THE ROLE OF STANDARDIZED MANGIFERA INDICA L. LEAF EXTRACTS IN ANTIOXIDATION AND NEUROPROTECTION

By

Kanistha Kaewpoomhae

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Program of Pharmaceutical Sciences

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บทบาทของสารสกัดมาตรฐานใบมะม่วงในการต้านออกซิเดชันและการป้องกันการตายของเซลล์ประสาท

โดย

นางสาวขนิษฐา แก้วภูมิแห่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชา วิทยาการทางเภสัชศาสตร์
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
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The Graduate School, Silpakorn University has approved and accredited the thesis title of “The role of standardized *Mangifera Indica* L. leaf extracts in antioxidation and neuroprotection” submitted by Miss Kanistha Kaewpoomhae as a partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

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KANISTHA KAEWPOOMHAE: THE ROLE OF STANDARDIZED *MANGIFERA INDICA* L. LEAF EXTRACTS IN ANTIOXIDATION AND NEUROPROTECTION. THESIS ADVISORS: ASST. PROF. MONRUDEE SUKMA, Ph. D., AND ASSOC. PROF. THEERASAK ROJANARATA, Ph. D. 149 pp.

This study was performed in order to evaluate antioxidant and neuroprotective effects of standardized *Mangifera indica* L. (mango) leaf extracts. First, the effect of cultivar on free radical scavenging (FRS) effect by diphenyl-1-picryhydrazyl (DPPH) assay and the extracts was standardized by quantification of total phenolic and tannic acid contents as well as quantification of three main phenolic components, mangifern, methyl gallate and gallic acid. We found that cultivar had far less effect on free radical scavenging activities. The IC_{50} of Namdokmai, Aokrong, Muntawai and Fahlun were 4.94 ± 0.63, 5.09 ± 1.4, 5.59 ± 1.59 and 7.26 ± 1.05 µg/ml, respectively. Mangiferin was found as the main bioactive compound. Then the standardized methanol extract of *M*. *indica* cv. Namdokmai leaf was selected for further antioxidant and neuroprotective experiments. To study mechanisms of antioxidation, the extract were evaluated for FRS and metal ion chelating effects. We found that the IC_{50} value for the 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) assay was 1.33 ± 0.13 µg/ml. The EC_1 value for ferric reducing antioxidant power (FRAP) was 202.92 ± 3.39 µg/ml. The IC_{50} value for superoxide anion and hydrogen peroxide were 0.07 ± 0.0049 and 70.89 ± 6.56 µg/ml. In addition, The extract also chelated metal ion with IC_{50} = 0.89 ± 0.81 µg/ml. To study neuroprotective effect, the protective activities on oxidant- and neurotoxin-induced cell death were evaluated by measuring the protection of neuroblastoma (NG 108-15) cells from H_2O_2-, glutamate–and 6-hydroxyldopamine (6-OHDA)–induced neurotoxicity. The extracts at 30-50 µg/ml and 100 µg/ml effectively protected the cells from H_2O_2- and 6-OHDA-induced cell death, respectively. However, the extract could not protect them from glutamate-induced cell death. Mangiferin at 100 µM effectively protected the cells from H_2O_2-induced cell death. From our findings, it can be concluded that the standardized methanol extract of *M.indica* L. cv Namdokmai leaf acted as an antioxidant. The mechanisms of antioxidant result from 1) the FRS abilities which partly due to the sigle electron transfer (SET) capabilities and 2) metal ion chelating effect. In addition, the extract and mangiferin, the major bioactive content, could protect cells from oxidative damage. Thus, the extract as well as mangiferin may be valuable in the treatment of neurodegenerative diseases in which free radical generation is implicated.

Program of Pharmaceutical Sciences Graduate School, Silpakorn University Academic year 2009
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Thesis Advisors' signature 1 …… 2……

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ขนิษฐา แก้วภูมิแห่ : บทบาทของสารสกัดมาตรฐานใบมะม่วงในการต้านออกซิเดชันและการป้องกันการตายของเซลล์ประสาท. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภญ.ผศ. ดร. มนฤดี สุขมา และ ภก.รศ.ดร. ธีรศักดิ์ โรจนราธา. 149 หน้า.

การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านออกซิเดชันและการป้องกันการตายของเซลล์ประสาทของสารสกัดมาตรฐานใบมะม่วง เริ่มจากการศึกษาผลของสายพันธุ์ที่มีต่อฤทธิ์ต้านอนุมูลอิสระด้วยวิธี Diphenyl-1 picryhydrazyl (DPPH) และทำมาตรฐานสารสกัดโดยการหาปริมาณ total phenolic content และ tannic acid content หาปริมาณสารโพลีฟีนอลที่สำคัญได้แก่ mangifern methyl gallate และ gallic acid จากผลการทดลองไม่พบว่าสายพันธุ์มะม่วงมีผลต่อฤทธิ์ต้านอนุมูลอิสระ ค่า IC_{50}จากสารสกัดของสายพันธุ์ น้ำดอกไม้ อกร่อง มันทะวาย และฟ้าลั่น เท่ากับ 4.94 ± 0.63, 5.09 ± 1.4, 5.59 ± 1.59 และ 7.26 ± 1.05 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารโพลีฟีนอลที่พบมากที่สุดในสารสกัดคือแมนจิเฟอริน ต่อมาได้เลือกสารสกัดเมทานอลจากใบมะม่วงน้ำดอกไม้เพื่อทำการศึกษากลไกการต้านออกซิเดชันและฤทธิ์ปกป้องเซลล์ประสาท ทำการศึกษากลไกการต้านออกซิเดชันโดยการศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์คีเลตโลหะ ผลการทดลองพบว่า ค่า IC_{50} ของวิธี 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) เท่ากับ 1.33 ± 0.13 ค่า EC_1 ของวิธี ferric reducing antioxidant power (FRAP) คือ 202.92 ± 3.39 ไมโครกรัมต่อมิลลิลิตร ค่า IC_{50} ของการต้านอนุมูล superoxide anion และ hydrogen peroxide คือ 0.07 ± 0.0049 และ 70.89 ± 6.56 ไมโครกรัมต่อมิลลิลิตร และค่า IC_{50} จากการคีเลตโลหะเท่ากับ 0.89 ± 0.81 ไมโครกรัมต่อมิลลิลิตร จากนั้นทำการทดสอบฤทธิ์ป้องกันเซลล์ประสาทตายจากการเหนี่ยวนำด้วยสารออกซิแดนซ์และสารพิษ โดยทดสอบฤทธิ์การปกป้องเซลล์เพาะเลี้ยงเซลล์ประสาท (NG 108-15) จากการเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ กลูตาเมท และ 6-ไฮดรอกซิลโดปามีน (6-OHDA) พบว่าที่ความเข้มข้น 30-50 และ 100 ไมโครกรัมต่อมิลลิลิตร สารสกัดสามารถปกป้องเซลล์ประสาทจากการเหนี่ยวนำให้เกิดการตายจากไฮโดรเจนเปอร์ออกไซด์และ 6-OHDA ตามลำดับ แต่ไม่สามารถป้องกันการตายของเซลล์จากการเหนี่ยวนำด้วยกลูตาเมทได้ และพบว่าแมนจิเฟอรินที่ความเข้มข้น 100 ไมโครโมลาร์ สามารถปกป้องเซลล์ประสาทจากการเหนี่ยวนำให้เซลล์ตายด้วยไฮโดรเจนเปอร์ออกไซด์ได้ จากการทดลองพบว่าสารสกัดมาตรฐานใบมะม่วงมีฤทธิ์ต้านอออกซิเดชัน ซึ่งกลไกการต้านออกซิเดชันเป็นผลจาก 1) การต้านอนุมูลอิสระซึ่งส่วนหนึ่งอาจเกิดจากกลไกการถ่ายโอนอิเลคตรอนเดี่ยว และ 2) การคีเลตโลหะ สารสกัดรวมถึงแมนจิเฟอรินซึ่งเป็นองค์ประกอบหลักมีฤทธิ์ในการป้องกันการตายของเซลล์ประสาทจากการเกิดออกซิเดชัน ดังนั้นสารสกัดมาตรฐานใบมะม่วงรวมถึงแมนจิเฟอรินจึงอาจมีประโยชน์สำหรับการรักษาโรคของการเสื่อมของเซลล์ประสาทที่เกี่ยวข้องกับการเกิดอนุมูลอิสระได้

สาขาวิชาวิทยาการทางเภสัชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2552

ลายมือชื่อนักศึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1..... 2.....

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CHAPTER 1
INTRODUCTION

1. Statement and significance of the research problem

Mangos belong to the genus *Mangifera* of the family Anacardiaceae. Most of mangos belong to the species *Mangifera indica*. There are over 1000 mango cultivars throughout the world. The genus *Mangifera* originates in tropical Asia, with the greatest number of species was found in Borneo, Java, Sumatra, and the Malay Peninsula. The most-cultivated *Mangifera* species has its origins in India and Myanmar (Bally 2006 : 145).

Mangos are the economic fruit of Thailand. In addition, they showed many biological activities. A standardized aqueous extract from the stem bark of *M. indica* L. exhibited anti-inflammatory activity (Garrido et al. 2004 : 145). The extract composed of a variety of bioactive components such as phenolic acids, phenolic esters, flavanols and xanthone (Rodrguez et al. 2006 : 1335).

Mangiferin (C_2- β –D –glucopyranosyl-1, 3, 6, 7-tetrahydroxyxanthone) was suggested as the principle bioactive component of mango (*M. indica* L.) (Sanchez et al. 2000 : 567). Mangiferin and the extracts from mango leaves, bark, and flowers exhibited a wide range of pharmacological effects: antioxidant, anticancer, antimicrobiology, antiatherosclerosis, anti-allergic, anti-inflammatory, analgesic, and immunomodulatory. Mangiferin protected hepatocytes, lymphocytes, neutrophils, and macrophages from oxidative stress; reduce artherogenicity in streptozotocin-induced diabetic rats; reduced the streptozotocin-induced oxidative damage in cardiac and renal tissues (Muruganandan et al. 2002 : 95). M. Gottlieb et al. (Gottlieb et al. 2006 : 377) assayed the neuroprotective efficacy of mangiferin. It ameliorated damage caused by ischemia to peripheral organs. They observed that mangiferin reduced oxidative stress and neuronal death due to excitotoxicity in culture.

Free radicals cause depletion of the endogeneous antioxidants, change in gene expression, and induce abnormal proteins. These ultimately results in degenerative diseases and aging. Recently the therapeutic potential new antioxidants, especially those originate from nature are needed. In this regard, flavonoids and other polyphenols present as bioactive molecules in vegetables, fruit and red wine have been shown to be strong antioxidant. They potentially benefit to neurodegenerative diseases associated with oxidative stress (Mandel et al. 2004 : 1559). A variety of experimental and epidemiological data documented that selected natural polyphenols exerted protective action on a number of pathological conditions including cardiovascular diseases, cancer, infections, and neurodegenerative disorders (e.g. Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis, multiple sclerosis) (Middleton et al. 2000 : 675; Rotondo et al. 2000 : 98; Ramassamy 2006 : 55; Schaffer et al. 2006 : 90). Phenolic compounds have the capacities to quench lipid peroxidation, prevent DNA from oxidative damage, and scavenge free radicals (Cao 1999 : 381). Antioxidant nutrients inhibit the oxidation in living cells by protecting the lipids of the cell membranes through free radical scavenging, blocking the initiators of free radical attack, neutralizing or converting free radicals into less active, stable products thus breaking the chain reaction and assisting in salvaging oxidized antioxidants enabling them to continue to be of benefit (Halliwell et al. 1992 : 561). There are 2 main antioxidant defense mechanisms developed by living organisms: enzymatic and nonenzymatic components defense systems. An array of small molecules including polyphenols falls under the later system (Shahidi et al. 1992 : 73; Rice-Evans et al. 1997 : 155). Polyphenols have the ability to scavenge free radicals via hydrogen donation or electron donation (Shahidi et al. 1992 : 80). The antioxidant activity of polyphenols is governed by the number, reactivity, and location of their aromatic hydroxyl groups (Chen et al. 1996 : 159).

The mango plant has been the focus in research field for the new source of potent antioxidants. LT Ling showed antioxidative effects of *M. indica* L. leaf extracts. The standardized ethanol and aqueous extracts were reported to contain free radical scavenging activities and protective effect on DPPH and ABTS radical-induced human

keratinocyte and human fibroblast cells death (Ling et al. 2009 : 1156). In area of neuroscience, the methanol extract of *M. indica* L. could prevent glutamate-induced excitotoxicity in primary cultured neurons of the rat cerebral cortex (Lemus-Molina et al. 2009 : 57).

From the aforementioned data, *M. indica* Linn. leaf extracts may have antioxidative and cytoprotective properties. However, trough our knowledge, little is known for underlying mechanism of antioxidation as well as protective effect against oxidant- and neurotoxin-induced neuronal cell death. In addition, As cultivars had influences on taste of mango fruit and may had effect on quantity of some potential bioactive polyphenols, then first, we evaluated effect of cultivars on free radical scavenging effect and quantify content of main polyphenols i.e. mangiferin, gallic acid and methyl gallate. Then, the most powerful extract on free radical scavenging activity were selected for further experiments. Therefore, the underlying mechanisms of antioxidation were evaluated. Finally, the neuroprotective activities of standard *M.indica* Linn. leaf against oxidant- and neurotoxin-induced neuronal cell death were investigated. The results from this study may provide new insight of new antioxidants and neuroprotective agents.

2. Objective of this research

2.1 To study effect of cultivars on free radical scavenging effect (Diphenyl-1 picryhydrazyl (DPPH) assay)

2.2 Standardization of *M. indica* leaf extracts by quantify content of some bioactive components (mangiferin, gallic acid and methyl gallate) by High performance liquid chromatography (HPLC) method and quantify total phenolic content and tannic acid content of methanol extracts of *M. indica* leaf

2.3 To study underlying mechanisms of antioxidative effect of standardized *M. indica* leaf extracts

2.4 To study neuroprotective effects of standardized *M. indica* leaf extracts from oxidant- and neurotoxin-induced cell death

CHAPTER 2

LITERATURE REVIEW

1. Free radicals and active oxygen species

Free radicals are chemical species, which have unpaired electrons. Molecules are composed of atoms and electrons. Electrons are present generally in pairs. However, under contain conditions, molecules have unpaired electrons and as such they are called free radicals. Unpaired electrons usually seek other electrons to become paired. Thus, free radicals are in general reactive and attack other molecules, although some radicals are not reactive but stable enough to have long life. Examples of reactive free radicals are the hydroxyl ($OH^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals, while the nitric oxide ($^{\bullet}NO$), vitamin E (tocopheroxyl), and vitamin C (dehydroascorbate) radicals are examples of stable radicals.

Active oxygen species (also known as reactive oxygen species) denote oxygen-containing molecules, which are more active than the triplet oxygen molecule present in air. Superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen ($^1O_2$) are accepted as typical active oxygen species, but in broader sense, other species such as alkoxyl radical, proxy radical ($LO_2^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), lipid hydroperoxide (LOOH), protein hydroperoxide, and hypochlorite (HOCl) are also considered as active oxygen species. Some of them have unpaired electrons are free radicals, but others are not. Table 1 summarizes the active oxygen species, which are relevant to lipid peroxidation and oxidative stress in *vivo*. Nitric oxide and thiyl radical ($RS^{\bullet}$), which do not bear unpaired electrons on oxygen are also included.

Table 1 Active oxygen and related species

Radicals	Non-radicals
$O_2^{\bullet -}$ superoxide	H_2O_2 hydrogen peroxide
$HO^{\bullet}$ hydroxyl radical	$^1O_2$ singlet oxygen
$HO_2^{\bullet}$ hydroperoxyl radical	LOOH lipid hydroperoxide
$L^{\bullet}$ lipid radical	Fe=O iron-oxygen complexes
$LO_2^{\bullet}$ lipid peroxyl radical	HOCl hypochlorite
$LO^{\bullet}$ lipid alkoxyl radical	
$NO_2^{\bullet}$ nitrogen dioxide	
$NO^{\bullet}$ nitric oxide	
$RS^{\bullet}$ thiyl radical	
$P^{\bullet}$ protein radical	

2. Physiological functions and effects

Active oxygen and related species play an important physiological role and, at the same time, they may exert toxic effects as well. The active oxygen species are essential for production of energy, synthesis of biologically essential compounds, and phagocytosis, a critical process of our immune system. They also play a vital role in signal transduction, which is important for cell communication and function. On the other hand, there is now increasing evidence which shows that these active oxygen species may play a causative role in a variety of diseases including heart disease and cancer, and aging. Consequently, the role of antioxidants, which suppress such oxidative damage, has received increased attention. It is important to elucidate the mechanisms and dynamics of the oxidative damage in order to understand its biological significance and develop strategies to prevent it. Both active oxygen species and antioxidants are double-edged swords and the balance of their beneficial and toxic effects is determined by the relative importance of many competing biological reactions.

3. The Role of Active Species in Pathological Processes.

In addition to environmental causes such as oxygen, light, or ionizing radiation three physiological circumstances result in extraordinarily high local fluxes of radical species: (1) activation of the P-450-centered mixed function oxidase systems of endoplasmic reticulum, (2) activation of NADPH oxidase in phagocytes in response to antimicrobial defense and inflammation and (3) the presence of extraordinarily high levels of compounds which can reduce oxygen directly in autooxidation reactions. Under such circumstances, the rate of active species generation may exceed the local capacity of the antioxidant defense and may contribute to injury.

4. Oxidative damage to DNA, lipids and proteins

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko et al. 2006 : 1162). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge 1999 : 212). The most extensively studied DNA lesion is the formation of 8-OH-G. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing. It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems, Grune and Esterbauer 1995 : 786). Once formed, peroxyl radicals ($ROO^{\bullet}$) can be rearranged *via* a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy- 2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide

radicals are formed. The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS. Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation. A number of highly sensitive methods have been developed for the assay of protein carbonyl groups advanced glycation end products (AGEs) is a class of complex products. They are the results of a reaction between carbohydrates and free amino group of proteins. The intermediate products are known, variously, as Amadori, Schiff Base and Maillard products, named after the researchers who first described them. Most of the AGEs are very unstable, reactive compounds and the end products are difficult to be completely analysed. The brown colour of the AGEs is probably related to the name of melanoidins initially proposed by Maillard, and well known from food chemistry. The best chemically characterized AGEs compounds found in human are pentosidine and carboxyl methyl lysine (CML).

5. Formation of free radicals and active oxygen species

Free radicals and active oxygen species are formed by various extrinsic and intrinsic sources such as light, heat, and metals. They are formed in *vivo* by various ways at different times and sites as summarized in Table 2.

6. Reactions of free radicals and active oxygen species and their reactives

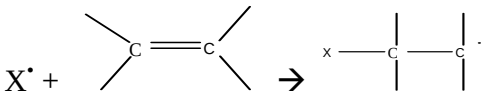
Free radicals and active oxygen species attack lipids, sugars, proteins, and DNA and induce their oxidation, which may result in oxidative damage such as deterioration of foods, membrane dysfunction, protein modification, enzyme inactivation, and break of DNA strands and modification of its base. The important reactions of free radicals underlying these events may be classified into the following categories:

1. Hydrogen atom transfer reaction

$$X^{\bullet} + RH \rightarrow XH + R^{\bullet} \qquad (1a)$$

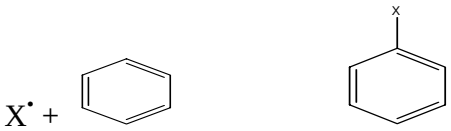
$$X^{\bullet} + RH \rightarrow X^{\bullet -} + RH^{\bullet +} \rightarrow XH + R^{\bullet} \qquad (2a)$$

2. Addition reaction



(2)

3. Aromatic substitution reaction



(3)

4. β-Scission reaction

$X^{\bullet} \rightarrow Y^{\bullet}$ + products (4)

5. Coupling reactions

$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$ (5)

The hydrogen atom transfer reaction is an important key step in lipid peroxidation, protein modification, and DNA damage. It may proceed by direct hydrogen atom abstraction by free radical (reaction 1a) or by electron transfer reaction followed by proton transfer (reaction 1b). The hydrogen atom abstraction from lipids by peroxyl radicals is the key step in determining the rate and product distribution in lipid peroxidation. They are determined by the reactivity of the attacking radicals as well as those of lipids. The reactivity of free radicals toward lipids vary quite extensively by a factor of 10^{10}. The reactivity of the free radical $X^{\bullet}$ can be estimated by the bond dissociation energy of the X-H bond, BDE(X-H). The larger the bond dissociation energy of the X-H bond, the higher the reactivity of $X^{\bullet}$ radical. The bond dissociation energy of HO-H bond is 119 kcal/mol (498 kJ/mol), while that of LOO-H is 88 kJ/mol (368 kJ/mol), the $HO^{\bullet}$ radical is much more reactive than peroxyl radical in hydrogen atom abstraction.

The bond dissociation energy of the C-H bond being attacked is also important in determining the rate. The weaker the C-H bond, the faster the hydrogen is abstracted. Hydroxyl radical is so reactive that it is capable of abstracting any type of hydrogen from lipids rapidly, whereas peroxyl radical is much less reactive and it selectively abstracts only reactive hydrogen such as bisallylic hydrogen of polyunsaturated lipids.

Table 2 Production of active oxygen species

Active oxygen species	Formation
Superoxide (Hydoperoxyl radical) $O_2^{\bullet-}$ ($HO_2^{\bullet}$)	Enzymatic and non-enzymatic one electron reduction of oxygen $O_2 + e \rightarrow O_2^{\bullet-}$ $HO_2^{\bullet}$ (pK=4.8)
Hydroxyl radical, $HO^{\bullet}$	Radiolysis of water, metal-catalyzed Decomposition of hydrogen peroxide, interaction of NO and superoxide $NO + O_2^{\bullet-} \xrightarrow{H^+} ONOO^- \rightarrow HO^{\bullet} + NO_2$
Alkoxyl and peroxyl radicals $LO^{\bullet}$, $LO_2^{\bullet}$	Metal-catalyzed decomposition of hydroperoxides
Hydrogen peroxide, H_2O_2	Dismutation of superoxide, oxidation of sugars
Iron-oxygen complex, Fe=O, etc	Hemoglobin, myoglobin, etc.
Singlet oxygen, $^1O_2$	Photosensitized oxidation, bimolecular interactions between peroxyl radicals, reaction of hypochlorite and hydrogen peroxide
Lipid and protein	Oxidation of lipids and proteins hydroperoxides
Nitrogen dioxide, $NO_2^{\bullet}$	Reaction of peroxyl radicals and NO, polluted air and smoking
Nitric oxide, $NO^{\bullet}$	Nitric oxide synthase, nitroso thiol, and polluted air
Thiyl radical, $RS^{\bullet}$	Hydrogen atom transfer from thiols
Protein radical	Hydrogen atom transfer from protein

7. Lipid peroxidation

The reactions of free radicals and active oxygen species are considered in this section taking lipid peroxidation as a model reaction. Lipid peroxidation is important in, for example, food deterioration and oxidative modification of low density lipoprotein (LDL) which is now accepted as a key initial event in the progression of atherosclerosis. Lipid peroxidation proceeds by three different pathways: (1) non-enzymatic, free radical-mediated chain reaction (2) non-enzymatic, non-radical oxidation, and (3) enzymatic reaction.

1. Non-Enzymatic, free radical-mediated chain oxidation

The characteristic of this oxidation is that it proceeds by a chain reaction as illustrated in Figure1

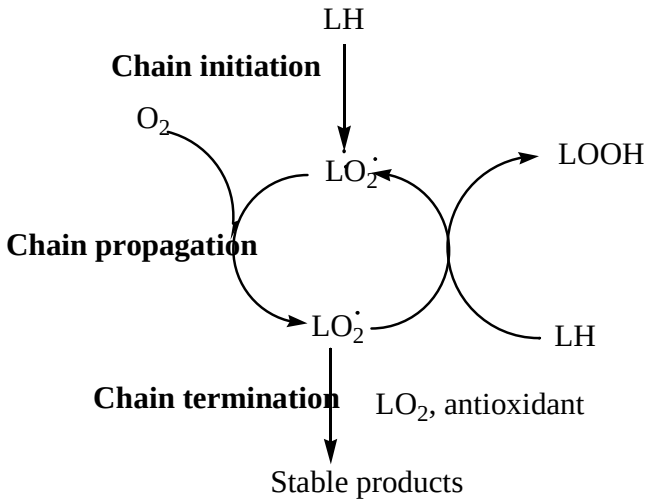


Figure 1 Oxidation of lipids by free radical chain mechanism. LH: lipid; $L^{\bullet}$: carbon centered lipid radical; $LO_2^{\bullet}$: lipid peroxyl radical; LOOH: lipid hydroperoxide.

It consists of three steps, namely, chain initiation, chain propagation, and chain termination. In the chain initiation step, the free radical is formed and attacks the lipid. The free radical (reaction 6) may be formed by various sources such as light, heat, metal,

and some physiological reactions. Foods are stored in the dark and at low temperature to suppress the formation of free radicals. If free radicals are not formed, lipids are stable and not oxidized. However, if radicals are formed, they may attack lipid and generate lipid radicals (reaction 7). The rate and site of this reaction depend on the attacking radical and the lipids. As described above, the hydroxyl radical attacks lipid quite rapidly and randomly, while the peroxyl radical selectively abstracts bisallylic hydrogen. This is true for different classes of lipids such as free fatty acids, phospholipids, triglycerides, and cholesterol esters.

Chain initiation $\longrightarrow$ radical $X^{\bullet}$ (6)

$X^{\bullet} + LH \text{ (lipid)} \longrightarrow XH + L^{\bullet} \text{ (lipid radical)}$ (7)

Chain propagation:

$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet} \text{ (lipid peroxyl radical)}$ (8)

$LO_2^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$ (9)

The lipid radical $L^{\bullet}$ reacts with oxygen molecule quite rapidly to give lipid peroxyl radical (reaction 8), although under low oxygen concentration the lipid radical may live longer. The lipid peroxyl radical attacks another lipid molecule and abstracts hydrogen atom to yield lipid hydro peroxide and at the same time another lipid radical (reaction 9), which reacts with oxygen and continues the second oxidation sequence. Thus, the chain is propagated and one molecule of chain-initiating radical may cause the oxidation of many molecules. The number of chain propagations per each chain initiation is called kinetic chain length and shows how long the chain reaction continues. It has been show that the *in vitro* oxidation of erythrocytes and LDL proceed by a chain reaction, but it is not clearly known how long the chain continues *in vivo.* It is noteworthy that the chain is propagated by lipid peroxyl radical independent of the chain initiating species, whether it is initiated by hydroxyl radical or NO_2, and that polyunsaturated lipids having two or more double bonds are selectively oxidized.

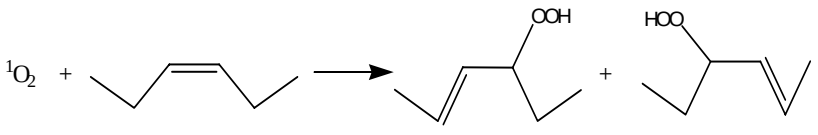
The chain propagation does not continue forever, but the chain oxidation is terminated when lipid radical or lipid peroxyl radical is scavenged by an antioxidant such

as vitamins E and C or when two lipid peroxyl radicals react to give non-radical products such as ketones and alcohols (reaction 10).

$$2LO_2^{\bullet} \rightarrow \text{ketone} + \text{alcohol} + O_2 \qquad (10)$$

2. Non-enzymatic, non-radical oxidation

Some active oxygen species oxidize lipids by non-radical mechanisms. A typical example is the oxidation by singlet oxygen, which adds to the double bond rapidly to give hydroperoxide and/or cyclic peroxide instead of abstracting hydrogen from lipids to produce free lipid radical. For example, oleic acid is oxidized by singlet oxygen it give two kinds of hydroperoxides (reaction 11).



(11)

It should be noted that, in the oxidation by singlet oxygen, the oxidation by singlet oxygen, the double bond migrates. Free cholesterol is also oxidized by singlet oxygen to give a specific hydroperoxide. As shown above, free radicals oxidize predominantly polyunsaturated lipids, whereas singlet oxygen oxidizes mono-olefins as well. It may be also noted that, although singlet oxygen oxidizes lipidathan the peroxyl radical, this oxidation is stoichiometric and one molecule of singlet oxygen oxidizes only one lipid molecule.

3. Enzymatic reaction

Lipids are oxidized by lipoxygenases and cyclooxygenases. The so called arachidonate cascade is well known and its physiological importance has been well established. Lipoxygenases are non-heme iron containing a 1Z,4Z-pentadiene system to their 1-hydroperoxy-2(E),4(Z)-pentadiene product. In contrast to the non-enzymatic, free radical-mediated lipid peroxidation, the lipooxygenase reaction is in general regiospecific and enantiospecific with respect to the initial hydrogen abstraction and subsequent oxygen insertion. Originally, it was believed that lipoxygenase can oxidize only free

polyenoic fatty acids. However, more recent studies indicated that certain plant and mammalian lipoxygenases are capable of specifically oxidizing phospholipids and cholesterol esters in biomembranes and lipoproteins. Thus, a stereospecific pattern of oxidation products in cells and tissues may be regarded as indicator for the *in vivo* action of a lipoxygenase. The detection of stereospecific products of oxidized polyenoic fatty acids in atherosclerotic lesions suggested that 15-lipoxygenases may contribute to lipid peroxidation in the early stages of atherogenesis.

8. Antioxidants

The word "antioxidant" is increasingly popular in modern society as it gains publicity through mass media coverage of its health benefits. The dictionary definition of antioxidant is rather straightforward but with a traditional annotation (Merriam-Webster 2002) "a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances (as the tocopherols) being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging)". A more biologically relevant definition of antioxidants is "synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene that are capable of counteracting the damaging effects of oxidation in animal tissues". The biologically relevant definition fits better to the concept of antioxidants known to the general public as people are more aware of their health than prevention of rubber autoxidation. Depending on the scientific discipline, the scope and protection targets are significantly different. In the chemical industry, antioxidants often refer to compounds that retard autoxidation of a chemical product such as rubber and plastics. The autoxidation is caused primarily by radical chain reactions between oxygen and the substrates. Effective antioxidants are radical scavengers that break down radical chain reactions. Sterically hindered phenols and amines are often used as antioxidants in the rubber and plastic industries. In food science, antioxidants have a broader scope, in that

they include components that prevent fats in food from becoming rancid as well as dietary antioxidants “a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans” (National Academy of Science: Washington 2000), as defined by the Institute of Medicine. Like the other definitions, this definition does not provide limitation on the mechanism(s) of antioxidant action. Therefore, a dietary antioxidant can (sacrificially) scavenge reactive oxygen/nitrogen species (ROS/RNS) to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (preventive). Dietary antioxidants often broadly include radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors. Selenium is a cofactor of selenoproteins (e.g., glutathione peroxidase), which reduce peroxides to alcohols and water. Whereas autoxidation of a lifeless matter occurs by radical chain reactions, oxidation in a biological system is primarily mediated by a host of redox enzymes. Nonetheless, nonenzymatic lipid autoxidation by radical chain reaction may still occur and lead to oxidative stress. Consequently, biological antioxidants include enzymatic antioxidants (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic antioxidants such as oxidative enzyme (e.g., cyclooxygenase) inhibitors, antioxidant enzyme cofactors, ROS/RNS scavengers, and transition metal chelators. Halliwell defined biological antioxidants as “molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules” (Halliwell 1990 : 15). Figure 2 outlines the scope of antioxidants in three fields. Despite the difference in scope, a radical chain reaction inhibitor is commonly regarded as an antioxidant and also the most extensively studied.

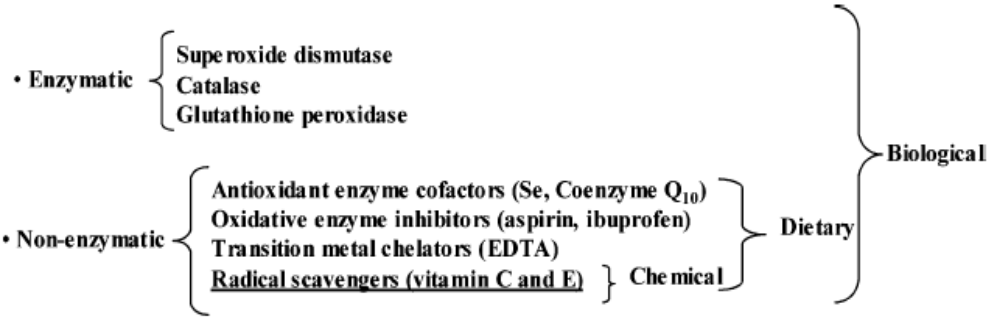


Figure 2 Broad scopes of antioxidants

Aereobic organisms are protected from oxidative stress induced by free radicals and active oxygen species by an array of defense systems. As summarized in Table 3, various kinds of antioxidants with different functions play an important role in these defense systems. The preventive antioxidants acting in the first defense line suppress the formation of free radicals and active oxygen species. The radical scavenging antioxidants are responsible in the second defense line and inhibit chain initiation and/or break the chain propagation. The antioxidant enzymes such as phospholipases, proteases, DNA repair enzymes, and transferases act as the third line defense. In addition, the apprppriate antioxidant is generated and transferred to the right site at the right time and at the right concentration when the oxidative stress takes place. This adaptation mechanism is also important in the total defense system.

With increasing experimental, clinical, and epidemiological evidence which shows the involvement of free radicals and active oxygen species in a variety of diseases, cancer, and aging, the role of antioxidants has received increasing attention. For example, recent epidemiological studies showed that high intake of vitamin E, a potent radical-scavenging antioxidant, reduces the risk of coronary heart disease and that low levels of vitamin E seem to correlate with an increased incidence of myocardial infarction. Furthermore, probucol, a synthetic radical-scavenging antioxidant, is widely used as a drug in the treatment of hypercholesterolemia and atherosclerosis.

9. **Mechanism of action of antioxidants**

Inhibition of lipid peroxidation

The role and action of antioxidants will be reviewed briefly using lipid peroxidation as an example. Lipid peroxidation can be inhibited by suppressing chain initiation and chain propagation and/or by enhancing chain termination. Metals often play an important role in radical generation. For this reason, proteins such as ferritin and ceruloplasmin, which sequester metal ions, are also important antioxidants. Hydroperoxide and hydrogen peroxide are precursors of oxygen radicals and, hence, peroxidases such as glutathione peroxidase (GPX) also act as antioxidants. Superoxide dismutase (SOD) acts as an antioxidant by dismutating superoxide to triplet oxygen and hydrogen peroxide. Various carotenoids may act as quenchers of singlet oxygen.

Table 3 Defense systems *in vivo* against oxidative damage

1. Preventive antioxidants: suppress the formation of free radicals	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide $H_2O_2 \rightarrow 2H_2O+O_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2+2GSH \rightarrow 2H_2O+GSSG$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholpid hydroperoxides $PLOOH+2GSH \rightarrow PLOH+ H_2O+GSSG$
Phospholipid hydroperoxide	Decomposition of phospholipid hydroperoxide glutathione peroxidase peroxides
Peroxidase	Decomposition of hydrogen peroxide and lipid

Table 3 (continue)

Hydroperoxides	
	$LOOH+AH_2 \rightarrow LOH+ H_2O+A$
	$H_2O_2+AH_2 \rightarrow 2H_2O+A$
Gkutathione S-transferase	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of acting of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide
	$2O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+ O_2$
Carotenoids, vitamin E	Quenching singlet oxygen

1. Radical-scavenging antioxidants; scavenge radicals to inhibit chain initiation and break chain propagation
   Hydrophilic: Vitamin C, uric acid, bilirubin, albumin
   Lipophilic: Vitamin E, ubiquinol, carotenoids, flavonoids

2. Repair and *de novo* enzymes : Repair the damage and reconstitute membranes
   Lipase, protease, DNA repair enzymes, transferase

3. Adaptation; Generate appropriate antioxidant enzymes and transfer them to the right site at the right time and in the right concentration
   Multifaceted nature of antioxidants

10. Neurotoxicity

Compounds that cause neural toxicity many be categorized as those that cause central nervous system (CNS) toxicity, those that cause peripheral nervous system (PNS) toxicity and those that cause a mixture of the two. While the body contains many enzymes to protect against potential toxins, further steps are taken to protect arguably the most fragile organ in the body, and the centre of the nervous system, the brain. As body functioning is wholly dependent upon the brain then it is vital that no impairment of its function occurs. Therefore, in addition to enzymes that react to potentially harmful molecules, the brain uses a physical barrier to prevent toxin entry into the brain: the blood-brain barrier.

Transport across most membranes in the body is aided by the presence of multiple transport proteins and ion channels within the membrains, allowing routes for transport of molecules that are not sufficiently lipophillic to cross membranes by passive diffusion. In addition there are collections of "spacer proteins" between the individual cells forming "gab junctions" through which further transport can occur. In comparison, the cells of the blood-brain barrier have a very restricted set of membrane proteins/ion channels and individual cells are connected by "tight junctions" which do not allow transport. The net result of this is that only a very restricted set of chemicals has ready access to the brain, and this acts as the first line of defence to protect the brain from potential toxins. (Nick Plant, "Neurotoxin" In Molecular Toxicology 2003, 1)

11. Antioxidants and neurological diseases

For other major neurologic disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS), genetic defects, not related to vitamin E deficiency, are believed to be the cause. Oxidative stress may play a role either in triggering the defect when it is not inherited, or accelerating the onset of the disease after the defect occurs. Extensive postmortem and other studies support the involvement of oxidative stress in the development of these diseases probably due, at least in part, to impaired mitochondrial function. Evidence supporting oxidative stress includes increased brain iron content, decline of superoxide dismutase (SOD) and

glutathione (GSH), and oxidative damage to lipids, proteins, and DNA. In familial forms of ALS, genetic defects in cytosolic SOD have been demonstrated. In addition, neuroleptic drugs such L-beta-3,4-dihydroxyphenylalanine hydrochloride (L-DOPA), used in the treatment of Parkinson's disease, may increase the production of free radicals. In the last decade, several epidemiological and clinical studies evaluated the role of antioxidants, primarily vitamin E, in delaying the onset or for treatment of these diseases with mixed results.

12. Antioxidant capacity assays

Terms one can find include total antioxidant "capacity" (or efficiency, power, parameter, potential, potency, and activity). The "activity" of a chemical would be meaningless without the context of specific reaction conditions such as pressure, temperature, reaction media, co reactants, and reference points. Because the "antioxidant activity" measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay, it is inappropriate and misleading to generalize the data as indicators of "total antioxidant activity". The other terms listed above are more independent of specific reactions and have similar chemical meanings. To be consistent in the review, we use "capacity" to refer to the results obtained by different assays. Oxidant-specific terms such as "peroxyl radical scavenging capacity", "superoxide scavenging capacity", "ferric ion reducing capacity" and the like would be more appropriate to describe the results from specific assays than the loosely defined terms "total antioxidant capacity" and the like.

On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. HAT- and ET-based assays are intended

to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample. Because the relative reaction rates of antioxidants (or substrates) against oxidants, particularly peroxyl radicals, are the key parameters for sacrificial antioxidant capacity, we will analyze autoxidation and its inhibition kinetics before in-depth analysis of the individual assays. ET-based assays measure an antioxidant's reducing capacity, and the HAT-based assays quantify hydrogen atom donating capacity. It is apparent that the hydrogen atom transfer reaction is a key step in the radical chain reaction. Therefore, the HAT based method is more relevant to the radical chain-breaking antioxidant capacity.

Table 4 *In Vitro* Antioxidant Capacity Assays

Assays involving hydrogen atom transfer reactions	
	ORAC (oxygen radical absorbance capacity)
$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$	TRAP (total radical trapping antioxidant parameter)
$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	Galvinoxyl scavenging
Assays by electron-transfer reaction	
	TEAC (Trolox equivalent antioxidant capacity)
$M(n)+ e \text{ (from AH)} \rightarrow AH^{\bullet+} + M(n-1)$	
	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl)
	Total phenols assay by Folin-Ciocalteu reagent
	Copper (II) reduction capacity

13. Antioxidant assays

13.1 Hydrogen atom transfer (HAT)

13.1.1 Oxygen radical absorbance capacity assay (ORAC)

The improved ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals (Huang et al. 2002 : 4440). As the reaction progresses, fluorescein is consumed and FL intensity decreases. In the presence of antioxidant, the FL decay is inhibited. A typical ORAC assay kinetic curve is shown in Figure 6. Data reduction from the ORAC assay is achieved by (1) calculating of the area under the kinetic curve (AUC) and net AUC (AUC_{sample} - AUC_{blank}), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (linear or quadratic fit between 0.78 and 12.6 μM Trolox), and (3) calculating the Trolox equivalents of a sample using the standard curve. It should be noted that in some cases, antioxidant samples have different curves of concentration versus AUC from that of Trolox standard.

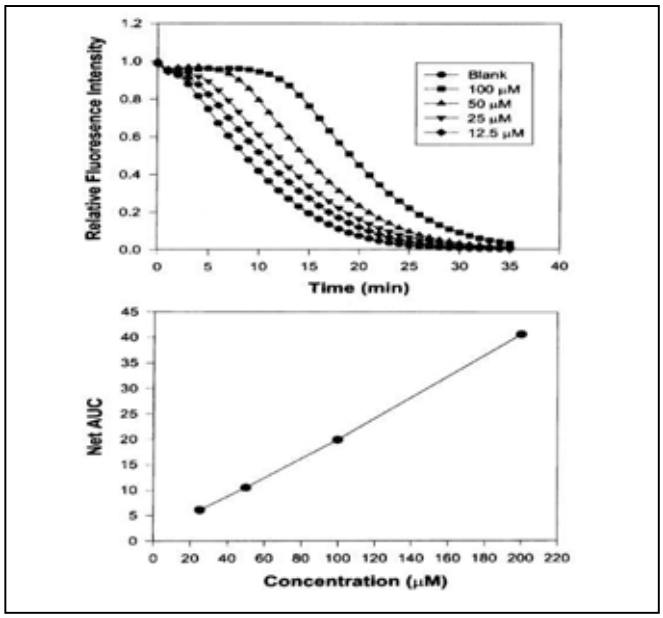


Figure 3 Fluorescence decay curve of fluorescein in the presence of R-tocopherol and AAPH and Linear plot of the net AUC versus α-tocopherol concentration.

Source: Huang Dejian et al. "Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated cyclodextrin as the solubility enhancer," J. Agric. Food Chem 50 (2002) : 1816.

The advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. There is a direct linear correlation of AUC and a broad range of sample types, including raw fruit and vegetable extracts, plasma, and pure phytochemicals (Cao et al. 1993 : 308). Therefore, the ORAC assay has been broadly applied in academics and the food and supplement industry as a method of choice to quantify antioxidant capacity. In fact, an antioxidant database has been generated applying the ORAC assay in combination with the total phenols assay (Caldwell et al. 2003 : 4592; Prior et al. 2003 : 3275; Wu et al. 2004 : 4029). Many antioxidants are lipophilic, and it is also known that the antioxidant capacity of a compound is dependent upon reaction media (Abraham et al. 1989 : 705; Howard et al. 1964 : 1047; Foti et al. 2001 : 345). Therefore, an organic solvent based ORAC assay would be particularly useful for lipophilic samples. However, fluorescein is

not sufficiently lipid soluble, and its fluorescence intensity in nonpolar organic solvent is rather low.

13.1.2 Total peroxyl radical-trapping antioxidant parameter assay

The TRAP assay uses R-phycoerythrin (R-PE) as a fluorescent probe (Ghiselle et al. 1995 : 32). The reaction progress of R-PE with AAPH was monitored fluorometrically (λ_{ex}=495 nm and λ_{ex} =575 nm). A typical kinetic curve of fluorescence decay is shown in Figure 4. The antioxidant capacity of an unknown sample was expressed as Trolox equivalence (X) by the equation where C_{Trolox} is Trolox concentration, T_{Trolox} is the lag time of the kinetic curve of R-PE in the presence of Trolox, X is the antioxidant capacity of plasma, and T_{plasma} is the lag time of the kinetic curve in the presence of plasma. X is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by the dilution factor of the sample to give the TRAP value (μmol/L). To obtain the T_{Trolox} from the same kinetic curve of the sample, trolox was added to the reaction mixture when R-PE fluorescence was ~50% of the initial value. (Wayner et al. 1985 : 35)

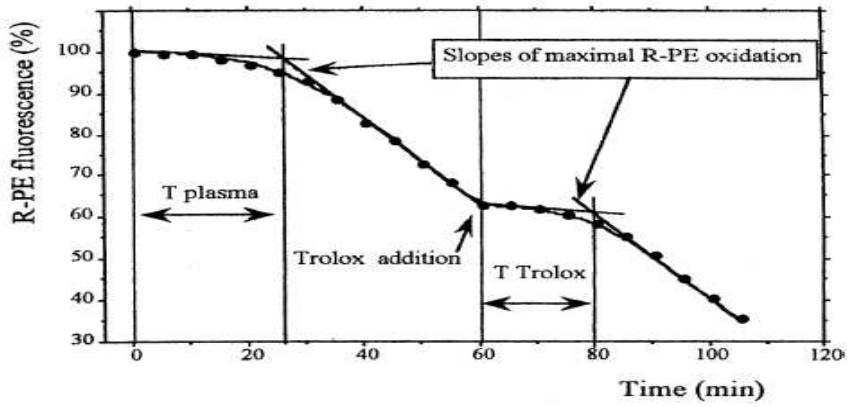


Figure 4 Kinetics of R-PE oxidation initiated by 5 mM AAPH in the presence of plasma (8 μL) before and after Trolox addition (1.8 μM final solution). The antioxidant capacity of each plasma sample is calculated by comparing the two lag phases obtained in the presence and in the absence of trolox.
Source: Franco Tubaro et al., "Analysis of plasma antioxidants capacity by competition kinetics," Free Radical Biol. Med. 24 (1998) : 1230.

The reaction was followed until the fluorescence decay rate resumed to the level before the Trolox addition. The lag phase was then calculated by extrapolating the curves of maximal R-PE oxidation before and after Trolox addition (Figure 4). The increase of fluorescence signal is an indication of oxidation progress. How the assay results relate to the antioxidant capacity of a sample. To make the correlation, it is assumed that antioxidant capacity is equal to reducing capacity (Benzie and Strain 1999 : 20).

13.1.3 Crocin Bleaching Assay

This assay measures the inhibition capacity of antioxidants in protecting the bleaching of crocin, a naturally occurring carotenoid derivative, by the free radical generator AAPH (W. Bors et al. 1984 : 315). The crocin bleaching assay has found limited applications in food samples so far. Reaction rate constants between $ROO^{\bullet}$ and phytochemicals may vary greatly, and some of them have rates comparable to that of crocin (thus, no lag phase), whereas others will give a lag phase. In this case, the inhibited bleaching rates are very small and are not sensitive to the concentration changes of antioxidants. This could be the reason vitamin C has an unusually large antioxidant capacity value. Crocin absorbs at a rather short wavelength (450 nm), and many food pigments, such as carotenoids, absorb light at the same wavelength. To avoid the interference for each sample, a sample blank (a mixture containing only AAPH and food sample) must be tested at the same time. Finally, crocin is a mixture of natural pigments extracted from saffron and is subject to lot-to-lot variability, which limits its industrial application in a quantitative procedure.

13.1.4 Inhibited oxygen uptake (IOU) method

Experimentally, the measurement of the rate of oxygen uptake or conjugated diene peroxide formation was applied to derive the k_6 values of a pure antioxidant compound (Burton and Ingold 1981 : 2226). Using styrene as a substrate and azoisobutrylnitrile (AIBN) as a radical initiator, Ingold and co-workers measured the oxygen consumption rates in the presence or absence of tocopherols in chlorobenzene

using a pressure transducer system under one atmospheric pressure of oxygen. The k_6 value was calculated by applying the equation: $[O_2]_0 - [O_2]_t = - (k_3 [RH]/ k_6[\ln(1 - t/\pi)]$

Where π is the induction period, or lag time, and *t* is any time point before the acceleration phase. The k_6 values of the tocopherols were 2.35x106 (α), 1.66x106 (β), 1.59x106 (γ), and 6.5x105 (σ) $M^{-1} \bullet s^{-1}$. The IOU method has not found broad usage. The reason could be that (1) the experimental data were collected under unrealistically high oxygen pressure; (2) accurate measurement of oxygen uptake rates may be difficult, especially at the inhibition period when the uptake rate is slow; (3) food samples normally have lower antioxidant concentrations, and the sensitivity of this method may not be sufficient; and (4) phase transition between inhibited oxidation and uninhibited oxidation may not be as distinct as that of the tocopherols and may lead to ambiguous π values (W. A. Pryor and others 1988 : 2226).

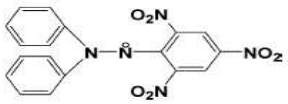
13.1.5 Galvinoxyl scavenging assay

Galvinoxyl, another stable phenoxyl radical can be reduced by hydrogen-donating free radical scavengers. Galvinoxyl are stable radicals with a low deterioration rate and reactivity towards most compounds. Consequently, only good H-atom donors are likely to react with stable radicals in a stoichiometric way. Concentration of extracts and standards required to achieve 50% phenoxyl radical scavenging activity was determined according to the method of Shi and Niki (Shi and Niki 1998 :). A total of 900 μl of 1 mM galvinoxyl methanol solution was added to 90 μl of test samples to make a final volume of 990 μl and the mixture was allowed to react at 37 ºC. After 20 min, the absorbance value was measured at 420 nm. Methanol was used as negative controls while L-ascorbic acid served as the positive controls. The phenoxyl radical scavenging activity and IC_{50} of the extracts and positive controls were calculated as described for the DPPH assay.

13.2 Singlet electron transfer (SET)

13.2.1 Free radical scavenging activity for DPPH

DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm. Upon reduction, the solution color fades; the reaction progress is conveniently monitored by a spectrophotometer.



DPPH

Figure 5 2,2-diphenyl-1-picryl-hydrazyl (DPPH) structure

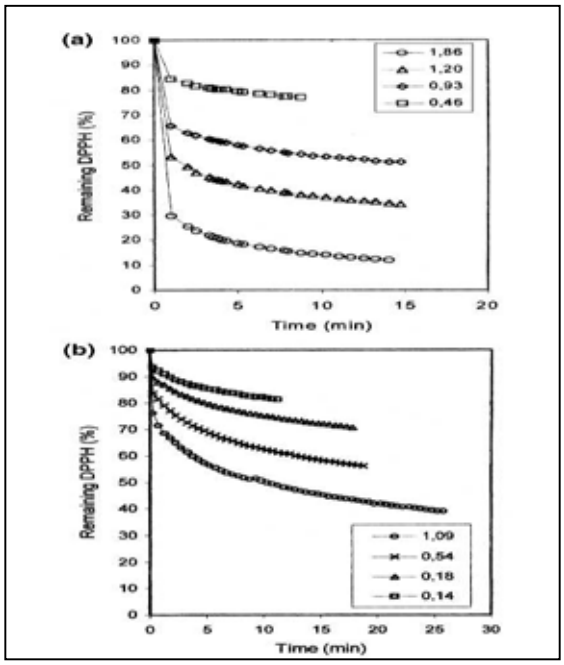


Figure 6 DPPH bleaching kinetics in the presence of different concentrations of α -carotene (**a**) and β-xanthophylls (**b**).

Sourcee: Jimenez Escrig et al. “Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picrylhydrazyl,” J.Sci. Food Agric. 80 (2000) : 1688.

The percentage of the DPPH remaining is calculated as:

$\%DPPH_{rem} = 100 \times [DPPH]_{rem}/[DPPH]_{T=0}$

$\%DPPH_{rem}$ is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as T_{EC50}. A representative kinetic curve of a DPPH assay is shown in Figure 5. Sanchez-Moreno and co-workers classified the kinetic behavior of the antioxidant compound as follows: <5 min (rapid), 5-30 min (intermediate), and >30 min (slow). They further proposed a parameter, called "antiradical efficiency (AE)" (Jimenez-Escrig et al. 2000 : 1688), to express the antioxidant capacity of a certain antioxidant. AE is calculated as: $AE = (1/ EC_{50}) T_{EC50}$

The DPPH assay is technically simple, but some disadvantages limit its applications. Besides the mechanistic difference from the HAT reaction that normally occurs between antioxidants and peroxyl radicals, DPPH is a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. This is evident from the T_{EC50} values ranging from 1.15 min (ascorbic acid) to 103 min (rutin). Consequently, the antioxidant capacity is not properly rated. The reaction kinetics between DPPH and antioxidants are not linear to DPPH concentrations (Figure 8). It is thus rather arbitrary to express antioxidant capacity using EC_{50}. Finally, it was reported that the reaction of DPPH with eugenol was reversible (Bondet et al. 1997 : 611). This would result in falsely low readings for antioxidant capacity of samples containing eugenol and other phenols bearing a similar structure type (*o*-methoxyphenol).

The DPPH assay was believed to involve hydrogen atom transfer reaction, but a recent paper suggested otherwise. On the basis of the kinetic analysis of the reaction between phenols and DPPH (Foti et al. 2004 : 2311), Foti and co-workers suggested that the reaction in fact behaves like an ET reaction. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path, because

it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. In addition, the author found that adventitious acids or bases present in the solvent may dramatically influence the ionization equilibrium of phenols and cause a reduction or an enhancement, respectively, of the measured rate constants. This renders the DPPH assay much less chemically sound as a valid assay for antiradical activity of measurement.

13.2.2 Scavenging activity for ABTS radicals

In the improved version, $ABTS^{\bullet-}$, the oxidant, was generated by persulfate oxidation of 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$). Specifically, 7 mmol of ABTS ammonium was dissolved in water and treated with 2.45 mmol of potassium persulfate, and the mixture was then allowed to stand at room temperature for 12-16 h to give a dark blue solution. This solution was diluted with ethanol or buffer (pH 7.4) until the absorbance reached 0.7 at 734 nm. The difference of the absorbance reading is plotted versus the antioxidant concentrations to give a straight line. The concentration of antioxidants giving the same percentage change of absorbance of the $ABTS^{\bullet-}$ as that of 1 mM Trolox was regarded as TEAC. Due to its operational simplicity, the TEAC assay has been used in many research laboratories for studying antioxidant capacity, and TEAC values of many compounds and food samples are reported. The TEAC values for pure antioxidant compounds do not show clear correlation between TEAC values and the number of electrons an antioxidant can give away. Apparently, the reaction rate differences between antioxidants and oxidants are not reflected in the TEAC values because the TEAC assay is an end-point assay.

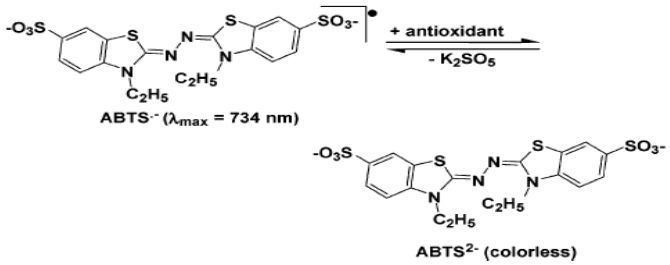


Figure 7 Reaction in TEAC assay

Source: Dejian Huang et al. "The chemistry behind antioxidant capacity assay," J. Agric. Food Chem 53 (2005) : 1849.

13.2.3 The ferric reducing/antioxidant potential (FRAP)

The FRAP assay also takes advantage of electron-transfer reactions. Here in a ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ = 2,4,6-tripyridyl*s*- triazine), is used as an oxidant (Benzie and Strain 1996 : 73).

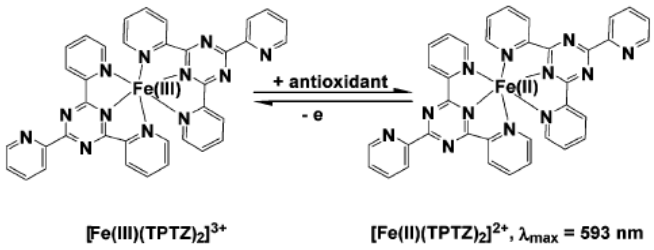


Figure 8 Reaction in FRAP assay

Source: Dejian Huang et al. "The chemistry behind antioxidant capacity assay," J. Agric. Food Chem 53 (2005) : 1849**.**

Therefore, the TPTZ is deficient as the ideal reaction stoichiometry between Fe(III) and TPTZ is 1 to 2. The oxidant is not just $Fe(III)(TPTZ)_2$, it also contains other Fe(III) species which can lead to potential problems as many metal chelators in food extract could bind Fe(III) and form complexes that are also capable of reacting with antioxidants. To measure FRAP value, 300 μL of freshly prepared FRAP reagent is warmed to 37 °C and a reagent blank reading is taken at 593 nm. The change

of absorbance ($\Delta A = A_{4min} - A_{0min}$) is calculated and related to ΔA of an Fe(II) standard solution. ΔA is linearly proportional to the concentration of antioxidant. One FRAP unit is arbitrarily defined as the reduction of 1 mol of Fe(III) to Fe(II). The FRAP values for ascorbic acid, α-tocopherol, and uric acid are identical (2.0). The FRAP value of bilirubin is 1-fold higher than that of ascorbic acid. These results suggest that 1 mol of vitamin C can reduce 2 mol of Fe(III) and that 1 mol of bilirubin can reduce 4 mol of Fe(III). This is in conflict with the fact that both vitamin C and bilirubin are two-electron reductants. It is known that when bilirubin is oxidized, it is transformed to beliverdin (by losing two hydrogen atoms, not just electrons), which happens to have an absorption at 593 nm with sufficiency ($\varepsilon_{593} = 1x10^4$) comparable with that of Fe(II)(TPTZ) 2.50 Pulido and co-workers (Pulido et al. 2000 : 3398) measured the FRAP values of several polyphenols in water and methanol. However, the absorption (A_{593}) does not stop at 4 min; instead, it slowly increased even after several hours. Polyphenols with such behaviors include caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin. The FRAP values of these compounds cannot be obtained accurately if 4 min reaction time was followed.

13.2.4 Total phenols assay by Folin-ciocalteu Reagent

FCR was initially intended for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue (Folin and Ciocalteu 1927 : 627). The FCR-based assay gained popularity and is commonly known as the total phenols (or phenolic) assay. The FCR actually measures a sample's reducing capacity, but this is not reflected in the name "total phenolic assay". Numerous publications applied the total phenols assay by FCR and an ET-based antioxidant capacity assay (e.g., FRAP, TEAC, etc.) and often found excellent linear correlations between the "total phenolic profiles" and "the antioxidant activity". This is not surprising if one considers the similarity of chemistry between the two assays. One of the assays may just be redundant. A recent report of using polyphenol oxidase for assaying total phenols in tea may be more specific to phenolic compounds (Stevanato et al. 2004 : 6289). Contamination of reductants leads to a green color, and the addition of

oxidants such as bromine can restore the desired yellow color. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates-molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI): Mo (VI) + e → Mo(V)

Obviously, the FC reagent is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds [e.g., vitamin C, Cu (I), etc.]. Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH~10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electrontransfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds. Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible. As a result, a large body of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants.

13.2.5 Total antioxidant potential assay using Cu(II) as an oxidant

The method is based on reduction of Cu(II) to Cu(I) by reductants (antioxidants) present in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), forms a 2:1 complex with Cu(I), which has a maximum absorbance at 490 nm (Schilt 1966). It was found that 1 mol of R-tocopherol can reduce 2 mol of Cu(II) to Cu(I) (Yamashita et al. 1998 : 858).

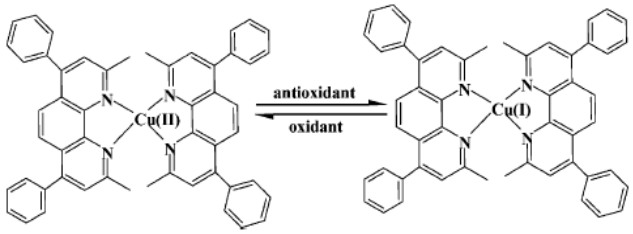


Figure 9 Reaction in total antioxidant potential assay using Cu(II) as an oxidant assay

Source: Dejian Huang et al. "The chemistry behind antioxidant capacity assay," J. Agric. Food Chem 53 (2005) : 1849.

More recently, Zaporozhets et al. reported a method for measuring the antioxidant power of herbal products based on solid-phase spectrophotometry using tetrabenzo[*b*,*f*,*j*,*n*][1,5,9,- 13]tetraazacyclohexadecine-Cu(II) complex immobilized on silica gel. The absorbance of the modified sorbent (712 nm) increases when the Cu(II) is reduced (Zaporozhets et al. 2004 : 23).

13.3 Assays measuring other ROS scavenging capacity

Experimental evidence has directly or indirectly suggested that there are six major reactive oxygen species causing oxidative damage in the human body. These species are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$). To counteract the assault of these ROS, living cells have a biological defense system composed of enzymatic antioxidants that convert ROS/RNS to harmless species. For example, $O_2^{\bullet-}$ is converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD) or reacts with nitric oxide ($NO^{\bullet}$) to form peroxynitrite. H_2O_2 can be converted to water and oxygen by catalase. In contrast, no enzymatic action is known to scavenge $ROO^{\bullet}$, $HO^{\bullet}$, $^1O_2$, and $ONOO^-$. Therefore, the burden of defense relies on a variety of nonenzymatic antioxidants such as vitamins C and E and many phytochemicals that have the property of scavenging oxidants and free radicals. To comprehensively evaluate the oxidant-scavenging capacity of a food sample, assays have to be designed to include these ROS.

However, so far the majority of assays are designed to measure a sample's capacity to react with one oxidant (either organic radical or redox active compounds). The peroxyl radical has been the most frequently used ROS in the assays because it is a key radical in autoxidation and it can be generated conveniently from the thermal decomposition of azo compounds. There are also limited numbers of papers describing assays for the scavenging capacity of other ROS. In this section, we briefly describe some of the assays.

13.3.1. $O_2^{\bullet-}$ scavenging capacity assay

Classically, the SOD activity assay uses the competition kinetics of $O_2^{\bullet-}$ reduction of cytochrome *c* (probe) and $O_2^{\bullet-}$ scavenger (sample). Cytochrome *c* can be reduced directly by antioxidants, which can also inhibit the xanthine oxidase. Therefore, this method is not suitable for quantifying nonenzymatic antioxidant (Yamashita et al. 1998 : 860). Ewing and Janero reported a high-throughput assay using a nonenzymatic (phenazine methosulfate/NADH/ O_2) $O_2^{\bullet-}$ generator and nitroblue tetrazolium (NBT) as a probe (Ewing et al. 1995 : 245). This assay takes advantage of the reducing property of $O_2^{\bullet-}$. Because many dietary antioxidants can also exhibit reducing capacity, as demonstrated in the TEAC and FRAP assays, this improved method cannot be applied to nonenzymatic samples. More recently, hydroethidine has been used as the probe in measuring $O_2^{\bullet-}$ scavenging capacity (Zhao et al. 2003 : 1362). Nonfluorescent hydroethidine is oxidized by $O_2^{\bullet-}$ (generated from xanthine oxidase and xanthine mixture) to form a species of unknown structure that exhibits a strong fluorescence signal at 586 nm. Addition of SOD inhibits the hydroethidine oxidation. This approach can avoid the problem of direct reduction of the probe by antioxidant, but possible inhibition of xanthine oxidase by antioxidants remains an issue.

13.3.2 H_2O_2 scavenging capacity assay

H_2O_2 is rather inert at low concentrations. Under physiological conditions, H_2O_2 oxidation power is believed to be observed in combination with Fe (II) (Fenton reaction). Biologically, H_2O_2 is converted to oxygen and water by catalase. A common assay that claims to measure H_2O_2 scavenging capacity of dietary antioxidants

uses horseradish peroxidase to oxidize scopoletin to a nonfluorescent product. In the presence of antioxidants the oxidation is inhibited. The nature of the inhibition is ambiguous because there are several potential inhibition pathways. The antioxidants can inhibit the reaction by (a) reacting directly with H_2O_2, (b) reacting with intermediates formed fro m enzyme and H_2O_2, or (c) inhibiting the horseradish peroxidase from binding H_2O_2. Therefore, it is difficult to explain the actual chemical meaning of the data (Martinez-Tome et al. 2001 : 1022).

13.3.3 Hydroxyl radical ($HO^{\bullet}$) scavenging assay

Biologically, the hydroxyl radical is widely believed to be generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction). However, the Fe (II)/ H_2O_2 mixture has disadvantages in a scavenging assay because many antioxidants are also metal chelators. When the sample is mixed with Fe (II), it may alter the activity of Fe (II) by chelation. As a result, it is impossible to distinguish if the antioxidants are simply good metal chelators or $HO^{\bullet}$ scavengers. Antioxidants in food (such as vitamin C) may act as pro-oxidants by reducing Fe(III) to Fe(II) and make the $HO^{\bullet}$ generation catalytic. In fact, ascorbic acid has been used in combination with catalytic Fe (II) and excess H_2O_2 to generate a constant flux of $HO^{\bullet}$ radicals. The hydroxylation is markedly inhibited by hydroxyl radical scavenging agents such as dimethyl sulfoxide and ethanol. The inhibited reaction was not affected by iron chelators, such as diethylenetriaminepenta acetic acid (DTPA), bathophenanthroline disulfonic acid, phytic acid, and bathocuprione disulfonic acid. The putative hydroxyl radical is an extremely reactive and short-lived species that can hydroxylate DNA, proteins, and lipids. Therefore, the direct scavenging of the hydroxyl radical by dietary antioxidants in a biological system is unrealistic as the cellular concentration of dietary antioxidants is negligible compared with other biological molecules. The rate constants for $HO^{\bullet}$ reactions have been determined by pulse radiolysis through the deoxyribose method (Halliwell 1995 : 1341). Therefore, the ability of antioxidants to scavenge the $HO^{\bullet}$ radical is not unlikely to provide any protection to biological molecules as the opportunity for $HO^{\bullet}$ and antioxidants to react is extremely small. On the other hand, it is possible to prevent the formation of hydroxyl radicals by

either deactivating free metal ions [e.g., Fe(II)] through chelation or converting H_2O_2 to other harmless compounds (such as water and oxygen). Catalase converts H_2O_2 to O_2, and H_2O and metal chelators bind metal ions so that they become inert toward H_2O_2. The quantitation method is the same as that of the ORAC assay except gallic acid is used as the standard. This method has been rigorously validated for linearity, precision, accuracy, and ruggedness. A wide range of phenolic antioxidants can be analyzed. The hydroxyl radical prevention capacity is mainly due to their metal-chelating capability.

13.3.4. Singlet oxygen scavenging capacity assay

Singlet oxygen is normally generated in the presence of light and photosensitizers. It is believed that $^1O_2$ is often responsible for UV light-dependent damage to skin (Sies and Stahl 2004 : 750), cataract formation in the lens of the eyes (Zigman 2000 : 162), macular degeneration (Rozanowska et al. 1998 : 1110), and photosensitivity resulting from ingestion or absorption of phytochemicals, pharmaceuticals and pesticides that act as photosensitizers (Ebermann et al. 1996 : 96). In the absence of light, $^1O_2$ production can be ambiguous in a biological system. It was suggested that the extracellular $^1O_2$ production by the spontaneous dismutation of superoxide anion has some physiological significance (Tarr and Velenzeno 2003 : 357). On the other hand, chemically, $^1O_2$ can be conveniently generated through non-photochemical decomposition of hydrogen peroxide by metals or hypochlorite(Martinez et al. 2000 : 10212; (a) Aubry et al. 1985 : 5846; (b) Busby et al. 1999 : 7455). Rate constants of singlet oxygen reaction with various compounds have been compiled by Wilkinson and co-workers (Wilkinson et al. 1995 : 673). Singlet oxygen can be quenched through physical means by transferring its excitation energy to another molecule (which is excited), or it can add to antioxidants forming endoperoxides. β-Carotene is an excellent physical quencher of $^1O_2$. Singlet oxygen emits characteristic phosphorescence at 1270 nm. The decay rates of the light intensity were used to measure the $^1O_2$ quenching activity of a compound. The singlet oxygen quenching rates vary by 6 orders of magnitude. Quenching of the visible SOSDF may provide a highly sensitive method for the measurement of $^1O_2$ quenching capacity using commonly available

apparatus or in systems where the 1270 nm luminescence is difficult to detect. This method is not yet widely applied.

13.3.5. Peroxynitrite ($ONOO^-$) scavenging capacity assay

Superoxide and nitric oxide react under diffusion control rate (k > 109 $M^{-1}\bullet s^{-1}$) to form peroxynitrite (Moncada and Higgs 1995 : 1319). $O_2^{\bullet-}$ and NO are not potent oxidants; it's adduct, $ONOO^-$, is not a strong oxidant either. Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant. Under physiological pH, ONOOH (pK_a=8.0) rearranges to form much less oxidizing nitrate (Pryor and Squadrito 1995 : 268). At pH 7.4, the ratio of peroxyntrite and peroxynitrous acid is 4 to 1. $ONOO^-$ and ONOOH often cause the nitration or hydroxylation of aromatic compounds, particularly tyrosine (to nitrotyrosine). Under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid. The adduct is believed to be responsible for the oxidative damage of proteins (Squadrito and Pryor 2002 : 891). There are a few papers on the scavenging capacity of antioxidants against $ONOO^-$. Two methods are used for $ONOO^-$ scavenging measurements: (1) inhibition of tyrosine nitration by $ONOO^-$ (Pannala et al. 1998 : 598) and (2) inhibition of dihydrorhodamine (DHR) 123 oxidation (Kooy et al. 1994 : 152). Pannala reported the peroxynitrite quenching capacity of catechin and other polyphenols by measuring their inhibition capacity on reaction between peroxynitrite and tyrosine. The method relies on HPLC separation and quantification of nitrotyrosine, and it is thus rather time consuming.

The traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. Experimental evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases (Richards and Sharma 1991; Niwa 1991). As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new

compounds with antioxidant activity. In the past few years, there has been increasing interest in the study of mango phenolics from mango fruits, peels, seeds, leaves, flowers, and stem bark due to their antioxidative and health promoting properties that make

consumption of mangoes and derived products a healthy habit. Bioactive compounds found in the mangos, among other plants and herbs have been shown to have possible health benefits with antioxidative, anticarcinogenic, antiatherosclerotic, antimutagenic, and angiogenesis inhibitory activities (Cao 1999 : 381). Interestingly, many herbs, fruits, and vegetables are known to contain large amounts of phenolic antioxidants other than the well-known vitamins C, E, and carotenoids.

***Mangifera indica* L.**

Family: Anacardiaceae

Syn.: *Mangifera anisodora* Blanco

Mangifera fragrans F.-Vill.

Mangifera rostrata Blanco

*Mangifera syl*6*atica* F.-Vill.

Habitat

India, south-east of Asia, Malaysia, Himalayan regions, Sri Lanka, Africa, America, Australia and, in general, in tropical and monsoon climate. Fruits ripen in hot and dry season (Calabrese 1993 : 172; Kirtikar and Basu 1993b; Sahni 1998b).

Used parts

Ripe and unripe fruits, root, bark, leaves, flowers, resin from bark.

Chemical constituents present in different parts of the plant

In whole plant

Friedelin, β-sitosterol, mangiferin (6.9%) mp. 278°, molecular weight 422.35, catechin, protocatechuic acid, ellagic acid, gallic acid, m-digallic acid, trigallic acid, gallotannin, butin, fisetin, leucocyanidin, quercetin, triterpenic acid-mangiferolic acid mp. 181° C, isomangiferolic acid, hydroxymangiferolic acid, mangiferonic acid, hydroxymangiferonic acid, ambonic acid and ambolic acid, exudate from stem yield gum (16.0%) and resin (81.0%), a triterpenoid pentacyclic-hopan- 1β, 3β,22-triol- and four tetracyclic triterpenoids 3α, 22(R:S)-, 3β, 22(R:S)-, 3β, 23(R:S)-, 3α, 27 dihydroxycycloart-24(E)-en-26-oic acids.

Fruits

Cycloartenol, 3β-hydroxycycloart-24-en-26-al, 24-methylene-cycloartan-3β, 26-diol, C-24 epimers of cycloart-25-en-3β,24-diol, α-amyrin, β-amyrin, dammarenediol II, ψ-taraxastane-3β, 20-diol, ocotillol, methyl mangiferonate, methyl mangiferolate, methyl isomangiferolate, sitosterol, a mixture of 5-(12-*cis*-heptadecenyl)- and 5-pentadecyl-resorcinols, vitamins A and C.

Roots

Friedelin, friedelan-3β-ol, α-amyrin, β-amyrina, cycloartenol, β-sitosterol.

Leafes

Flavonoids, phenolic, glucose, galactose, arabinose, xylose, rhamnose, tannins, leucine, tyrosine, valine, protocatechuic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid, methylic, ethyl, propyl, butyl, amyl and iso-butyl alcohols, α-pinene, bpinene, camphene, myrcene, car-3-ene, limonene, β-ocimene, γ-terpinene, α-terpinolene, linalool, estregole, δ-elemene, β-elemene, α-cubebene, methyleugenol, β-caryophyllene, humulene, alloaromadendrene, α-guaiene, β-bulnesene, α-farnesene, δ-cardinene, elemicin, chinomin, protocatechuic acid, gallic acid, methylchinomin, isochinomin, quercetin, hyperin, taraxerone, taraxerol, friedelin, lupeol, β-sitosterol.

Unripe fruits

Polysaccharides, a triterpene, acetates of cycloartanol, amyrin, lupeol, homomangiferin-2C-b- D-glucopyranosyl-3-methoxy-1,6,7-trihydroxyxanthone.

Bark

Protocatechuic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid, tetracyclic triterpenoids, cycloart-24-en-3b,26-diol, 3 ketodammar-24(E)-en-20S,26- diol, C-24 epimers of cycloart-25-en-3β,24,27-triol and cycloartan-3β,24,27-triol.

Seeds

Stearic acid, α-pinene, β-pinene, myrcene, limonene, oleic (86,0%), arachidonic, linoleic, linolenic and palmitic acids.

Pharmacological action

Mangiferin is a C-glucosylxanthone and it has cardiotonic and diuretic properties. Gallic acid and quercetine show a strong antiviral activity. Mangiferin stimulates after 48 h the proliferation of thymocytes and spleenic lymphocytes with a peak response at 5.0 mg:ml and 20.0 mg:ml, respectively. Mangiferin has a remarkable anti-inflammatory activity.

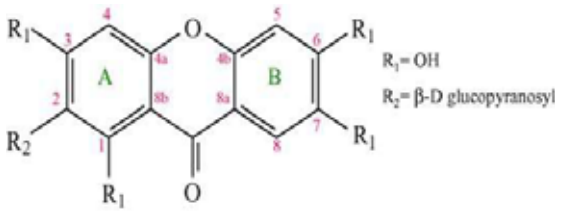


Figure 10 Mangiferin structure

Mangiferin is an antioxidant at different levels of oxidation sequence able to:

• prevent the lipoperoxidation by decreasing the O_2 concentration and generating mangiferin phenoxy radicals.

• Bind metal ions like Fe^{3+}, Fe^{2+} preventing the generation of hydroxyl radicals and : or oxo-ferryl groups.

• Regulate the polymer chain initiation by interaction with ROS to produce feebly-reactive oxo-radical.

• Act like a scavenger to lipid peroxy and alkoxy radicals and prevent the abstraction of H from cellular lipids.

• Maintain the balance of cellular oxidant : antioxidant (Ghosal 1996a : 562). (Kirtikar, “Medicinal Plants” In Indian Medicinal Plants 1993, 3; Lalit, “Indian Materia Medica” In Popular Prakashan Private 1993 a-f).

CHAPTER 3
MATERIALS AND METHODS

1. Materials

1.1 Tissue culture reagents

DMEM (Dulbecco's modified Eagle medium) (GIBCO™ Grand Island, NY, USA)

Fetal bovine serum (GIBCO™ Grand Island, NY, USA)

Polyornithine (GIBCO™ Grand Island, NY, USA)

Sterile water (GIBCO™ Grand Island, NY, USA)

Trypan Blue Stain 0.4% (GIBCO™ Grand Island, NY, USA)

HAT (GIBCO™ Grand Island, NY, USA)

1.2 All other Chemicals

2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich Chem, GmbH, Germany)

2,2-azinobis 3 ethylbenzothiazoline 6-sulfonate (ABTS) (Sigma-Aldrich Chem, GmbH, Germany)

Trolox®, ferrous sulfate (Sigma-Aldrich Chem, GmbH, Germany)

NADH (Sigma-Aldrich Chem, GmbH, Germany)

Polyornithine hydrobromide (Sigma-Aldrich Chem, GmbH, Germany)

Bovine serum albumin (Sigma-Aldrich Chem, GmbH, Germany)

Trypan blue (Fluka)

Potassium persulfate (Fluka)

2,4,6,-tripyridyl-s-triazine complex (TPTZ) (Fluka)

N-methylphenazonium methyl sulfate (PMS) (Fluka)

Nitrotetrazolium blue chloride (NBT) (Fluka)

Folin–Ciocalteu (Fluka)

Gallic acid (Fluka)

Tannic acid **(**Merck, Darmstadt, Germany)

Sodium carbonate **(**Merck, Darmstadt, Germany)

Dimethyl sulfoxide (DMSO) **(**Merck, Darmstadt, Germany)

Hydrogen peroxide (H_2O_2) **(**Merck, Darmstadt, Germany)

Acetic acid **(**Merck, Darmstadt, Germany)

Ethanol absolute (Scharlau® ET0016 Scharlau®Chemie SPAIN analytical reagent grade and HPLC grade)

Chloroform (VWR International Ltd. England analytical reagent grade)

Sodium dodecylsulfate (Fisher Scientific)

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) (Sigma-chemical company, St. Louis, MO, USA)

2. Equipment

Inverted Microscope (ECLIPSE TE 2000-U ; Model : T-DH Nikon® Japan)

Fusion universal microplate analyzer (Model No: AOPUS01 and A153601; A Packard bioscience company)

Laminar air flow (BIO-II-A)

CO_2 Incubator (HERA Cell 240 Heraeus)

Centrifuge (Hermle Z300K; Labnet®; Lab Focus CO., Ltd.)

Shaking incubator (GFL 3031)

TERUMO® Syringe 50 mL

Fusion universal microplate analyzer (Model No : AOPUS01 and A153601 ; A Packard BioScience Company)

Automatic autoclave (Model : LS-2D ; SCIENTIFIC PROMOTION CO.,LTD)

pH meter (HORIBA COMPACT pH METER B-212)

Rotary evapolator (V-850/R-205, BÜchi Vac®; Postfach, Switzerland)

Spray dryer (Minispray Dryer, BÜchi 190, Postfach, Switzerland)

HPLC (Agilent Technologies®; Agilent 1100 series)

Column (Apollo; Alltech)

Micropipette 0.1-2 μl, 2-20 μl, 20-100 μl, 100-1000 μl ,1-5 ml (masterpette ; Bio-Active Co.,Ltd.)

Micropipette tip

Pipette aid (POWERPETTE Plus ; Bio-Active Co.,Ltd.)

Measuring pipettes (2, 5, 10 ml)

25 cm^2, 75 cm^2 Cell Culture Flask (CORNING® ; Corning Incorporated)

24-well tissue culture testplates (TPP®)

96-well cell Culture Cluster (Costar®; Corning Incorporated)

15 ml, 50 ml centrifuge tubes-Sterile (Biologix research company)

Eppendorf tubes Filter (Sartorius BORO 3. 3 Goettingen, Germany)

Cellulose acetate filter 0.2 μm (Sartorius AG . 37070 Goettingen, Germany)

Heater and Magnetic stirrer (Heidolph®, Germany)

Water bath (Hetofrig CB60; Heto High Technology)

3. Plant material

Fresh leaves of mango (Namdokmai, Aoklong, Munthaway and Fahlun) from Nakornpathom, Thailand were selected in August 2008. Too old leaves in all cultivars were discorded.

4. Cell line and culture

The NG108-15 cells were a gift from Associate Professor Tohda Michihisa, Institute of Natural Medicine, University of Toyama. Cells were grown in DMEM containing 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine and 10% fetal bovine serum. Cell cultures were maintained in a humidified incubator with 5% CO_2 – 95% air at 37 ^oC.

5. Methods

5.1 The extraction of mango leaves

The leaves were washed with water and allowed to air dry at room temperature. They were then placed in an oven at 40^o C until completely dried, after which they were

powderized using a blender. A sample of 30 g was extracted with 300 ml of distilled water, methanol and chloroform. Extraction was carried out at room temperature by continuous shaking for 24 hrs. The suspension obtained was filtered using pump and the filtrate was collected. Aqueous filtrate was concentrated using a freeze drier while methanol and chloroform filtrate were concentrated using a rotary evaporator at 40 ºC.

5.2 Standardization of extract

5.2.2 Determination of total phenolic content

The amounts of phenolic compounds in the extracts were determined using the Folin-Ciocalteu method, and gallic acid was used as the standard phenolic compound. Fifty microliter aliquots of the extracts (1 mg/ml) were added to a mixture of 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of 7.5% Na_2CO_3. After incubation at 45º C for 30 min, the absorbance was measured at 765 nm. A linear dose-response regression curve was generated using absorbance reading of gallic acid (0.1 - 1.0 mg/ml). The content of total polyphenols in the extract is expressed as milligrams of gallic acid equivalent per gram of dried weight (mg GAE/g) of extracts. C = A/B where C is expressed as mg GAE/g dried weight of the extract; A is the equivalent concentration of gallic acid established from the calibration curve (mg); and B is the dried weight of the extract (g).

5.2.3 Determination of tannic acid content

The amounts of tannic acid content in the extracts were determined using $FeCl_3$. Two mg of bovine serum albumin was mixed with 1 ml of the sample extract at 1 mg/ml and then kept at room temperature for 20 min. Then, the mixtures were centrifuged and the sediment was dissolved with 0.1% sodium dodecyl sulfate (2 ml), triethanolamine (2 ml) and 10 mM $FeCl_3$ (1 ml). The suspensions were measured at wavelengh of 510 nm. The calibration curve was established using standard solution of tannic acid (1.6 - 2.4 mg/ml).

5.2.4 Determination of the major polyphenolic compounds contents

The amounts of main polyphenolic compounds content in the extracts were quantify using high performance liquid chromatography (HPLC) (Jacline 2008 :

5601): The analyses were performed on a HPLC (Agilent Technologies®; Agilent 1100 series). Chromatographic separation was achieved on a performance C18 5 μm column, 4 mm x 250 mm. The mobile phase consisted of solvent A: 2% acetic acid in water and solvent B: methanol; starting from 95% A for 2 min, 75% A for 8 min, 60% A for 10 min, 50% A for 10 min ,finally 100% B for 5 min and continuing at 100% B until completion of run for washing and recondition the column. The detection wavelength was 250, 278 and 340 nm, with flow rate 1 ml/min and an injection volume of 5 μl. Commercially available pure mangiferin, methyl gallate and gallic acid were used as standard substances and was identified using the above condition giving the retention time of 21.25, 15.26 and 7.38 min, respectively. Identification of phenolic compounds in the extracts was performed by comparison of their retention times with those from standards substances and characteristics of the UV spectra.

5.3 Antioxidant assays

5.3.1 Antioxidant capacity assays on chemical principles (mechanism)

5.3.1.1 Singlet electron transfer (SET)

5.3.1.1.1 Free radical scavenging activity for DPPH

DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm. Upon reduction, the solution color fades; the reaction progress is conveniently monitored by a spectrophotometer.

The method was modified from that of previous study. (Choi 2002 : 1172) A 200 μM DPPH solution (100 μl) was added to 100 μl of sample extracts. The mixture was left standing at room temperature for 30 min; the absorbance was then measured spectrophotometrically at the wavelength of 550 nm (Fusion Universal Microplate Analyser Model: A153601). The results of the assay were expressed as IC_{50}, which represents the concentration of the extract (μg/ml) required to inhibit 50% of the free radical scavenging activity. The free radical scavenging activity was assessed using the equation:

% inhibition = $(1-A_{sample}/A_{control}) \times 100$ …….(1)

Where A_{sample} and $A_{control}$ are the absorbance in the presence of the extracts and control, respectively. The IC_{50} values were calculated by linear regression of plots where the x-axis represented the various concentrations (μg/ml) of test plant extracts while the y-axis represented the % inhibition.

5.3.1.1.2 Scavenging activity for ABTS radicals

ABTS (2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate) is oxidized to the colored nitrogen-centered radical cation $ABTS^{\bullet+}$ in a persulfate system. $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS solution with 4.95 mM potassium persulfate. The mixture was kept in the dark at room temperature for 12-16 hrs before used. Prior to the assay, the solution was diluted with phosphate buffer pH 7 to give an absorbance of 0.7 ± 0.02 at 734 nm in a 1 cm cuvette. Then, 3.9 ml of the working solution was mixed with 0.1 ml of extracts at various concentrations or negative controls (phosphate buffer) or positive controls (Trolox). After keeping for 10 min at room temperature, the sample were measured at the wavelength of 734 nm. The inhibition percentage was calculated following equation (1).

5.3.1.1.3 The ferric reducing/antioxidant potential (FRAP) assay

The reducing ability of extracts was determined by FRAP assay. FRAP solution was freshly prepared and consisted of acetate buffer (pH 3.6), ferric chloride solution (20 mM) and TPTZ solution (10 mM). Two hundred microliters of the diluted sample extract (or for the blank 200 μl methanol and for the positive control 200 μl Trolox) was added to FRAP reagent and sodium acetate buffer to a final volume of 4 ml. The tubes were vortexed and left for exactly 30 min, and the absorbance was measured at the wavelength of 593 nm. The measurement was compared to a standard curve of $FeSO_4 \cdot 7H_2O$ solutions and expressed as an EC_1 value, which means the concentration of antioxidant in the reactive system having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$. Determination of each sample was performed in triplicate.

5.3.1.2 Hydrogen atom transfer (HAT)

5.3.1.2 .1 Scavenging activity onto galvinoxyl radicals

Galvinoxyl, another stable phenoxyl radical can be reduced by hydrogen-donating free radical scavengers. Concentration of extracts and standard substances required to achieve 50% phenoxyl radical scavenging activity was determined according to the method of Shi and Niki (Shi and Niki 1998 : 367). A total volume of 900 µl of 1 mM galvinoxyl methanol solution was added to 90 µl of test samples to make a final volume of 990 µl and the mixture was allowed to react at 37 ºC. After 20 min, the absorbance value was measured at the wavelength of 420 nm. Methanol was used as negative controls while L-ascorbic acid served as the positive controls. The phenoxyl radical scavenging activity and IC_{50} of the extracts and positive controls were calculated as described in the DPPH assay.

5.3.2 Scavenging capacity on biologically relevant oxidants

5.3.2.1 Superoxide anion scavenging

Non enzymatic superoxide anion radicals (Pontiki and Hadjipavlou-Litina 2007 : 195) were generated. The superoxide producing system was set up by mixing phenazine methosulfate (PMS), nicotinamide-adenine-dinucleotide (NADH) and air–oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method.

The samples were added into reaction mixtures containing PMS, NBT, 20 m MPBS and NADH. After incubation for 5 min at room temperature, the absorbance was measured at 550 nm. The inhibition percentage was calculated using the following equation (1).

5.3.2.1 Hydroxyl Radical (˙OH) scavenging

It is generally assumed that ˙OH is generated in biological systems from H_2O_2 by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ whereby Fe^{2+} is regenerated by superoxide anion $O_2^{\bullet-}$, giving rise to an overall Fe(II,III)-catalyzed Haber–Weiss reaction producing ˙OH from H_2O_2 and $O_2^{\bullet-}$, potentially available

in aerobic cells (Chen 1996 : 381). When $^{\bullet}OH$ radicals generated by iron-EDTA + H_2O_2 in the presence of ascorbic acid oxidize deoxyribose and the reaction products yield a pink chromogen absorbing at 532 nm upon heating with thiobarbituric acid (TBA), hydroxyl radical scavengers added to the medium compete with the deoxyribose probe and diminish chromogen formation, enabling the calculation of second-order rate constants of $^{\bullet}OH$ scavenging (Halliwell 1981 : 347-352). The inhibition percentage was calculated using the following equation (1).

5.3.2.3 Hydrogen peroxide scavenging

Method was modified by that of Bahorun 1996. Hydrogen peroxide scavenging assay is measure of the amounts of hydrogen peroxide in samples. It has a UV absorption maximum at the wavelength of 230 nm. Hydrogen peroxide was prepared in phosphate buffer pH 7.4. The reduced of the absorbance indicated the increased of scavenging hydrogen peroxide radicals of extracts.

5.3.3 Metal ion chelating

Metal ion chelating is the method for analyzed reducing power of antioxidant by scavenges ferrous ion (ferrozine-Fe^{2+} complex) produced by reaction between ferrozine and $FeSO_4$. The absorbance was measured at the wavelength of 550 nm. Scavenging activity is expressed as an IC_{50} value.

The ferrous ions chelating by the extracts and standard substance were estimated by the method of Yen (Yen 2000 : 438). Briefly, the samples were added to a solution of NaCl, Ferrous sulfate and EDTA. The reaction was initiated by the addition of ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at the wavelength of 550 nm by using a UV-Visible Spectrophotometer. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation:

% inhibition = $[(A_0-A_1)/ A_0] \times 100$ where A_0 and A_1 were the absorbance of the control reaction and standard or sample, respectively. The control solution contained water and ferrozine complex formation molecules.

5.4 Cytotoxicity assay

The MTT assay was performed to assess the toxicity on NG 108-15 cells. This assay is used to measure the cell viability and based on the reduction of MTT to a blue formazan product by dehydrogenase enzymes of intact mitochondria.

NG 108-15 cells were plated out at $2x10^3$ cells/well in a polyornithine-coated 96-well plate. After 48 h, the sample extracts final the concentration of 1, 10, 30, 50 and 100 μg/ml were added to achieve. The plates were then incubated for 4 h. After incubation, cells were treated with MTT solution (final concentration, 1 mg/ml) for 3 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and then measured the absorbance at 550 nm was measured with a microplate reader (Fusion Universal Microplate Analyser Model: A153601). Percent cell viability was calculated, assuming that the absorbance of control cells was 100%.

5.5 Neroprotective properties on oxidant- and neurotoxin-induced cell death

5.5.1 Hydrogen peroxide–induced neurotoxicity and study of protection offered by extracts

NG108-15 cells ($2x10^3$ cells/well) were seeded into 96-well plates and incubated at 37^oC for 48 h. After plating, they were treated with various concentrations of the extracts for 2 h, and then a stock solution of H_2O_2 solution was added to yield a final concentration of 100 μM. Neuronal survival was quantified using MTT assay.

5.5.2 Glutamate–induced neurotoxicity and study of protection offered by extracts

NG108-15 cells ($2x10^3$ cells/well) were seeded into 96-well plates and incubated at 37^oC for 36 h. After plating, they were treated with various concentrations of the extracts for 30 min, for the excitotoxity insult, neurons were

exposed to 50 mM L-glutamic acid for 22 h. After that, neuronal survival was quantified using MTT assay.

5.5.3 6-Hydroxyldopamine (6-OHDA) –induced neurotoxicity and study of protection offered by extracts

NG108-15 cells ($2x10^3$ cells/well) were seeded into 96-well plates and incubated at 37^oC for 48 h. After plating, they were treated with various concentrations of the extracts 2 h, and a stock solution of 50 µM 6-OHDA solution was added and incubated at 37^oC for 2 h. Neuronal survival was quantified using MTT assay.

5.6 Statistical analysis

Each of the measurements described above was carried out in triplicate and the results were reported in terms of means and standard deviations. In addition, one way analysis of variance (ANOVA) was used to analyze the results with post-hoc analysis, and where appropriate, Turkey's test was employed, using a commercial statistical software package (SPSS). p values less than 0.05 were considered significant.

CHAPTER 4
RESULTS AND DISCUSSION

The effect of cultivars on free radical scavenging (FRS) ability by diphenyl-1-picryhydrazyl (DPPH) assay

First, a DPPH assay was used for screening the free radical scavenging (FRS) activities of methanol, aqueous and chloroform extracts from *M. indica* leaf (Namdokmai). The methanol and aqueous extracts exhibited much higher FRS activities compared to the chloroform extract. (IC_{50} = 4.94±0.63, 7.24±1.05 and 20.07±2.62 μg/ml respectively) (Table 5). Since the methanol extract seemed to have the highest FRS activities in DPPH assay, it was used for further experiments. In order to investigate effect of cultivar i.e. Namdokmai, Aokrong, Muntawai and Fahlun on FRS, their leaves were extracted with methanol. The IC_{50} values for the DPPH of the methanol extracts of Namdokmai, Aokrong, Muntawai and Fahlun were 4.94±0.63, 5.09±1.4, 5.59±1.59 and 7.26±1.05 μg/ml, respectively (Table 5). Their IC_{50} values for the DPPH assay were not statistically significantly different. Similar results were reported for different plants extract that cultivar had similar radical scavenging activities (Bae et al. 2006 : 961, : Jose et al. 2009 : 515). Antioxidant activity and phenolic content increased as the latitude increased. This finding agrees with that reported by McGhie et al. They found that the antioxidant content in different cultivars was influenced by the geographic region where they were grown (Mcghie et al. 2005 : 3066). They suggested that climatic variables, such as solar radiation and temperature, were important factors. Since Namdokmai's extract had the lowest IC_{50} value, the methanol extract of Namdokmai was used for subsequent experiments.

Table 5 Effect of extraction solvents and cultivars of *Mangifera indica* L. on DPPH radical scavenging activities

Cultivar	Extraction solvent	Radical scavenging effect (IC_{50}) (μg/ml)
Namdokmai	Chloroform	20.07±2.62*
	Water	7.24±1.05
	Methanol	4.94±0.63
Namdokmai	Methanol	4.94±0.63
Aokrong		5.09±1.4
Muntawai		5.59±1.59
Fahlun		7.26±1.05

Free radical scavenging activities by DPPH assay of mango leaf extracts. Data are expressed as means ± S.D. (n=3) of three independent replicates. * $p \leq 0.05$ compared to Namdokmai methanol extract

Standardization of *M. indica* leaf extracts by quantification of total phenolic content, tannic acid content and some major bioactive components

In order to standardize the extracts, total phenolic, tannic acid contents (Table 6-7) as well as amount of main phenolic compounds i.e. mangiferin, methylgallate and gallic acid (Table 8-9) were investigated. Total phenolic contents were determined using Folin-Ciocalteu reagent and calculated using a standard curve for gallic acid (R^2 = 0.9984); tannic acid content was calculated using the standard curve of tannic acid (R^2 = 0.9964). The main phenolic compounds i.e. mangiferin, methylgallate and gallic acid, were assayed by HPLC using a standard curve for mangiferin (R^2 = 0.9997); methyl gallate (R^2 = 0.9997) and gallic acid (R^2 = 0.9676). It was found that DPPH radical scavenging activities correlated with the total phenolic contents in some cultivars. For example, the chloroform extract of Namdokmai showed low FRS activity, which

corresponded to relatively low total phenolic contents. On the other hand, the methanol extract of Namdokmai which exhibited the highest total phenolic contents also had the highest FRS activity. Anyway, only total phenolic content was not an indication for the FRS activity. The antioxidant activities of the extracts were strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity (Soong et al. 2004 : 413). In some cultivars, we found the inconsistence between total phenolic content and FRS activity. For example, Muntawai had the lowest total phenolic content while Fhalan showed the lowest FRS activity. Our findings was in agreement with the report by Kahkonen et al. (Kahkonen et al. 1999 : 32) who found no relationship between antioxidant activity and phenolic content on some plant extracts. Ivanova (Ivanova 2005 : 23) proposed that not all phenolic compounds possessed radical quenching activity. Therefore, some main bioactive components related to antioxidant activity which were mangiferin, methylgallate and gallic acid, were determined. The methanol extract showed highest amount of mangiferin, methylgallate and gallic acid. In addition, all of cultivars extracts exhibit comparable amount of three bioactive components. These corresponded with their FRS activity. Therefore, the main bioactive component may be a better indication for FRS activity than total phenolic content for *M. indica* extract. In all of samples, mangiferin was found as the highest component. This result was similar to the work done by Sanchez where mangiferin was suggested as the principle bioactive component of mango (*M. indica* L.) (Sanchez et al. 2000 : 568). Numerous studies have reported the pharmacological effects of mangiferin. It could protect hepatocytes, lymphocytes, neutrophils, and macrophages from oxidative stress, reduce artherogenicity in streptozotocin-induced diabetic rats, reduce the streptozotocin-induced oxidative damage in cardiac and renal tissues (Muruganandan et al. 2002 : 95). In addition, it could protect the neuron from glutamate-induced cell death (Gottlieb et al. 2006 : 377). We here found that the methanol extract of Namdokmai had high amount of mangiferin and it also had high antioxidant activity by DPPH assay (Table 5). Moreover it had the lowest tannic acid content which are reported to be toxic (El-sissi et al. 1971 : 344). Therefore, it was selected for subsequent experiments.

Table 6 Percent yield of extracts and standardized of Namdokmai leaf extracts by total phenolic content and tannic acid content

Extract	Total phenolic contents (mg of GAE/g)	Tannic acid content (mg/ml)	% yield
Water	187 ± 3*	1.634 ± 0.0074	10%
Methanol	420 ± 4.3	1.611 ± 0.014	14.4%
Chloroform	96 ± 3*	1.85 ± 0.024*	4.4%

Data of total phenolic and tannic acid content are expressed as means ± S.D. (n=3) of three independent replicates. * $p \leq 0.05$ compared to the methanol extract.

Table 7 Percent yield of mango leaf extracts and standardization of various cultivars on total phenolic content and tannic acid content

Extract	Total phenolic content (mg of GAE/g)	Tannic acid content (mg/ml)	% yield
Namdokmai	420±4.3	1.611 ± 0.0139	14.4%
Aokrong	480±1.8*	1.85±0.004*	18.6%
Muntawai	380±2.5*	1.89±0*	20%
Fahlun	400±8.1	1.86±0.02*	25%

Data of total phenolic and tannic acid content are expressed as means ± S.D. (n=3) of three independent replicates. * $p \leq 0.05$ compared to Namdokmai extract

Table 8 Quantitation of major phenolic compounds of Namdokmai leaf extracts

Extract	Mangiferin (mg/g dried)	Gallic acid (mg/g dried)	Methyl gallate (mg/g dried)
Water	68.067	10.786	4.274
Methanol	149.222	9.545	13.873
Chloroform	nd	nd	30.403

nd= can not be determined

Table 9 Quantitation of major phenolic compounds in *Mangifera indica* L. leaf methanol extracts

Cultivar	Mangiferin (mg/g dried)	Gallic acid (mg/g dried)	Methyl gallate (mg/g dried)
Namdokmai	149.222	9.545	13.873
Aokrong	143.503	4.162	17.704
Muntawai	143.942	3.607	9.708
Fahlun	126.603	2.619	29.825

nd= can not be determined

The mechanisms of antioxidation of standardized *M. indica* leaf extracts

The use of simple ‘‘total antioxidant capacity” methods differ in their way of generating free radicals, the strategy to measure the end point of the inhibition reaction, and the sensitivity towards the different reducing molecules in the sample (Pellegrini et

al. 2003 : 32; Roginsky and Lissi 2005 : 62). Therefore, more than one method should be used to gain useful information about the total antioxidant capacity.

Three kinds of extracts of Namdokmai leaf prepared in methanol, water and chloroform were analyzed for the antioxidant activities by the assays based on hydrogen atom transfer (HAT) and singlet electron transfer (SET). The former assay was galvinoxyl radicals scavenging assay. The latter assay were 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS), DPPH radicals scavenging assay and ferric reducing antioxidant power (FRAP).

Table 10 Free radical scavenging activities based on chemical principles of antioxidant capacity assays of Namdokmai leaf extracts

Extract	Singlet electron transfer (SET)		Hydrogen atom transfer (HAT)	
	FRAP value (EC_1) (μg/ml)	DPPH (IC_{50}) (μg/ml)	ABTS (IC_{50}) (μg/ml)	Scavenging activity onto galvinoxyl radicals (IC_{50}) (mg/ml)
Water	298.55 ± 8.65*#	7.24 ± 1.05*	2.96 ± 0.05*	nd.
Methanol	202.92 ± 3.39*	4.94 ± 0.63	1.33 ± 0.13*	nd.
Chloroform	46,481.60 ± 1.4*#	20.07 ± 2.62*#	6.56 ± 0.49#	nd.
Trolox	269.11 ± 5.16	3.1 ± 0.5	7.28 ± 0.9	-
Vit. C	-	-	-	0.22±0.003

Data are expressed as IC_{50} for DPPH, ABTS and galvinoxyl radical scavenging for means ± S.D. (n=3) EC_1 for FRAP of three independent replicates. ***** indicates significant differences in the means compared to positive control, Trolox.($p \leq 0.05$) and # indicates significant differences in the means compared to the methanol extract.($p \leq 0.05$)**,** nd. = can not be determined.

Table 11 Scavenging capacities of biologically relevant oxidants and Metal ion chelating of Namdokmai leaf extracts

Extract/ Positive control	Scavenging capacity of biologically relevant oxidants			Metal ion chelating
	Superoxide scavenging (IC_{50}) (μg/ml)	Hydroxyl radical scavenging (IC_{50}) (mM)	Hydrogen peroxide scavenging (IC_{50}) (μg/ml)	(IC_{50}) (μg/ml)
Extract	0.07 ± 0.0049*	nd.	70.89 ± 6.56*	0.89 ± 0.81
Trolox	7.28 ± 0.90	-	-	-
Mannitol	-	66.88 ± 5.01	-	-
BHA	-	-	183.26 ± 11.34	-
EDTA	-	-	-	0.84 ± 0.62

Free radical scavenging activities of mango leaf extracts. Data are expressed as IC_{50} of superoxide anion, hydroxyl radical and hydrogen peroxide scavenging as well as metal ion chelating for means ± S.D. (n=3) of three independent replicates. ***** indicates significant differences in the means compared to positive control (Trolox, mannitol, BHA and EDTA respectively) ($p \leq 0.05$)**,** nd. = can not be determined.

For other FRS activity assays, we found that the methanol extract (EC_1 = 202.92 ± 3.39 and IC_{50} = 1.33 ± 0.13 μg/ml) exhibited significantly higher FRS activity than the water extract (EC_1 = 298.55 ± 8.65 and IC_{50} = 2.95 ± 0.047 μg/ml) and chloroform extract (EC_1 = 46,481.60 ± 1.4 and IC_{50} = 6.56 ± 0.49 μg/ml) in the FRAP and ABTS assays, respectively (Table 10). The methanol extract exhibited slightly higher but not statistically significant free radical scavenging activity than the water extract (IC_{50} = 4.94 ± 0.63 and 7.24 ± 1.05 μg/ml, respectively) in the DPPH assay. In addition, the methanol extract showed significantly higher FRS activity in DPPH assay than the chloroform extract (IC_{50} = 20.07 ± 2.62 μg/ml) (Table 6). The three extracts did not have abilities to scavenge galvinoxyl radicals. Taken together, the results suggested that 1) the extracts of *M. indica* leaves acted as antioxidants via single electron transfer (SET) mechanism and 2) the methanol extracts had high content of total phenolic compounds as well as major bioactive compounds, which corresponded to higher FRS activity. To support the last suggestion, it is found that numerous studies have shown that the antioxidant capacities of plant extracts correlate to the content of phenolics (Selles et al. 2002 : 764; Ribeiro et al. 2007 : 15). Overall of SET-based assays are a multitude for measuring the reducing capacity of antioxidants. HAT-based assay plays a key role in the unwanted lipid oxidation in food and biological systems. The role of antioxidants is to help fight excessive ROS in our bodies. By doing so, antioxidants will be sacrificed to protect the biomolecules from being oxidized (as the antioxidant definition states), and thus the antioxidant has fulfilled its function. It is of importance to study whether there is a correlation between the intake of high potent antioxidants and the level of oxidative stress. The *in vitro* nature of these assays should not compromise their value in guiding clinical research. On the contrary, a valid *in vitro* assay is an invaluable tool for clinical studies if it is combined with bioavailability data and valid oxidative stress biomarker

consumption on reducing oxidative stress markers (Serafini 2003 : 1013). The synergistic effects of such a combination assays will allow to investigate the impact of antioxidant in reducing oxidative stress and thus the implication for disease prevention (Halliwell 2003 : 787, Schroeter 2003 : 787).

During oxidative stress, reactive oxygen species, superoxide anion ($O^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) can elicit widespread damage to cell constituents such as membrane lipids. These free radical combines with the cellular lipid and proteins to produce lipid peroxidation. To study the scavenging capacity on biologically relevant oxidants, superoxide, hydrogen peroxide and hydroxyl radical scavenging of the methanol extract were used. We found that IC_{50} values of superoxide anion and hydrogen peroxide were 0.07 ± 0.0049 and 70.89 ± 6.56 μg/ml respectively. The extract (IC_{50} = 0.07 ± 0.0049 μg/ml) exhibited significantly higher superoxide radical scavenging activity than Trolox (IC_{50} = 7.28 ± 0.90 μg/ml). In addition, the extract (IC_{50} = 70.89 ± 6.56 μg/ml) also showed significantly higher hydrogen peroxide radical scavenging activity than BHA (IC_{50} = 183.26 ± 11.34 μg/ml). These results suggested the extremely strong biological ($O^{\bullet}_2$ and H_2O_2) radical scavenging activity of *M. indica* extract. However, the hydroxyl radical scavenging of the methanol extract of *M. indica* was not observed in this study while the positive control, mannitol, showed the scavenging effect with IC_{50} value of 66.88 ± 5.01 mM (Table 11).

Iron ion plays a vital role for generation $^{\bullet}OH$ radicals in hydroxyl radical scavenging assay. In this assay, we perform $^{\bullet}OH$ scavenging assay by measuring the effect on the degradation of 2-deoxyribose induced by Fe (III)-EDTA. We found that the methanol extracts did not show positive results on trapping $^{\bullet}OH$ radicals which was generated by the oxidation of ferrous ion by H_2O_2. However, in the ferrous ion chelating assay, the methanol extracts had strong chelating activities (IC_{50} value = 0.89 ± 0.81 μg/ml). This finding could be used to explain why the extract showed poor activity in the Fenton reaction (Table 11) which used for generation hydroxyl radical in hydroxyl radical scavenging experiment (Table 11).

Neuroprotective effects of standardized *M. indica* leaf extracts on oxidant- and neurotoxin-induced cell death

Neurodegenerative diseases are characterized by the loss of neuronal cells in the brain. Reactive oxygen species (ROS) may involve in the etiologies of these diseases. Antioxidants in plants or herbs may be useful in delaying or preventing oxidation damage (Barnham et al. 2004 : 209; Casetta et al. 2005 : 2036; Knight et al. 1997 : 21; and Valko et al. 2007 : 51). The potent antioxidant activity of *M. indica* has been reported in various studies (Nithitanakool et al. 2009 : 1120; Barreto et al. 2008 : 5561; Ajila et al. 2008 : 305; Rocha Ribeiro et al. 2007 : 15; Akila et al. 2008 : 175; Martinez et al. 2000 : 426; Martinez et al. 2001 : 582; Pardo-Andreu et al. 2008 : 80; Pardo-Andreu et al. 2006 : 121 and Remirez et al. 2005 : 21). However, little is known about protective activity in the brain (Campos-Esparza et al. 2009 : 360; Martinez-Sanchez et al. 2001 : 469). Our results demonstrate that the extracts had strong radical scavenging effect on both non biological and biological free radicals i.e. superoxide anion and hydrogen peroxide. Therefore, this study was performed to reveal the protective effect of *M. indica* extracts on oxidant-induced cell death in NG108-15 cells. Since NG108-15 cells are a neuroblastoma-glioma hybrid cell line, they have both neuronal and glial properties and have been widely used as a neuron model in electrophysiology and pharmacology research (Brown and Higashida 1988 : 168, Schmitt and Meves 1995 : 182.). Prior to the neuroprotection study, the cytotoxic effect of the extract was evaluated. We found that the extract at 1-100 μg/ml was toxic to the cells (Fig. 11C).

On the experiment of *M. indica* extract on neuroprotection, we used H_2O_2 to induce oxidative cell damage because: 1) oxidative stress is believed to be an important mediator of neuronal cell death and has been postulated to contribute to the pathogenesis of various neurodegenerative diseases; 2) H_2O_2 is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems; 3) among a great variety of reactive oxygen species, H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress; and 4) exogeneous H_2O_2 can enter cells and induce cytotoxicity due to its high membrane permeability.

For the cytoprotective study, NG108-15 cells were treated with various concentrations of H_2O_2 (50-800 µM) in order to find the concentration of H_2O_2 which caused about 50% cell death. Following 2 h of incubation after H_2O_2 challenge, cell viability was determined. The results showed that H_2O_2 significantly reduced cell viability in a concentration-dependent manner with an IC_{50} value of about 100 µM (Fig. 11A). Therefore, 100 µM H_2O_2 was used for the cell treatment in the subsequent experiments.

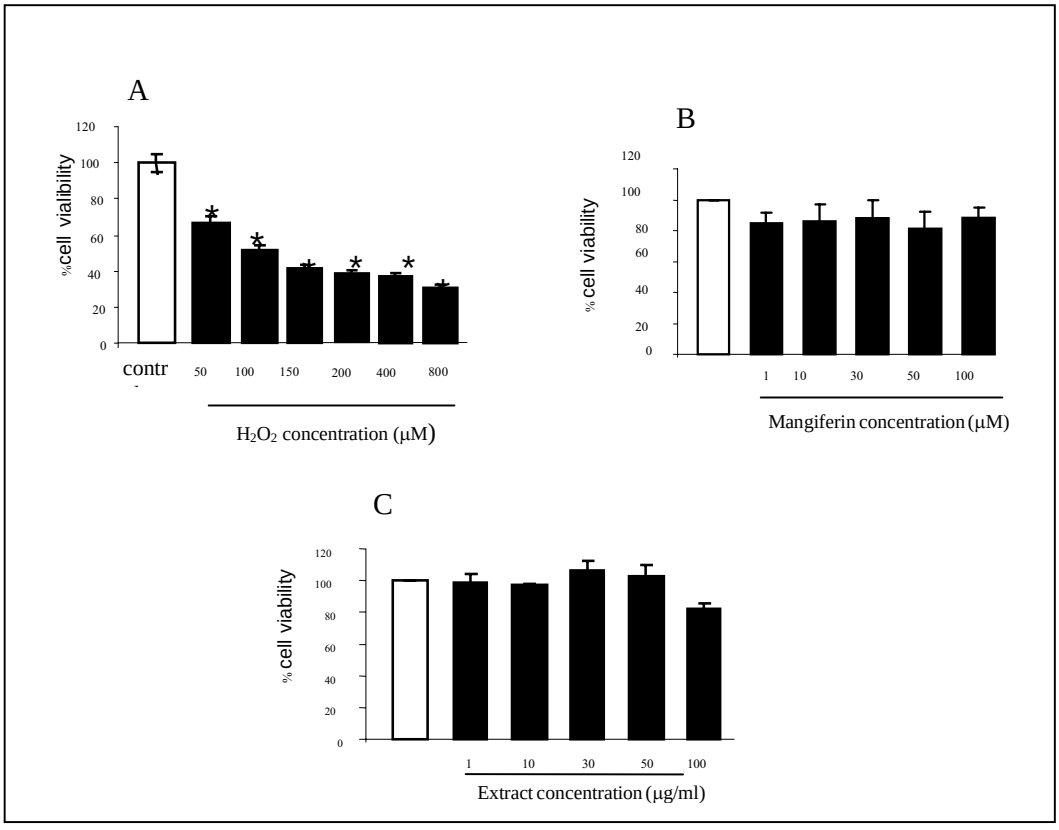


Figure 11 Effect test compounds on NG108-15 cells viability. NG108-15 cells were treated with various concentrations of hydrogen peroxide (A), mangiferin (B) and methanol extracts from *M. indica* leaf (Namdokmai) (C). Data are

expressed as means of cell viability ± S.D. (n=5) of three independent experiments. * $p \leq 0.05$ compared to the control group

For neuroprotection experiments, when a reference standard Trolox at different concentrations was added after H_2O_2 100 µM treatment for 2 hr, the cell viability increased in a concentration-dependent manner from 10 to 1000 µM (Fig. 12B). In a similar way, the methanol extract of *M. indica* (1-100 µg/ml) significantly increased the viability of NG108-15 cells in a concentration-dependent manner compared to the H_2O_2 treated group without the extract. The neuroprotective effects of methanol extracts were observed at 30-50 µg/ml but the extract at 100 µg/ml did not make significant change in cell viability in comparison with the H_2O_2 treated group (Fig. 12C). These results suggest that the protective effects of these extracts against H_2O_2-induced oxidative damage in NG108-15 cells are directly correlated with their antioxidant ability occurring within the concentrations range used. Thus, it is conceivable that the antioxidant properties of extract have the optimal concentration range and that further decrease in its concentration are inefficient or even counterproductive as a consequence of alterations in the redox equilibrium in neurons. Mangiferin is a famous biologically active phenolic compound that is present in large amounts in the leaves of *M. indica*. Mangiferin has been shown to have cytoprotective activities (Campos-Esparza et al. 2009 : 359; Rodriguez et al. 2006 : 1336). Therefore we evaluated cytoprotective effect of mangiferin on H_2O_2-induced oxidative damage. We here found that mangiferin could protect the cell from H_2O_2-induced cell death without effect on cell viability (Fig. 2B and 1B). Previous studies have shown that mangiferin rescued neurons from cell death in acute injury and reduced neurological deficits caused by ischemic damage to rat brain (Gottlieb et al. 2006 : 375). The results reported here indicate that the antioxidant and neuroprotective activity of extracts from *M. indica* leaves may also contribute to those properties, similar to that of mangiferin. However, this does not exclude the possibility that other components present in the extract may also contribute to those properties. Indeed, other constituents of extracts obtained from *M. indica* species are also neuroprotective (Nunez-Selles et al. 2002 : 764). Hydrogen peroxide is believed to cause

cell damage by reacting with the cell membrane, resulting in lipid peroxidation of the membrane. In addition, H_2O_2 can easily cross the cell membrane and exerts injurious effects on tissues by a number of different mechanisms, such as perturbing intracellular calcium homeostasis, (Hyslop et al. 1986 : 357) increasing intracellular ATP, (Hyslop 1988 : 1667) inducing DNA damage, (Barbouti et al. 2002 : 692) and inducing apoptosis (Chandra et al. (2000) : 324). It is possible that the protective effect of extracts from *M. indica* leaves against the H_2O_2-induced decrease in cell viability may at least partly result from its antioxidant and free radical scavenging properties. Their antioxidant activities at least partly due to their $O^{\bullet}_2$ and H_2O_2 radical scavenging as well as metal ion chelating activities. The iron-complexing ability of mangiferin was reported as a primary mechanism for protection of liver mitochondria against Fe^{2+} citrate-induced lipid peroxidation (Halliwell and Gutteridge 1986 : 506) and protect mitochondria through its direct free radical scavenging ability and acting as a chain-breaking antioxidant (Prado-Andreu et al. 2005 : 48). By this mechanism, the anti-radical properties of polyphenols lead to the reduction of superoxide radicals (Rice-Evans 2001 : 798). They also showed that *in vitro* antioxidant activity of mangiferin related to its iron-chelating properties and not merely due to the scavenging activity of free radicals. Iron chelators such as mangiferin could be an important approach to reduce iron-induced oxidative damage in pathologies related to abnormal intracellular iron distribution and/or iron overload, such as hereditary hemochromatosis, h-thalassemia, Friedreich's ataxia, and sideroblastic anemia (Britton et al. 2002 : 221).

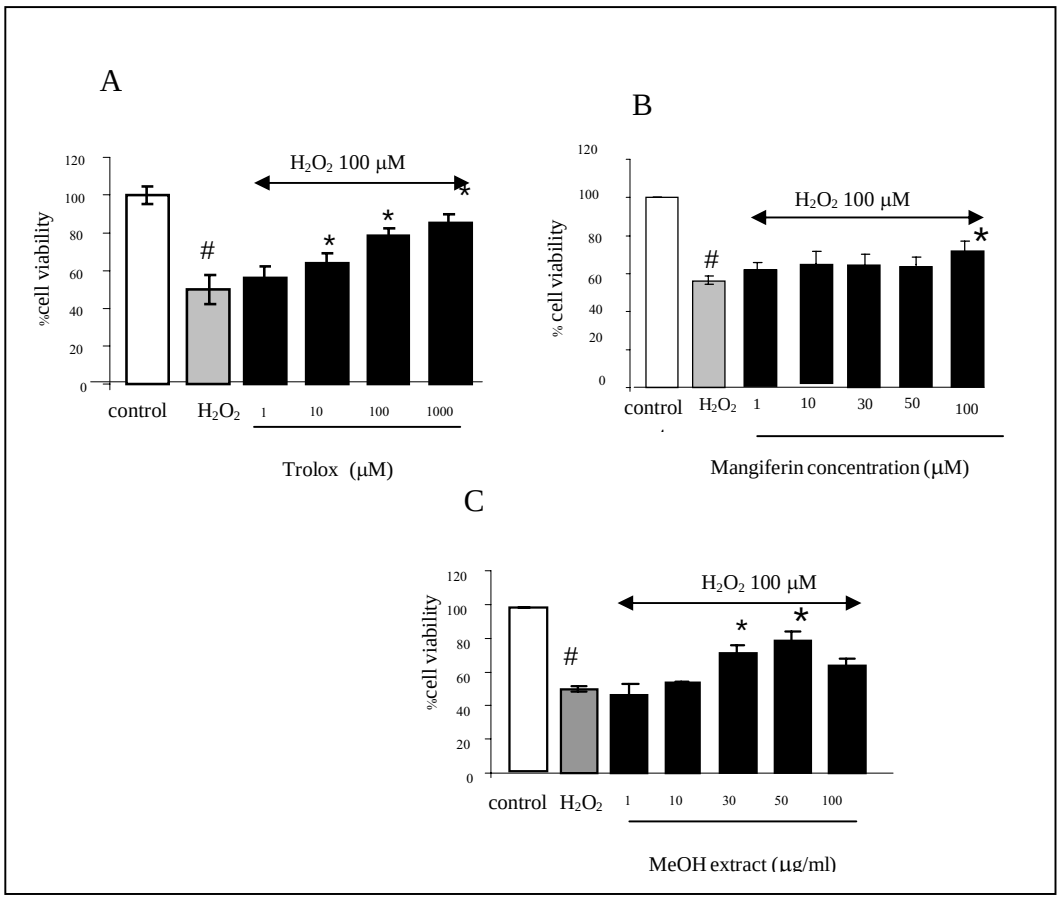


Figure 12 Effects of test compounds on H_2O_2-induced cell damage in NG108-15 mangiferin (B) and methanol extracts from *M. indica* leaf (Namdokmai) (C) for 2 h, then stock solution of H_2O_2 was added. After 2 h of incubation, cell viability was measured by the MTT assay. Data are expressed as means ± S.D. (n = 5). # $p \leq 0.05$ compared to the control group. * $p \leq 0.05$ compared to the H_2O_2-treated group.

L-Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system but excessive amounts of glutamate in the synaptic cleft cause over-activation of postsynaptic neurons. This process is known as excitotoxicity and provokes a massive influx of calcium, alterations in the mitochondrial membrane potential and increase the production of reactive oxygen species (ROS), which ultimately kills neurons and glia cells (Choi 1995 : 59; Matute et al. 2002 : 241). It has been suggested that excitotoxicity is the major mechanism contributing to the neurodegeneration involved in stroke and trauma, and other progressive neurological disorders such as Parkinson's disease and Alzheimer's disease (Mattson et al. 1999 : 156; Michaelis 2003 : 899). However, glutamate receptor antagonists have not been effective in clinical trials and new therapeutic approaches have to be developed. Glutamate induced neuronal death in a concentration-dependent manner at 10 and 100 mM (Fig. 13A) but the extract at 1-100 μg/ml could not protect the cell against the neurotoxicity when the cells were pretreated with the extract for 30 min and then exposured to 10 mM glutamate for 22 h (Fig. 13B and C). Moreover, the extract at 100 μg/ml significantly decreased the cell viability compared to glutamate-treated group (Fig. 13 C). It might result from its own toxicity or synergistic effect of the extract and glutamate. Further experiments especially those on cytotoxicity of the extract in this protocol should be performed.

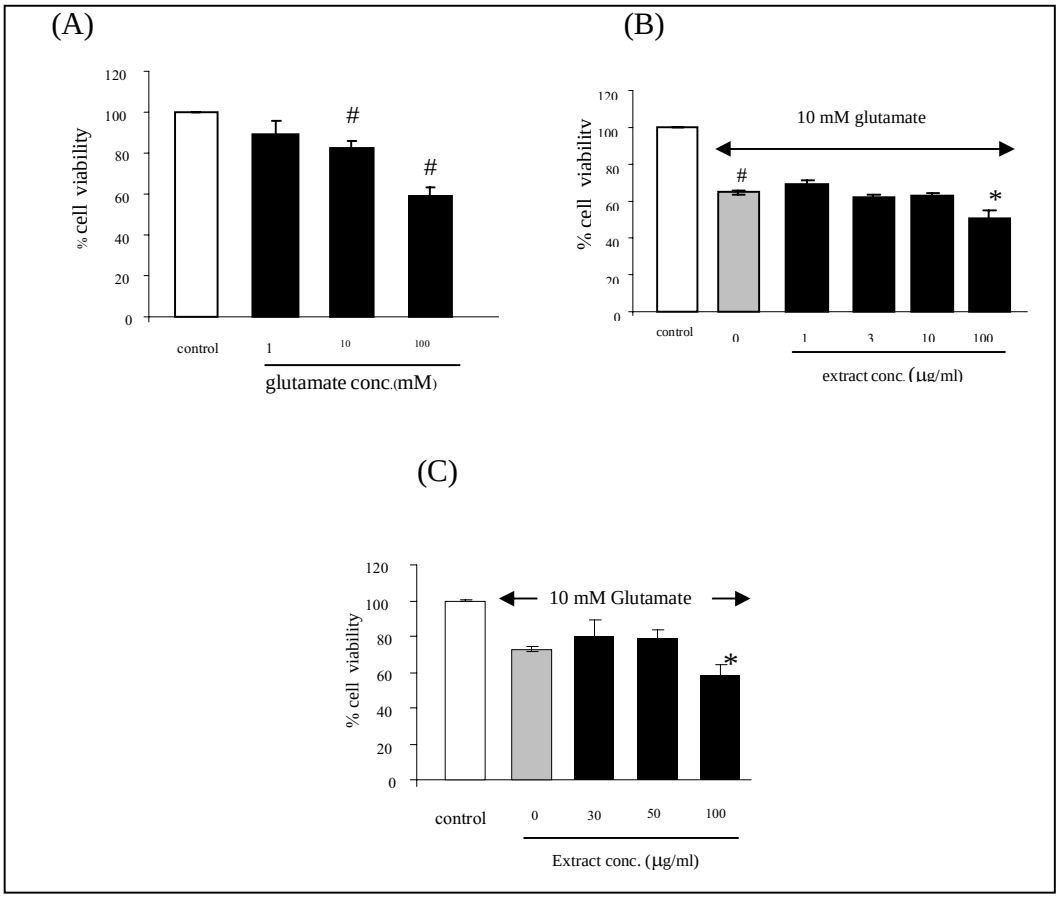


Figure 13 Effects of the extract on glutamate-induced cell damage in NG108-15 cells. NG108-15 cells were reated with various concentrations of glutamate (A) and the methanol extracts from *M. indica* cv.Namdokmai leaf with glutamate (B and C). The cells were pretreated with the extract for 30 min, and then stock solution of glutamate was added. After 22 h of incubation, cell viability was measured by the MTT assay. * $p \leq 0.05$ compared to the glutamate-treated group # $p \leq 0.05$ compared to the control group.

Oxidative stress-induced neuronal cell death appears to involve in certain neurodegenerative disorders such as Alzheimer's and Parkinson's disease (PD) (Fahn and Cohen 1992 : 808; Behl et al. 1994 : 820). 6-OHDA, a hydroxylated derivative of dopamine that, possibly, is formed endogenously in patients with PD (Andrew et al. 1993 : 1178; Jellinger et al. 1995 : 301) induces common features of PD (Blum et al. 2001). 6-OHDA lesion triggers nigral cell degeneration in rats with the production of high levels of ROS (Permual et al. 1989 : 139; Kumar 1995 : 1705). In this study treatments of NG108-15 cells with 6-OHDA resulted in significant neuronal death (Fig. 14A), however the pretreatment with methanol extract (100 μg/ml) for 2 hr prior to the exposure to 6-OHDA increased neuronal viability ranging from 51.46% to 63.75% (0, 100 μg/ml) (Fig. 14B). Anyway, more studies and information are needed to reveal the protective effect of the extract on 6-OHDA-induced cell death because 1) the cytoprotective effect of the extract at 100 μg/ml was a little bit weak 2) at this concentration, it could not protect cell from hydrogen peroxide-induced cell death and 3) lack of information of the extract at 30-50 μg/ml which showed the highest cytoprotective effect against hydrogen peroxide-induced cell death. The further investigation should be performed using the extract at 10-100 μg/ml in order to verify neuroprotective effect of extract on 6-OHDA-induced cell death.

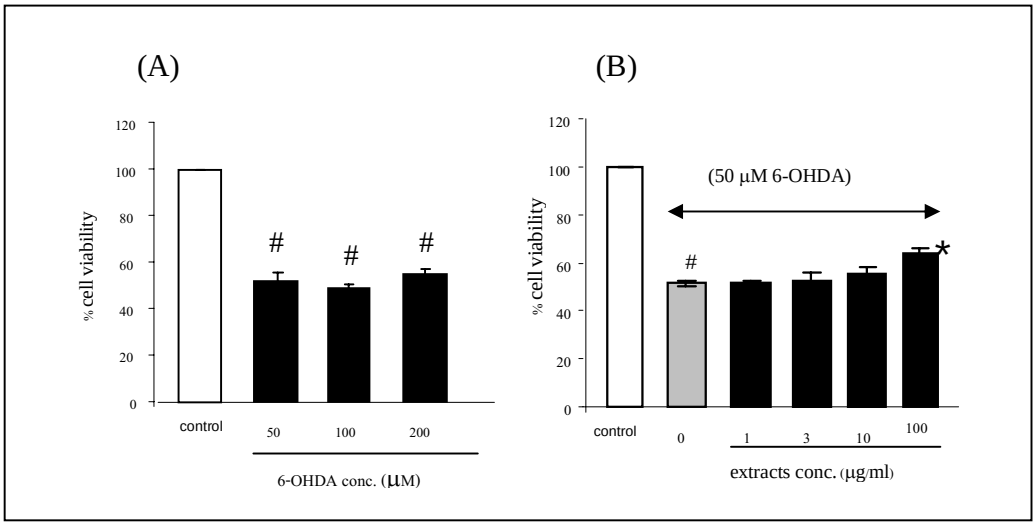


Figure 14 Effects of the extract on 6-OHDA -induced cell damage in NG108-15 cells. NG108-15 cells were treated with various concentrations of 6-OHDA (A) and the methanol extracts from *M. indica* cv. Namdokmai leaf (B). The cells were pretreated with the extract for 2 h, then stock solution of 6-OHDA was added. After 2 hr of incubation, cell viability was measured by MTT assay. # $p \leq 0.05$ compared to the control group. * $p \leq 0.05$ compared to the 6-OHDA -treated group

We here first reported that methanol extract was able to protect H_2O_2-induced neuronal death. The neuroprotective effect may be due to its antioxidant activity. Our findings suggest that these extracts may be benefit for further investigation on neurodegenerative disorders in which oxidative damage is implicated.

CHAPTER 5
CONCLUSIONS

The effect of cultivars on free radical scavenging (FRS) ability based on diphenyl-1-picryhydrazyl (DPPH) assay

In the preliminary studies on the effect of extraction solvent on FRS activity, methanol, water and chloroform extracts of *M. indica* leaf (Namdokmai) were evaluated. The methanol and water extracts exhibited much higher FRS activity than the chloroform extract. The methanol extract had the lowest IC_{50} value for the DPPH assay but was not statistically significantly different from the water extracts. Therefore, methanol was used as the extraction solvent for the investigation of the effect of cultivar i.e. Namdokmai, Aokrong, Muntawai and Fahlun on FRS activity. The IC_{50} values for the DPPH assay of these cultivars were not significantly different. The extract of Namdokmai leaves had the lowest IC_{50} value in the DPPH assay; therefore the methanol extract of *M. indica* (Namdokmai) leaves was used for the subsequent experiments.

Standardization of *M. indica* leaf extracts by quantification of total phenolic content and tannic acid content and some major bioactive components

It was found that the extract had high total phenolic and tannic acid content. By HPLC analysis of some bioactive compounds i.e. mangiferin, methylgallate and gallic acid, mangiferin was found as the highest component in all extracts. The methanol extracts had the highest total phenolic content, mangiferin and gallic acid, which correlated with the highest FRS activity. Anyway, while Aokrong extract had the highest amount of total phenolics, it did not show the highest FRS activity on DPPH assays. Thus, FRS activity may depend on bioactive components other than total phenolic content.

The mechanisms of antioxidative effect of standardized *M. indica* leaf extracts

The extract was not able to scavenge galvinoxyl radicals. Therefore, it had not hydrogen atom transfer (HAT) capability. However, it showed positive effects on DPPH, FRAP and ABTS assay, indicating that the extract had single electron transfer (SET) capabilities. For biological oxidant scavenging effect, we found that the extract could scavenge superoxide anion and hydrogen peroxide. In addition, it also exhibited metal ion chelating activity. However, it could not scavenge hydroxyl radical.

The neuroprotective effects of standardized *M. indica* leaf extracts on oxidant- and neurotoxin-induced cell death

The *M. indica* extracts could protect the neuronal cells from H_2O_2- and 6-OHDA -induced oxidative damage. This effect may be due to its antioxidant. These results suggest that the extract may be beneficial for the treatment of Parkinson's disease and neurodegenerative diseases associated with oxidative damage or stress.

In addition, one of biologically active component in *M. indica* leaf extract, mangiferin, could protect cells from H_2O_2-induced cell death. Thus, mangiferin may be the active component in preventing cells from H_2O_2-induced cell death.

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APPENDIX

APPENDIX A

To study effect of cultivars on free radical scavenging (FRS) ability by Diphenyl-1-picryhydrazyl (DPPH) assay

Calculation of free radical scavenging activities by DPPH assay of varies cultivars mango leaf extracts

The free radical scavenging activities by DPPH assay was expressed as IC_{50}. The IC_{50} were calculated by using the following example: For Figure 3, the following calculations were performed

$y = 21.741Ln(x) + 8.8843$ $(y = 50)$

$\ln (x) = (50-8.8843)/21.741$

$x = e^{(50-8.8843)/21.741}$

$x = 5.655$ μg/ml

$y = 21.741Ln(x) + 8.8843$, $R^2 = 0.9186$

Table 12 Data of DPPH assay of methanol extract of Namdokmai leaf in first experiment

Extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.705	0.702	0.723	0.710	
1	0.626	0.631	0.633	0.630	19.44
10	0.213	0.227	0.215	0.218	80.52
30	0.101	0.099	0.101	0.100	94.84
50	0.096	0.118	0.094	0.103	95.82
100	0.094	0.099	0.124	0.106	97.51
150	0.109	0.111	0.132	0.117	95.73
200	0.127	0.104	0.12	0.117	96.15

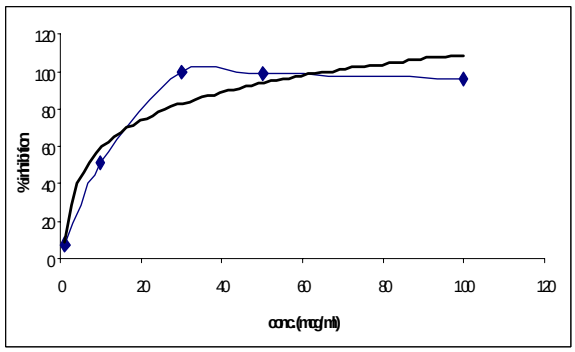
Table 13 Data of DPPH assay of methanol extract of Namdokmai leaf in second experiment

Methanol extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.694	0.706	0.735	0.712	
1	0.606	0.605	0.614	0.608	22.20
10	0.181	0.192	0.188	0.187	82.48
30	0.101	0.155	0.106	0.121	91.29
50	0.093	0.116	0.104	0.104	95.32
100	0.095	0.097	0.094	0.095	97.33
150	0.125	0.125	0.109	0.120	96.39
200	0.119	0.122	0.118	0.120	96.53

Table 14 Data of DPPH assay of methanol extract of Namdokmai leaf in third experiment

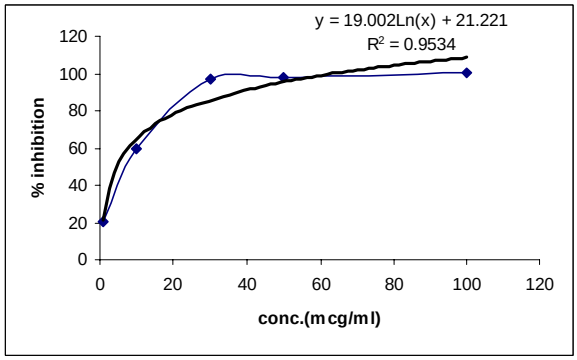
Methanol extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.694	0.706	0.735	0.712	
1	0.606	0.605	0.614	0.608	22.20
10	0.181	0.192	0.188	0.187	82.48
30	0.101	0.155	0.106	0.121	91.29
50	0.093	0.116	0.104	0.104	95.32
100	0.095	0.097	0.094	0.095	97.33
150	0.125	0.125	0.109	0.120	96.39
200	0.119	0.122	0.118	0.120	96.53

(a)



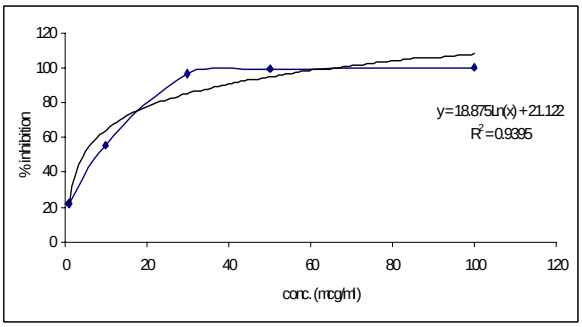
IC_{50} =5.655 µg/ml

(b)



IC_{50} = 4.547 µg/ml

(c)



IC_{50} = 4.618 µg/ml

Figure 15 IC_{50} data of DPPH assay of methanol extract of Namdokmai leaf; (a) first, (b) second and (c) third experiments

Table 15 Data of DPPH assay of water extract of Namdokmai leaf in first experiment

Water extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.689	0.685	0.677	0.684	
1	0.718	0.656	0.657	0.677	8.43
10	0.41	0.355	0.335	0.367	56.90
30	0.309	0.277	0.265	0.284	69.30
50	0.152	0.138	0.13	0.140	91.50
100	0.108	0.105	0.117	0.110	96.06
150	0.133	0.126	0.131	0.130	96.81
200	0.131	0.13	0.129	0.130	97.42

Table 16 Data of DPPH assay of water extract of Namdokmai leaf in second experiment

Water extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.674	0.675	0.666	0.672	
1	0.693	0.679	0.682	0.685	7.44
10	0.482	0.402	0.356	0.413	49.18
30	0.303	0.247	0.229	0.260	70.77
50	0.2	0.148	0.124	0.157	87.54
100	0.126	0.112	0.114	0.117	94.44
150	0.15	0.141	0.126	0.139	93.90
200	0.14	0.133	0.13	0.134	95.58

Table 17 Data of DPPH assay of water extract of Namdokmai leaf in third experiment

Water extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.694	0.706	0.735	0.712	
1	0.606	0.605	0.614	0.608	22.20
10	0.181	0.192	0.188	0.187	82.48
30	0.101	0.155	0.106	0.121	91.29
50	0.093	0.116	0.104	0.104	95.32
100	0.095	0.097	0.094	0.095	97.33
150	0.125	0.125	0.109	0.120	96.39
200	0.119	0.122	0.118	0.120	96.53

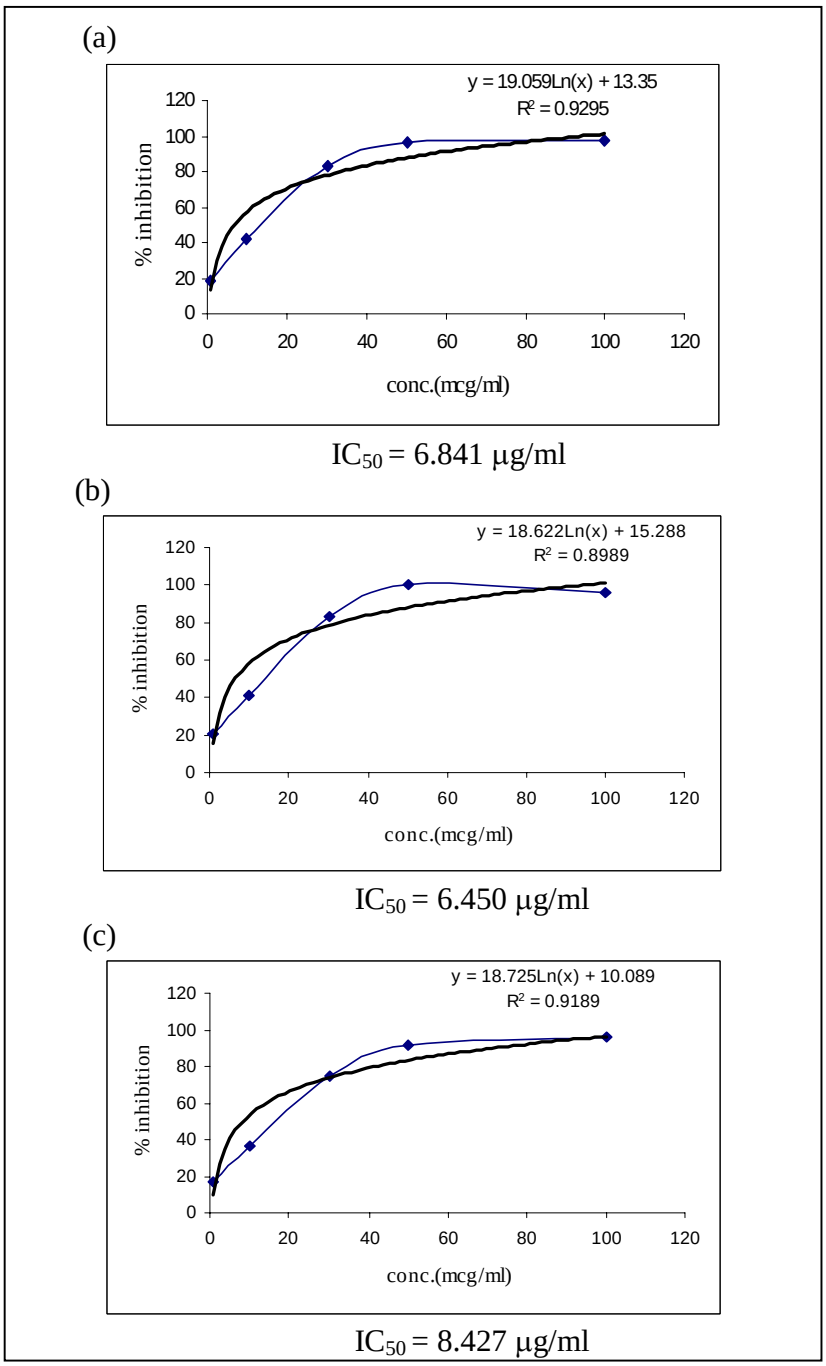


Figure 16 IC_{50} data of DPPH assay of water extract of Namdokmai leaf; (a) first, (b) second and (c) third experiments

Table 18 Data of DPPH assay of chloroform extract of Namdokmai leaf in first experiment

Chloroform extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.541	0.538	0.554	0.544	
1	0.483	0.492	0.505	0.493	22.05
10	0.398	0.396	0.4	0.398	41.46
30	0.334	0.333	0.331	0.333	53.64
50	0.243	0.233	0.243	0.240	74.65
100	0.147	0.138	0.147	0.144	96.75
150	0.19	0.174	0.19	0.185	95.77
200	0.19	0.194	0.219	0.201	98.22

Table 19 Data of DPPH assay of chloroform extract of Namdokmai leaf in second experiment

Chloroform extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.55	0.535	0.547	0.544	
1	0.724	0.735	0.721	0.727	-16.54
10	0.632	0.621	0.617	0.623	5.51
30	0.553	0.543	0.548	0.548	16.73
50	0.445	0.414	0.405	0.421	44.36
100	0.384	0.365	0.36	0.370	57.29
150	0.23	0.205	0.185	0.207	93.50
200	0.221	0.218	0.205	0.215	96.45

Table 20 Data of DPPH assay of chloroform extract of Namdokmai leaf in third experiment

Chloroform extract conc.(μg/ml)	sam+DPPH				%inhibition
	1	2	3	average	
control	0.694	0.706	0.735	0.712	
1	0.606	0.605	0.614	0.608	22.20
10	0.181	0.192	0.188	0.187	82.48
30	0.101	0.155	0.106	0.121	91.29
50	0.093	0.116	0.104	0.104	95.32
100	0.095	0.097	0.094	0.095	97.33
150	0.125	0.125	0.109	0.120	96.39
200	0.119	0.122	0.118	0.120	96.53

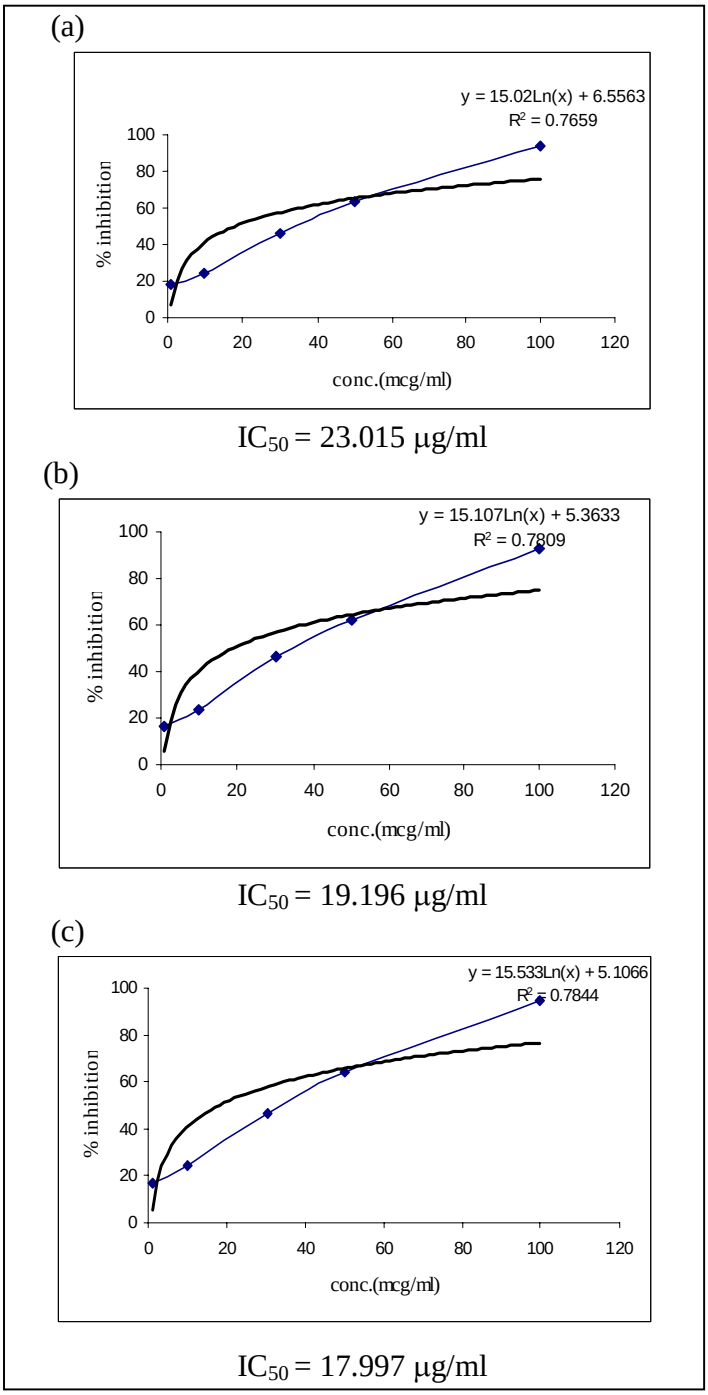


Figure 17 IC_{50} data of DPPH assay of chloroform extract of Namdokmai leaf; (a) first, (b) second and (c) third experiments

Table 21 Data of DPPH assay of Muntawai extract in first experiment

Muntawai conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.411	0.398	0.39	0.400	
1	0.051	0.054	0.062	0.056	10.16667
5	0.057	0.055	0.059	0.057	23.08333
20	0.051	0.056	0.067	0.058	61.41667
40	0.077	0.057	0.065	0.066	89.33333
100	0.094	0.063	0.064	0.074	90.08333

Table 22 Data of DPPH assay of Muntawai extract insecond experiment

Muntawai conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.384	0.383	0.418	0.395	
1	0.044	0.047	0.048	0.046	15.6962
5	0.048	0.055	0.049	0.051	31.22363
20	0.045	0.048	0.048	0.047	69.19831
40	0.06	0.055	0.053	0.056	86.75105
100	0.055	0.056	0.058	0.056	92.1519

Table 23 Data of DPPH assay of Muntawai extract third experiment

Muntawai conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.381	0.381	0.389	0.384	
1	0.046	0.049	0.055	0.050	11.37153
5	0.052	0.049	0.05	0.050	20.39931
20	0.056	0.048	0.048	0.051	57.20486
40	0.047	0.048	0.056	0.050	87.23958
100	0.055	0.055	0.086	0.065	88.02083

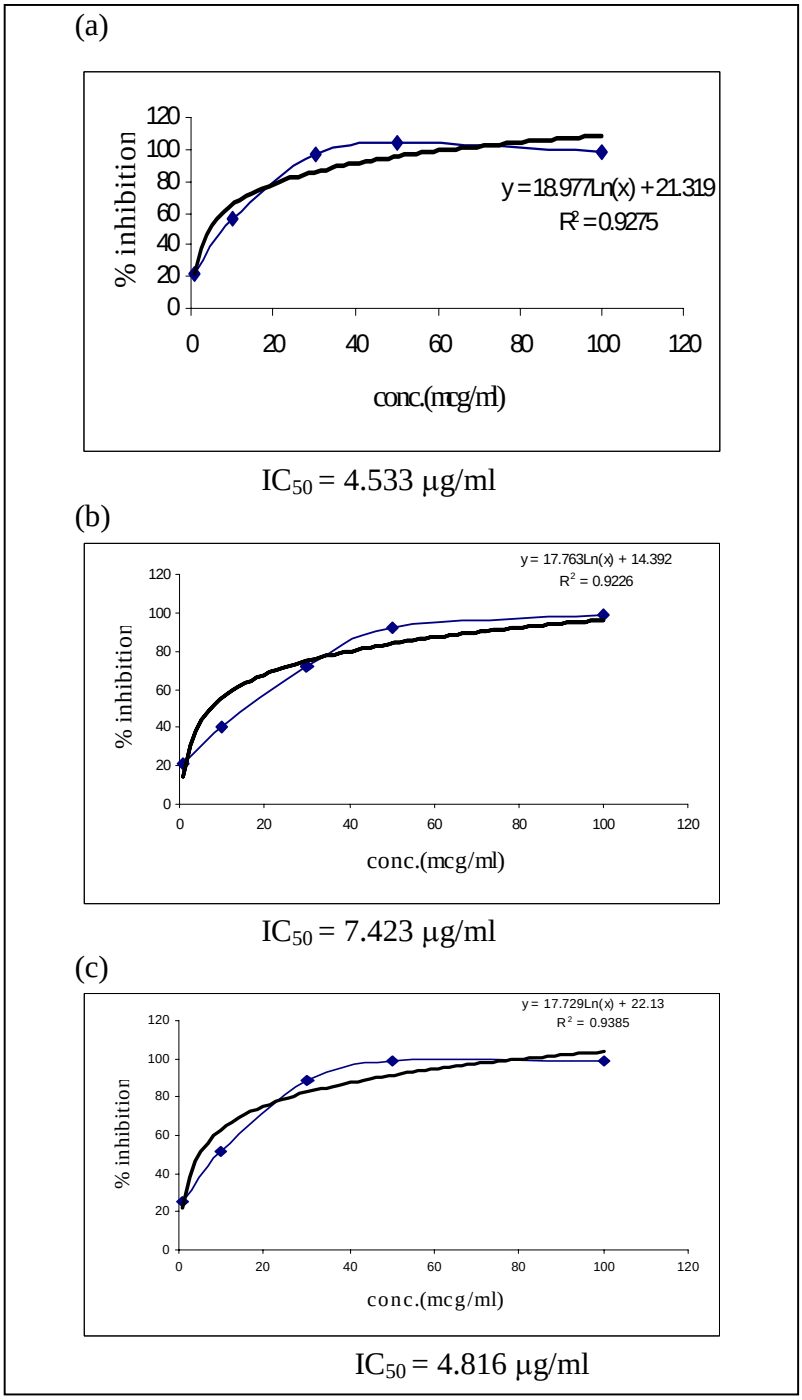


Figure 18 IC_{50} data of DPPH assay of methanol extract of Muntawai leaf; (a) first, (b) second and (c) third experiments

Table 24 Data of DPPH assay of Fahlun leaf extract in first experiments

Fahlun conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.411	0.398	0.39	0.400	
1	0.067	0.052	0.062	0.060	21.66667
5	0.063	0.062	0.061	0.062	35.75
20	0.077	0.06	0.063	0.067	68.08333
40	0.094	0.058	0.059	0.070	83.41667
100	0.068	0.068	0.063	0.066	92.08333

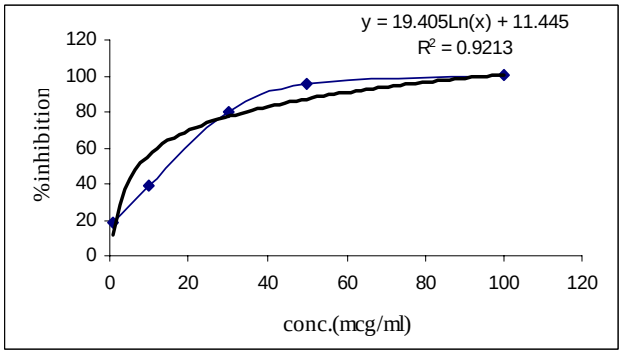
Table 25 Data of DPPH assay of Fahlun leaf extract in second experiments

Fahlun conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.384	0.383	0.418	0.395	
1	0.049	0.052	0.047	0.049	19.57806
5	0.053	0.054	0.051	0.053	33.83966
20	0.06	0.064	0.056	0.060	71.64557
40	0.05	0.052	0.058	0.053	87.173
100	0.054	0.06	0.093	0.069	86.16034

Table 26 Data of DPPH assay of Fahlun leaf extract in third experiments

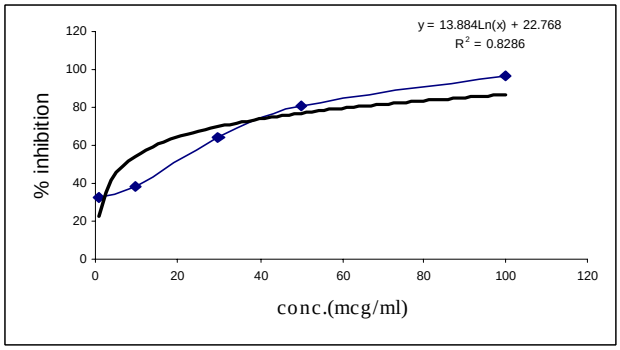
Fahlun conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.381	0.381	0.389	0.384	
1	0.048	0.046	0.045	0.046	17.36111
5	0.048	0.047	0.059	0.051	28.99306
20	0.05	0.046	0.047	0.048	65.625
40	0.07	0.058	0.076	0.068	90.27778
100	0.063	0.055	0.082	0.067	96.18056

(a)



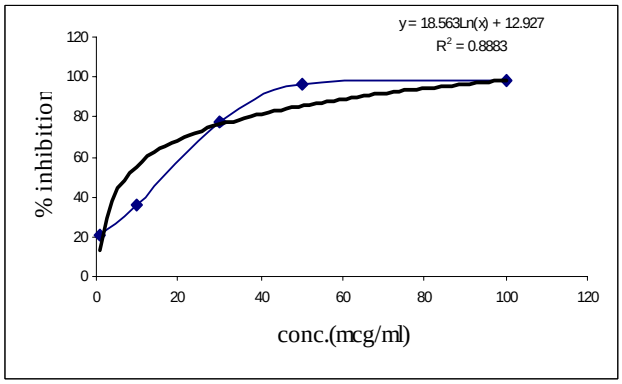
IC_{50} = 7.293 µg/ml

(b)



IC_{50} = 7.109 µg/ml

(c)



IC_{50} = 7.368 µg/ml

Figure 19 IC_{50} data of DPPH assay of methanol extract of Fahlun leaf; (a) first, (b) second and (c) third experiments

Table 27 Data of DPPH assay of Aoklong leaf extract in first experiments

Aoklong conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.411	0.398	0.39	0.400	
1	0.046	0.049	0.05	0.048	19.25
5	0.048	0.049	0.051	0.049	38.58333
20	0.052	0.048	0.066	0.055	83.83333
40	0.053	0.049	0.062	0.055	93.25
100	0.058	0.057	0.059	0.058	94.41667

Table 28 Data of DPPH assay of Aoklong leaf extract in second experiments

Aoklong conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.384	0.383	0.418	0.395	
1	0.054	0.049	0.059	0.054	18.14346
5	0.057	0.053	0.049	0.053	45.40084
20	0.049	0.061	0.059	0.056	89.70464
40	0.066	0.093	0.064	0.074	99.91561
100	0.054	0.061	0.068	0.061	97.29958

Table 29 Data of DPPH assay of Aoklong leaf extract in third experiments

Aoklong conc. (μg/ml)	sam+EtOH				%inhibition
	1	2	3	average	
control	0.381	0.381	0.389	0.384	
1	0.043	0.046	0.046	0.045	13.88889
5	0.047	0.053	0.048	0.049	37.15278
20	0.044	0.045	0.046	0.045	84.11458
40	0.047	0.046	0.094	0.062	96.875
100	0.051	0.055	0.058	0.055	94.79167

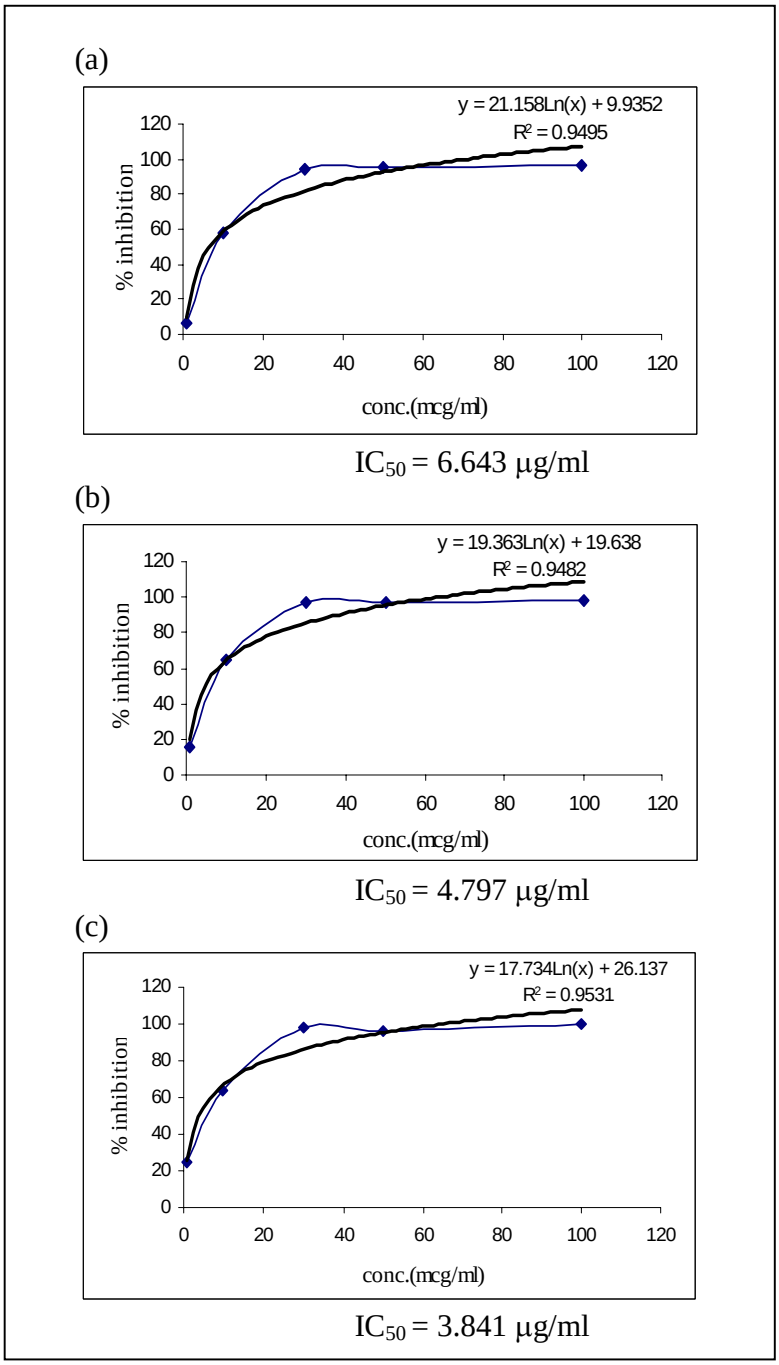


Figure 20 IC_{50} data of DPPH assay of methanol extract of Aokrong leaf; (a) first, (b) second and (c) third experiments

APPENDIX B

Standardization of *M. indica* leaf extracts by quantification of total phenolic content and tannic acid content and some major bioactive components

Calculation of Phenolic acid content by Folin' ciocuceu reagent method

Phenolic acid content was expressed as mg/ml. This calculated phenolic acid content by using the following example: For Table 13, the following calculations were performed

$y = 1.14x-0.0073$, $R^2= 0.9985$; linear equation of standard gallic acid

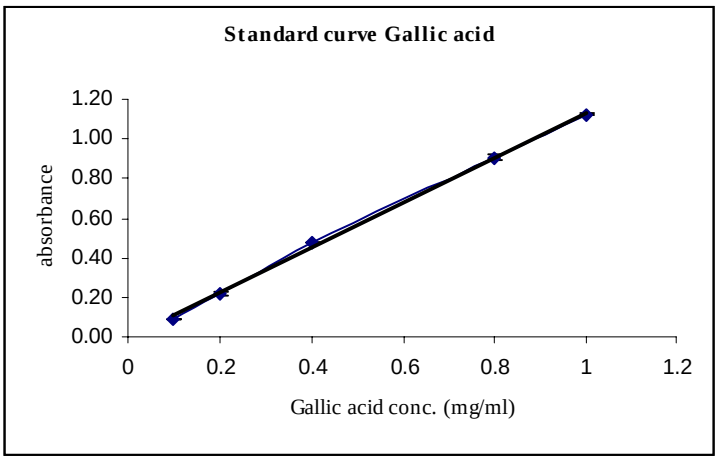
($y = 0.194$)

$x = (0.194+0.0073)/ 1.14$

$x = 0.176$ mg/ml

Table 30 Phenolic acid content of standard gallic acid by Folin' ciocuceu reagent method

Gallic acid	absorbance			Average	SD
conc. (mg/ml)	1	2	3		
0.1	0.090	0.087	0.089	0.089	0.00
0.2	0.209	0.232	0.219	0.220	0.01
0.4	0.476	0.479	0.476	0.477	0.00
0.8	0.919	0.888	0.903	0.904	0.02
1	1.123	1.126	1.124	1.124	0.00



$y = 1.14x-0.0073$, $R^2= 0.9985$

Figure 21 Standard curve of gallic acid by Folin'ciocultue reagent method

Table 31 Absorbance of Namdokmai leaf extracts of 3 solvents by Folin' ciocuceu reagent method for calculate total phenolic content

Extracts	abs.1	abs.2	abs.3	Average
Water	0.197	0.203	0.203	0.201
Water	0.204	0.214	0.207	0.209
Water	0.206	0.208	0.210	0.208
Methanol	0.426	0.483	0.489	0.466
Methanol	0.441	0.485	0.494	0.473
Methanol	0.442	0.492	0.492	0.475
Chloroform	0.095	0.101	0.101	0.099
Chloroform	0.100	0.108	0.107	0.105
Chloroform	0.097	0.104	0.106	0.102

Table 32 Total phenolic content of Namdokmai leaf extracts of 3 solvents by Folin'ciocuceu reagent method

Extracts	Gallic acid content (mg/ml)			Average	SD
	1	2	3		
water	0.183	0.189	0.188	0.187	0.004
methanol	0.415	0.422	0.423	0.420	0.004
chloroform	0.093	0.098	0.096	0.096	0.003

Table 33 Absorbance of mango leaf extracts of varies cultivars by Folin'ciocuceu reagent method

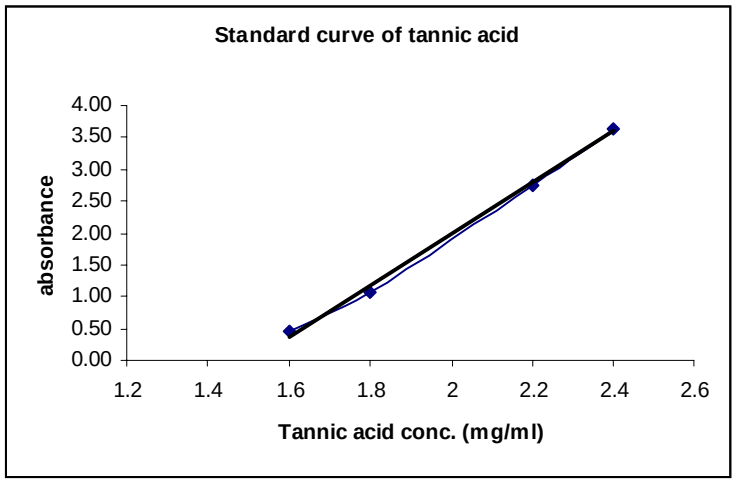
Cultivars	abs.1	abs.2	abs.3	Average
Namdokmai	0.426	0.483	0.489	0.466
	0.441	0.485	0.494	0.473
	0.442	0.492	0.492	0.475
Aokrong	0.454	0.449	0.448	0.450
	0.493	0.485	0.484	0.487
	0.486	0.483	0.482	0.484
Fahlun	0.289	0.290	0.905	0.495
	0.330	0.328	0.328	0.329
	0.342	0.342	0.342	0.342
Muntawai	0.327	0.324	0.324	0.325
	0.381	0.376	0.375	0.377
	0.373	0.371	0.370	0.371

Table 34 Total phenolic content of mango leaf extracts of varies cultivars by Folin'ciocuceu reagent method

Cultivars	Gallic acid content (mg/ml)			Average	SD
	1	2	3		
Namdokmai	0.415	0.422	0.423	0.420	0.004
Aoklong	1.843	1.844	1.853	1.846	0.004
Muntawai	1.898	1.899	1.887	1.895	0.006
Fahlun	1.892	1.839	1.848	1.860	0.023

Table 35 Tannic acid content of standard tannic acid

Tannic acid conc. (mg/ml)	Absorbance			Average	SD
	1	2	3		
1.6	0.4529	0.4529	0.4527	0.4528	0.00012
1.8	1.0642	1.065	1.0644	1.0645	0.00042
2.2	2.7453	2.7449	2.745	2.7451	0.00021
2.4	3.646	3.6458	3.6456	3.6458	0.00020



$y = 4.0332x-6.0894$, $R^2= 0.9964$

Figure 22 Standard curve of tannic acid

Table 36 Tannic acid content of Namdokmai leaf extracts of 3 solvents

Extracts	Tannic acid content (mg/ml)			Average	SD
	1	2	3		
water	1.626	1.644	1.633	1.634	0.0074
methanol	1.612	1.627	1.593	1.611	0.0139
chloroform	1.869	1.863	1.814	1.849	0.024

Table 37 Tannic acid content of mango leaf extracts of varies cultivars

Cultivars	Tannic acid content (mg/ml)			Average	SD
	1	2	3		
Namdokmai	1.612	1.627	1.593	1.611	0.014
Aoklong	1.843	1.844	1.853	1.846	0.004
Muntawai	1.898	1.899	1.887	1.895	0.006
Fahlun	1.892	1.839	1.848	1.860	0.023

Quantitative main polyphenol of mango leaves extracts by HPLC

Calculation of main polyphenol compounds by HPLC of varies mango leaf extracts

Main polyphenol compounds**,** Gallic acid, methyl gallate and mangiferin, was expressed as mg/ml.

This calculated mangiferin (MW. = 422.34 g/mol) by using the following example: For Table 21, the following calculations were perform

$y = 4568.8x - 0.4756$; $R^2 = 0.997$ (linear equation of mangiferin, y = 1558.593)

x = (1558.593+0.4756)/ 4568.8 x = 0.353 mM

stock solution of extract for injection is 1 mg/ml

solution 1000 ml have mangiferin 0.353 x 422.34 mg

if solution 1 ml " (0.353 x 422.34) /1000 = 0.149 mg x 1000

= 149.22 mg/g (dried)

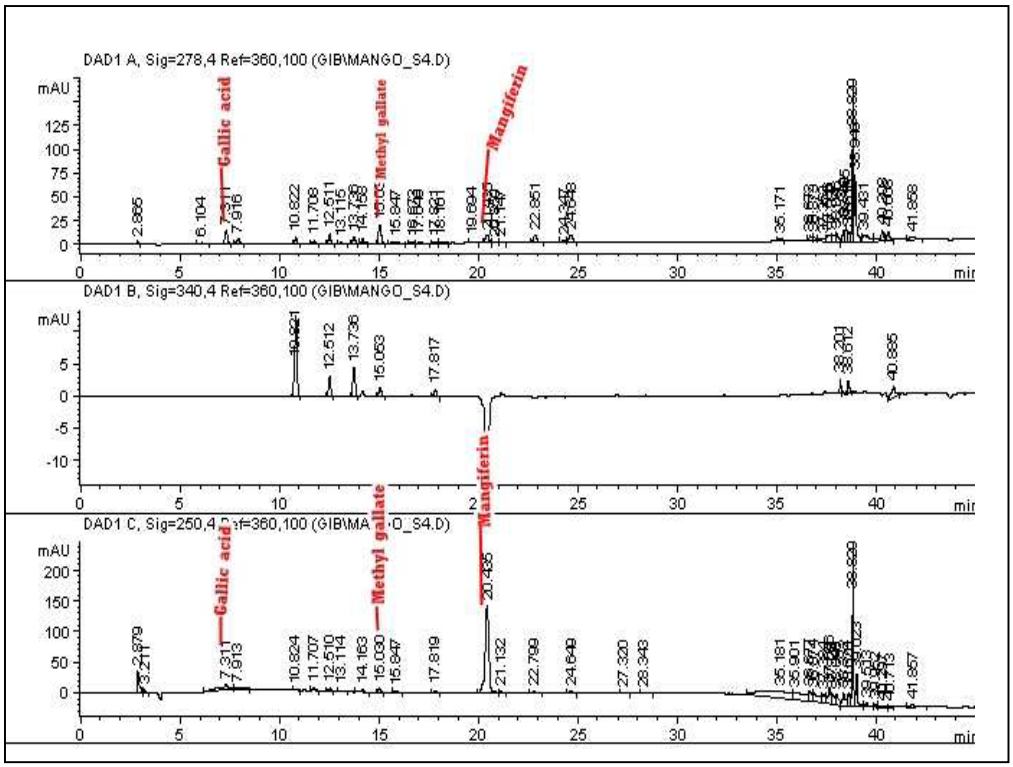


Figure 23 Chromatogram of Namdokmai methanol extract (1 mg/ml) by HPLC

Table 38 Quantitative main polyphenolic compound of Namdokmai methanol extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	113.382	0.056	9.545
Methyl gallate	195.766	0.075	13.873
Mangiferin	1558.593	0.353	149.222

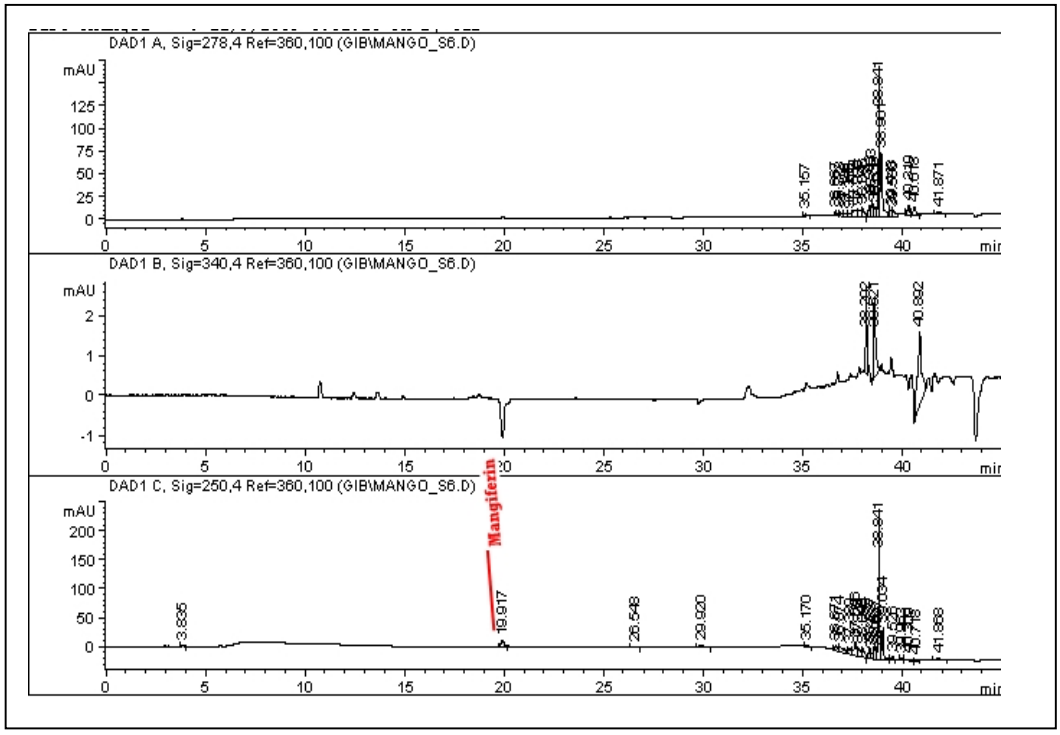


Figure 24 Chromatogram of Namdokmai chloroform extract (1 mg/ml) by HPLC

Table 39 Quantitative main polyphenolic compound of Namdokmai chloroform extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	7.252	nd	nd
Methyl gallate	6.423	nd	nd
Mangiferin	321.170	0.072	30.403

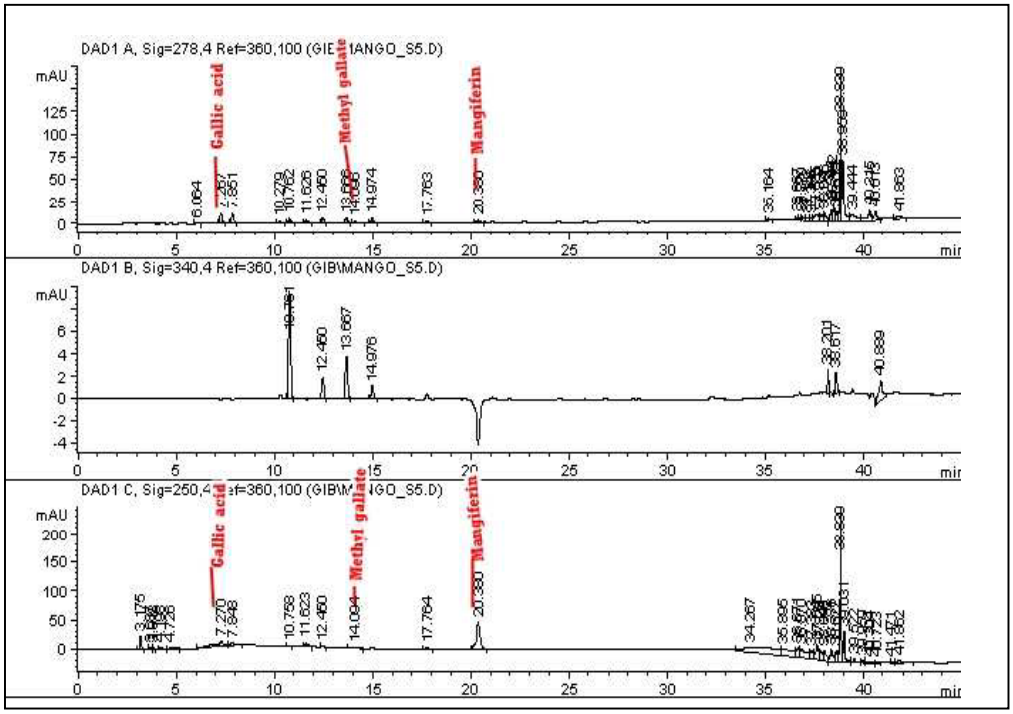


Figure 25 Chromatogram of Namdokmai water extract (1 mg/ml) by HPLC

Table 40 Quantitative main polyphenolic compound of Namdokmai water extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	129.446	0.063	10.786
Methyl gallate	63.794	0.023	4.274
Mangiferin	713.422	0.161	68.067

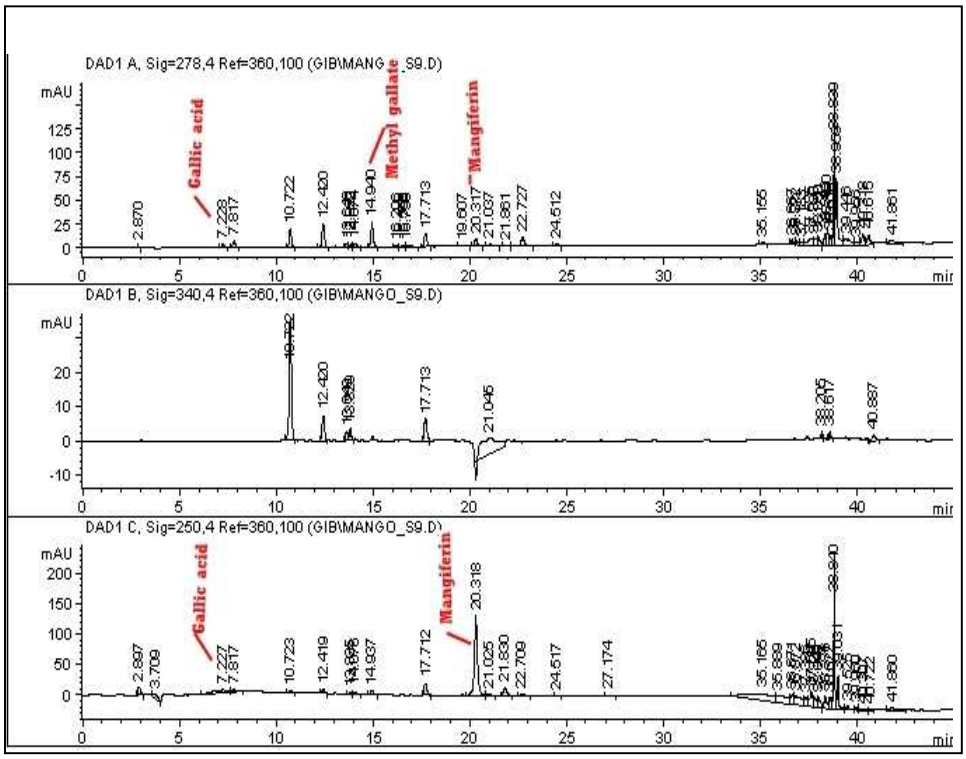


Figure 26 Chromatogram of Aoklong methanol extract (1 mg/ml) by HPLC

Table 41 Quantitative main polyphenolic compound of Aoklong methanol extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	43.734	0.024	4.162
Methyl gallate	248.434	0.096	17.704
Mangiferin	1499.031	0.340	143.503

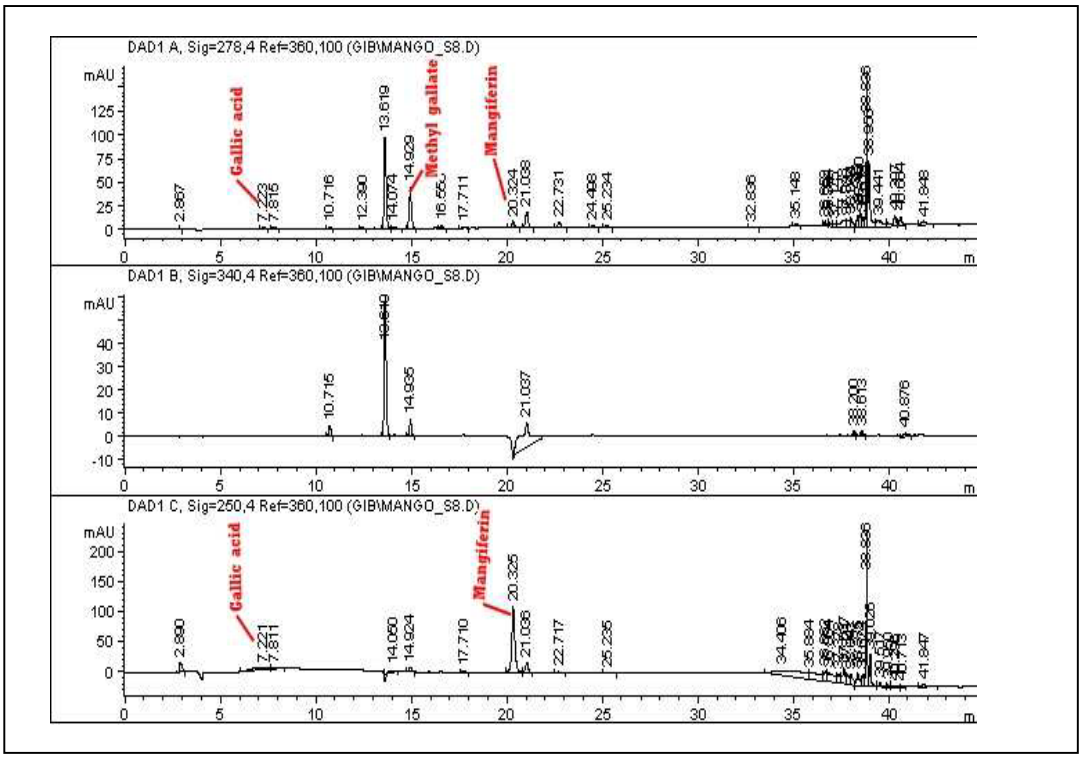


Figure 27 Chromatogram of Fahlun methanol extract (1 mg/ml) by HPLC

Table 42 Quantitative main polyphenolic compound of Fahlun methanol extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	23.769	0.015	2.619
Methyl gallate	415.062	0.162	29.825
Mangiferin	1323.032	0.300	126.603

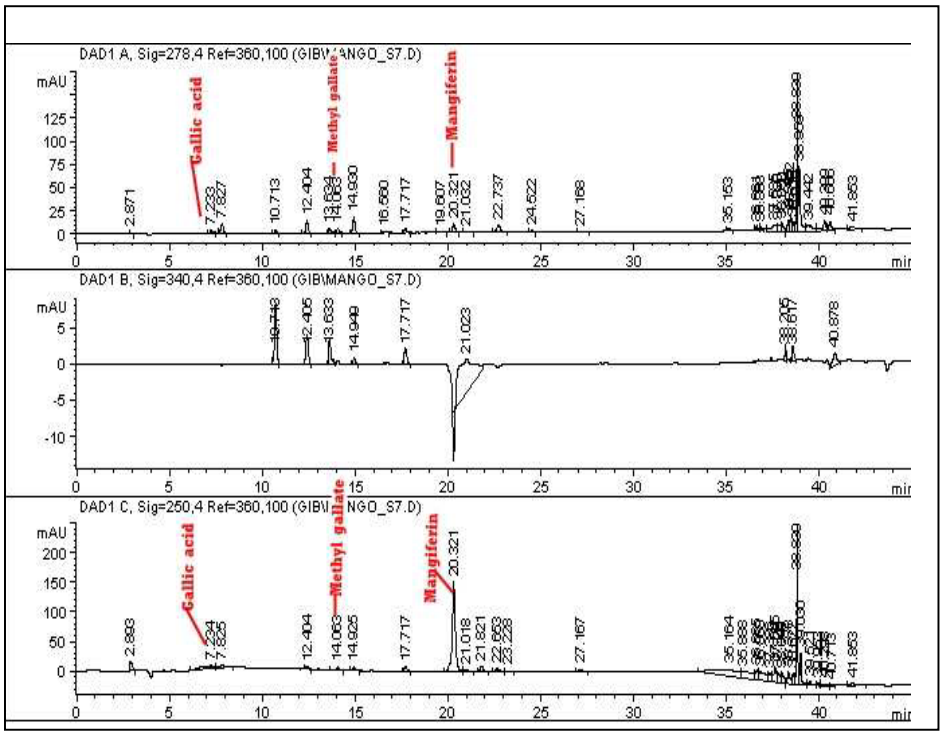


Figure 28 Chromatogram of Muntawai methanol extract (1 mg/ml) by HPLC

Table 43 Quantitative main polyphenol of Muntawai methanol extract by HPLC

Substrate	Area	content	
		mM	mg/g
Gallic aid	36.560	0.021	3.607
Methyl gallate	138.494	0.053	9.708
Mangiferin	1503.611	0.341	143.942

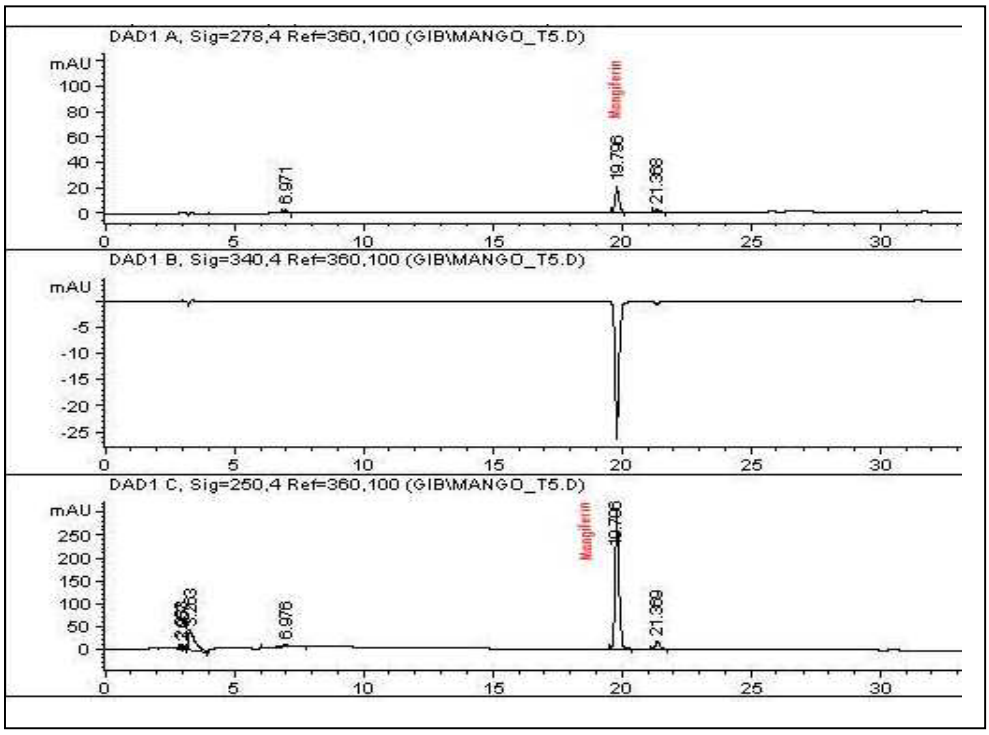
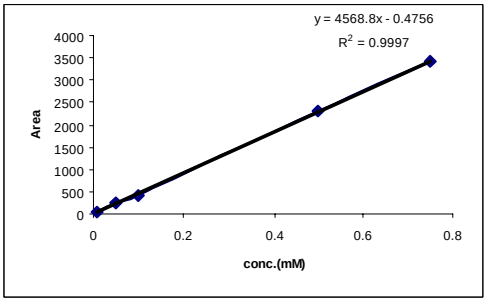


Figure 29 Chromatogram of standard mangiferin 0.75 mM by HPLC

Table 44 Area under the peak of standard mangiferin for standard curve by HPLC

Substrate	conc.(mM)	Area
Mangiferin (250 nm)	0.01	54.442
	0.05	250.396
	0.1	415.01
	0.5	2301.404
	0.75	3418.351



y= 4.568.8x-0.4756; R^2= 0.9997

Figure 30 Calibration curve of standard mangiferin by HPLC

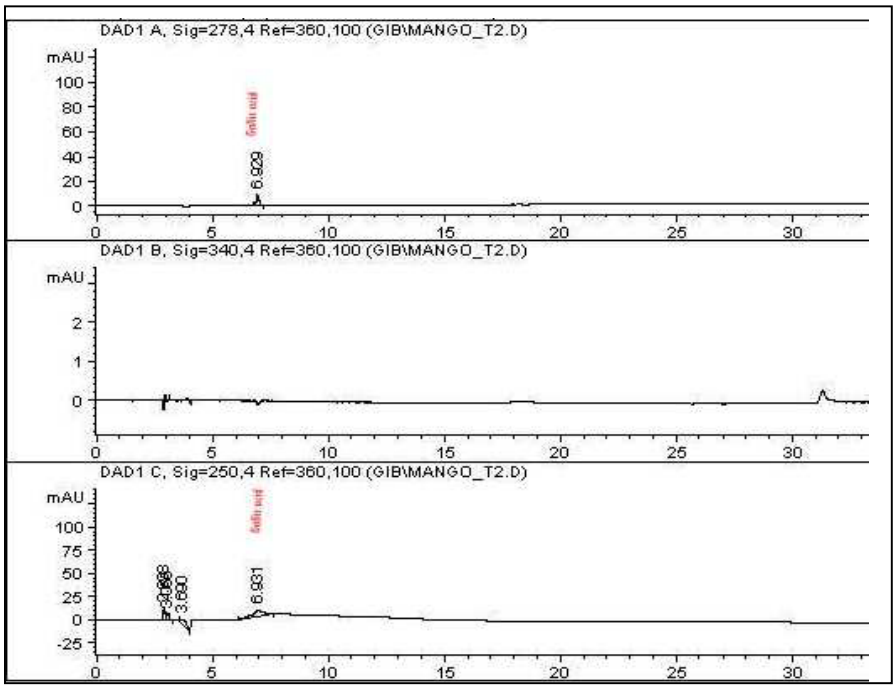
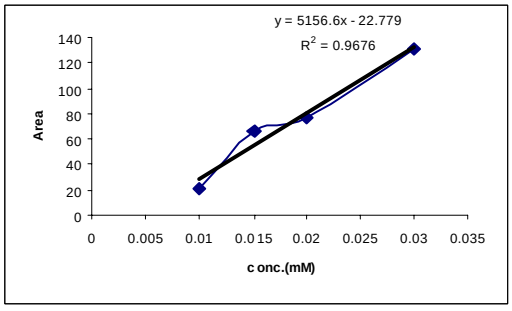


Figure 31 Chromatogram of standard gallic acid 0.03 mM by HPLC

Table 45 Area under the peak of standard gallic acid for standard curve by HPLC

substrate	conc.(mM)	Area
Gallic acid (278 nm)	0.01	21.781
	0.015	66.104
	0.02	77.065
	0.03	130.68



$y = 5156.6x - 22.779$; $R^2 = 0.9676$

Figure 32 Calibration curve of standard gallic acid by HPLC

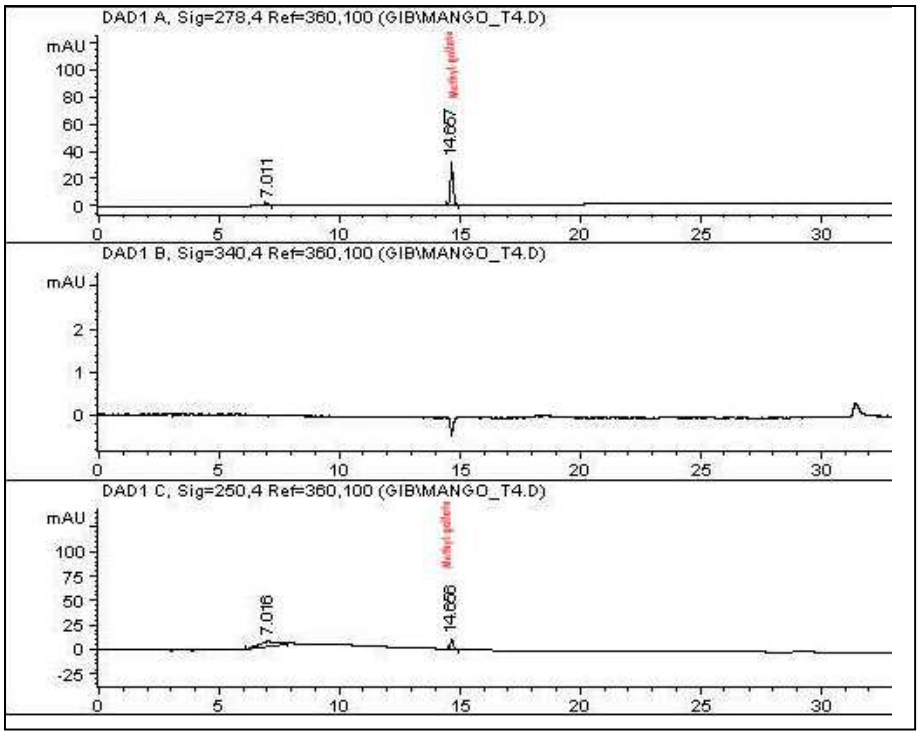
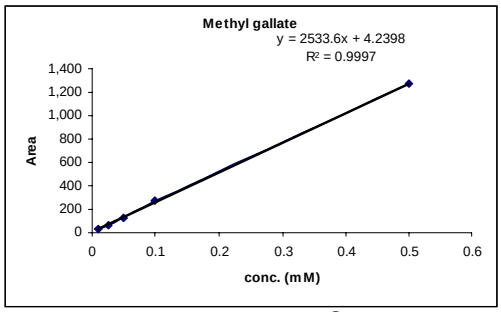


Figure 33 Chromatogram of standard methyl gallate 0.01 mM by HPLC

Table 46 Area under the peak of methyl gallate for standard curve by HPLC

substrate	conc.(mM)	Area
Methyl gallate (278 nm)	0.01	27.729
	0.025	59.367
	0.05	126.964
	0.1	274.072
	0.5	1268.607



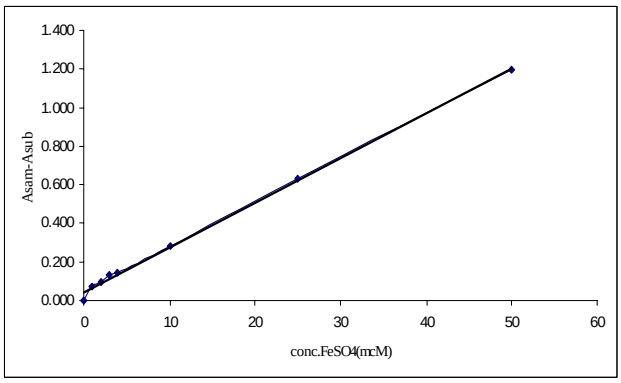
y= 2533.6x+4.2398; R^2= 0.9997

Figure 34 Calibration curve of standard methyl gallate by HPLC

APPENDIX C

To study underlying mechanisms of antioxidative effect of standardized *M. indica* leaf extracts

Calculation of free radical scavenging activities by FRAP assay of varies mango leaf extracts



$y = 0.00232x+0.044$, $R^2= 0.9978$

Figure 35 Standard curve of $FeSO_4$ for FRAP assay

The free radical scavenging activities by FRAP assay was expressed as EC_1, concentration of antioxidant that reduced Fe^{3+} -TPTZ equal 1 mM $FeSO_4$.

Calculated absorbance of 1mM $FeSO_4$ for Figure 9

$y = 0.00232(1000) + 0.044$

$y = 23.244$

The EC_1 were calculated by using the following example: For Figure 10a, the following calculations were performed

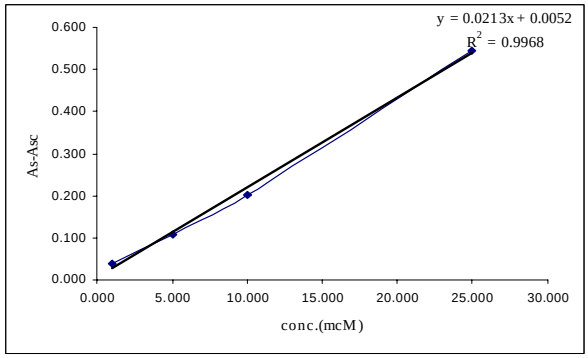
$y = 0.00213x + 0.0052$ ($y = 23.244$)

$x = (23.244-0.0052)/ 0.00213$

$x = 1091.023$ μM

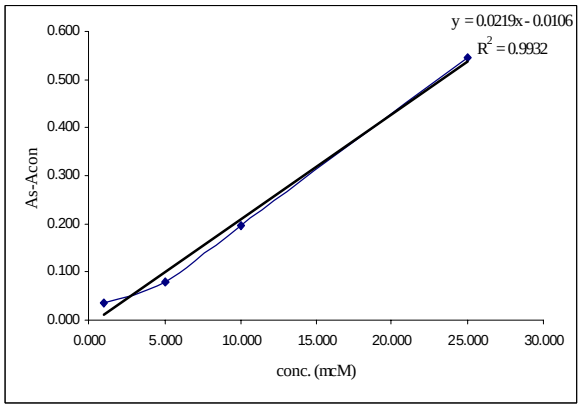
$= 272.756$ μg/ml (MW. Trolox = 250.29 mg/mol)

(a)



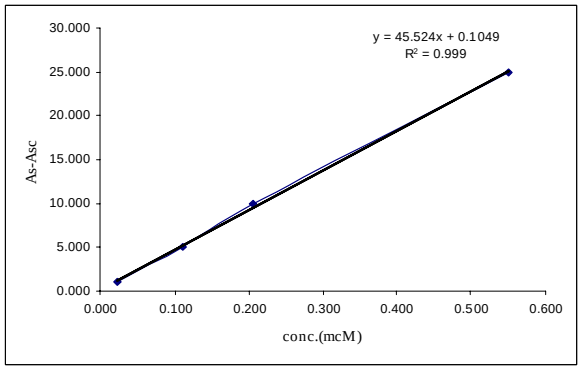
EC_1 = 272.756 µg/ml

(b)



EC_1 = 265.464 µg/ml

(c)



EC_1 = 267.942 µg/ml

Figure 36 Graph of Trolox for calculated EC_1 for FRAP; (a) first, (b) second and (c) third experiments

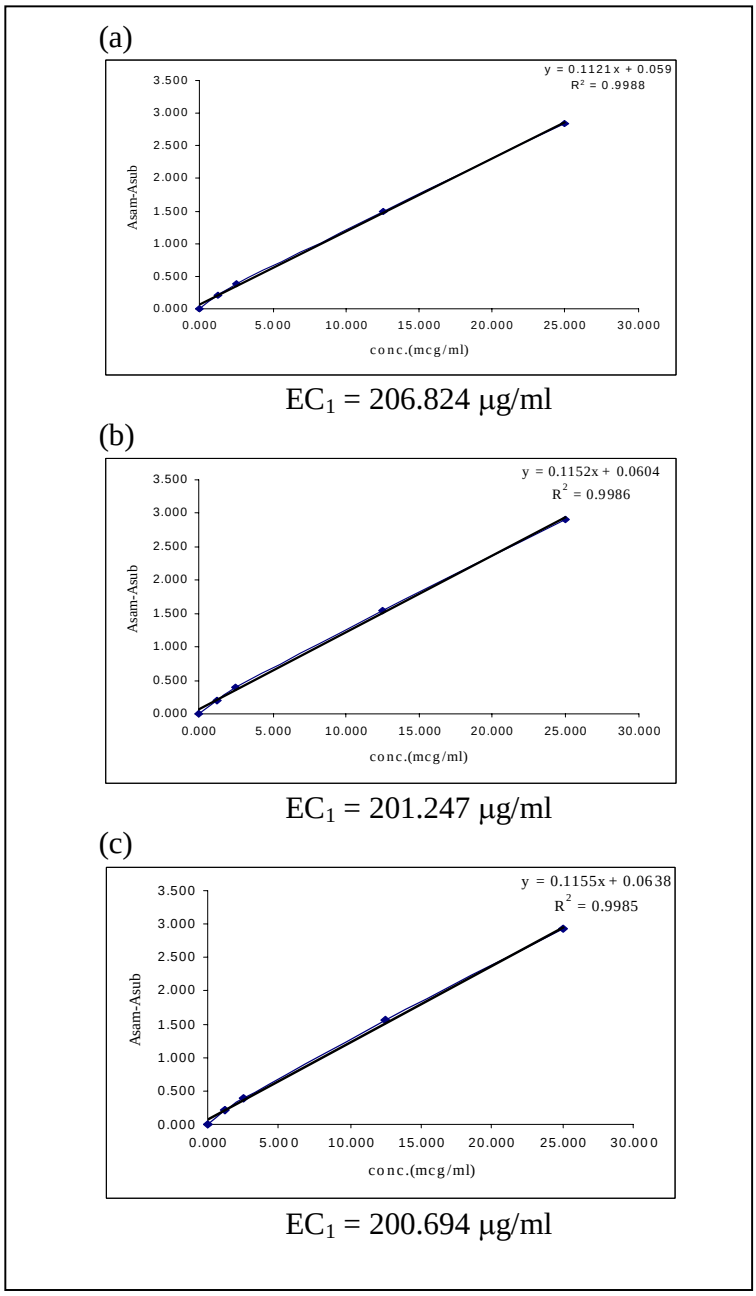
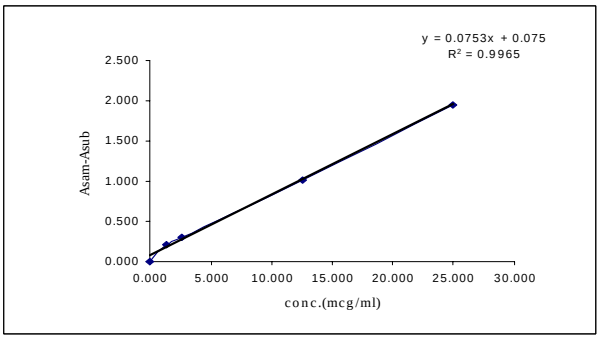


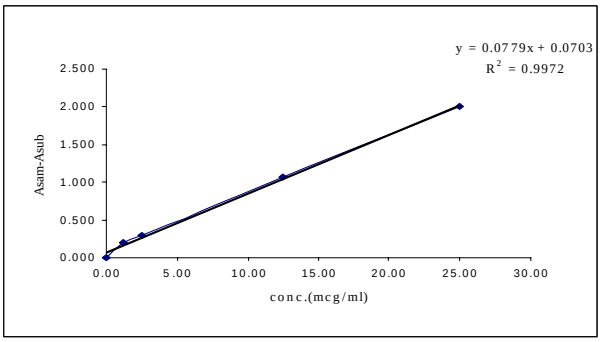
Figure 37 Graph of methanol extract of Namdokmai for calculated EC_1 for FRAP assay ; (a) first, (b) second and (c) third experiments

(a)



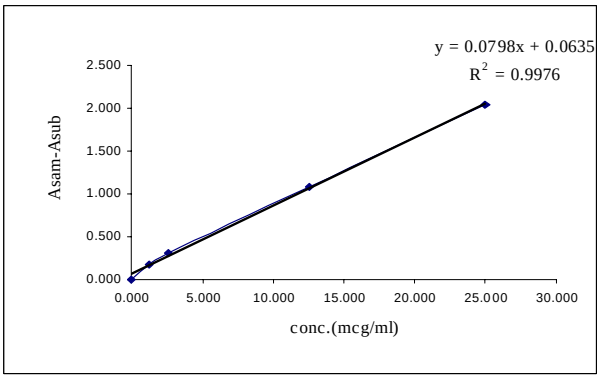
EC_1 = 307.689 µg/ml

(b)



EC_1 = 297.480 µg/ml

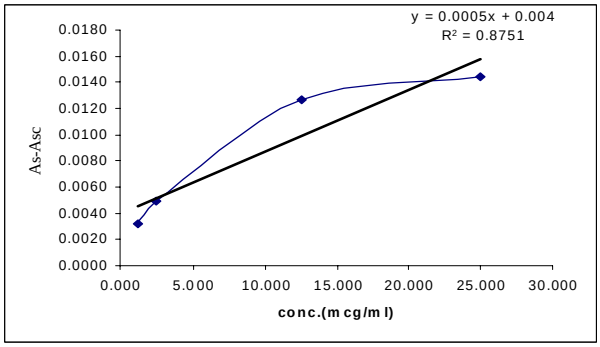
(c)



EC_1 = 290.483 µg/ml

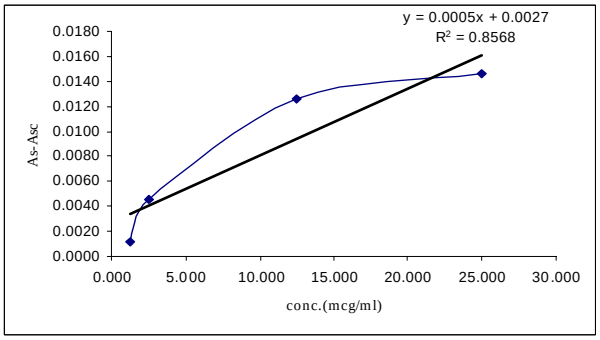
Figure 38 Graph of water extract of Namdokmai for calculated EC_1 for FRAP assay ; (a) first, (b) second and (c) third experiments

(a)



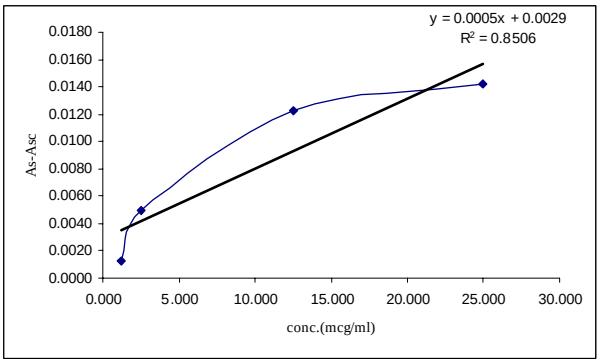
EC_1 = 46480.00 µg/ml

(b)



EC_1 = 46482.60 µg/ml

(c)



EC_1 = 46482.20 µg/ml

Figure 39 Graph of chloroform extract of Namdokmai for calculated EC_1 for FRAP assay; (a) first, (b) second and (c) third experiments

Calculation of free radical scavenging activities by ABTS assay of varies mango leaf extracts

The free radical scavenging activities by ABTS assay was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

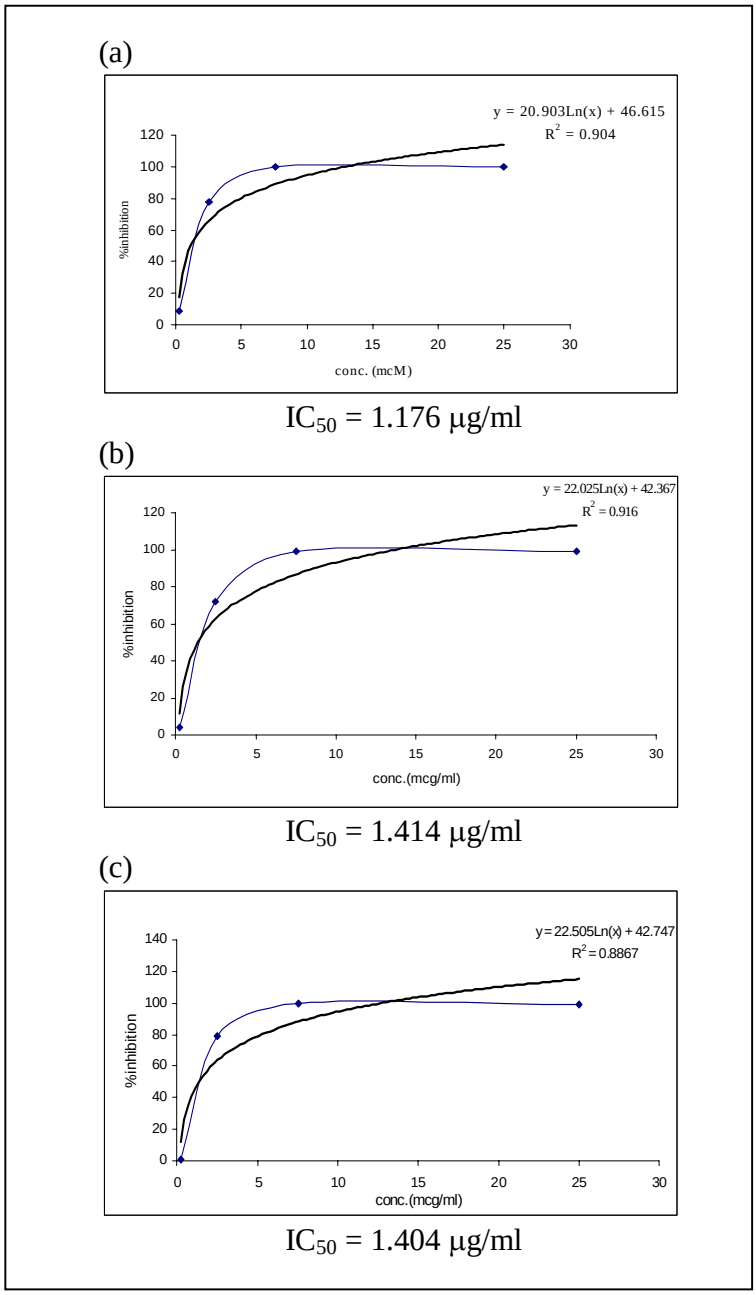
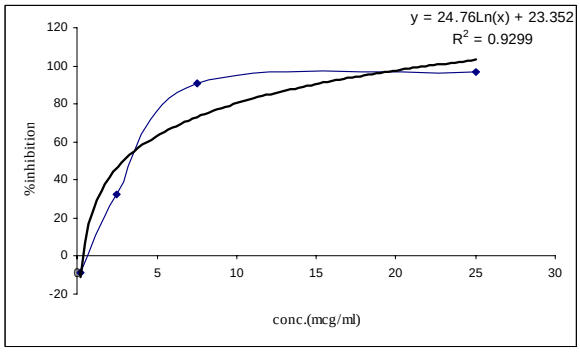


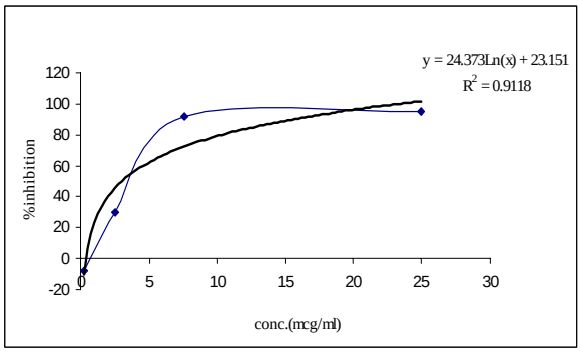
Figure 40 Graph of methanol extract of Namdokmai for calculated IC_{50} for ABTS scavenging assay; (a) first, (b) second and (c) third experiments

(a)



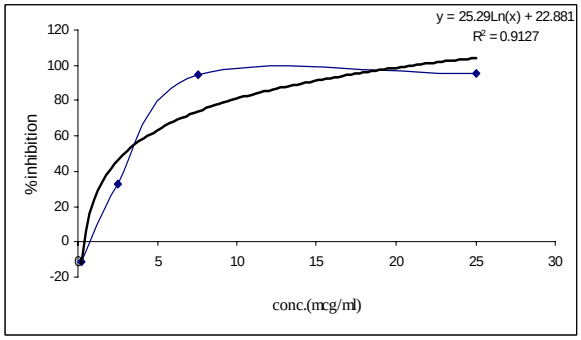
IC_{50} = 2.934 µg/ml

(b)



IC_{50} = 3.009 µg/ml

(c)



IC_{50} = 2.922 µg/ml

Figure 41 Graph of water extract of Namdokmai for calculated IC_{50} for ABTS scavenging assay; (a) first, (b) second and (c) third experiments

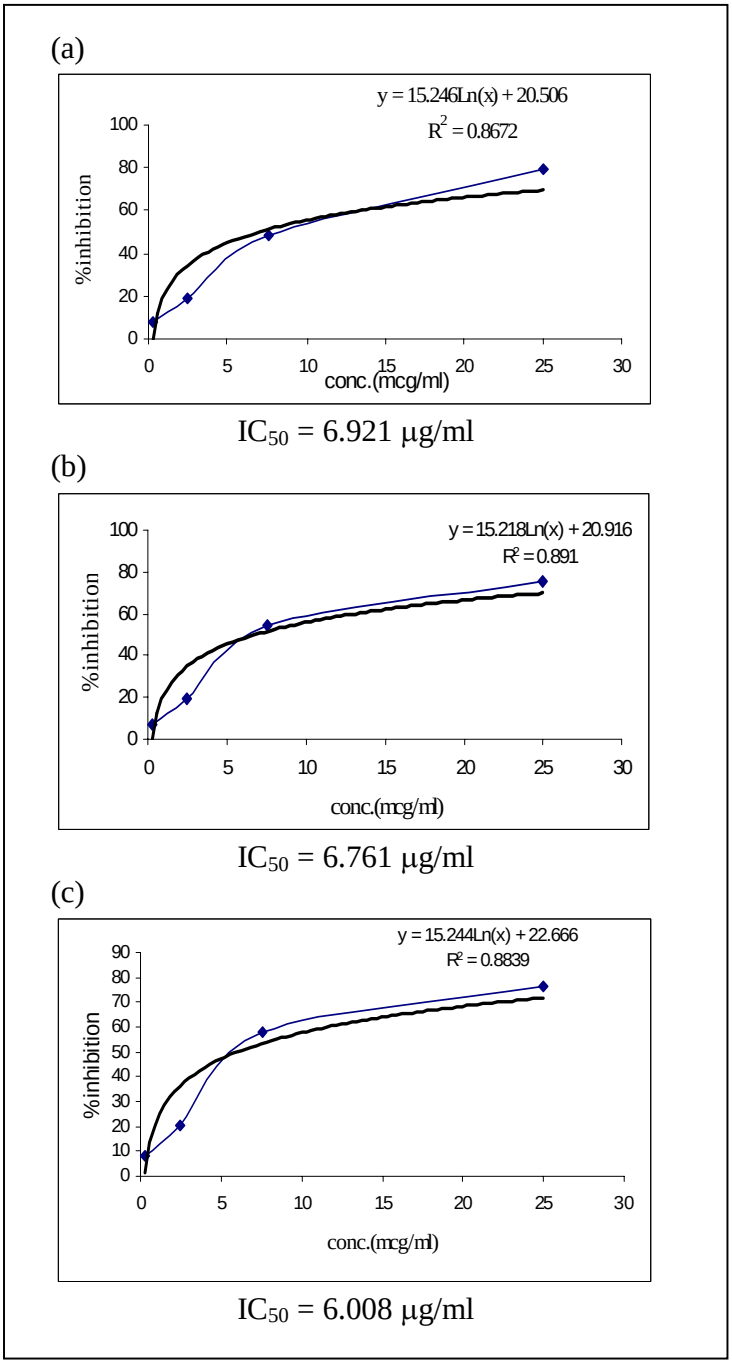


Figure 42 Graph of chloroform extract of Namdokmai for calculated IC_{50} for ABTS scavenging assay; (a) first, (b) second and (c) third experiments

Free radical scavenging activities by galvinoxyl scavenging assay of extract of Namdokmai leaf

The free radical scavenging activities by galvinoxyl scavenging assay was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

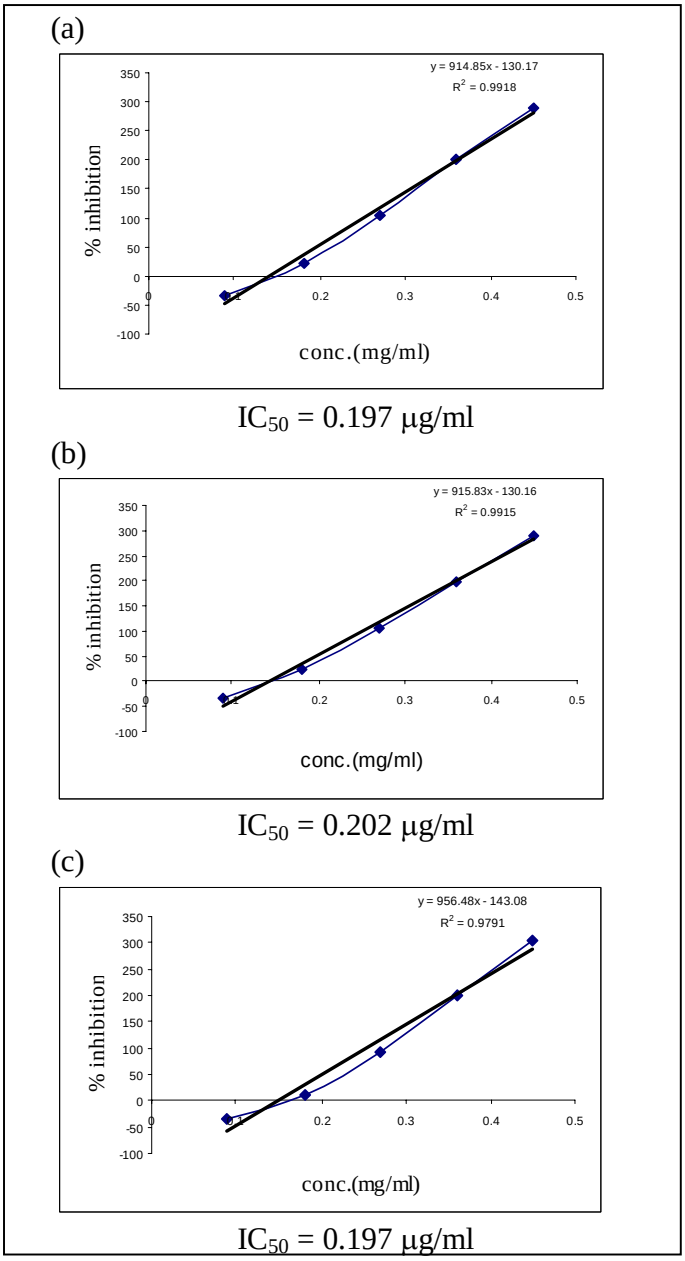


Figure 43 Graph of vitamin c for calculated IC_{50} for galvinoxyl scavenging assay ;(a) first, (b) second and (c) third experiments

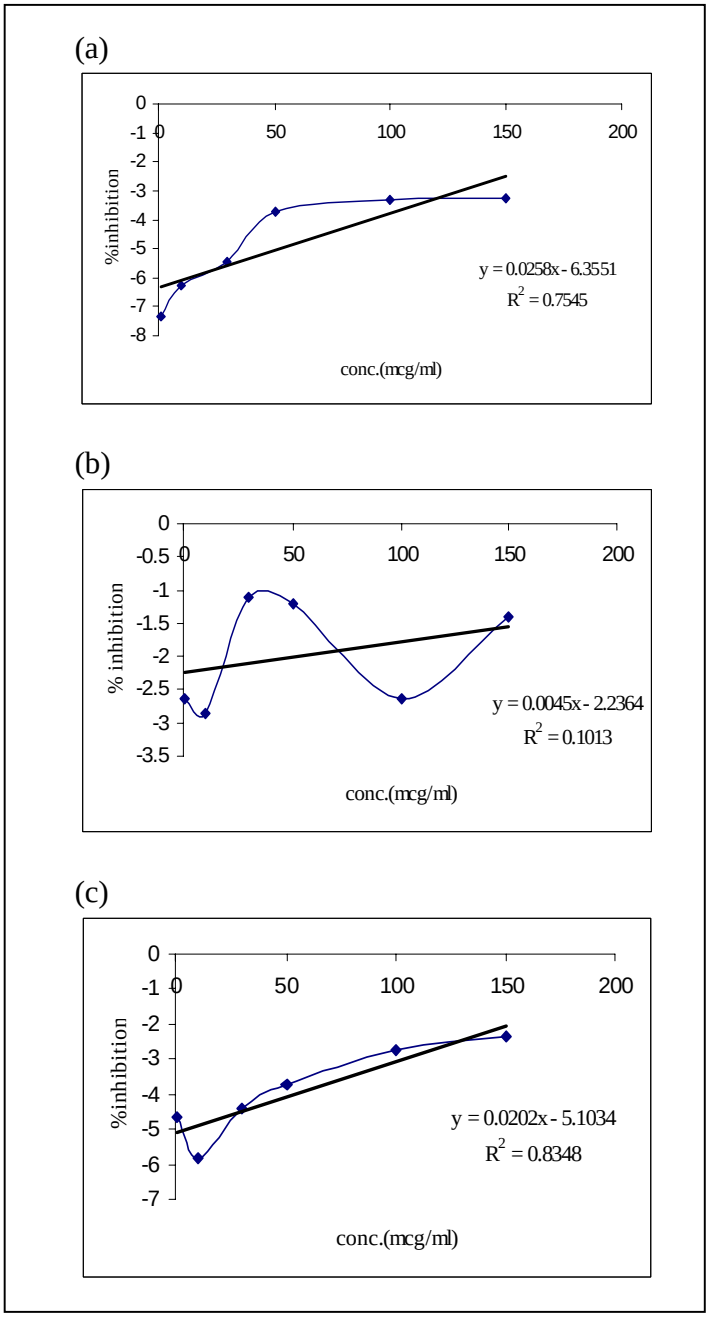


Figure 44 Graph of methanol extract of Namdokmai for galvinoxyl scavenging assay ; (a) first, (b) second and (c) third experiments

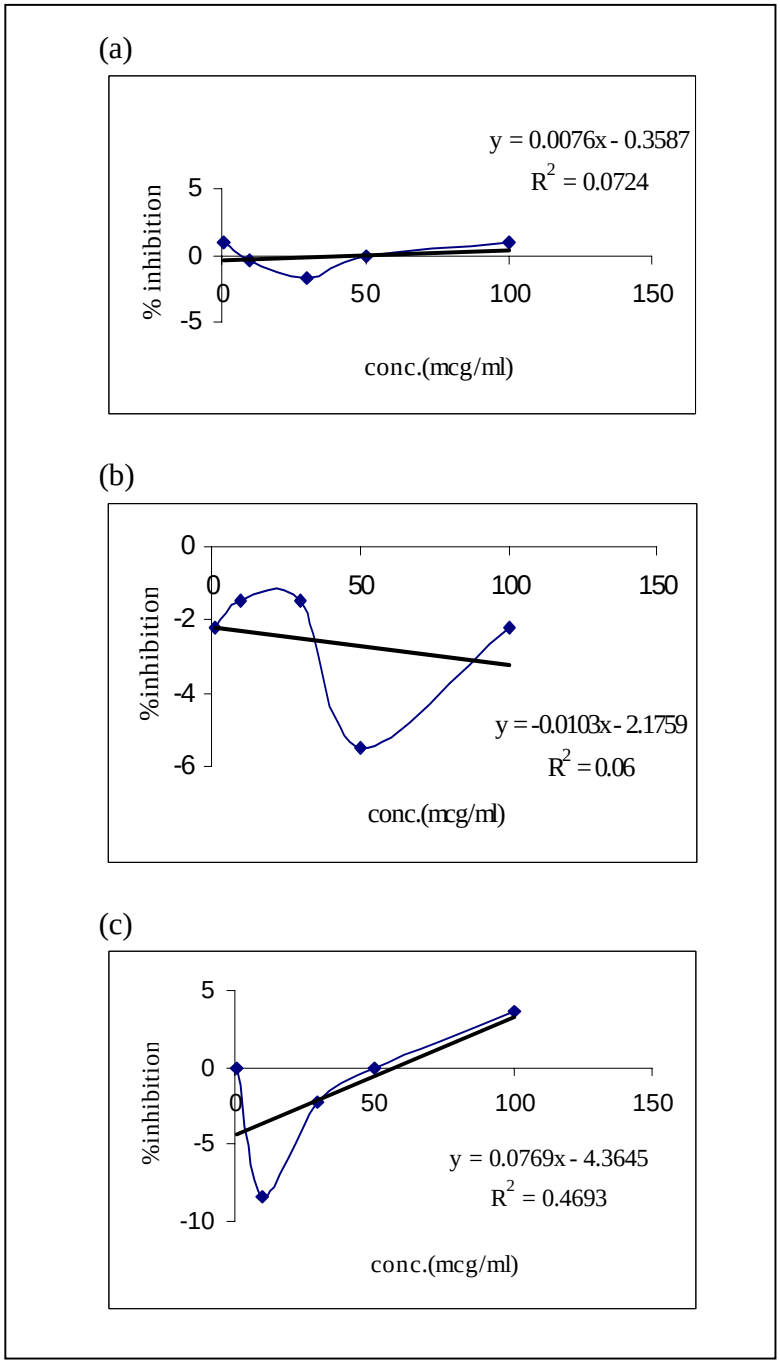


Figure 45 Graph of water extract of Namdokmai for galvinoxyl scavenging assay ; (a) first, (b) second and (c) third experiments

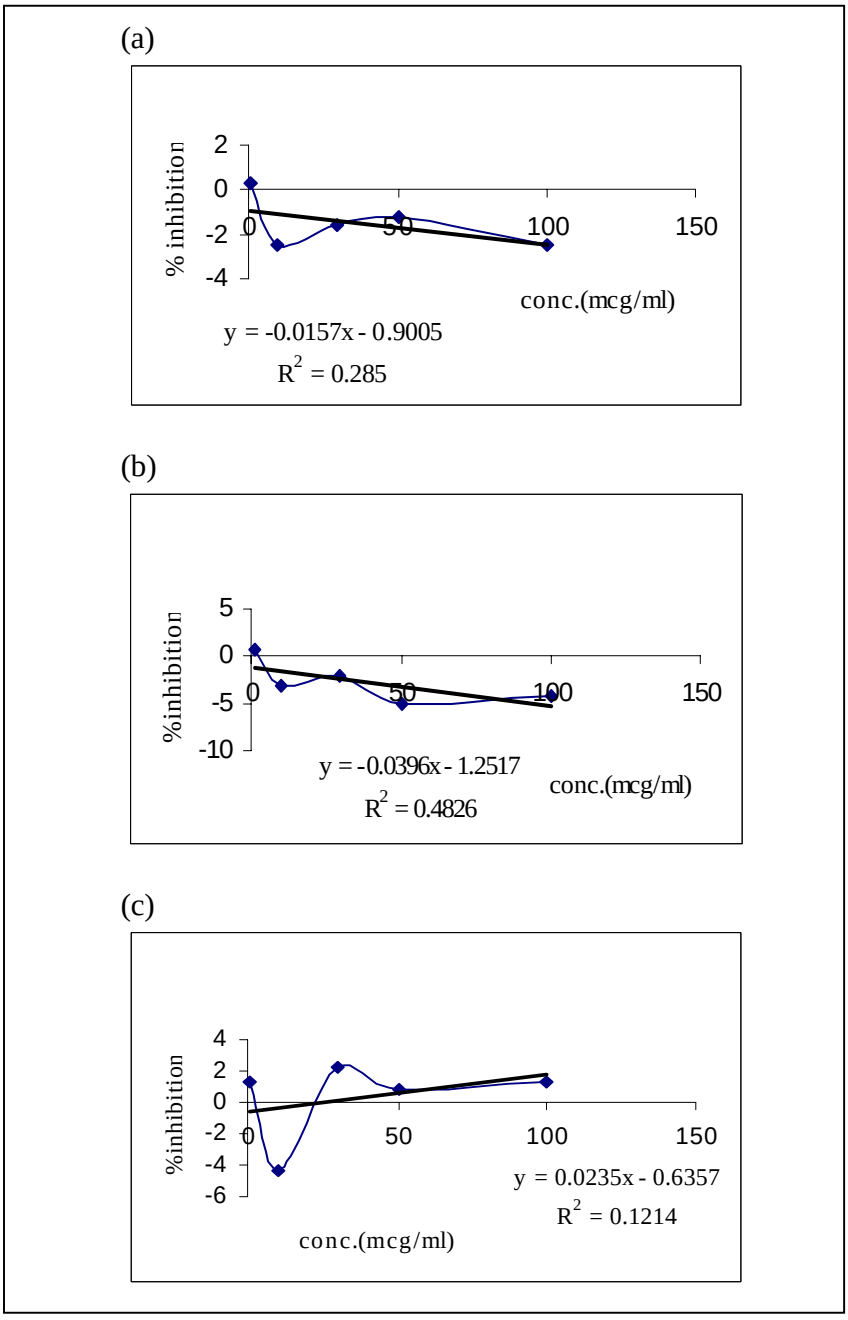


Figure 46 Graph of chloroform extract of Namdokmai for galvinoxyl scavenging assay; (a) first, (b) second and (c) third experiments

Free radical scavenging activities by super oxide scavenging assay of methanol extract of Namdokmai leaf

The free radical scavenging activities by super oxide scavenging assay was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

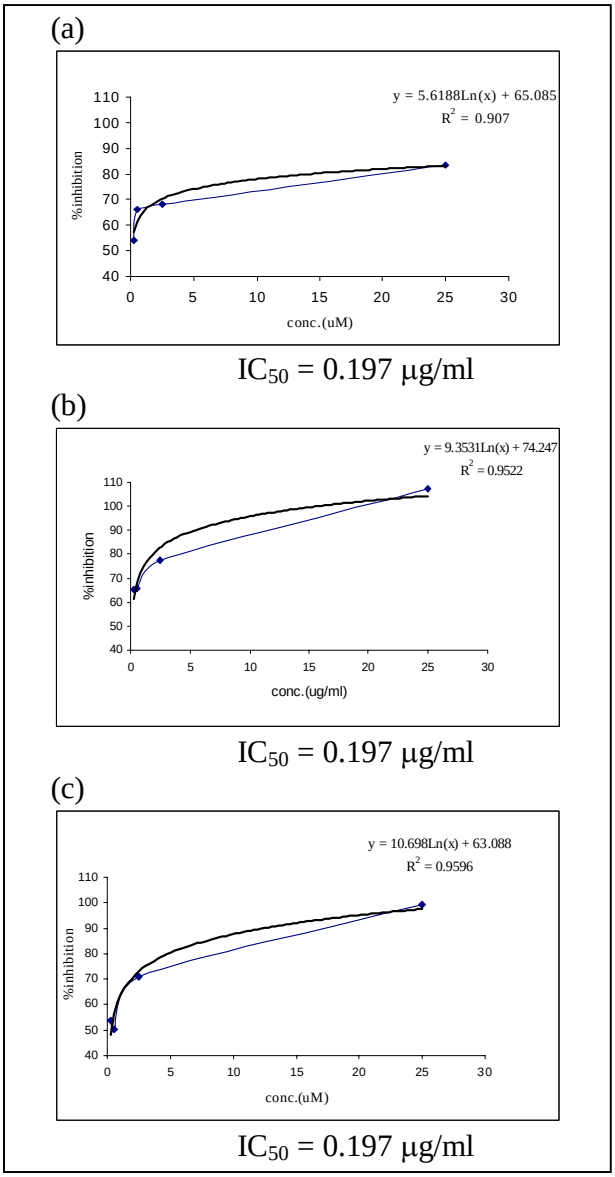


Figure 47 Graph of methanol extract of Namdokmai for super oxide scavenging assay; (a) first, (b) second and (c) third experiments

Free radical scavenging activities by hydroxyl radical scavenging assay of methanol extract of Namdokmai leaf

The free radical scavenging activities by hydroxyl radical scavenging assay was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

Table 47 Data of manitol for hydroxyl radical scavenging assay of the first experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	-4.135	1.87518	2.25983	0
1000	88.7297	85.287	87.7007	87.2392
300	74.9014	77.8248	77.1901	76.6388
100	57.8806	57.7171	57.5632	57.7203
10	19.4826	16.8862	19.7231	18.6973

Table 48 Data of manitol for hydroxyl radical scavenging assay of the second experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	-5.3706	2.21856	3.15203	0
1000	89.6571	90.3852	89.7598	89.934
300	79.4449	78.8195	79.3142	79.1929
100	58.8618	57.5082	58.6658	58.3453
10	20.2813	20.2439	20.1226	20.2159

Table 49 Data of manitol for hydroxyl radical scavenging assay of the third experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	-7.7835	1.7791	6.00445	0
1000	82.4907	82.7131	82.298	82.5006
300	72.6909	73.3284	73.447	73.1554
100	56.1453	56.4715	56.4418	56.3529
10	21.4529	21.5567	21.6308	21.5468

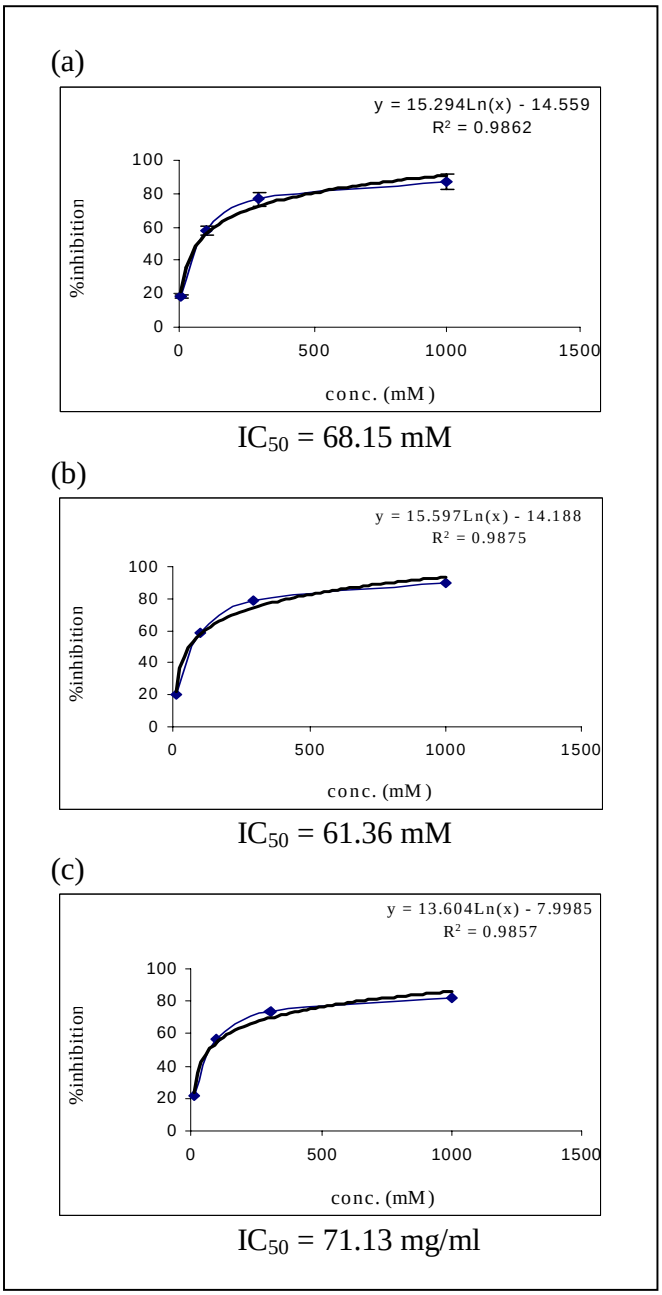


Figure 48 Graph of manitol for hydroxyl radical scavenging assay; (a) first, (b) second and (c) third experiments

Table 50 Data of methanol extract of Namdokmai for hydroxyl radical scavenging assay of the first experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	1.34566	-0.60867	-0.73699	0
100	-6.43219	-8.11015	-8.2286	-7.59031
10	18.09568	18.26347	18.47075	18.27663
1	10.91005	10.54484	10.81134	10.75541
0.1	7.652826	7.524511	7.060604	7.412647

Table 51 Data of methanol extract of Namdokmai for hydroxyl radical scavenging assay of the second experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	0.344564	-0.37328	0.028714	0
100	5.484303	4.881317	4.910031	5.091884
10	14.01225	14.29939	14.69181	14.33448
1	7.953675	7.685681	7.293262	7.644206
0.1	5.187596	3.857198	4.689893	4.578229

Table 52 Data of methanol extract of Namdokmai for hydroxyl radical scavenging assay of the third experiments

Conc. (μg/ml)	% inhibition			Average
	1	2	3	
control	2.726209	-0.87483	-1.85138	0
100	30.71054	31.09201	31.04623	30.9496
10	17.17614	17.93907	17.69493	17.60338
1	12.21708	11.88139	12.04923	12.04923
0.1	21.38752	19.89217	19.587	20.2889

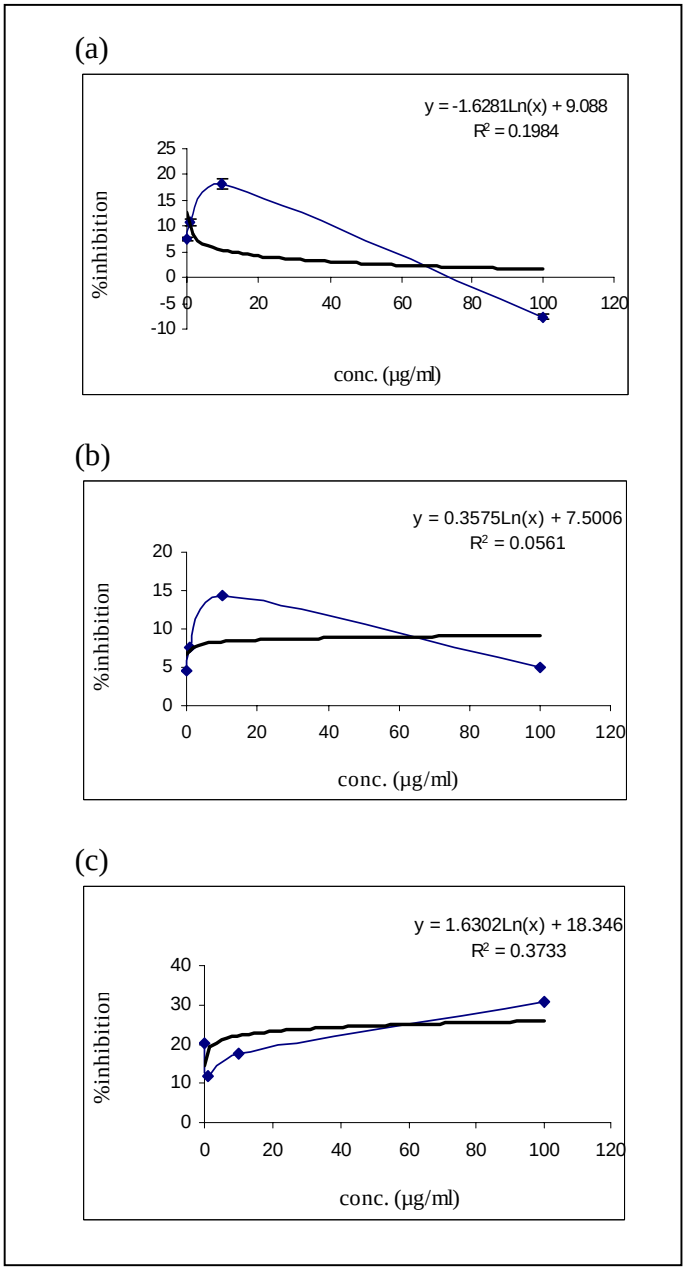


Figure 49 Graph of methanol extract of Namdokmai for hydroxyl radical scavenging assay; (a) first, (b) second and (c) third experiments

Free radical scavenging activities by hydrogen peroxide scavenging assay of methanol extract of Namdokmai leaf

The free radical scavenging activities by hydrogen peroxide scavenging assay was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

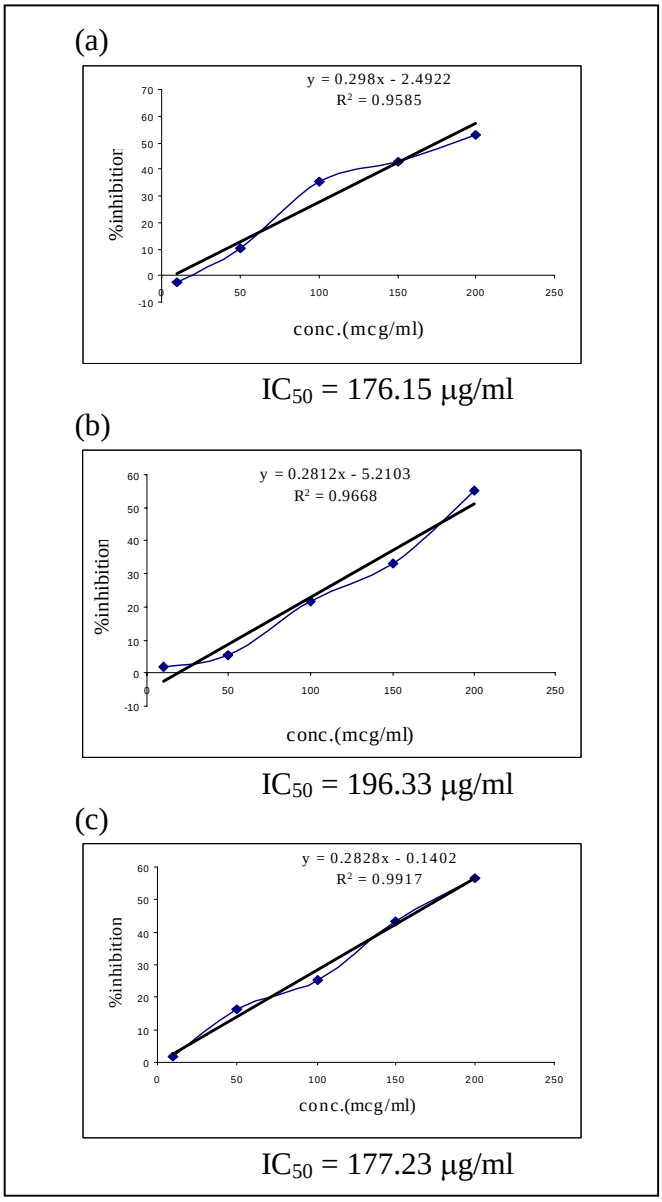


Figure 50 Graph of BHA for hydroxyl radical scavenging; (a) first, (b) second and (c) third experiments

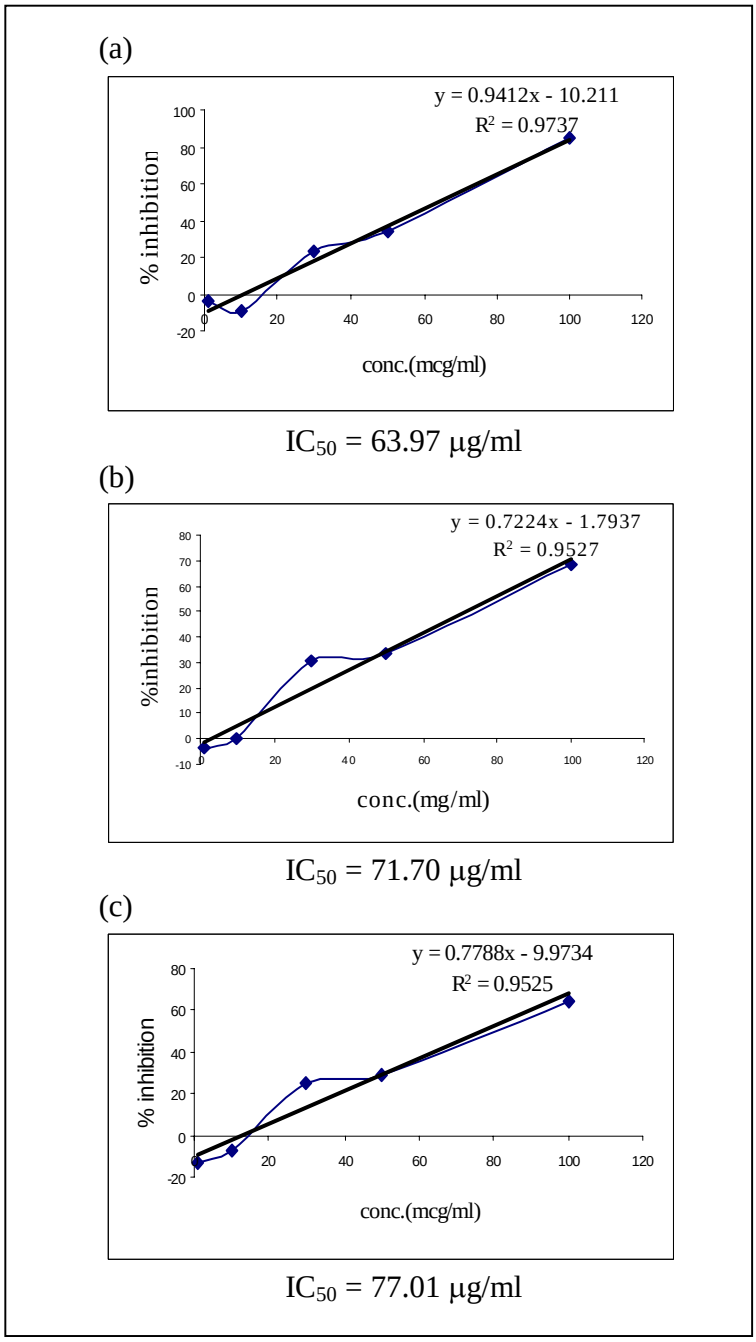


Figure 51 Graph of methanol extract of Namdokmai leaf for hydrogen peroxide scavenging assay; (a) first, (b) second and (c) third experiments

Free radical scavenging activities by metal ion chelating of methanol extract of Namdokmai leaf

The free radical scavenging activities by metal ion chelating was expressed as IC_{50}. The IC_{50} were calculated by using the following as DPPH assay.

Table 53 Data of EDTA for metal ion chelating of the first experiments

Concentration (µg/ml)	% ion chelating mean
control	0.000
10	152.941
5	88.235
1	36.975
0.5	56.303
0.1	25.210
0.05	-10.084

Table 54 Data of EDTA for metal ion chelating of the second experiments

Concentration (µg/ml)	% ion chelating mean
control	0.000
10	120.863
5	113.669
1	47.482
0.5	18.705
0.1	32.374
0.05	20.144

Table 55 Data of EDTA for metal ion chelating of the third experiments

Concentration (µg/ml)	% ion chelating mean
control	0.000
10	81.503
5	78.613
1	39.884
0.5	10.983
0.1	13.873
0.05	-1.734

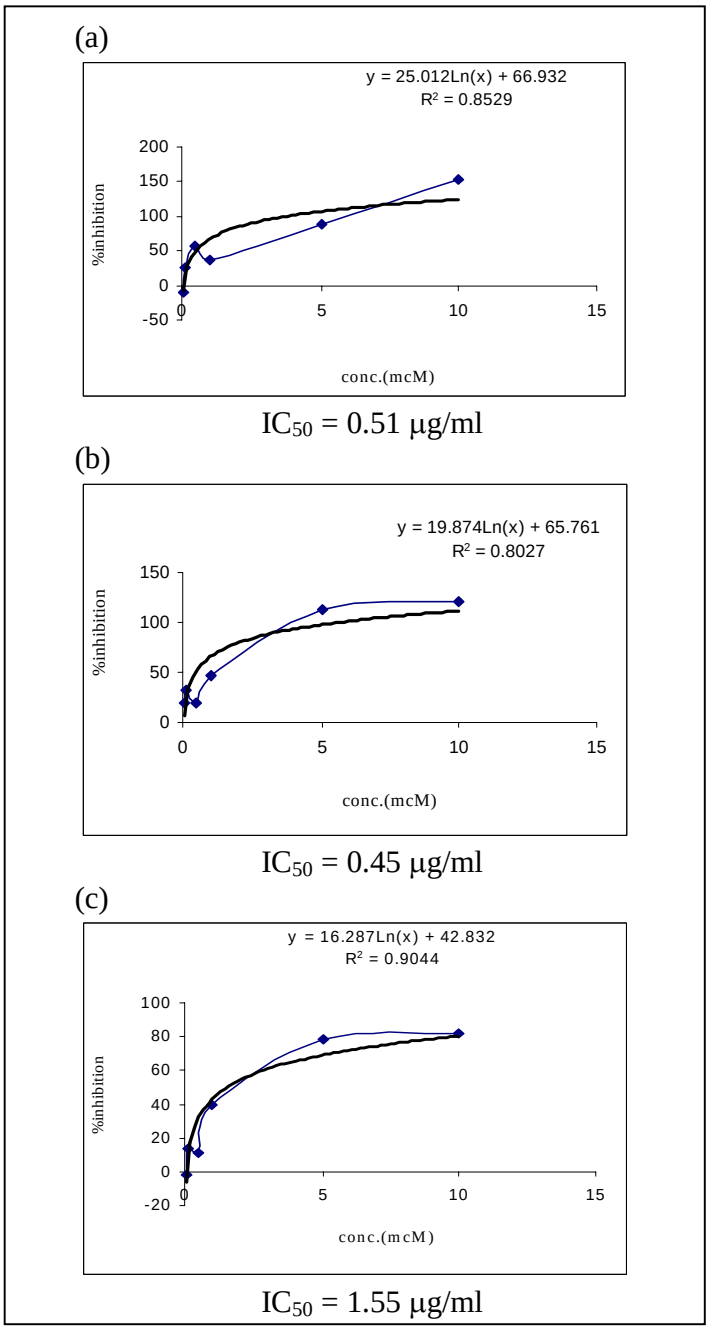


Figure 52 Graph of EDTA for metal ion chelating; (a) first, (b) second and (c) third experiments

Table 56 Data of methanol extract of Namdokmai leaf for metal ion chelating of the first experiments

concentration (µg/ml)	%inhibition
control	0
100	108.98
50	73.47
30	73.47
10	66.53
1	48.16

Table 57 Data of methanol extract of Namdokmai leaf for metal ion chelating of the second experiments

concentration (µg/ml)	%inhibition
control	0.00
100	129.27
50	108.94
30	121.95
10	90.65
1	68.70

Table 58 Data of methanol extract of Namdokmai leaf for metal ion chelating of the third experiments

concentration (µg/ml)	%inhibition
control	0.00
100	130.08
50	106.02
30	114.29
10	94.36
1	56.39

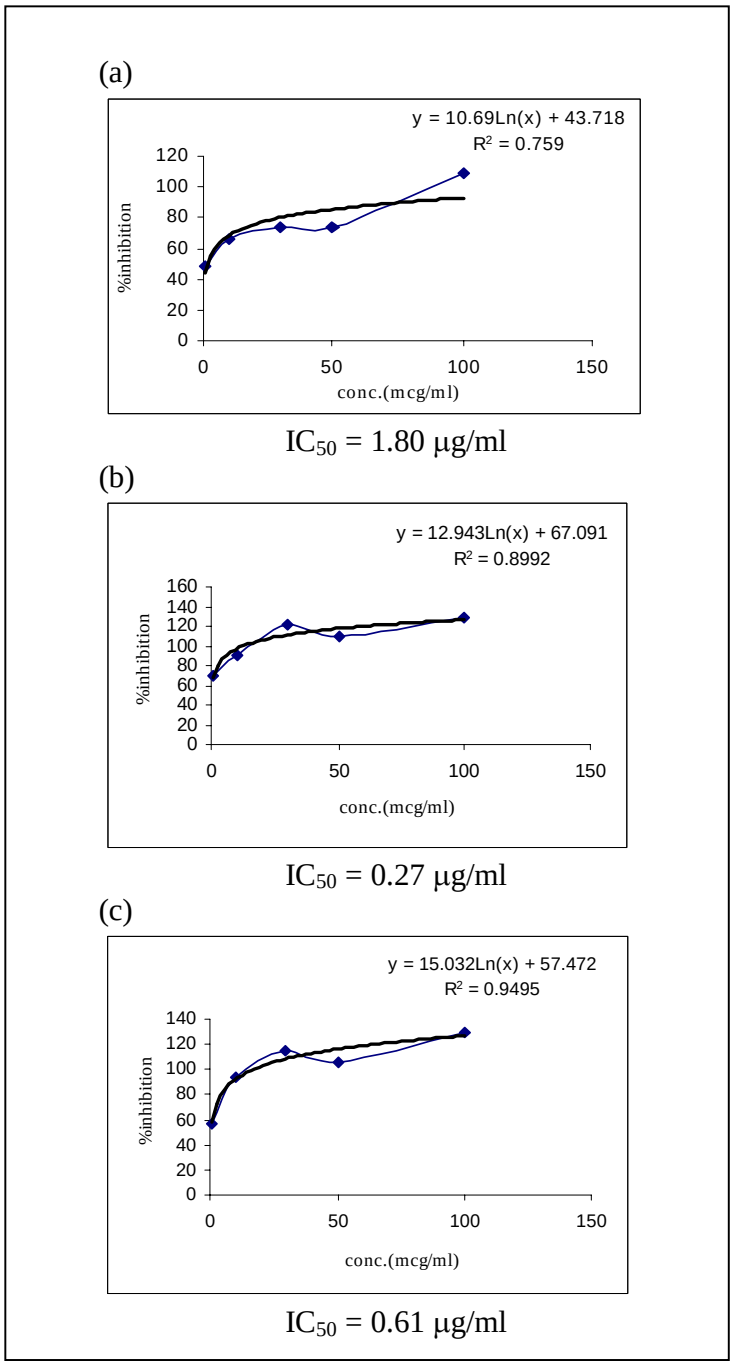


Figure 53 Graph of methanol extract of Namdokmai leaf for metal ion chelating; (a) first, (b) second and (c) third experiments

APPENDIX D

Cytotoxicity

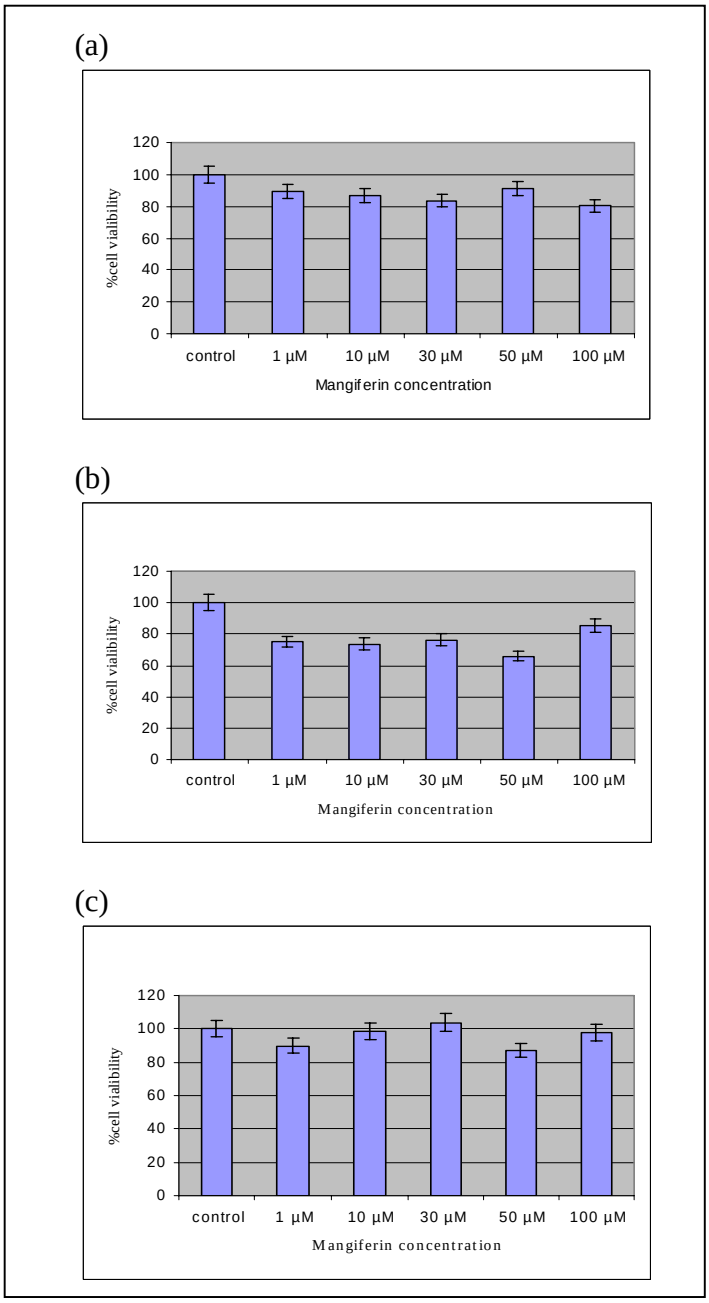


Figure 54 Cytotoxic of mangiferin; (a) first, (b) second and (c) third experiments

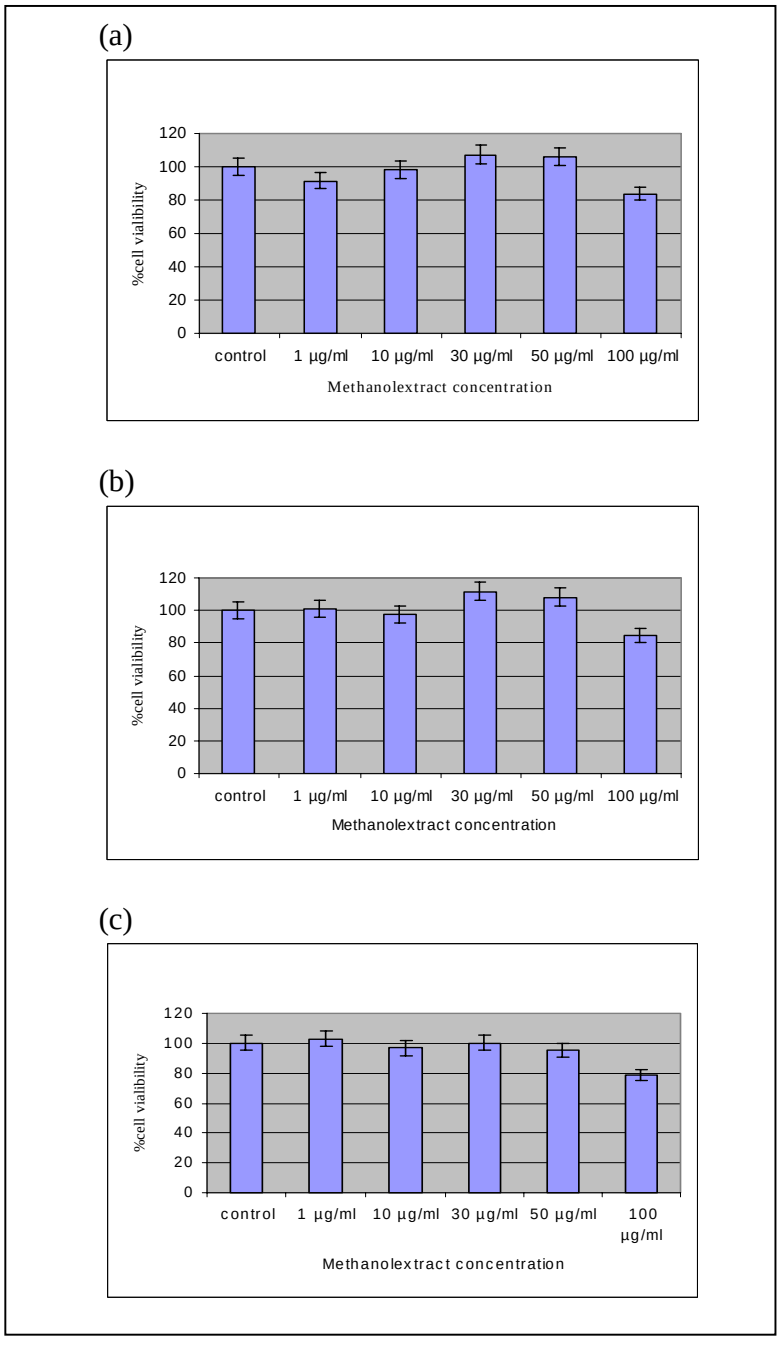


Figure 55 Cytotoxic of methanol extract of Namdokmai leaf; (a) first, (b) second and (c) third experiments

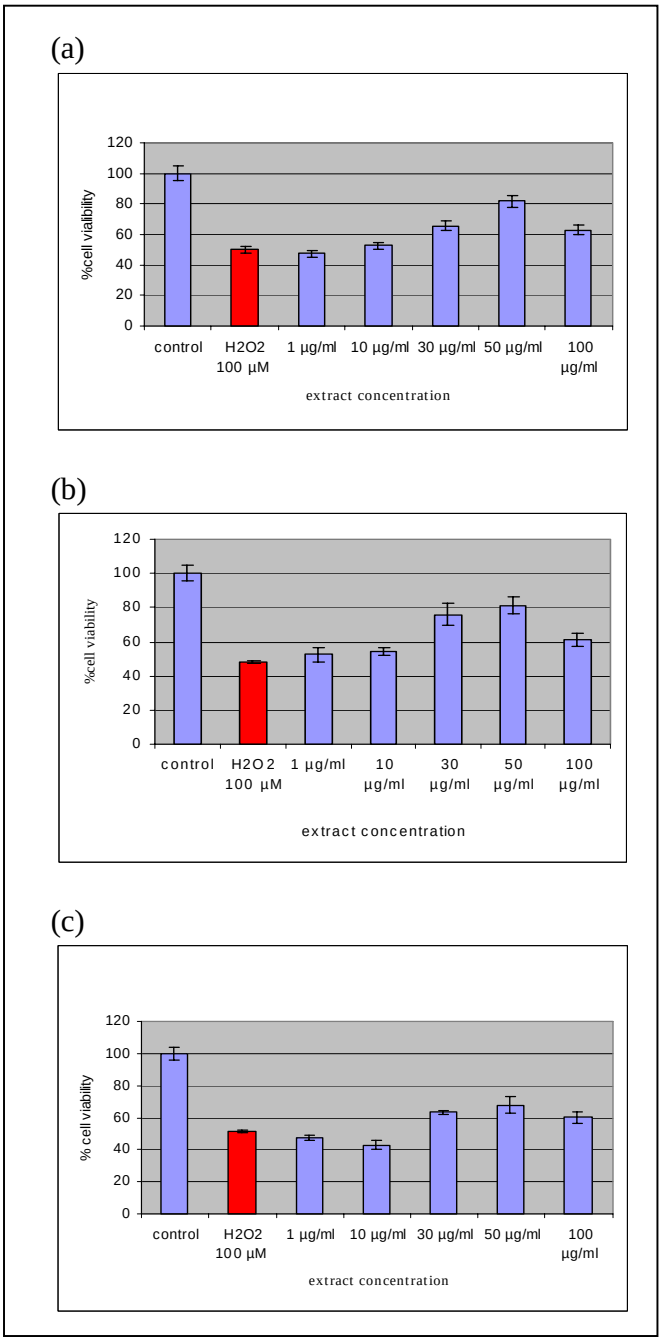


Figure 56 Hydrogen peroxide-induce cell death of methanol extract of Namdokmai leaf; (a) first, (b) second and (c) third experiments

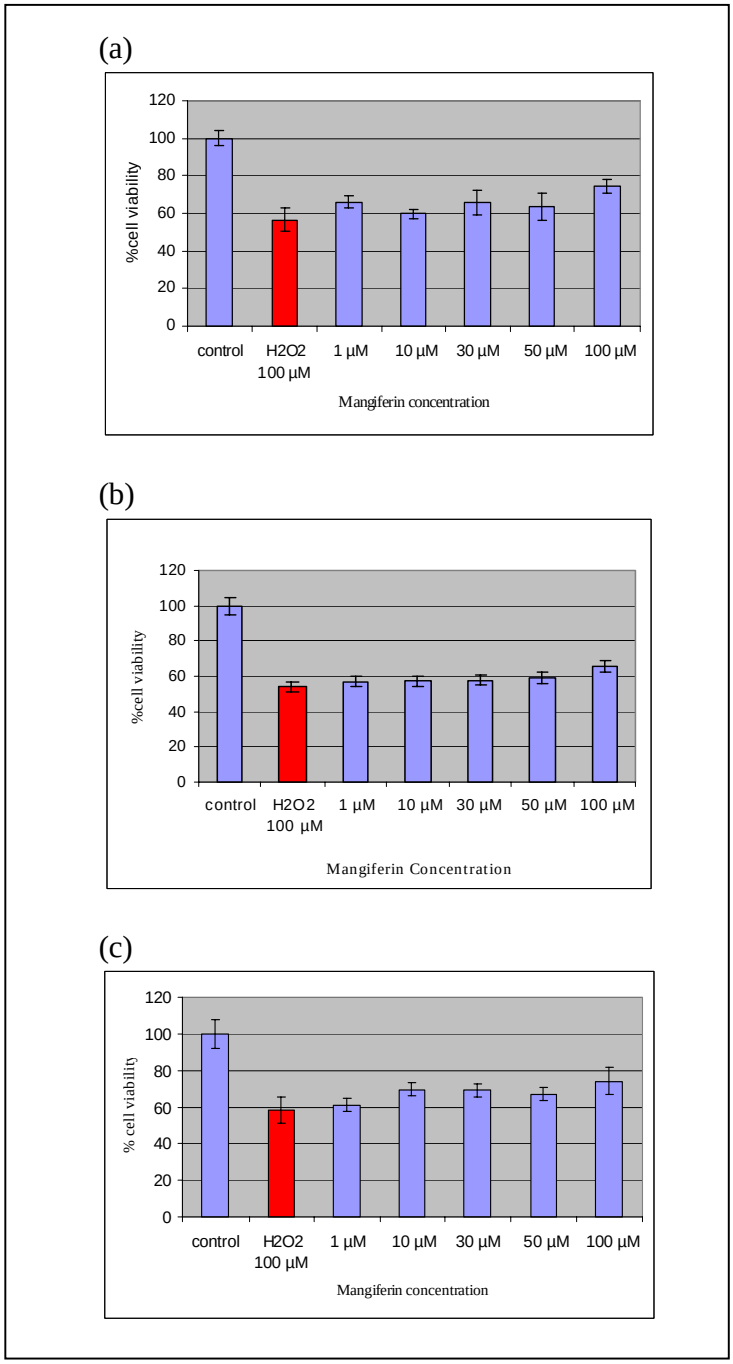


Figure 57 Hydrogen peroxide-induce cell death of mangiferin; (a) first, (b) second and (c) third experiments

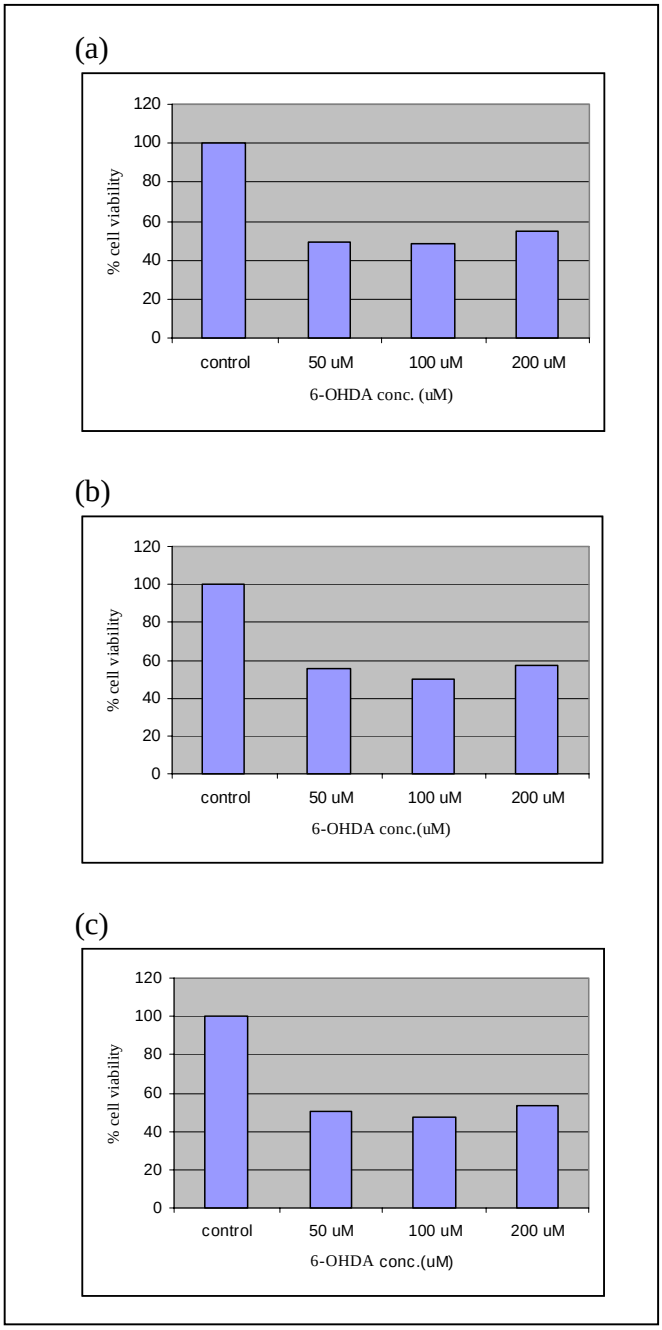


Figure 58 Cytotoxic of 6-OHDA dose dependent; (a) first, (b) second and (c) third experiments

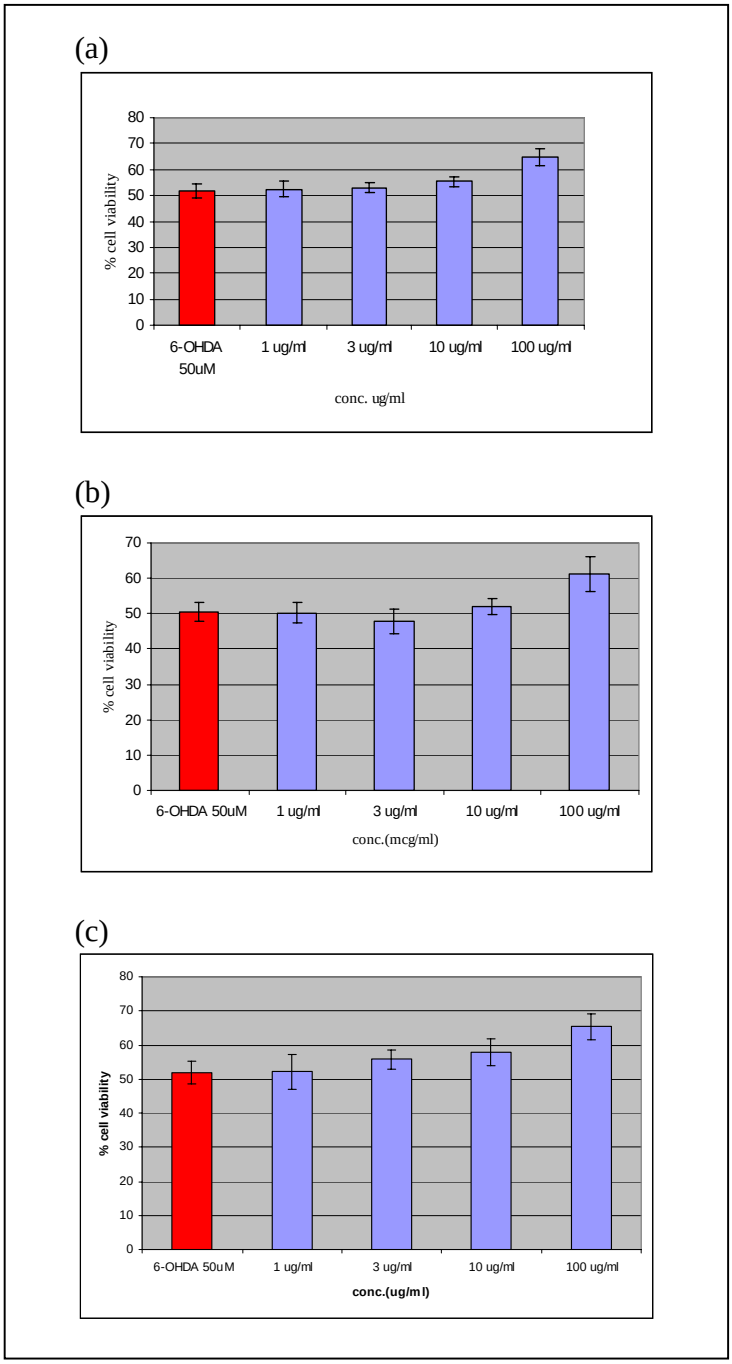


Figure 59 6-OHDA-induce cell death of methanol extract Namdokmai leaf extract; (a) first, (b) second and (c) third experiments

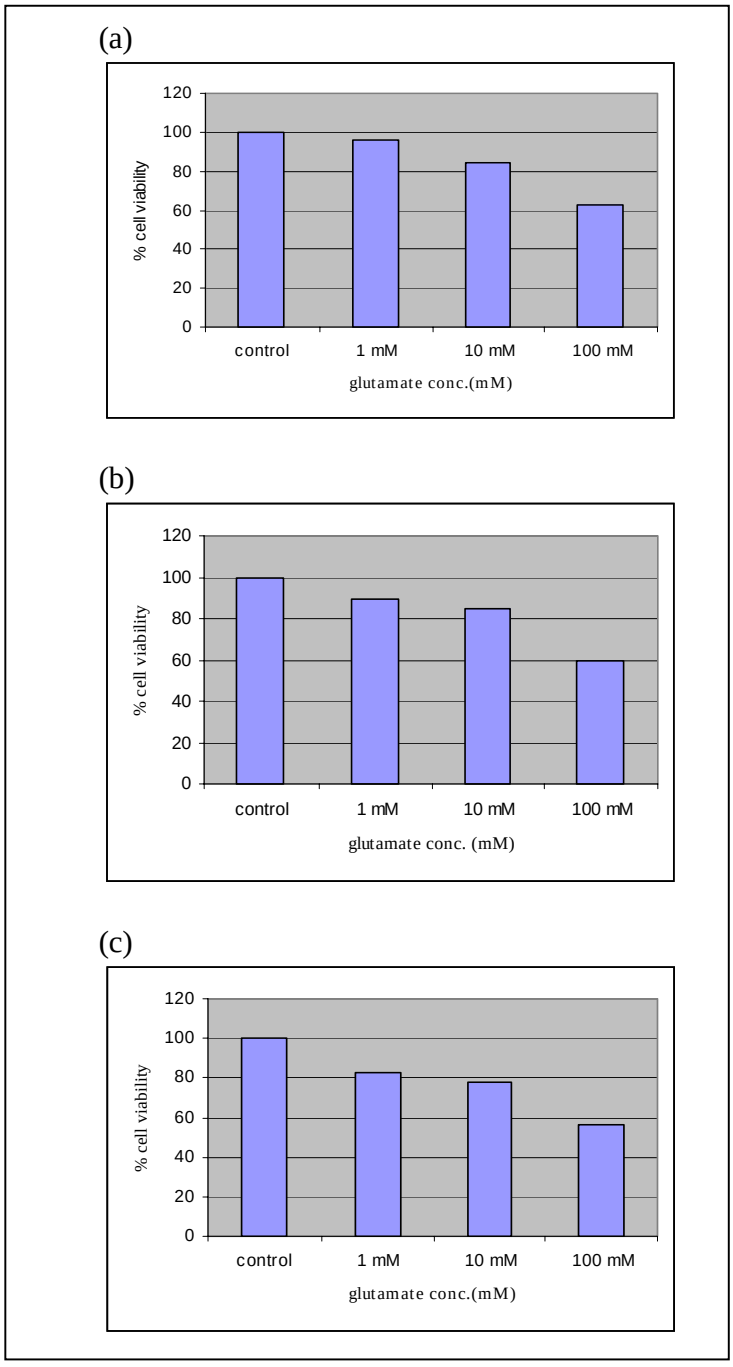


Figure 60 Cytotoxic of glutamate dose dependent; (a) first, (b) second and (c) third experiments

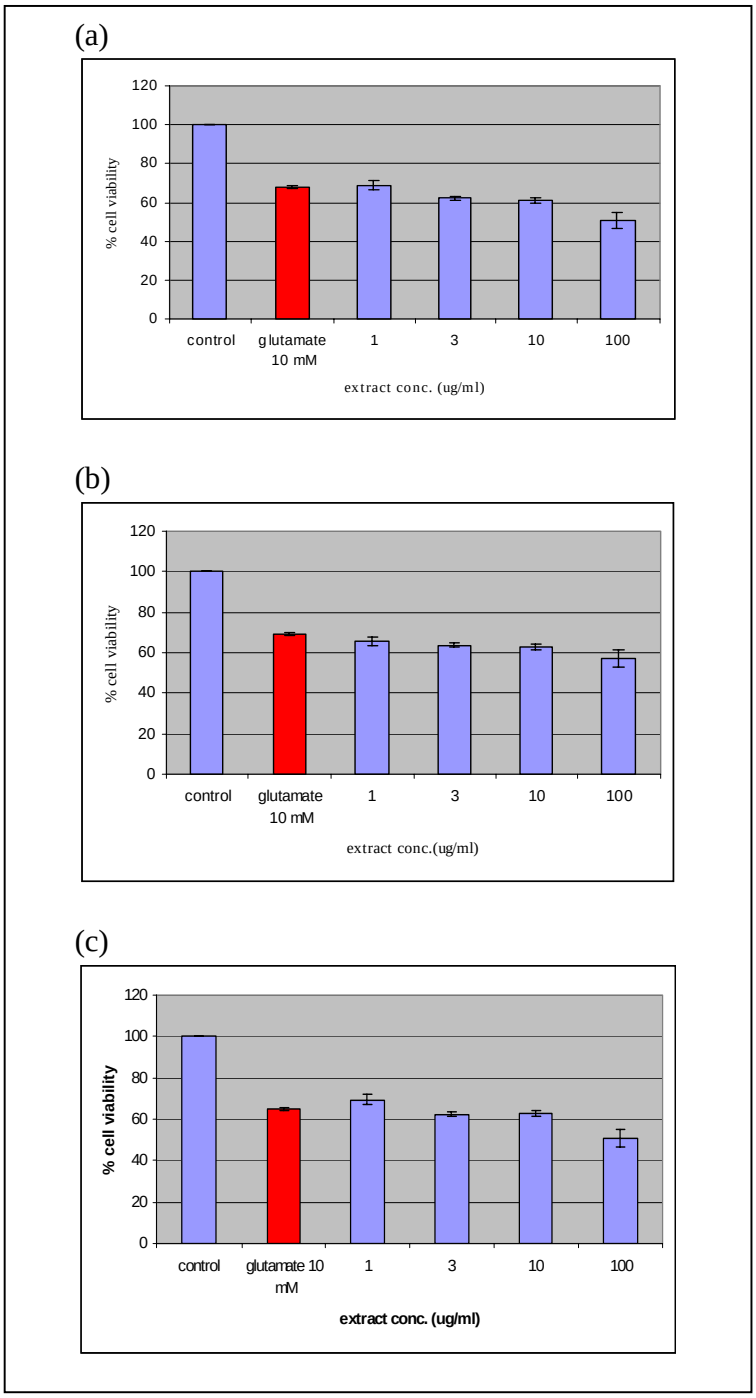


Figure 61 Glutamate-induce cell death of methanol extract Namdokmai leaf extract; (a) first, (b) second and (c) third experiments

Table 59 List of abbreviations

Symbol	Definition
°C	degree Celsius
>	more than
<	less than
%	percent
mV	milivolt
MW	molecular weight
pK_a	minus logarithm base 10 of K_a, -log K_a
et al.	and others
Etc.	for example, such as
v/v	volume by volume
w/w	weight by weight
cm^2	square centimeter
g	gram
mg	milligram
µg	microgram
mL	milliliter
µL	microliter
nm	nanometer

Table 59 (continue)

Symbol	Definition
min	minute
h	hours
EC_1	Concentration of antioxidant reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$
IC_{50}	Inhibition concentration fifty
PBS	phosphate-buffered saline
pH	The negative logarithm of the hydrogen ion concentration
R^2	coefficient of determination
rpm	revolution per minute
SD	standard deviation
AVG	Average
UV	ultraviolet

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Presentation

1. Kanistha Kawpoomhae, Monrudee Sukma, Tanasait Ngawhirunpat, Praneet Opanasopit, Theerasak Rojanarata and Areerut Sripattanaporn "Antioxidant of standardized extracts of *Mangifera indica* leaf protect cutured neuroblastoma cells from hydrogen peroxide-induced oxidative injury" The 26th Annual Research Conference in Pharmaceutical Sciences, 4 December 2009, Bangkok, Thailand, Poster presentation.

2. Kanistha Kawpoomhae, Monrudee Sukma and Areerut Sripattanaporn "Antioxidative and Cytoprotective Effect of Mangiferin" The 3rd Annual Research Conference in Silpakorn Research, 28-29 January 2009, Nakornpathom, Thailand, Poster presentation.

3. Kanistha Kawpoomhae, Monrudee Sukma, Tanasait Ngawhirunpat, Praneet Opanasopit and Areerut Sripattanaporn "Antioxidant and neuroprotective effects of standardized extracts of *Mangifera indica* leaf" Thai J. Pharm. Sci. 34 (2010) 32-43.