Chemistry**. 128: 889-894.

Shukla, S., Mehta, A., Mehta, P., and Bajpai, V. K. (2012). Antioxidant ability and total phenolic content of aqueous leaf extract of *Stevia rebaudiana* Bert. **Experimental and Toxicologic Pathology**. 64: 807-811.

Shukla, Y., and Taneja, P. (2002). Antimutagenic effects of garlic extract on chromosomal aberrations. **Cancer Letters**. 176: 31-36.

Shukla, Y., Arora, A., and Taneja, P. (2002). Antimutagenic potential of curcumin on chromosomal aberrations in Wistar rats. **Mutation Research**. 515: 197-202.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. **Experimental Physiology**. 82: 291-295.

Singleton, V. L. and Rossi, J. A. Jr. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. **American Journal of Enology and Viticulture**. 16: 144-158.

Sommer, C. (2005). Inflammation and healing. In C. Porth (ed.). **Pathophysiology** (7th ed., pp 387-398). Philadelphia, PA: Lippincott Williams & Wikins.

Stadtman, E. R. (1992). Protein oxidation and aging. Science. 28(257): 1220-1224.

Stadtman, E. R. (2001). Protein oxidation in aging and age-related diseases. **Annals of the New York Academy of Sciences.** 928: 22-38.

Stahl, W., and Sies, H. (2003). Antioxidant activity of carotenoids. **Molecular Aspects of Medicine.** 24: 345-351.

Surh, Y. (2003). Cancer chemoprevention with dietary phytochemicals. **Nature Review.** 3(10): 768-780.

Suzuki, M., Nakamura, T., Iyoki, S., Fujiwara, A., Watanabe, Y., Mohri, K., Isobe, K., Ono, K., and Yano, S. (2005). Elucidation of anti-allergic activities of curcumin-related compounds with a special reference to their anti-oxidative activities. **Biological and Pharmaceutical Bulletin.** 28(8): 1438-1443.

Tem Samittanan (2001). **Thai Plant Names.** Royal Foreat Department, Bankok, pp. 20.

Teresa Serra, A., Duarte, R. O., Bronze, M. R., and Duarte, C. M. M. (2011). Identification of bioactive response in traditional cherries from Portugal. **Food Chemistry**. 125: 318-325.

Tewtrakul, S., and Itharat, A. (2006). Anti-allergic substances from the rhizomes of *Dioscorea membranacea*. **Bioorganic and Medicinal Chemistry.** 14: 8707-8711.

Tewtrakul, S., and Subhadhirasakul, S. (2007). Anti-allergic activity of some selected plants in the Zingiberaceae family. **Journal of Ethnopharmacology.** 109: 535-538.

Theoharides, T. C., Alysandratos, K. D., Angelidou, A., Delivanis, D. A., Sismanopoulos, N., Zhang, B., Asadi, S., Vasiadi, M., Weng, Z., Miniati, A., and Kalogeromitros, D. (2012). Mast cells and inflammation. **Biochimica et Biophysica Acta**. 1822(1): 21-33.

Thomas, D. D., Ridnour, L. A., Isenberg, J. S., Flores-Santana, W., Switzer, C. H., Donzelli, S., Hussain, P., Vecoli, C., Paolocci, N., Ambs, S., Colton, C. A., Harris, C. C., Roberts, D. D., and Wink, D. A. (2008). The chemical biology of nitric oxide: implications in cellular signaling. **Free Radical Biology and Medicine**. 45: 18-31.

USP<88> (2005). **United States Pharmacopeia.** *In vivo* biological reactivity tests. USP 28:2269-2273.

Van Snick, J. (1990). Interleukin-6: an overview. **Annual Review of Immunology**. 8: 253-278.

Vane, J. R., Bakhle, Y. S., and Botting, R. M. (1998). Cyclooxygenase 1 and 2. **Annual Review of Pharmacology and Toxicology**. 38: 97-120.

Wang, D., and DuBois, R. N. (2010). The role of COX-2 in intestinal inflammation and colorectal cancer. **Oncogene.** 29: 781-788.

Wang, P., Henning, S. M., and Heber, D. (2010). Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. **PLoS One**. 5: e10202.

Winchester, B., and Fleet, G. W. J. (1992). Amino-sugar glycosidase inhibitors: versatile tools for glycobiologists. **Glycobiology.** 2: 199-210.

Wink, D. A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M. W., and Mitchell, J. B. (1998). The multifaceted roles of nitric oxide in cancer. **Carcinogenesis**. 19(5): 711-721.

World Health Organization (1981). Some antineoplastic and immunosuppressive agents. **International Agency for Research on Cancer**. France: Lyon.

Yamamoto, T., Yoshimura, M., Yamaguchi, F., Kouchi, T., Tsuji, R., Saito, M., Obata, A., and Kikuchi, M. (2004). Anti-allergic activity of naringenin chalcone from a tomato skin extract. **Bioscience Biotechnology and Biochemistry.** 68(8): 1706-1711.

Yen, G. C., Chiang, H. C., Wu, C. H., and Yeh, C. T. (2003). The protective effects of Aspergillus candida metabolites against hydrogen peroxide-induced oxidative damage to Int 407 cells. **Food Chemical Toxicology**. 41(11): 1561-1567.

Yokoyama, C., Takai, T., and Tanabe, T. (1988). Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. **FEBS Letters**. 231: 347-351.

Yun, S., Kang, M., Park, J., and Nam, S. (2010). Comparison of anti-allergenic activities of various polyphenols in cell assays. **Applied Biological Chemistry**. 53: 139-146.

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°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. Aluminum Chloride Colorimetric Method

• 5% sodium nitrite

- Sodium nitrite 5 gm
- Distilled water 100 ml

• 10% aluminum chloride

- aluminum chloride 10 gm
- Distilled water 100 ml

3. Vanillin assay

• 4% vanillin-methanol

- Vanillic acid 4 gm
- Methanol 100 ml

• 4% HCl-methanol

- HCl 4 ml
- Methanol 96 ml

4. 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.3H2O 3.1 gm
- Glacial acetic acid 16 ml
- Distilled water to 1 L

(Store at 4oC)

• Dilute HCl: 40 mM

- Conc. HCl (1 M) 1.46 ml
- Distilled water to 1 L

(Store at Room temperature)

• TPTZ (10 mM)

- TPTZ 0.062 gm
- HCl (40 mM) 20 ml

(Freshly prepared)

• Ferric chloring (20 mM)

- FeCl3.6H2O 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. Chromosome aberration assay

• KCl (75mM) 1 L

- KCl 5.62 gm
- Distilled water 1000 ml

• Fixative solution 800 ml

- Methanol 600 ml
- Glacial acetic acid 200 ml

• Gurr buffer 1 L

- Gurr tablet 1 tablet
- Distilled water 1 L

• Giemsa solution 1L

- KARYOMAX® Giemsa Stain 100 ml
- Gurr buffer 900 ml

• Cyclophosphamide 100 ml

- Cyclophosphamide 0.5 gm
- Distilled water 100 ml

(Freshly prepared)

• Colchicine 100 ml

- Colchicine 0.035 gm
- Distilled water 100 ml

5. β-hexosaminidase assay

• PIPES buffer 1X 100 ml

- NaCl (119 mM) 6.954 gm
- KCl (5 mM) 0.373 gm
- Glucose (5.6 mM) 1.008 gm
- $MgCl_2.6H_2O$ (0.4 mM) 0.081 gm
- PIPES (25 mM) 7.560 gm
- NaOH (40 mM) 0.160 gm
- $CaCl_2$ (anhydrous) (1 mM) 0.011 gm
- BSA (0.1% W/V) 0.100 gm

• anti-DNP IgE (50 ng/ml) 50 ml

- anti-DNP IgE 2.5 µl
- MEM complete media 50 ml

(Freshly prepared)

• DNP-BSA 100 ml

- DNP-BSA 0.010 gm
- PBS 100 ml

• 0.1 M citrate buffer pH 4.5, 50 ml

- citric acid (monohydrate) 1.05 gm
- sodium citrate (dihydrate) 1.47 gm

Adjusted pH to 4.5, 50 ml

• 1 mM p-NAG (substrate) 50 ml

- p-NAG 0.0173 gm
- citrate buffer 50 ml

• 0.1 M Na_2CO_3/$NaHCO_3$ (stop solution) pH 10.0, 100 ml

- Na_2CO_3 1.06 gm
- $NaHCO_3$ 0.84 gm

Adjusted pH to 10.0 , 100 ml

6. 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°C)

7. Western blot

• RIPA buffer

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

(Store at 4°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°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°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°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°C)

• Running buffer (10 X)

- Tris-base 30 gm
- Glycine 14.4 gm
- SDS 10 gm
- distilled water to 1 L

(Filtrate, store at 4°C)

• Running buffer (1X)

- Running buffer (10 X) 100 ml
- distilled water 900 ml

(Filtrate, store at 4°C)

• Blotting buffer (1X)

- Tris-base 3.03 gm
- Glycine 14.4 gm
- Methanol 200 ml

- distilled water to 1 L

(Filtrate, store at 4°C)

• TPBS 0.1% Tween 20

- PBS 1X 1 L
- Tween 20 1 ml

(Filtrate, store at 4°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°C)

• Reagent B (Lowry method)

- CuSO4.5H2O 0.05 gm
- Sodium citrate 0.1 gm
- distilled water 10 ml

(Store at 4°C)

• 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)

APPENDIX B

REAGENTS FOR CELL CULTURE

1. Reagents for cell culture

• FBS/FCS (inactivated)

- Slowly thaw the frozen FBS in a breaker filled with water.
- Put in water bath at 37°C till completely thaw.
- Heat inactivate (56°C, 20 min), gentle mix every 5-10 min
- Aliquot 50 ml each tube

(Store at -20°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 DI.water

Adjust pH to 0.2-0.3 below desired (7.4)

(Filter sterile, store at 4°C)

• RPMI 1640 1X (complete media)

- RPMI-1640 1X	175 mL
- FBS (inactivated)	20 mL
- HEPES buffer 1 M	3 mL
- Penicillin+Streptomycin	2.5 mL

(Store at 4°C)

• MEM 1X

MEM 1X with L-glutamine, with phenol red 1 pack

- NaHCO3	2.2 gm

Adjust volume to 1,000 ml with DI.water

Adjust pH to 0.2-0.3 below desired (7.4)

(Filter sterile, store at 4°C)

• MEM 1X (complete media)

- MEM 1X	165 ml
- FBS (inactivated)	30 ml
- HEPES buffer 1 M	3 ml
- Penicillin+Streptomycin	2 ml

(Store at 4°C)

• PBS 10X

- KH2PO4 0.288 gm
- NaCl 18 gm
- $Na2HPO_4.7H_2O$ 1.5 gm
- DI.water 200 ml

(Autoclave 121°C, 15 min, store at 4°C)

• **PBS 1X**

- PBS 10X 20 ml
- DI.water 180 ml

(Store at 4°C)

• **Hepes buffer (1M)**

- Hepes 23.83 gm
- DI.water 100 ml

(Store at 4°C)

• **Penicillin/Streptomycin (100X)**

- Penicillin 0.6 gm
- Streptomycin 1.34 gm
- PBS 1X 100 ml

(Aliquot, store at -20°C)

• **Trysin/EDTA**

- Trysin 0.25 gm
- EDTA 0.04 gm
- PBS 1X 100 ml

(Aliquot, store at -20°C)

APPENDIX C

MATERIALS

Chemicals and reagents

The chemicals employed in the present studies were summarized in Table 1.

Table 1 List of chemicals used in the studies.

Name	**Source**
Acetic acid	Merck
Acrylamide	Sigma
Albumin, dinitrophenyl (DNP-albumin)	Sigma
Aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$)	Sigma
Ammonium persulphate	Bio-rad
Ascorbic acid	Fluka
(+)-Catechin hydrate	Fluka
Bromphenol blue	Sigma
Bovine serum albumin (BSA)	BDH
Colchicine ($C_{22}H_{25}NO_6$)	Sigma
Coomassie blue G250	Sigma
Cyclophosphamide monohydrate	Sigma
2', 7'-Dichlorofluorescin-diacetate (DCFH-DA)	Sigma
Dimethylsulfoxide (DMSO)	Gibco

Table 1 (Continued).

Name	Source
3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT)	Gibco
1,1-Diphenyl-2-picrylhydrazyl (DPPH)	Sigma
ECL plus western blot detection reagent	Thermo
Ethyl alcohol	BDH
Ethylenediaminetetraacetic acid (EDTA)	Amresco
Folin-Ciocalteu's phenol reagent	Sigma
Ferric-2, 4, 6-tripyridyl-s-triazine (Fe^{III}-TPTZ)	Acros
Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Sigma
Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)	Carlo Erba
Fetal bovine serum (FBS)	Gibco
Gallic acid	Sigma
Glycine	BDH
GURR buffer tablets	Gibco
Hank's balance salts solution (HBSS)	Sigma
Hydrochloric acid (HCl)	BDH
4-(2-Hydroxyethyl) piperazine-1-ethanesulonic acid (HEPES)	Fluka
6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox)	Aldrich
Interferon gamma (γ-IFN)	Pierce
KaryoMax®Giemsa stain	Gibco
Lipopolysaccharide (LPS)	Sigma
2-Mercaptoethanol	Sigma

Table 1 (Continued).

Name	**Source**
Methyl alcohol	BDH
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma
4-Nitrophenyl N-acetyl-β-D-glucosaminide (p-NAG)	Sigma
Nonidet P-40	Bio basic
Penicillin G	Sigma
Permount™	Fisher
Phosphoric acid	BDH
1, 4-Piperazinediethanesulfonic acid (PIPES)	Sigma
Quercetin dehydrate	Sigma
RPMI medium 1640	Gibco
Sodium bicarbonate ($NaHCO_3$)	Sigma
Sodium carbonate anhydrous (Na_2CO_3)	Carlo Erba
Sodium dodecyl sulfate	Fluka
Sodium hydroxide (NaOH)	Carlo Erba
Sodium nitrite ($NaNO_2$)	Sigma
Streptomycin sulphate	Sigma
Sulfanilamide	Sigma
TEMED	usb
Tert-butyl hydroperoxide (*t*-BuOOH)	Sigma
Trisma base	Sigma
Trypan blue	Gibco
Trypsin	Sigma

Table 1 (Continued).

Name	Source
TWEEN20	usb
TWEEN80	Sigma
Xylene	Merck

Antibodies

The antibodies employed in the present studies were summarized in Table 2.

Table 2 List of antibodies used in the studies.

Name	Source
Anti-COX-2 (murine polyclonal) (160106)	Cayman
Anti-DNP (mouse monoclonal IgE) (SPE-7)	Sigma
Anti-iNOS (mouse monoclonal IgG1) (C-11)	Santa cruz
Goat-anti-mouse-IgG-HRP conjugate (Sc-2005, lot L1304)	Santa cruz
Goat-anti-rabbit-IgG-HRP conjugate (10004301)	Cayman
α-Tubulin (mouse monoclonal IgG) (B-7, Sc5286, lot J2505)	Santa cruz

Instruments, plastic and glassware

The instruments, plastic and glassware employed in the present studies were summarized in Tables 3.

Table 3 List of instruments used in the studies.

Name	Source
6-well plate (flat bottom)	Corning

Table 3 (Continued).

Name	Source
96-well plate (flat bottom)	Corning
Blender	Phillips
Centrifuge machine	SORVALL
CO_2 incubator	Thermo
Conical tubes	Corning
Electrophoresis separately system (model SE-250)	Hoefer
Electrophoresis transfer system (model TE-22)	Hoefer
ELISA plate reader (model E6504)	BIORAD
Fluorescence microplate reader	Molecular Device
Pyrex Co.	Glasswares
Haemocytometer and cover slip	Fisher
Inverted microscope	Olympus
Laminar flow hood (model SG 600E)	Holten
Light microscope	Olympus
Lyophilizer	Labconco
Microcentrifuge	SORVALL
Microscope slides	Fisher
Multichannel pipette	Gibco
Pasteur pipettes	Fisher
Petri dish	Nunc

Table 3 (Continued).

Name	**Source**
Rotary evaporator with vacuum (model R205)	Buchi
Single frosted end slide	Fisher
Snap cap tubes	SARSTEDT
Soxhlet extraction apparatus	Buchi
Water bath	memmert

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