ANTIOXIDANT AND CYTOTOXICITY ACTIVITIES OF *VEITCHIA MERRILLII* FRUITS

ALI VAFAEI

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2013

ANTIOXIDANT AND CYTOTOXICITY ACTIVITIES OF *VEITCHIA MERRILLII* FRUITS

ALI VAFAEI

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2013

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Ali Vafaei (I.C/Passport No:L95235477)

Registration/Matric No: SGF080045

Name of Degree: Master of Biotechnology

Title of Dissertation: ANTIOXIDANT AND CYTOTOXICITY ACTIVITIES OF *VEITCHIA MERRILLII* FRUITS

Field of Study: Biotechnology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:

Designation:

ABSTRACT

Veitchia merrillii (Arecaceae family) is commonly known as the "Christmas Palm" because its fruits become bright scarlet and tend to be that color in winter. A study was conducted to evaluate *Veitchia merrillii* fruits for the presence of total phenolic and flavonoid contents and determine antioxidant activity as well as cytotoxicity effects of extracts with solvents methanol, ethyl acetate and water. Further more, qualitative and quantitative composition of phenolics and flavonoid compounds in all extracts were also analyzed using RP-HPLC.

The results of the study showed that methanol extract gave the highest yield compared to the other solvents used. The analysis showed that a 5 g powdered dried fruit sample of *Veitchia merrillii*, resulted in 28.25 ±2.12%, 21 ±1.31% and 14.75 ±1.83% yield of extracts in methanol, ethyl acetate and water, respectively. Results of analysis on phenolics and flavonoids in the *Veitchia merrillii* fruit extracts also showed significant differences ($P<0.05$). The total phenolic content in methanolic, ethanolic and water extracts were found to be 17.8, 7.6 and 2.22 mg GAE/g DW, respectively. On the other hand the total flavonoid content in the methanolic, ethanolic and water extracts were found to be 5.43, 3.12 and 1.11 mg Rutin/g DW, respectively. Meanwhile the results of the HPLC analysis clearly showed gallic acid, pyrogallol, caffeic acid, vanillic acid syringic acid, as the major phenolic acid whereas naringin and rutin are flavonoid compounds present in extracts of *Veitchia merrillii* fruits.

Antioxidant activity determined using DPPH radical scavenging, NO scavenging and ABTS scavenging assays indicated that methanolic extracts exhibited higher levels of antioxidant activity compared to ethyl acetate and water extracts. The IC_{50} concentrations of methanolic extract for DPPH, NO scavenging and ABTS scavenging

activity were found to be >1000 µg/ml, 616.5 µg/ml and 884.8 µg/ml, respectively. Compared with the standards these activities were not very strong.

The extracts exhibited moderate to weak cytotoxic activity against two Human hepatocytes cells (Chang liver cells) and NIH/3T3 (Fibroblast cells). The compounds present in the extracts were non-toxic, which render them as suitable potential therapeutics to develop an anticancer drug.

ABSTRAK

Veitchia merrillii (famili Arecaceae) biasanya dikenali sebagai "Christmas Palm" kerana buahnya menjadi skarlet cerah dan cenderung pada warna itu pada musin dingin. Kajian ini dijalankan untuk menilai kehadiran jumlah kandungan fenol dan flavonoid didalam buah *Veitchia merrillii* dan aktiviti antioksidannya serta kesan sitotoksik ekstrak yang diperolihi dari pelarut berlainan polar menggunakan metanol, etil asetat dan air. Disamping itu, komposisi kualitatif dan kuantitatif sebatian fenolik dan flavonoid dalam kesemua ekstrak telah dianalisa menggunakan system RP-HPLC.

Keputusan yang diperolihi menunjukkan metanol memberikan hasil tertinggi ekstrak berbanding dengan pelarut lain yang digunakan. Ia menunjukkan bahawa dari 5 g berat sampel kering serbuk buah *Veitchia merrillii* metanol, etil asetat dan air telah memberikan hasil 28.25 ±2.12%, 21 ±1.31% dan 14.75 ±1.83% dari ekstrak masing-masing. Keputusan kandungan fenol dan flavonoid dalam buah *Veitchia merrillii* menunjukkan perbezaan signifikan ($P<0.05$). Kandungan jumlah fenol ekstrak metanol, etanol dan air telah diperhatikan dengan nilai 17.8, 7.6 dan 2.22 mg GAE/g DWmasing-masing. Manakala jumlah kandungan flavonoid ekstrak methanol, etanol dan air diperhatikan dengan nilai 5.43, 3.12 and 1.11 mg Rutin/g DW masing-masing. Sementara itu keputusan analisa HPLC jelas menunjukkan asid galik, pirogalol, asid cafeik, asid vanilik, asid syringik hadir sebagai asid fenolik utama manakala naringin dan rutin adalah sebatian flavonoid didalam ekstrak buah *Veitchia merrillii.*

Aktiviti antioksidan ditentukan menggunakan asai pemerangkap radikal bebas DPPH, pemerangkap aktiviti NO dan pemerangkap ABTS menunjukkan ekstrak methanol menunjukan aktiviti antioksidan yang tinggi berbanding dengan ekstrak etil asetat dan air. Kepekatan IC_{50} ekstrak methanol dalam DPPH, pemerangkap NO dan pemerangkap

ABTS aktiviti adalah didapati > 1000 ug/ml, 616.5 ug/ml dan 884.8 ug/ml berbanding dengan piawai adalah tidak begitu kuat.

Akhir sekali, aktiviti ketoksikan ekstrak terhadap dua hepatosit manusia (Chang liver cells) dan NIH/3T3 (Fibroblasts cell) menunjukan aktiviti ketoksikan adalah moderat kepada lemah oleh ekstrak berlainan dan kehadiran sebatian didalam ekstrak adalah tidak toksik yang menyebabkan ia berpotensi dan sesuai sebagai bahan terafeutik untuk dibangunkan sebagai dadah antikanser.

ACKNOWLEDGEMENT

It would not have been possible to write this master thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

In the first place I would like to record my gratitude to Dr. Jamaludin Bin Mohamad for his supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, he provided me unflinching encouragement and support in various ways. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be. I am indebted to him more than he knows.

I would like to acknowledge the financial, academic and technical support of the University of Malaya.

Words fail me to express my appreciation to my wife Elaheh whose dedication,
love and persistent confidence in me, has taken the load off my shoulder. I owe her for being unselfishly let her intelligence, passions, and ambitions collide with mine.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVATIONS

ATP	adenosine tree phosphate
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	ethylene-diamine-tetraacetic acid
FRAP	ferric reducing antioxidant power
H_2O	distilled water
H_2SO_4	sulphuric acid
HbA1c	glycosylated haemoglobin
HCI	hydrochloric acid
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory activity
KCI	potassium chloride
L	litter
ml	milliliter
µl	microliter
m	meter
cm	centimeter
Kg	kilogram

g	gram
mg	milligram
µg	microgram
M	molarity
mM	millimolar
µM	micromolar
mmol	millimole
min	minute
MW	molecular weight
MeOH	methanol
MgCI2	magnesium chloride
NaOH	sodium hydroxide
%	percentage
≤	Less than or equal to
≥	More than or equal to
BHA	Butylated hydroxianisole
DW	Dry weight
BHT	Butylated hydroxytoluene
DNA	Deoxyribonucleic acid

NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBHQ	*Tert*-butyldroquinone
PGs	Prostaglandins
UV	Ultra violet
CVD	Cardiovascular disease
H NMR	Proton nuclear magnetic resonance
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MCF-7	Breast cancer cells
HT-29	Colon cancer cells

CHAPTER 1

INTRODUCTION

In the past decade, there has been high demand to apply secondary metabolites and bioactive compounds of plants as medicinal agents since synthetic drugs have indicated a great deal of different side effects on the human body (Manian *et el.*, 2008). A variety of biological activities such as antioxidant, anticancer and anti microbial properties have been demonstrated by natural phytochemicals derived from plants. The role of flavonoid and phenolic compounds have been revealed by recent studies. These have been confirmed as the major secondary metabolites with biological activities in plant extracts (Ao et al., 2008). Plant phenols have been shown to be multifunctional antioxidants that may act as singlet oxygen quenchers, hydrogen donating antioxidants and reducing agents (Rice-Evans, 2001). A number of vital biological effects have been confirmed for flavonoids, including antioxidative, antitumor, antiviral, antifungal actions, antibacterial anti-inflammatory (inhibition of lipoxygenase and cyclooxygenase) and as effective inhibitors of platelet aggregation (Narayana et al., 2001; Nijveldt et al., 2001).

Antioxidants include vitamins, phytochemicals and certain nutrients. They can inhibit a wide range of diseases of the heart, cardiovascular system, kidneys, muscles, lungs and brain, and they are also very helpful in retarding the process of aging (Miguel, 2010). The formation of lipid peroxidation and free radicals in human bodies can be prevented or delayed by these chemical substances (Miguel, 2010). All in all, synthetic and natural antioxidants are the two basic categories of these agents. Rich sources of natural antioxidants are provided by plants. There are a variety of nutrients that have antioxidant properties and they are very helpful for the protection of proteins, DNA and lipids in cells from damaged by oxygen. These nutrients include phenolics, carotenoids,

flavonoids, and also vitamins E and C as well as selenium (Surai, 2005). The most diverse groups of phytochemicals are phenolics and flavonoids which are able to protect the human body against reactive oxygen species. The tissue structure and body cells may be ruined by reactive oxygen and free radicals that are normally generated during oxygen metabolism and by exogenous damage (Galindo et al., 2010).

The Arecaceae family includes approximately 200 genera and 2,500 species. Palms range from towering trees to very small understory plants, and can be found in the subtropics and tropics. A monotypic genus of flowering plants among Arecaceae family is *Adonidia* which includes the species, *Veitchia merrillii* (the Manila Palm). The species is mostly known as the "Christmas Palm" since the fruits get bright scarlet in winter. Manila Palm is typically quite small, usually 15-25 feet in height, but it has grown up to 36 feet under greenhouse conditions. In recent years a number of biological properties have been reported in this family. The fruits of *Phoenix dactylifera* for instance, have been utilized as an astringent and detersive for intestinal troubles and also in treatment of colds, sore throat, bronchial asthma, to relieve fever, gonorrhea, cystisis, and liver, edema and abdominal troubles. It has been shown to possess a wide range of pharmacological activities useful in a variety of disorders and diseases.

New scientific knowledge on the medicinal properties of *Veitchia merrillii* fruits is very important to enhance the development of its biopharmaceutical potential. Currently, knowledge on the chemical constituents of this plant is quite limited.

1.1 Objectives of the study:

1. To determine and analyse the phenolic and flavonoid compounds present in *Veitchia merrillii* fruit extract using reversed-phased high performance liquid chromatography (RP-HPLC).
2. To evaluate antioxidant activity of *Veitchia merrillii* fruit extract.
3. To investigate the cytotoxic activity of *Veitchia merrillii* fruit extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal plants and their biological activities

Plants have always played an indispensable role to affecting human beings. Mankind has been also influencing the properties and forms of plants in order to make them more adaptable to the progress of human life. In the history of medicine, plants have been always beneficial and have been utilized as a primary medicinal source by the people in ancient times (Carper, 1988). There are several types of natural medicinal plants which are unique on the basis of their properties. Medicinal plants are becoming more popular than before because of several benefits to humans and the society, with respect to mostly pharmacological or medicinal aspects. The bioactive phytochemical constituents are the agents responsible for the production of definite physiological actions in the human body, and these determine the medicinal and pharmacological value of the plants. (Akinmoladun et al., 2007). In folk medicine there has always been the consumption and utilization of medicinal plants or the chemical constituents from these plants to treat a wide range of ailments (Castelucci et al., 2007). Tannins, alkaloids, flavonoids, essential oils, saponins, terpenoids and phenolic acids are some examples of bioactive phytochemical constituents (Edeoga et al., 2005).

Medicinal plants are utilized in many different forms. The most popular and common forms are extracts (powder, liquid or viscous forms), decoctions, mixtures (containing two or more than two medicinal herbs), macerations, medicinal essences, infusions or tea, juice, capsules, syrups, pills, tablets, ointments, tinctures, poultice and sometimes as suppositories (Gurib-Fakim, 2006; Halberstein, 2005). In spite of the high curative value, all of the forms mentioned are considered raw as per pharmaceutical standards

and as a matter of fact, researchers and investigators from a variety of companies which promote herbal products, such as the British Herbal Medicine Association have been trying to enhance the quality by chemical standardization of these products (Singh, 2006). Some of the drugs that have been derived from medicinal plants are illustrated in Table 2.1.

Table 2.1 Bioactive compounds in some traditional medicinal plants (Singh, 2006)

Botanical names	**English names**	**Indigenous use**	**Origin**	**Uses in biomedicine**	**Biologically active compounds**
Adhatoda vasica,	–	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Condrodendron tomentosum	–	Arrow poison	Brazil, Peru	Muscular relaxation	D-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies	Ginkgolides
Harpagophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podophyllotoxin and lignans
Prunus Africana	African plum	Laxative, 'Old man's disease'	Tropical Africa	Prostate hyperplasia	Sitosterol

2.2 *Veitchia merrillii* (Manila palm, Christmas palm)

Veitchia merrillii also known as "Christmas Palm" is normally very small and slender, and typically can grow up to 15-25 feet in height. However under greenhouse conditions it has been able to attain a height of 36 feet. Some palms which are very similar and sold as "adonidia", are in fact "Alexander" palms.

The Christmas palm is an exotic palm and it generates clusters of bright red colored fruits around winter every year. At the initial stage, the palm produces very small gray-green flowers, which develop into fruites by the end of autumn. These fruits become

bright red by Christmas time and appaear as decorations on the palms. However, animals are not attracted to these fruits. The form of the tree, fruits and the fruit powder of *Veitchia merrillii* are presented in Figure 2.1.

The taxonomy of *Veitchia merrillii* is as shown below:

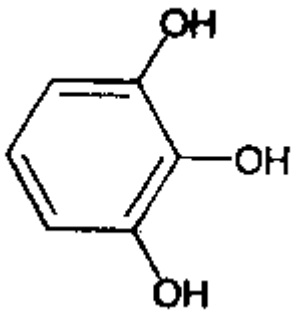
Kingdom	: Plantae
Sub division	: Angiospermae
Class	: Liliopsida
Order	: Arecales
Family	: Arecaceae
Genus	: *Adonidia* Becc
Species	: *A. merrillii*

Figure 2.1 Tree, fruits and fruit powder of *Veitchia merrillii.*

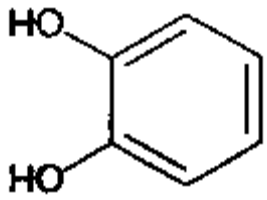
2.3 Phenolic Compounds

Phenolic compounds are phytochemicals that may be characterized by hydroxylated aromatic rings that have varying substitution patterns and functional derivatives (Francis, 2000; Shahidi and Naczk, 2003). Phenolic compounds in plants have diverse functions, including pollination encouragement, leaf and fruit colouring, repelling or attracting insects, protecting plants from herbivores, and seed dispersal and some agents that can protect it against UV light; as well as structural materials which are necessary for stability of plants (Shahidi and Naczk, 2003; Nichenametla et al., 2006). Phenolic compounds in foods are responsible for characteristics like taste, nutritional value, palatability, and also the acceptability of fresh or processed and pre-packaged foods (Francis, 2000). Based on the number of the subunits of phenol, two basic groups of phenolics are formed, which include simple phenols and polyphenols. Simple phenols also termed as 'phenolic acids' have a single phenolic ring with aldehyde, carboxylic acid or alcoholic groups that makes their function specific. Polyphenols have two or more phenol rings. Flavonoids belong to polyphenols (Harborne et al., 1994; Middleton et al. 2000). Some common phenolic compounds are illustrated in Figure 2.2 (Križková et al., 2000). In plants, the major polyphenol pigments include flavons, the yellow flavonols and anthocyanins. Anthocyanins are highly reactive species. The conversion of anthocyanins into other molecules during food processing, leads to loss or sometimes stabilization of the colour and may also increase the available hues range (Cheynier, 2005). Jakobek et al. (2009) reported that antioxidant activity in all fruits was increased by anthocyanins (~90%) followed by flavonols, flavan-3-ols and phenolic acids (~10%).

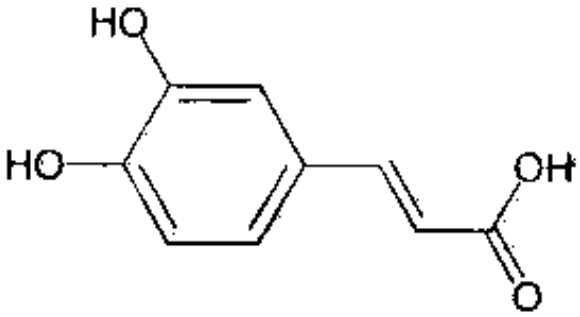
Phenolics have a wide range of biochemical activities including anti-mutagenic, anti-oxidant, anti-inflammation, anti-allergic, and anti-carcinogenic (Falleh et al., 2008), and also the ability to modify gene expression (Marinova et al., 2005).



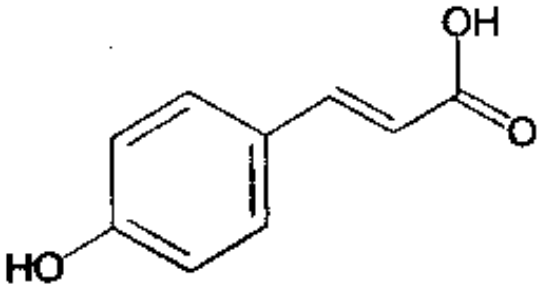
Pyrogallol



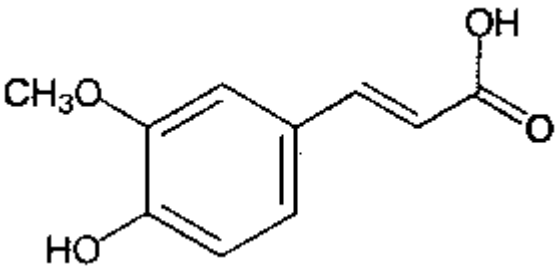
Catechol



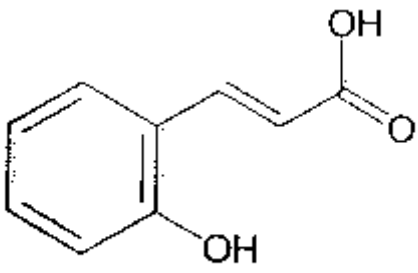
Caffeic acid



p-Coumaric acid



Ferulic acid



o-Coumaric acid

Figure 2.2 Chemical structure of some common phenolic compounds (Križková et al., 2000).

2.3.1 Phenolic Acids

Phenolic acids found in foods derived from plants are secondary metabolites. Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the main phenolic acids in plants, with hydroxycinnamic acids being the more common. The patterns of hydroxylation and methoxylation of the aromatic rings result in different derivatives (Boudet, 2007; Matilla and Hellstrom, 2007). Phenolic acids include one-third of dietary phenols that may exist in free and bound forms in plants (Fang et al., 2009), but commonly exist in bound form (Matilla and Hellstrom, 2007). Ferulic, *p*-coumaric and caffeic acids are the most common hydroxycinnamic acids, that occur frequently as simple esters in foods with glucose or quinic acid. Chlorogenic acid is the a bound hydroxycinnamic acid that comprises of quinic and caffeic acids. Unlike hydroxycinnamates, the derivatives of hydroxybenzoic acid commonly exist in the form of *p*-hydroxybenzoic, glucosides, procatechuic and vanillic acids in foods (Manach et al., 2004; Matilla and Hellstrom, 2007). Phenolic acids are effective antioxidants that have been reported to have antiviral, antibacterial, anti-inflammatory and anticarcinogenic activity (Matilla and Hellstrom, 2007). Faried *et al*., (2007) indicated that gallic acid selectively induces death in cancer cells such as gastric cancer (MKN-28), human oesophageal cancer (TE-2), breast cancer (MCF-7) and colon cancer (HT-29). Observation of apoptosis molecular mechanism demonstrated that gallic acid was able to up-regulate the pro-apoptosis protein and also to induce caspase activity.

2.3.2 Flavonoids

Flavonoids belong to a subfamily of polyphenols and they are a diverse group of secondary metabolites involved in reproduction, plant growth, seed germination, and protection against predators and pathogens (Treutter 2005). Structurally they are related

compounds with a chromane-type skeleton, and with a phenyl substituent in C2 or C3 position. There are over 4000 flavonoids identified from plant sources which are structurally unique (Middleton et al., 2000). Flavonoids are frequently present in photosynthesizing cells and as a matter of fact they are extremely ubiquitous among the plant kingdom. They can be found in nuts, fruits, seeds, vegetables, flowers, and stems, and also in wine, tea, honey and propolis, which are usual constituents of human diet (Cushnie and Lamb, 2005). The chief subclasses of flavonoids are flavonols, flavones, isoflavones, chalcones, flavanones, flavanonols and anthocyanidins (de-Rijke *et al*., 2006).

Flavonoids are the result of malonyl CoA addition to phenylpropanoid molecule of coumaroyl CoA (Figure 2.3; Pourcel et al. 2006). The main flavonoid structural feature is the flavane nucleus or the 2-phenyl-benzo[α]pyrane, that consist of two benzene rings (A and B) integrated through a heterocyclic pyrane ring (C) (Figure 2.4; Cushnie and Lamb, 2005; Kaiserová et al. 2007).

Flavonoids have been widely investigated because of their biological activities such as modulation of enzymatic activity, inhibition of cellular proliferation, and free-radical scavenging, and also for their potential utility as antiallergic, antibiotic, anti inflammatory, antiviral , and anti-diarrheal agents (Harborne and Williams, 2000; Cherng et al., 2007; Ramos, 2007). For instance flavonoids such as vitexin and orientin (C-glycosylflavonoid) have shown antiviral activity against para-influenza virus. To inhibit 50% of the cytopathic effect the required concentrations of vitexin and orientin were about 20.8 and 11.7 μg/ml respectively (Li et al., 2002).

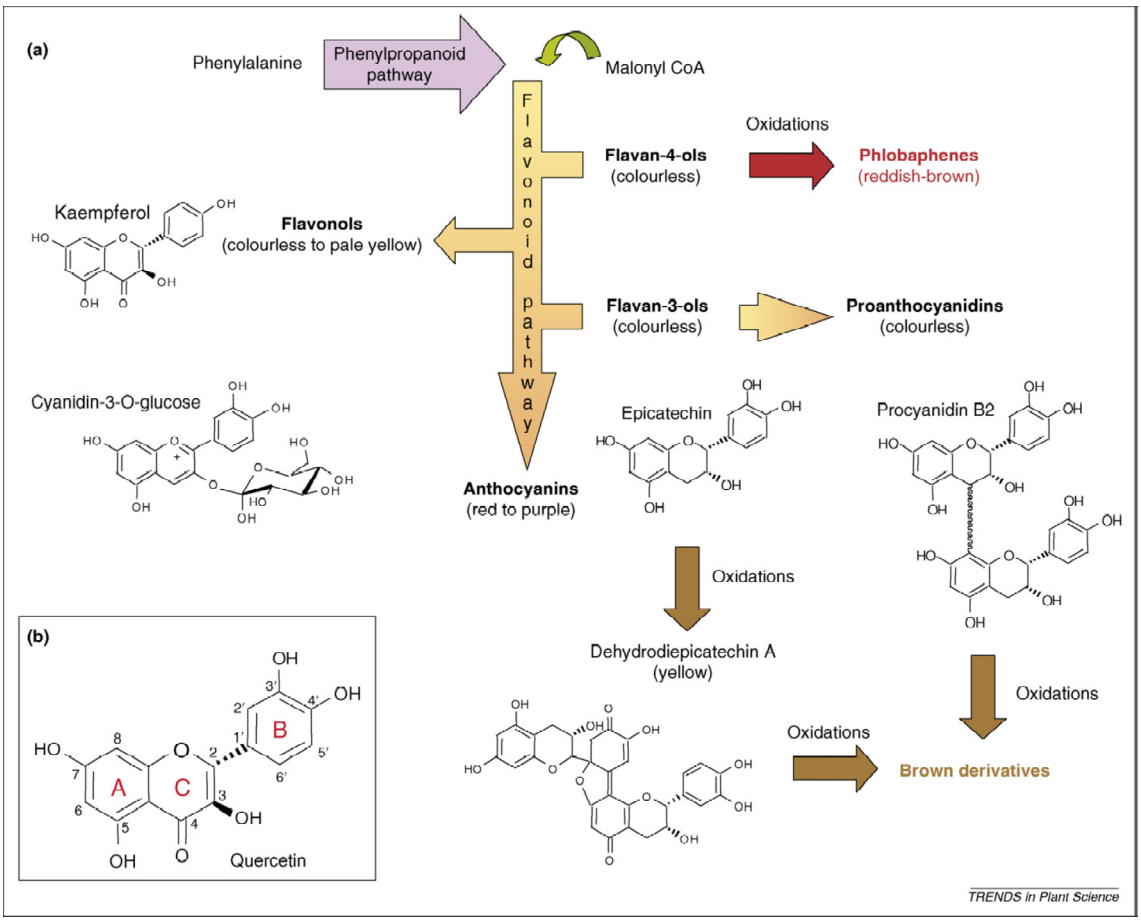
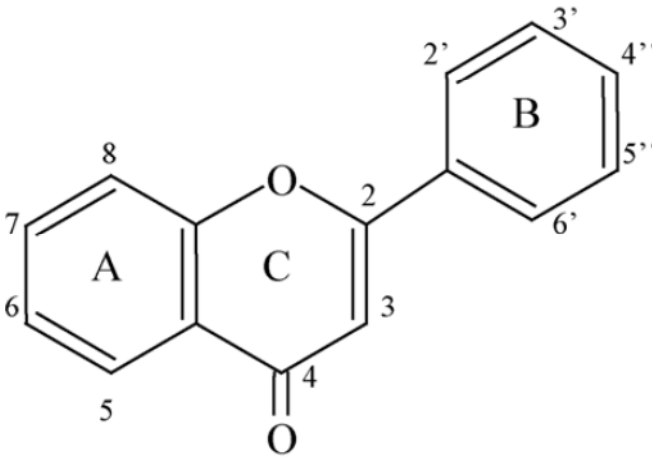


Figure 2.3 Simplified schematic flavonoid pathway [Main classes of end products are presented, and their molecular structure illustrated by one example for each class; Pourcel et al., 2006).



Flavones

	3	5	7	2'	3'	4'	5'
Quercetin	OH	OH	OH	-	OH	OH	-
Kaempferol	OH	OH	OH	-	-	OH	-
Myricetin	OH	OH	OH	-	OH	OH	OH
Rutin	OH	-	-	OH	-	-	-
Luteolin	-	OH	OH	-	OH	OH	-

Figure 2.4 Skeletal structure of flavones [A class of flavonoids, with ring named and positions numbered; The lower part of the figure shows some representative compounds where the hydroxyl group of ring B are shown; Cushnie and Lamb, 2005; Middleton et al., 2000)

2.4 Free Radicals

Certain molecules in the body which are called reactive nitrogen species (RNS) and reactive oxygen species (ROS) are commonly generated as part of the defense system and as by-products of cellular metabolic processes utilizing oxygen. The reactive species are molecules or free radicals that might be converted to oxidizing agents. A number of factors may cause the body to generate the reactive species more than required. These factors include drinking alcohol, smoking, excessive exposure to the sun, too much fat in the diet, excessive exercise and too many pollutants in the air (Simoes *et al.,* 1996). The oxidation procedure in the human body can damage cell membranes and also others such as DNA, lipids, and proteins. A wide range of free radicals and other reactive oxygen species like the hydroxyl radical ($OH^{\cdot}$), super oxide anion ($O_2^{\cdot}$), peroxyl (RO_2), and nitric oxide ($NO^{\cdot}$) may be formed in food systems and in the human body (Rice-Evans and Burdon, 1994).

Rice-Evans *et al.* (2001) and Amic *et al.* (2003) investigated the antioxidant activities of flavonoids and observed that the radical scavenging activity of flavonoids was dependent on the substitution pattern and the molecular structure of hydroxyl groups; in other words on the availability of phenolic hydrogens and on the probability of stabilization of resulting phenoxyl radical by expended electron delocalization or via the substitution pattern. The existence of a 3', 4'-dihydroxy in the B ring, having electron donating properties is necessary for flavonoids to be effective radical scavengers. Furthermore, the C2-C3 double bonds connected with a group of 4-keto, that is responsible or electron delocalization from the B ring, causes further radical scavenging (Van Acker *et al.,* 1995). The structural criteria which modulates the free radical scavenging activity of flavonoids is illustrated in Figure 2.5.

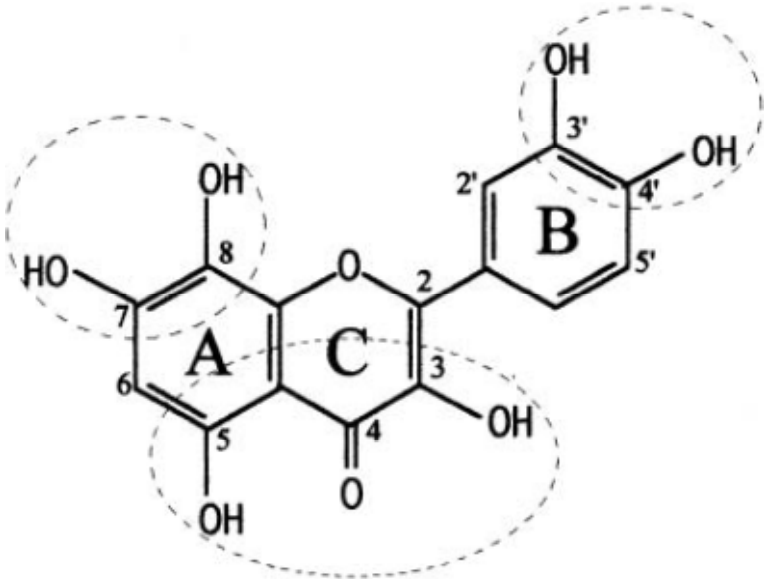


Figure 2.5 Structural features of flavonoids enabling high radical scavenging activity (Amic *et al.*, 2003).

2.4.1 Damage to Lipids, Proteins and DNA Caused by Free Radicals

There are many types of lipids in the body and one of them is responsible for maintaining the structural integrity of cell membranes. Free radicals can damage these cell membranes by oxidation, thereby making them leaky (Table 2.2). Free radical oxidation of another type of lipid, the low-density-lipoprotein (LDP), is considered to play a major role in the development of atherogenesis (Leonarduzzi *et al.*, 2000). Free radicals also cause damage to proteins and genes. Some proteins serve as enzymes that regulate metabolic reactions. Free radicals can interfere with these protein functions via various methods, including by cross linking, leading to irregular and abnormal metabolism (Table 2.2). DNA is the genetic material responsible for heredity. Oxidative damages to DNA can cause changes to both structure and function of chromosomes. These changes in the genetic code may lead to cancer and other chronic disease (Loft and Poulson, 1996).

Table 2.2 Free radicals and their effects (Namiki, 1990)

Radicals	**Effects**
Hydroxyl($OH^{\blacksquare}$)	Highly reactive radicals which attack all biological molecules.
Super oxide ($O_2^{\blacksquare}$)	Less reactive radicals which can travel in the blood and attack a number of biological targets.
Nitric Oxide($NO^{\blacksquare}$)	Act on smooth muscle cells in vessel walls causing relaxation.
H_2O_2	Cross cellular membranes easily and may cause expression of virus genes e.g. HIV infected cells. They have only a few cellular targets but can result in the production of hydroxyl radicals.

2.4.2 Lipid Oxidation

Lipids are present in almost all food raw materials and their major classes are phospholipids that exist in biological membranes and triglycerides, which are present in fat storage cells in animals and plants. In the processing of a variety of foods, fats are considered as part of the formulation of the food. The added fats are a chief component of some foods such as frying oils, margarine, and mayonnaise. The fats are triglycerides that are significant components as potential sources of oxidative off-flavors in foods. The phospholipids that are present in plant or animal tissues used as foods might be a significant and vital substrate for oxidative deterioration (Pokorny and Korczak, 2001).

An indispensable mechanism in free radical mediated cell injury is lipid peroxidation. It may cause direct damage to cell membranes and also the products of reactive carbonyl can spread the damage to sites far from the original location of production of the radical. This has been considered as a factor which involves a wide range of toxic tissue injuries as well as certain processes of specific diseases such as cancer. The oxidation of lipids

has a pivotal role in the health issues of the human body. The free radical oxidation of lipids is involved in the pathogenesis of some diseases including cancer and cardiovascular heart disease as well as the aging process (Chan et al., 1987). Lipid oxidation is quite usual in cells and adipose tissues, organelle membranes, brain, lipoproteins and tissues in which poly unsaturated fatty acids (PUFA) abundantly exist. The main mechanism of the oxidation of lipids can be explained in 3 different steps: initiation, propagation and termination (Figure 2.6). Initiation happens in contact with oxygen and in this reaction hyperoxides and peroxy radicals are formed. The initiation step is mediated by a number of agents and mechanisms such as high oxygen tension, radiation, and xenobiotic metabolism. The propagation reaction provides peroxy radicals and free radical $R^{\cdot}$ which can start chain reactions with some molecules. Termination reactions, in which a reduction of the unsaturated lipids happens, may stop the chain from self propagation. There is a tendency for the radicals to bond with each other leading to the production of some agents which are not useful to the propagation reaction. Peroxides cause cellular lesions in the major organs which happens by damaging the cellular compounds, such as phospholipids, poly unsaturated fatty acids, proteins, DNA, and free cholesterol. It has been proven that active oxygen species may oxidise lipid rich regions, subsequently demonstrating the presence of cross-linked sulphydryl in proteins and some other macromolecules, and cause instability in lipid bilayer from liberating free fatty acids (Fabbi et al., 2004; Tai et al., 2004). Moreover, extensive experimental support currently exists for early occurrence and pathophysiological significance of oxygen radicals and peroxidation of cell membrane lipids in the nervous system that has been injured (Balu et al., 2005). Lipid oxidation starts with free radical production. The substances which contain one or more unpaired electrons and are able to exist independently are called free radicals. Free radicals include superoxide ($O_2^{\bullet}$), trichloromethyl ($CCl_3^{\bullet}$), hydroxyl ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$),

and peroxyl ($ROO^{\bullet}$), and are known as agents which can be generated in living organisms metabolically. The derivatives of oxygen molecules which are non-radicals including hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) may be produced in biological systems and foods (Sanchez-Moreno, 2002).

The significance of utilizing more than a single method for determination of prooxidant or antioxidant activity is emphasized for evaluating lipid oxidation, because of the various test systems and the oxidizable substrates generated (Lim et al., 2001).

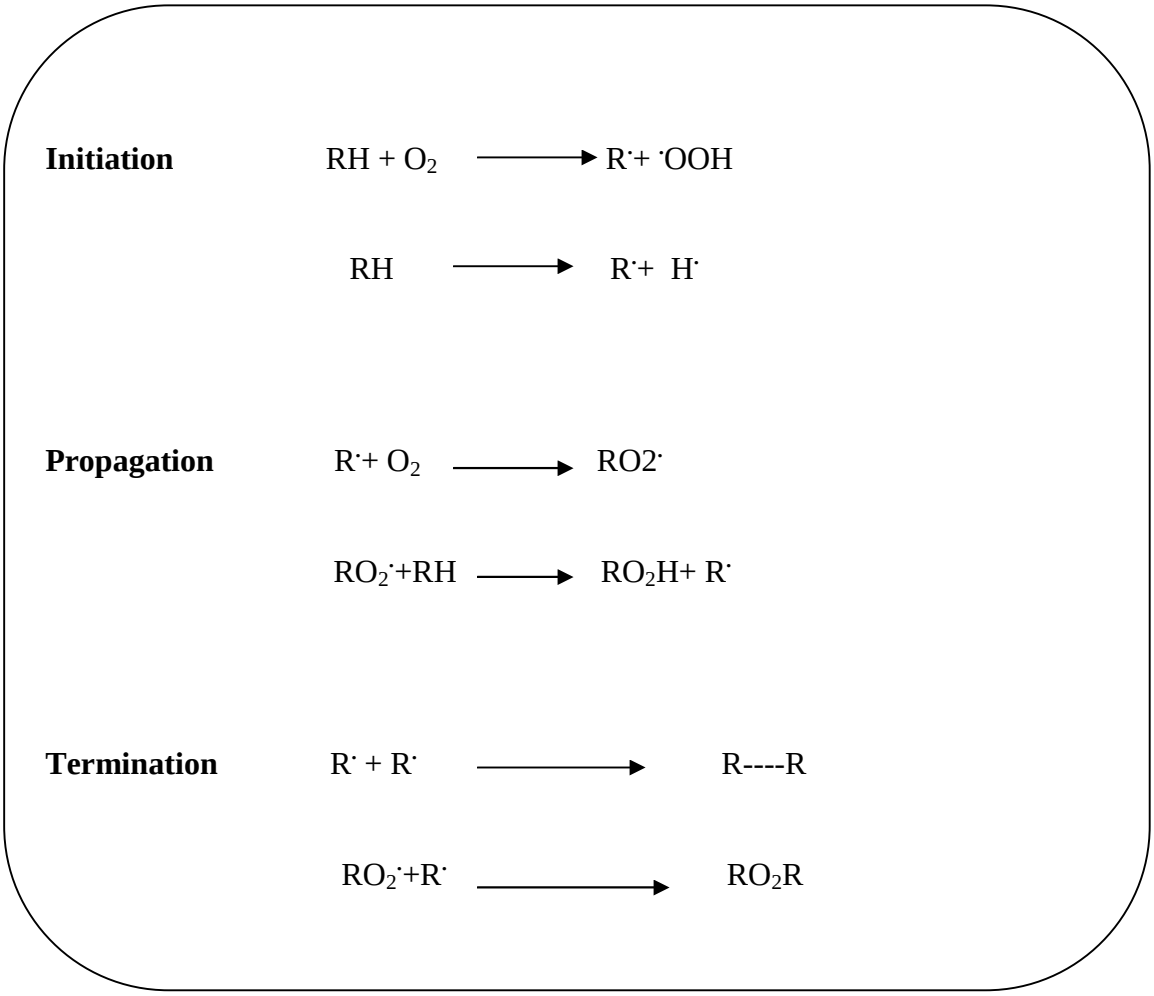


Figure 2.6 Steps in lipid oxidation (Halliwell, 2002).

2.5 Antioxidants

Antioxidants, including phenolic compounds and flavonoids with biological activities, are secondary metabolites which are derived from plants. The biological activities of the products in the inhibition of lipoxygenase led to antioxidant activity investigations (Castelluccio et al., 1995). Antioxidant is an old term, that was first used to describe inhibitors of oxidative processes, that were capable of reacting with peroxyl radicals (Denisov et al., 2005). Antioxidants are considered as agents with a very indispensable role against reactive oxygen species (ROS) in the defence system of the body. The reactive oxygen species (ROS) are injurious by-products produced during normal cell aerobic respiration (Ou et al., 2002). These harmful by-products are able to oxidize lipids, nucleic acids and cellular protein. The peroxidation of lipids is a free-radical mediated propagation of oxidative insult on polyunsaturated fatty acids (PUFAs) involving many types of free radicals. Termination may occur by enzymatic means or through free radical scavenging using antioxidants (Korkina and Afans'ev, 1997). ROS can contribute to mutagenesis (Takabe et al, 2001), cellular aging (Sastre et al., 2000), DNA damage (Takabe et al., 2001), coronary heart disease (Khan and Baseer, 2000) and carcinogenesis (Kawanishi et al., 2001). The most common antioxidants which exist among plants are carotenoids (Stahl and Sies, 2002), Vitamin E and C (Tsao and Deng, 2004), flavonoids (McCune and Johns, 2007), thiol (SH) compounds (Ou et al., 2002) and polyphenols (Scalbert et al., 2005).

2.5.1 Natural Antioxidants

Epidemiological evidence has demonstrated that consumption of fresh fruits is generally advantageous for health and helps to prevent some of the degenerative diseases like

cardiovascular diseases and cancer. The capacity of the antioxidants in vegetables and fruits is a determinative factor (Hertog et al., 1993). The first investigation to point out that the consumption of fruits and vegetables was inversely associated with breast cancer was a study from Greece (Katsouyanni et al., 1986). The antioxidant vitamins that occur naturally, include the vitamin E family of compounds (tocopherols and tocotrienols), vitamin C and carotenoids (which may also be pro-vitamin A). Some metal elements that are found in the diet may exert *in vivo* antioxidant influences as metallo-enzymes like selenium (a part of the glutathione peroxidase). A number of the compounds that exist in vegetables and fruits which promote body health are considered as strong antioxidants. Tocopherols and ascorbic acid are the most substantial natural antioxidants that are commercially available (Table 2.3) (Casimir and David, 2002). The natural antioxidant products are commercially quite important and they are absolutely desired by consumers. The basic and most prominent advantage of substances which exist in foods naturally is that it is much easier to prove the safety of the products in comparison to synthetic products. A number of the natural antioxidants that have been derived from a variety of herbs, such as Maillard reactions and spices may be listed as flavorants instead of antioxidants, due to a technical distinction that can serve to exempt these substances from the requirements of testing for safety (Casimir and David, 2002).

Table 2.3: Antioxidant Components in Food (Aruoma, 1999)

Common name	Compound	Food source
Vitamins	**Vitamin C** (ascorbic acid) **Vitamin E** (tocopherols and tocotrienols) Beta carotene and other carotenoids	Citrus fruit, berries, papaya Seed-like cereal grains, nuts and oils derived from plants. Orange pigmented, and green leafy vegetables
Elements	**Copper** (as part of superoxide dismutases) **Selenium** (as part of glutathione peroxidase	Cocoa, wheat bran, yeast grains, meats.
Macronutrient –derived	Peptides e.g. glutathione	Whey protein
Phytochemicals(food components of plant origin)	**Isoflavone** e.g. genistein and daidzen **Flavonols** e.g. quercetin and kaempferol. **Polyphenols** e.g. rosmarinic acid **Catechins** e.g. epigallocatechin gallate	Soy, Tea, red wine, onions, apples. herbs, oregano, thyme. Green tea, meats
Zoochemicals (food components of animal origin)	**Ubiquinone** (coenzyme Q_{10}).	Meats especially meat organs, fish

2.5.2 Synthetic Antioxidants

Synthetic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are widely utilized in food industries. However, synthetic antioxidants are reported to be responsible for causing or promoting some negative health problems like lung, liver, gastrointestinal tract damage and also carcinogenesis among laboratory animals (Grice, 1986; Wichi, 1988; Sasaki et al., 2002). Hence, more strict restrictions are currently imposed for their application. For instance Butter Yellow (p-dimethylaminoazobenzene), which is an azo compound, has been removed from the list of antioxidants used for food in the United State of America owing to the fact that it has been implicated as a carcinogen in a large number of animal species (Sasaki et al., 2002). The dietary supplement of BHT can inhibit a number of hepatic cytochrome P450s-linked activities and also activate aflatoxin to exo-aflatoxin-8,9-epoxide in turkey

liver. Thus, BHT is able to process chemoprotection (Klein et al., 2003) and act as anticarcinogen in food additive usage (William et al., 1999).

There is therefore a trend to substitute them with naturally occurring antioxidants. Antioxidant activities have been in spices, including tropical ginger, pepper, medicinal plants, and herbs (Mau et al., 2003; Perucka and Materska, 2001), and also in some fruits like cherries, berries, grape, pear, citrus, kiwi, plums and olives (McDonald et al., 2001; Netzel et al., 2007; Negro et al., 2003; Kim et al., 2003; García -Alonso et al., 2004; Ponce et al., 2004; Kuti, 2004), as well as in vegetables like onion, mushroom, cucumber, potato, pea, spinach, garlic and tomato (Nuutila et al., 2003; Cheung et al., 2003; Jing et al., 2003; Singh and Rajini, 2004**;** Osman et al., 2004). Antioxidant activities have been noted in beverages like wines, beer (Gorinstein et al., 2000), and also in green and black teas (Morsy and Khaled, 2002; Bonnely et al., 2003).

2.5.3 Methods in evaluating antioxidant activity

Several *in vivo* methods and *in vitro* models have been developed to evaluate antioxidant activity. Some examples of simple in vitro models that have been used frequently to evaluate total antioxidant activity (Tsao and Deng, 2004), include:

- β-Carotene-linoleic acid model system (β-CLAMS)

The method is based on β-carotene decolouration which is performed by peroxides produced during linoleic acid oxidation at an increased temperature. The readings are recorded at 490 nm at time intervals of 15 minutes for 100-300 min. The existence of stronger antioxidants is indicated by flatter decaying curves (Tsao and Deng, 2004).

- Ferric reducing/antioxidant power (FRAP)

The FRAP assay is considered as a new method to assess antioxidant power. Ferric reducing ability of plasma (FRAP) happens at a low pH. A complex of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) can be reduced to ferrous (Fe^{2+}) form, with an intense blue colour and with an absorption maximum at 593 nm. This is a non-specific reaction. Any half-reaction with a less-positive redox potential, under the conditions of the reaction, may favour the complex reduction and, as a matter of fact, the development of the colour, indicates that an antioxidant (reductant) exists (Benzie and Strain, 1996).

- Oxygen radical absorption capacity (ORAC) method

It is utilized to evaluate the antioxidant capacity of water-soluble phytochemicals. A peroxyl radical generator, called AAPH (2,2'-azobis(2-amidinopropane) dihyrochloride and a fluorescent protein, called R-phycoerythrin (RPE) are applied in the assay. The emission and excitation wavelengths are set at 565 and 540 nm, respectively (Tsao and Deng, 2004). The ORAC assay is not considered as a "total antioxidant activity assay", owing to the fact that it is only able to measure antioxidant activity against peroxyl radicals (Ou *et al.*, 2002).

- Thiobarbituric acid reactive substance (TBARS) method

The assay is based on stable product detection, that is produced between thiobarbituric acid (TBA) and aldehydes in the aqueous phase. The generation of TBARS is spectrophotometrically measured at 535 nm after an incubation period of 20 minutes at 80 ^{o}C (Tsao and Deng, 2004).

- Trolox equivalent antioxidant capacity (TEAC) method

The TEAC assay is based on the relative capability of the antioxidants for scavenging the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{+}$) which is produced through the interaction of ABTS with the ferrlymyoglobin radical species, that is produced through the activation of metmyoglobin with H_2O_2.

Some other free radicals like 2,2-diphenyl-1-picrylhydrazyl (DPPH·) have also been utilized to measure the antioxidant activity. DPPH· indicates a maximum absorbance at 515 nm that disappears upon reduction by an antioxidant phytochemical with anti-radical properties (Tsao and Deng, 2004).

- Photochemiluminescence (PCL) method

This method is based on the photo-induced auto-oxidation inhibition of luminol through antioxidants mediated from the radical anion superoxide ($O_2^{\cdot -}$). Owing to the fact that the latter is a deleterious by-product from oxygen metabolism, and is responsible for significant damages related to reperfusion injuries, the values achieved by PCL method can be directly related to the health properties of a food or an ingredient. The PCL method is very easy and fast and offers a great deal of advantages (Sacchetti *et al*., 2005).

2.6 Cancer

Cancer is one of the most important causes of death in the world. The death rate of the world population due to cancer has been estimated at 12.8%. About 4.7 million women and 5.3 million men developed malignant tumors in the year 2000, and among them 6.2 million people died due to the disease.

The number of new cases is expected to grow by 50% which may lead to 15 million patients by the year 2020. There were 1,050,346 cases and 372,969 deaths reported for breast cancer worldwide (Stewart, 2008). Breast cancer has been the most prevalent cancer among women in the world. This cancer is one of the most life-threatening health problems which can happen to a woman in the duration of her lifetime. Approximately more than 1 million women in the world are facing the disease and more

than 400,000 women die because of it (Stewart, 2003). In 2002, the cancer represented 30.4% of all malignancies among women of all ethnicities in Malaysia, and the cumulative lifetime risk was reported as 1:19 (Lim, 2003). The Age Standardized Rate (ASR) of breast cancer among women has been reported as 52.8 per 100,000 population (Lim, 2003). The most recent statistics of the National Cancer Registry (NCR) indicate that breast cancer is proven to be the most frequent cancer among women in the country, whilst lung cancer is the most frequent cancer normally experienced by men. Lung cancer accounts for about 13.8 per cent of all cancer cases in men. Among women, breast cancer accounts for 31 per cent of all cases (Lim, 2003).

There is a wide range of lifestyle factors including obesity, weight gain, level of physical activity and fat intake associated with the risk of breast cancer. The women who are overweight are normally at higher risks of postmenopausal breast cancer. Obesity and also a high intake of fat, meat, alcohol and dairy products can enhance the risk and also a high intake of fruits, fiber, vegetables, phytoestrogens, and anti-oxidants can reduce the risk (Farah & Begum, 2003).

2.6.1 Phytomedicine

The products of plants have always been used to cure or even to prevent diseases throughout history. There are a wide range of natural compounds among plants, bacteria and fungi that have been utilized to plan and design novel drugs in the process of drug development. The cases of breast neoplasia that were reported over the last few decades have been clearly on the increase and it has led to the development of novel drug combinations, anticancer drugs, and chemotherapy strategies being performed through scientific exploration of a wide range of natural, biological, and synthetic products.

The requirement for exploring to find effective anti-cancer agents on the one hand, and the association of fruits and vegetables consumption with reduced cancer risk on the other hand, leads us to consider a variety of edible plants as valuable sources for anticancer drugs. An enormous amount of scientific evidence is available which indicate that medicinal plants as the most important source of novel healthcare products and pharmaceuticals, including medication for ethno-veterinary medicine. In recent years, chemoprevention of cancer by a wide range of strategies using medicinal herbs has been considered.

Currently investigations are mostly focused on antibiotics and drugs which are active against tropical diseases, anti-tumor drugs derived from plants, anti-inflammatory drugs, contraceptive drugs, drugs for psychiatric use, and also kidney protectors. Recent epidemiological investigations have reported that antioxidant supplements can decrease the risk of breast cancer-related mortality and also breast cancer recurrence. Consumption of beverages and food rich in poly-phenols such as anthocyanins, flavones, and catechins, might also reduce incidence of cancers. Experimental studies indicate that a number of plant extracts and agents had the potential as anticancer and antioxidant treatments in a variety of animal models and bioassay systems relevant to human diseases (Aziz et al., 2003).

2.6.2 Molecular Basis of Cancer

Cancer is a disease that includes a multi-step process. The application of epithelial tissues for instance, with mutation or even loss of the Adenomatous Polyposis Coli (APC) gene, a colon which is normal may change into a hyperplasic one. Hyperplasia can develop to adenoma, when one of the numerous proto-oncogenes gets activated as well. Later, if one of the genes which work as tumor suppressor such as p53 loses the

normal function, the resulting adenoma will advance into carcinoma and a process of invasion into some other locations in the body, metastasis, will start to happen (Coleman, 2008).

The whole procedure is associated with genome damage, and the genes which are involved in the development of cancer and the factors that might be the cause of the damage to those genes will be at the focus of attention. Several investigations have demonstrated that four different types of genes are normally involved in the development of cancer including tumor suppressor genes, proto-oncogenes, DNA repair genes, and apoptosis genes (Coleman, 2008).

Proto-oncogenes are genes which code for the growth factor receptors (CSF and EGFR), the growth factors (FGF and EGF), cell cycle regulators (cdks and cyclins), and signal transduction proteins (abl and *ras*), and when these genes mutate, they may change into oncogenes. A very important proto-oncogene is called “Ras” which is a signal transduction protein. When this gene is functioning in a normal and correct way, the protein which is GTP-binding will switch between two different states, or “on” and “off”. After it is bound to GTP in the “on” state, it may be hydrolyzed and will go back into the “off” state which is a GDP-binding state. However, if it is mutated it is not able to be hydrolyzed after binding GTP, and therefore Ras may not switch into the “off” state and will remain in the “on” state. If the signaling results in activating certain transcription factors, then the mutated Ras will lead to cancer (Coleman, 2008).

The genes considered as tumor suppressors are those that inhibit or disfavor division of cells. After the DNA is damaged, p53 will activate the transcription of p21, which is a cyclin kinase inhibitor (CKI). As a result, p21 will bind and then inhibit a variety of cyclin-CDK complexes, and stop the cell cycle in the G1-S phase. In the case where p53 has a mutation, p21 will not be activated, and the cell cycle will go on through mitosis,

and subsequently, the damaged DNA will be passed into the daughter cells, and the process can result in cancer.

There are different genes in charge of regulating apoptosis, including *bcl-xS, bad*, and *bax*, that drive programmed cell death, or apoptosis and *bcl-xL* and *bcl-2*, that favor cell survival. The loss of function for *bcl-xS, bad,* or *bax,* or the over-expression of *bcl-xL or bcl-2* might contribute to cancer development (Coleman, 2008).

Among the different types of damage that may cause the mutation of proto-oncogenes to oncogenes, the most frequent is chemical damage. Reactive oxygen species (ROS) have an indispensable role in DNA damage. There are more than 100 variable types of oxidative DNA lesions which have been described, and they range from base modification to single-strand and double-strand DNA breaks and also intra-strand cross-links. The resulting lesions may disrupt important processes like transcription and replication, which can lead to death of cells or even growth arrest. It might also induce mutations which may cause cancer (Hasty et al., 2003).

ROS may arise if the cell uses oxygen, just like when inflammatory cells undergo phagocytosis and generate hydrogen peroxide (Coleman, 2008). Furthermore, ROS inflammation may also enhance generation of cytokines. The cytokines may activate the pathway of signal transduction which might lead to phosphorylation, ubiquitination, and degradation of the inhibitor of NF-κB, I-κB. The activated NF-κB will exert anti-apoptotic activities and induce or enhance cancer development (May and Ghosh, 1997).

The cytoprotective antioxidants and enzymes, like superoxide vitamin E, dismutase, tea polyphenols, ascorbate, catalase, etc., can provide comprehensive protection against cancer development by down-regulating ROS production, scavenging ROS, inhibiting pro-oxidant enzymes, and also by eliminatimg radical precursors (Lin and Tsai, 1999).

Biological observations produced from the broader systemic cancer structure include: (i) self-sufficiency in growth signals; (ii) insensitivity to anti-growth signals; (iii) evading apoptosis; (iv) sustain angiogenesis; (v) limitless replicate potential; and (vi) tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Steroid hormones are very important for reproductive systems development. However, when the hormones or even their receptors are produced more than the normal amounts, the cancers of reproductive systems may lead to a simplified pathway of steroid biosynthesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant sample

Fresh fruits of *Veitchia merrillii* were collected from several locations inside the University of Malaya campus. The fruits were separated, washed and were then dried at room temperature for approximately three weeks. the dried fruits were ground into fine powder using a grinding machine (Model DF-20).

3.2 Reagents

Ethyl acetate, methanol, hydrochloric acid, sodium carbonate, Folin-Ciocateu reagent, gallic acid, sodium hydroxide, aluminium chloride, rutin, alpha-tocopherol, ascorbic acid, butylated hydroxytoluene (BHT), dimetyl sulfoxide, acetonitrile HPLC grade, and 1.1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Fisher Scientific, USA. Potassium ferricyanide, trichloroacetic acid, ferum chlorate and Difco ™ agar nutrient broth were purchased from Sigma Aldrich.

Dulbecco's Modified Eagle's Medium (DMEM) with or without phenol red, foetal calf serum (FCS) and *Escherichia coli* lipopolysaccharide (strain 055:B5), N-(1-naphtyl) ethylene diamine dihydrochloride, sulfanilamide, 85% phosphoric acid, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT powder) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.3 Reflux Extraction (Hydrolysis)

Solvents of different polarity (methanol, ethyl acetate and water) were used to extract the soluble constituents from *Veitchia merrillii* fruits. The hydrolysis extraction technique was performed according to the method described in Crozier et al. (1997). Half gram of the dried *Veitchia merrillii* fruit powder was weighed and transferred into a 100 ml conical flask. Forty ml of methanol or ethyl acetate (v/v) was added, followed by 10 ml of 6M HCl. The mixture was then stirred with the use of a magnetic stirrer. The mixture was then placed in a sample flask (250 ml) and refluxed for 2 hours at 90 ^{o}C. The mixture was then filtered through a Whatman No. 1 filter paper (Whatman, England) and evaporated to dryness in a vacuum rotary evaporator (Buchii, Switzerland) at 40 ^{o}C. The method described in Gulcin et al. (2004) was used for extraction with water. Five gram dried sample was mixed in 100 ml water using a magnetic stirrer for 15 min. The extract was then filtered through Whatman No. 1 paper and evaporated to dryness using a vacuum rotary evaporator (Buchii, Switzerland). The crude extracts were re-dissolved in 5 ml of respective solvent and retained for further tests.

3.4 Determination of Total phenolic and Flavonoid Content

The determination of total phenolic content was performed based on Folin-Ciocalteu's reagent (Halicia et al., 2005), while the total flavonoid content was determined using the aluminum chloride colorimetric assay (Ismail et al., 2010).

3.4.1 Total Phenolics

The Folin-Coicalteu's reagent was used for the determination of total phenolic compounds in the extracts (Halicia et al., 2005). A series of different concentrations of gallic acid in distilled water was initially prepared (0, 100, 150, 200, 250 and 300 µg/ml) and a standard curve was plotted with the absorbance at 765 nm versus the amount of standard phenolic (µg) concentrations applied.

To 500 micro liter of *Veitchia merrillii* fruit extracts were added 2.5 ml of Folin-Ciocalteu's reagent (diluted 1:10, v/v) and 2 ml of 7.5% sodium carbonate (w/v). The mixture was then vortexed and incubated at 30 °C for 90 minutes. The mixture was then diluted by five times and the absorbance was read at 765 nm. All samples were prepared in triplicate in the dark. The amount of total phenolics was calculated as mg of gallic acid standard solution and expressed as mg gallic acid equivalent (GAE) per gram dry weight (DW) of plant material.

3.4.2 Total Flavonoids

Determination of flavonoid compounds in the extracts was carried out using the aluminium chloride colorimetric assay (Ismail et al., 2010). A series of concentrations of rutin was initially prepared in distilled water (100, 150, 200, 250, 300 µg/ml). A standard curve was plotted with absorbance at 510 nm versus the amount of standard flavonoid (µg) used. An aliquot (0.1 ml) of extracts was added to 0.3 ml 5% $NaNO_2$. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6 min, 2 ml 1 M NaOH was applied and the total volume was made up to 5 ml with distilled water. The solution was then mixed well and the absorbance was finally measured at 510 nm.

3.5 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The phenolic and flavonoid compounds in the *Veitchia merrillii* fruit extracts were quantitatively measured by reversed-phase HPLC (Agilent 1100, Agilent) according to the method described in Schieber et al. (2001). The methanolic extracts were used for determination of biological activities. The samples were dissolved in 5 ml methanol HPLC grade and filtered through membrane-filters (0.45 µm). Phenolic standards were syringic acid, gallic acid, caffeic acid, vanillic acid and pyrogallol, and flavonoid standards were naringenin and rutin. Aliquots of sample extracts was loaded on to the HPLC system equipped with an Intersil ODS-3 (5 µm 4.6 x 150 mm, Gl Science Inc) analytical column. All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), prepared in methanol-dimethyl sulfoxide (DMSO) (v/v; 50:50), and stored at -18 $^{\circ}$C before use. The mobile phase was composed of (A) 2% acetic acid (aqueous) and (B) 0.5% acetic acid (aqueous)-acetonitrile (50:50 v/v), and gradient elution was performed as follows: 0 min 95:5; 10 min 90:10; 40 min 60:40; 55 min 45:55; 60 min 20:80; and 65 min 0:100. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate was 1 ml/min. UV absorbance for phenolic and flavoniod compounds were measured at 280 nm and 350 nm, respectively. The operating temperature was maintained at room temperature.

3.6 Antioxidant Assay

The antioxidant activity in methanol, ethyl acetate and hot water extracts of *Veitchia merrillii* fruits was determined using three different assays which were free radical scavenging activity (1,1-diphenyl-picryl-hydrazyl; DPPH), Nitric oxide scavenging activity, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS)

3.6.1 DPPH Free Radical Scavenging Activity

The free radical scavenging activity for each of the extracts was determined using the DPPH assay as described in Gulcin et al. (2004). One milliliter the methanolic extract of *Veitchia merrillii* fruits at the different concentrations were mixed with 3 mL 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazil (DPPH) in methanol. After incubation at room temperature for 30 min in the dark, the absorbance of the mixture was read using a spectrophotometer (Novaspec II Visblespectro) at 517 nm. Ascorbic acid and α-tocopherol were utilized as antioxidant standards. Free radical scavenging activity from the sample was calculated according to the following formula:

$$[(A0- A1)/A0] \times 100\%$$

A0 represents the absorbance of the control reaction

A1 represents the absorbance in the presence of the sample.

3.6.2 Nitric Oxide Scavenging Activity

The nitric oxide (NO) scavenging activity of each extract was determined by the method described in Tsai et al. (2007). Sixty microliters of two-fold diluted samples were mixed with 60 μL of 10 mM sodium nitroprusside in phosphate buffered saline (PBS) in a 96-well flat-bottomed plate and the plate was incubated under light at room temperature for 150 min. Finally, an equal volume of Griess reagent was added into each well and the NO content was measured. Ascorbic acid, BHT and α-tocopherol were included as controls. The NO-scavenging effect of extracts was expressed as IC50 which denotes the concentration of tested herbal tea extracts required to quench 50% of the NO radicals released by sodium nitroprusside.

3.6.3 ABTS radical cation-scavenging

The ABTS radical cation-scavenging activity was evaluated using the method described in Giao et al. (2007). ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline- 6-sulfonic) acid) was initially dissolved in water at 7 mM concentration. ABTS radical cation ($ABTS^{\cdot +}$) produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) was allowed to stand in the dark at room temperature for 16 h before use. To obtain an absorbance of 0.700 ± 0.005 at 734 nm, the stock solution was diluted with ultra-pure water as required. The absorbance was measured with a UV 1800 spectrophotometer (Shimadzu). A 100-µl sample in PBS was added to 900 µl of this diluted solution, and the absorbance was determined at 734 nm after 2 min initial mixing. The antioxidant solution reduced the radical cation to ABTS, which led to observing a reduction in the colour. The extent of decolorization was calculated as the percentage reduction in absorbance.

3.7 Cytotoxic effect of Veitchia merrillii fruit extracts

3.7.1 Cell Culture and Treatment

NIH/3T3 (Fibroblasts cells) and Human hepatocytes (Chang liver cells) obtained from the American Type Culture Collection (ATCC) were utilized in this study. The cells were subcultured in the ratio of 2:3 to 1:6 for two or three times and were then seeded in 96 well plates (1×10^4 cells/well) in serum-free medium and incubated for 24 h. The medium was then removed and treated with crude extracts at concentrations ranging from 3.12-200 µg/ml (Ahmad et al., 2005).

3.7.2 MTT Assay

The assay at each sample concentration was carried out in triplicate and the culture plates were kept at 37 ^{o}C under 5% CO_2 for 3 days. After incubating for 72 h at 37^{o}C, 100 µl of medium was removed from each of the wells. Subsequently, 20 µl of 0.5% w/v MTT (Sigma, USA) dissolved in phosphate buffer saline, was then added to each of the wells to dissolve the formazan crystals. Absorbance values were then measured at 550 nm with a microplate reader (Bio Tek EL 340, USA). The anticancer activity was expressed as IC_{50} (concentrations that show 50% inhibition of proliferation on any tested cell line). An anticancer drug, called Tamoxifen, was utilized as positive control in the current study (Ahmad et al., 2005).

3.8 Statistical Analysis

The experiment was carried out using a completely random design in three replicates. All data were analysed using the analysis of variance procedure, followed by separation of means at $p < 0.05$. All experimental results in the study are expressed as means ± standard deviation. The graphs were prepared using Graph pad version 5.0 and the statistical analysis was carried out using SAS Software version 9.1.

CHAPTER 4

RESULTS

4.1 Total phenolic and flavonoid contents in *Veitchia merrillii* fruits

Three different solvents were used for the extraction of *Veitchia merrillii* fruits. Table 4.1 clearly demonstrates the yields of the extracts of *Veitchia merrillii* fruits that had been extracted by water, ethyl acetate and methanol. Results obtained from the study indicated that in general, the highest yield of extracts was obtained by methanol extract compared to the other organic solvents applied. From 5g of dried weight of *Veitchia merrillii* fruits powder sample of methanol, ethyl acetate and water extract were able to give 28.25±2.12%, 21±1.31% and 14.75±1.83% yield of extract respectively. Total phenolic contents was expressed as gallic acid equivalent (GAE)/g dry weight (DW) by referring to a standard curve of gallic acid ($y=0.008x+0.106$, $R^2 = 0.980$) (AP 1). The results of the total flavonoid contents were expressed as rutin equivalents/ g dry weight (DW) by referring to a standard curve of rutin ($y=0.002x+0.009$, $R^2 = 0.99$) (AP 2). The results on the flavonoid and phenolic contents of *Veitchia merrillii* extracts obtained by the use of different solvents are clearly demonstrated in Table 4.2. *Veitchia merrillii* fruits contained flavonoid and phenolic compounds and different contents of total flavonoid and phenolic were obtained by the use of different solvents. Remarkable differences ($p < 0.05$) in the phenolic content of water, ethanolic and methanolic extracts were observed, with values of 2.22, 7.6 and 17.8 mg GAE/g DW, respectively. Similarly, the flavonoid contents were significantly higher in the methanolic extract, with a value of 5.43 mg rutin equivalent/g DW in comparison with the ethyl acetate extract at 3.12 mg and the water extract with a value of 1.1 mg rutin equivalent/g DW. This research clearly indicates that different extracting solvents influenced different

levels of total flavonoid and phenolic compounds in the present study.

Table 4.1 Percentage yield of extracts in the different solvents.

Extracts	Crude yield extract (%)
Methanolic extract	28.25 ± 2.12^{a}
Ethyl acetate extract	21 ± 1.31^{b}
Water extract	14.75 ± 1.83^{c}

Means in the same columns with the different superscript letters [a, b and c] are significantly different at $p< 0.05$ (Given by Duncan Comparison Test).

Table 4.2 Total phenolics and flavonoids extracted by the different solvent.

Samples	Total Phenolic Compounds[1] (mg/g DW)	Total Flavonoid Compounds[2] (mg/g DW)
Methanolic extract	17.8 ± 0.45^{a}	5.43 ± 0.33^{a}
Ethyl acetate extract	7.6 ± 0.37^{b}	3.12 ± 0.54^{b}
Water extract	2.22 ± 0.25^{c}	1.1 ± 0.63^{c}

[1]mg gallic acid eq./g DW
[2]mg rutin eq./g DW
Means within columns with the different superscript letters [a, b and c]are significantly different at $P<0.05$(Given by Duncan Comparison Test).

4.2 Quantification of phenolic and flavonoid compounds present in *Veitchia merrillii* fruits

From the results, it can be concluded that pyrogallol, gallic acid, caffeic acid, syringic acid, vanillic acid, rutin and naringin were present as the major flavonoid and phenolic acid compounds in the extracts of *Veitchia merrillii* fruits. The results obtained from the HPLC analysis clearly indicated that the methanolic extract exhibited a variable pattern of phenolic (Table 4.3) and flavonoid compounds (Table 4.4) compared to the water and ethyl acetate extracts. The methanolic extract of *Veitchia merrillii* fruits demonstrated that gallic acid and pyrogallol were the major phenolic compounds with values of 101.6 ±0.19 and 913.1 ±0.79 µg/g dried weight, respectively (Table 4.3). It was also

demonstrated that naringenin was the main flavonoid compound in the methanolic fruit extracts of *Veitchia merrillii* with a value of 895.4 ±0.36 µg/g dry sample (Table 4.4). The HPLC chromatograms of phenolic and flavonoids compounds in methanolic fruit extracts of *Veitchia merrillii* are illustrated in Figures 4.1 to 4.2.

Table 4.3 Concentration of major phenolic compounds in *Veitchia merrillii* fruit extracts.

Compound	Phenolic compounds (µg/g DW)		
	Methanolic extract	ethyl acetate extract	Water extract
Gallic acid	101.6±0.19	37.6±0.27	59.9±0.57
Pyrogallol	913.1±0.79	56.0±0.66	152.3±0.14
Caffeic acid	73.8±0.92	18.8±0.97	27.8±0.12
Vanillic acid	21.6±0.26	24.8±0.12	26.2±0.82
Syringic acid	22.9±0.38	20.5±0.47	16.8±0.79

All analyses were mean of triplicate measurements ± standard deviation.

Table 4.4 Concentration of major flavonoid compounds in *Veitchia merrillii* fruit extracts.

Compound	Flavonoid compounds (µg/g DW)		
	Methanolic extract	ethyl acetate extract	Water extract
Rutin	41.1±0.61	13.9±0.52	8.9±0.43
Naringenin	895.4±0.36	192.5±0.24	152.3±0.30

All analyses were mean of triplicate measurements ± standard deviation.

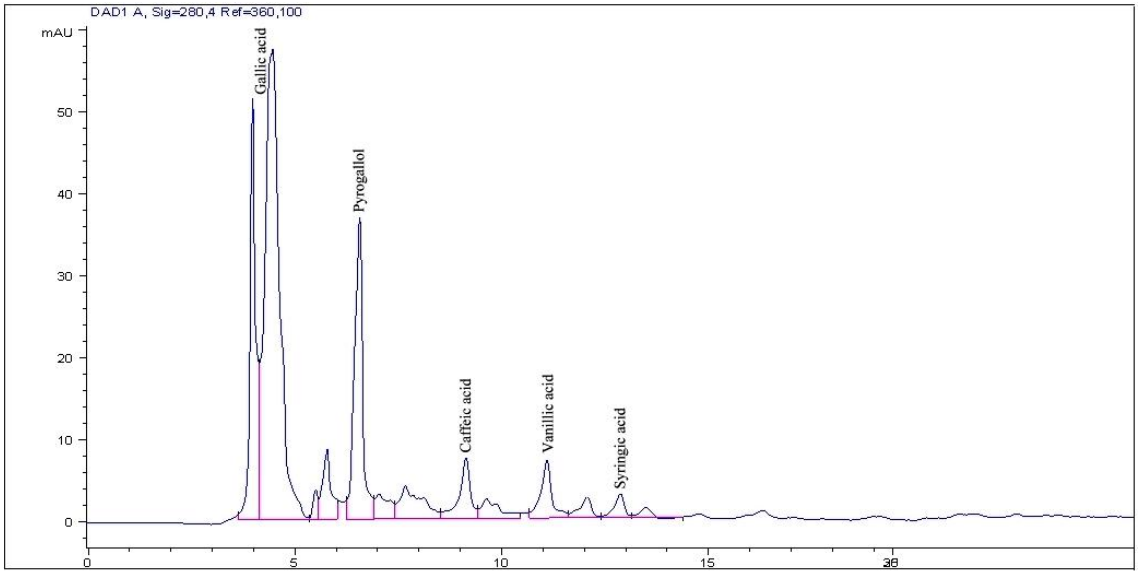


Figure 4.1 RP-HPLC chromatogram of phenolic compounds in methanolic extract of *Veitchia merrillii* fruits [Major compounds present: gallic acid, pyrogallol, caffeic acid, vanilic acid and syringic acid]

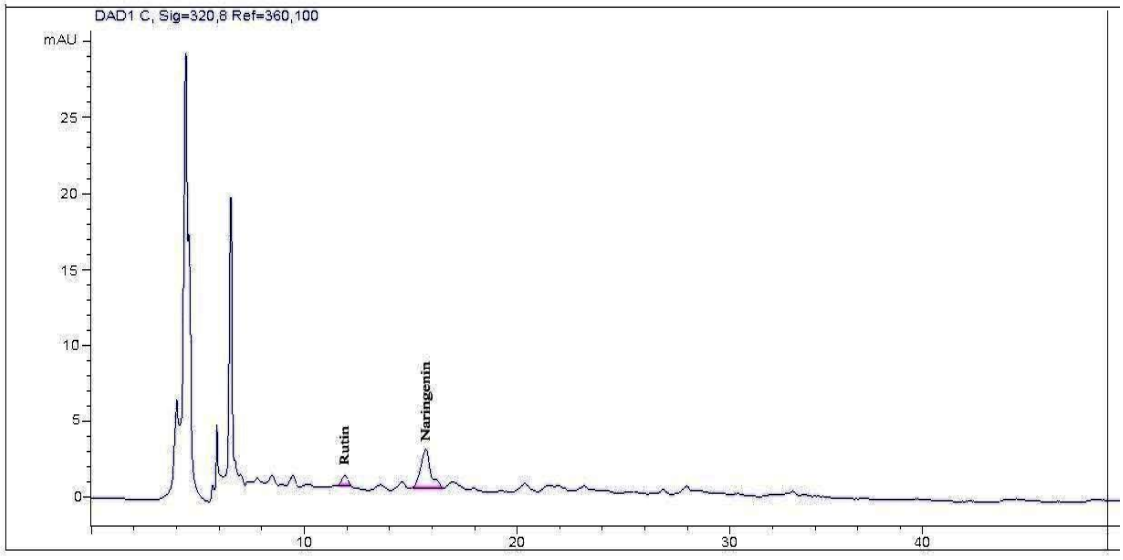


Figure 4.2 RP-HPLC chromatogram of flavonoid compounds in methanolic extract of *Veitchia merrillii* fruits [Major compounds present: rutin and naringenin]

4.3 Antioxidant activity assays

The antioxidant activity of *Veitchia merrillii* fruit extracts as determined by the three different assays including DPPH, NO scavenging activity and ABTS, showed significant responses.

4.3.1 DPPH scavenging activity assay

The results obtained with the DPPH assay indicated that the antioxidant activities of *Veitchia merrillii* fruit extracts were influenced by the nature of the solvent used. The free radical scavenging activity (DPPH) assay showed a steady increase in the free radical scavenging activity for all extracts and standards in the range of 0 to 1 mg/mL (Figure 4.3). However, the free radical scavenging activity of the methanolic extract was much stronger than that of the ethyl acetate extract, followed by the water extract. All of the values, however, were less than those obtained with Vitamin C, BHT and Vitamin E which were utilized as antioxidant standards. The IC_{50} (required concentration to inhibit 50% of DPPH radicals) of Vitamin C, BHT and Vitamin E were found to be 28.8, 31.3 and 25.1 µg/ml respectively (Table 4.5). The different antioxidant activity of the different extracts was associated with the total phenolics, and especially the flavonoid content, although both contents were influenced by the solvents depending on the lipophilic or hydrophilic nature of the solvent. The DPPH scavenging assays indicated that the methanol extract had the highest free radical scavenging activity in comparison to the ethyl acetate and water extracts (Figure 4.3).

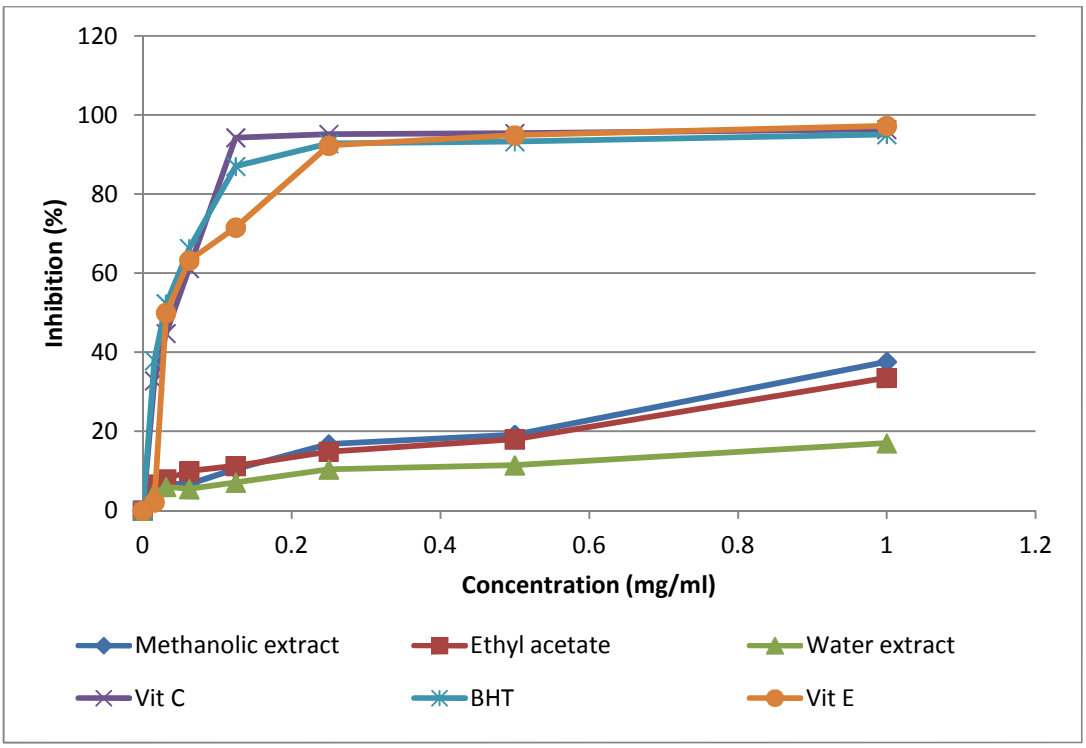


Figure 4.3 Free radical scavenging activity of the extracts and standards [Each value represents the mean of three replications]

Table 4.5 IC_{50} values of extracts and standards for free radical scavenging activity

IC_{50} (µg/ml)	
Extract and Standards	Free radical scavenging activity
Methanolic extract	>1000
Ethyl acetate extract	>1000
Water extract	>1000
Vitamin C	28.8 ± 1.47^{b}
Vitamin E	25.1 ± 3.04^{a}
BHT	31.31 ± 2.43^{c}

Means within columns with the same superscripts are not significantly different at $p < 0.05$.

4.3.2 NO scavenging activity assay

The antioxidant activities of methanol, ethyl acetate and water extracts as well as standards in the reactions with nitric oxide (NO) showed that the extracts inhibited the NO in a dose dependent manner (Figure 4.4). The IC50 concentrations indicated quite remarkable ($P < 0.01$) differences in NO scavenging activity among the samples, and the methanolic extract presented the lowest value followed by ethyl acetate and water extraction (Table 4.6). The findings showed that all fruit extracts of *Veitchia merrillii* fruits had remarkable effects on scavenging free radicals.

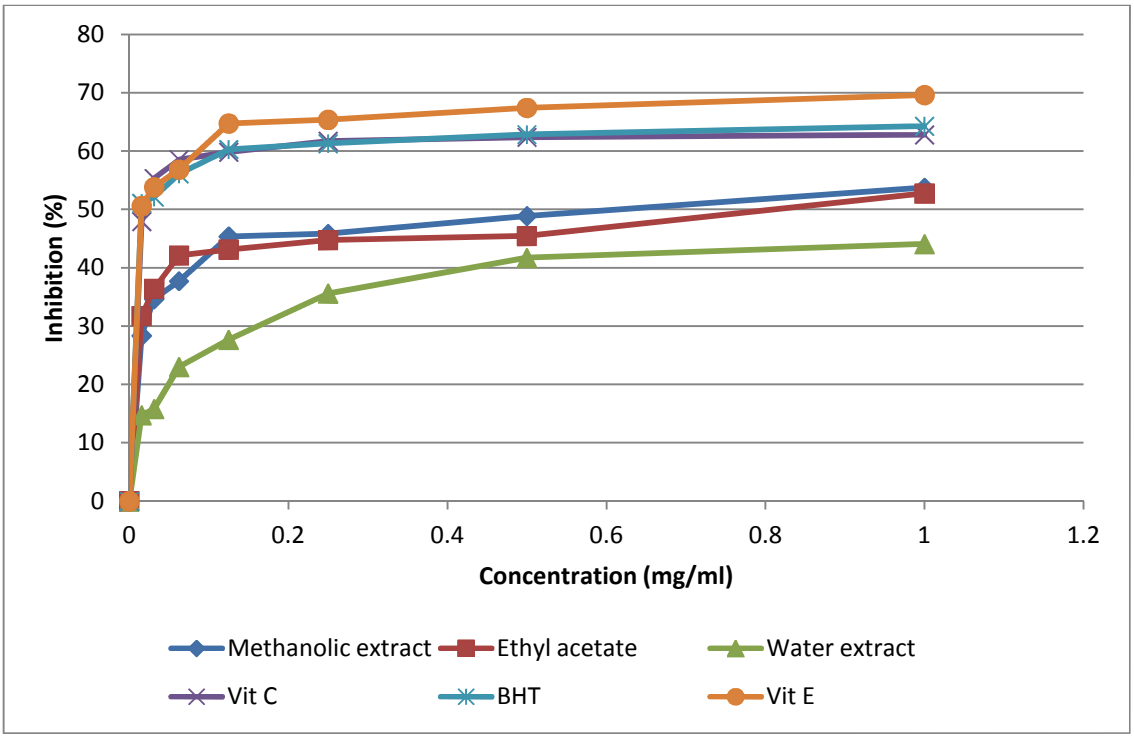


Figure 4.4 Nitric oxide scavenging activity of extracts and positive controls [Each value represents the mean of three replications]

Table 4.6 IC_{50} values of extracts and standards in NO radical scavenging activity

IC_{50} (µg/ml)	
Extract and Standards	Nitric oxide scavenging activity
Methanolic extract	616.5 ± 8.44^{d}
Ethyl acetate extract	812.7 ± 6.88^{e}
Water extract	>1000
Vitamin C	19.99 ± 3.42^{b}
Vitamin E	15.6 ± 2.62^{a}
BHT	28.73 ± 1.73^{c}

Means within columns with the same superscripts are not significantly different at $p< 0.05$.

4.3.3 ABTS radical scavenging assay

The ABTS radical cation-scavenging activities of different extracts *Veitchia merrillii* fruits are clearly illustrated in the Figure 4.5. Ethyl acetate and water extracts did not give ABTS radical cation- scavenging activity up to 1000 mg/ml, whereas methanolic extracts exhibited 51.49% radical scavenging activity at 1000 mg/ml. Trolox was applied as a positive control, and it represented higher activity than those of the tested extracts. The IC_{50} concentrations (Table 4.7) also indicated that ABTS scavenging activity of methanolic extract was much stronger than ethyl acetate and water extracts.

Table 4.7 IC_{50} values of extracts and standards in ABTS radical scavenging activity

IC_{50} (µg/ml)	
Extract and Standards	ABTS scavenging activity
Methanolic extract	884.8 ± 9.24^{b}
Ethyl acetate extract	>1000
Water extract	>1000
Trolox	40.47 ± 1.012^{a}

Means within columns with the same superscripts are not significantly different at $p<0.05$.

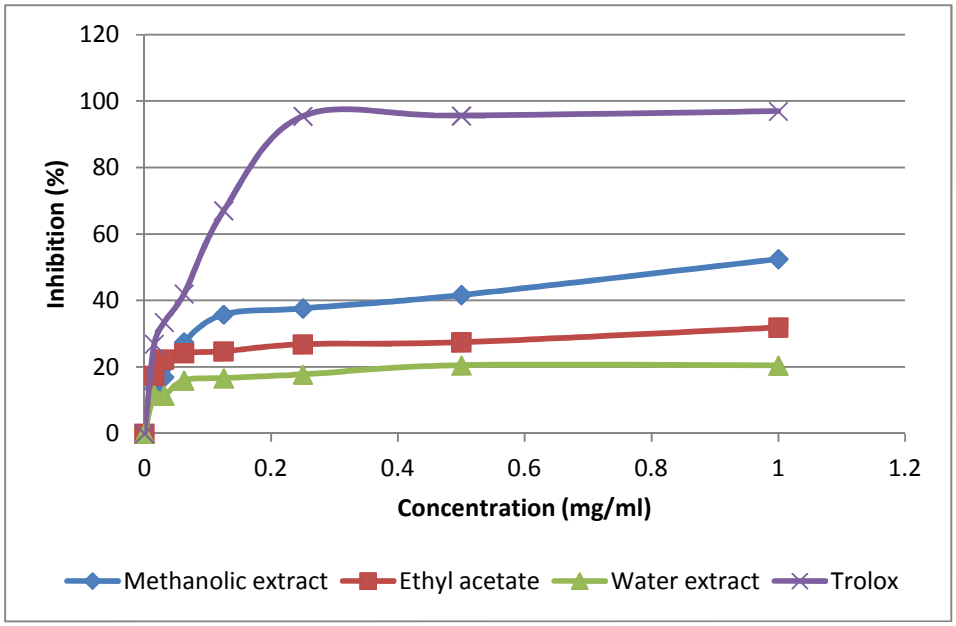


Figure 4.5 ABTS radical scavenging activity of extracts and positive controls [Each value represents the mean of three replications]

4.4 Cytotoxic effects of *Veitchia merrillii* fruit extracts

The results of cytotoxicity activity of methanolic, ethyl acetate and water extracts of Veitchia merrillii fruits showed that an increase in extract concentrations of up to 1000 µg/ml, reduced cell viabilities remarkably in a dose-dependent manner in the two cell lines that were tested (Figures 4.6 to 4.11). The IC_{50} values of the extracts utilized in the study are shown in Table 4.8. The results showed that all of the extracts inhibited Human hepatocytes (Chang liver cells) and NIH/3T3 (Fibroblasts cells) cells. A plant extract is commonly regarded for in vitro cytotoxic activity when IC50 < 100 µg/ml (Boyd, 1995). In the present study, all of the extracts showed in vitro cytotoxic activity to all cells with various IC50 values. Jonville et al (2010) mentioned that the definition of promising activity was reserved for extracts with an IC_{50} value of less than 50 µg/ml.. The study showed that the methanolic extract against Chang liver cells and NIH/3T3 cells indicated higher activity in comparison with ethyl acetate and water extract (Table

4.6). Moreover, Tamoxifen the positive control had the lowest IC50 value compared with the other extracts and it indicated remarkable differences in cytotoxic activity for all cells. It is known that different cell lines might exhibit different sensitivities when treated with different plant extracts. Hence, the need for more than one cell line to perform comprehensive screening of plant extracts for anti cancer activity.

Table 4.8 IC_{50} values of extracts and positive control on Chang liver and NIH-3T3 cell lines

Sample	IC_{50} value (µg/ml)	
	Chang liver cell	NIH-3T3
Methanolic extract	67.4±6.87	29.5±10.31
Ethyl acetate extract	369.8±5.66	62.5±8.44
Water extract	664.3±9.44	729±6.82
Tamoxifen	34.4 ± 0.12	36.2 ± 2.99

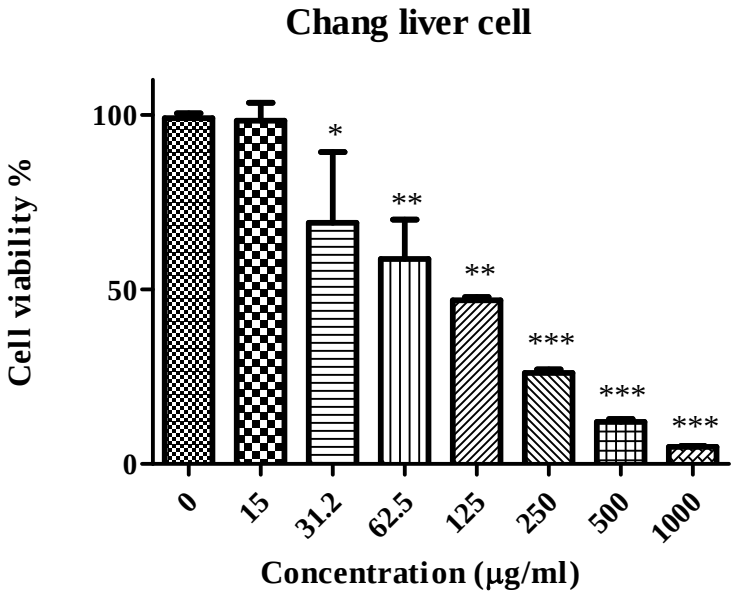


Figure 4.6 Effect of crude methanolic extract of *Veitchia merrillii* fruits on Chang liver cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

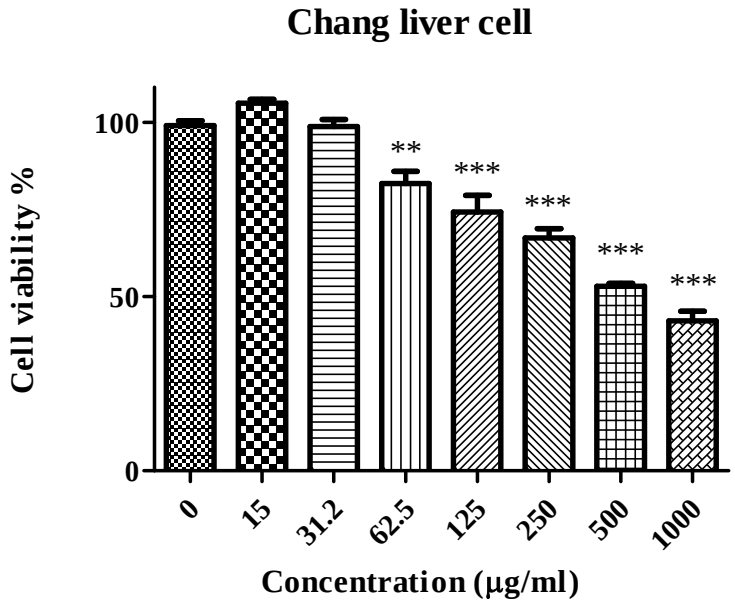


Figure 4.7 Effect of crude ethyl acetate extract of *Veitchia merrillii* fruits on Chang liver cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

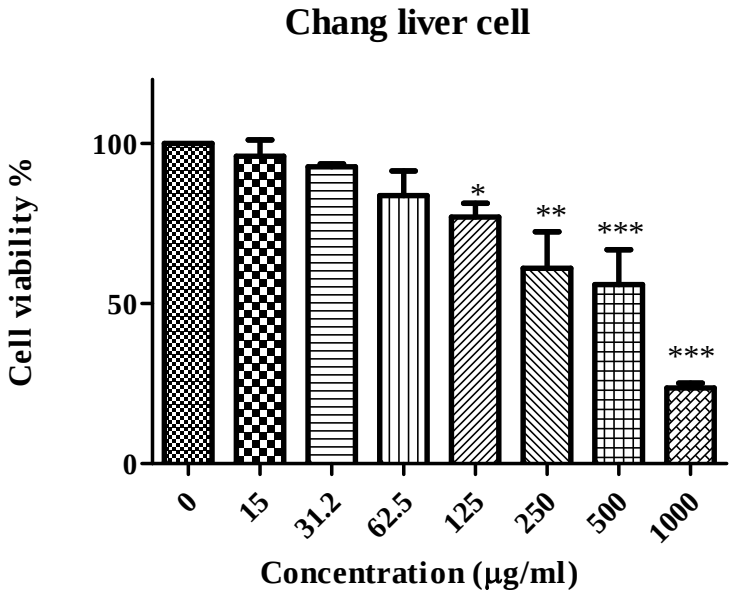


Figure 4.8 Effect of crude water extract of *Veitchia merrillii* fruits on Chang liver cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

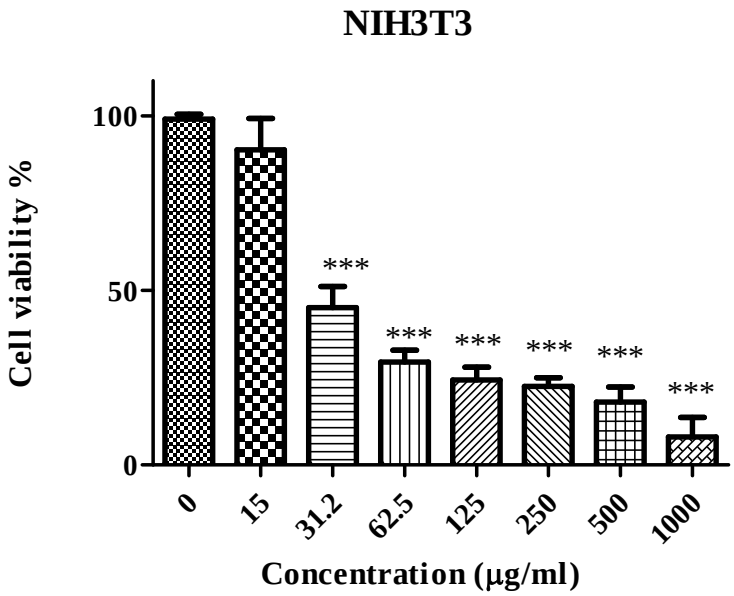


Figure 4.9 Effect of crude methanolic extract of *Veitchia merrillii* fruits on NIH 3T3 cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

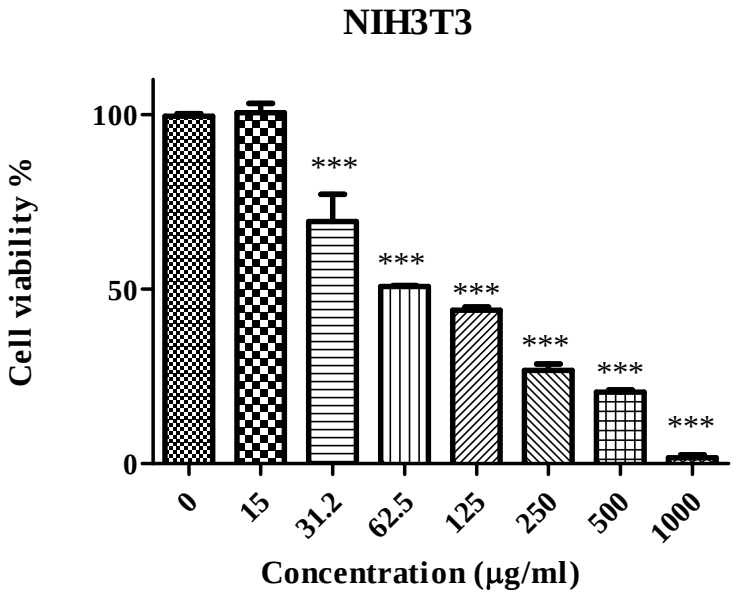


Figure 4.10 Effect of crude ethyl acetate extract of *Veitchia merrillii* fruits on NIH 3T3 cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

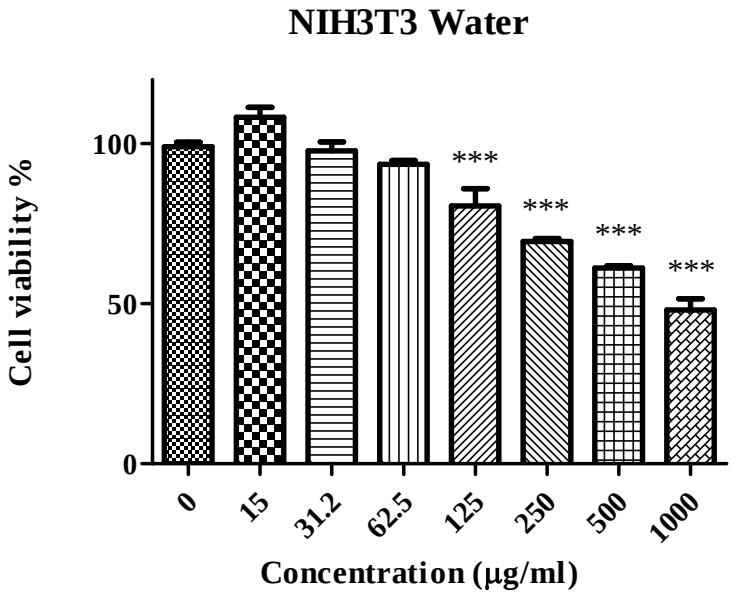


Figure 4.11 Effect of crude water extract of *Veitchia merrillii* fruits on NIH 3T3 cell viability [All values represent the mean ± S.D from three independent experiments. ***P < 0.001 indicates significant difference compared to the untreated control group]

CHAPTER 5

DISCUSSION

In this research *Veitchia merrillii* fruits were extracted using solvents of various polarity including water, ethyl acetate and methanol, followed by determination of total flavonoids and phenolics. The quantity of different phenolics and flavonoid compounds present were analysed using RP-HPLC. Antioxidant activity of all extracts were determined by ABTS, NO_2 scavenging and DPPH methods. And finally the cytotoxicity of all extracts were tested againt NIH/3T3 (Fibroblasts cells) and Human hepatocytes (Chang liver cells).

Investigations all over the world have provided a large body of knowledge on the immense potential of medicinal plants in various traditional systems. The medicinal value of the plants is attributed to their phytochemical components that produce definite physiological actions in the human body (Dahanukar et al., 2000).

The most significant factor about these bioactive compounds is that they constitute a variety of secondary metabolites including polyphenols, phenols, flavonoids, flavones, flavonols terpenoids, saponin, tannins, terpenes, terpenoids, coumarins, quinines, essential oils, lectins, polypeptides and alkaloids that have pharmacological applications (Harvey, 2000; Cowan, 1999). One of these secondary metabolites called eugenol, which is a simple phenolic compound, is widely utilized in dentistry as a local anaesthetic, and as an anti-inflammatory and anti-bacterial agent (Gurib-Fakim, 2006). Flavonoids have also been reported to provide effective protection against cardiovascular diseases (Erlund, 2004). These bioactive compounds are mostly found in

various parts of plants including seeds, fruits, nuts, flowers and stems (Cushie and Lamb, 2005).

Successful detection of bioactive compounds from plant materials largely depends on the type of solvents used in the extraction procedure. The solvents and the procedure must be chosen very carefully in order to optimize the extraction process. Antioxidant activity and the yield of extracts are highly dependent on solvent polarity, which affects the extraction of antioxidant compounds both qualitatively and quantitatively (Karimi et al., 2010). Characteristics of a good solvent include ease of evaporation at low heat, low toxicity, preservative action, rapid absorption of extracts, and inability of the solvent to form complexes or to dissociate extract components (Eloff, 1998).

Economou et al. (1991) had suggested that solvents with high polarity are the most suitable for the extraction of bioactive compounds from higher plants. The findings of the present study on *Veitchia merrillii* fruits are in concurrence with the results reported by Chang et al. (1993), where methanol was found to be the most effective solvent for the extraction of the bioactive compounds. Polar organic solvents are generally better for the extraction of organic compounds from plants when compared to water. Palma and Taylor (1999) and Karimi et al. (2010) also reported methanol as the most efficient solvent for the extraction of flavonoids and phenolic compounds. The results on the extraction of flavonoids are in agreement with Havsteen (2002) who also observed that polar solvents increased extraction efficiency. It has been widely reported that the recovery percentage directly depends on the type of solvent and the extraction method utilized (Sun and Ho, 2005; Turkmen et al., 2006; Hayouni et al., 2007). Oskoueian et al. (2011) also reported highest phenolic and flavonoid contents in crude methanolic extracts of *Jatropha curcas* compared to ethanol or water. Sun et al. (2005) reported that methanol was the most effective solvent for the extraction of phenolic components from oat bran. The phenolic components extracted with methanol were approximately 3 times

higher than with acetone and 4 times higher than that extracted using hexane. Thurkmen et al. (2006) observed that the difference in polarity of the solvents had a significant effect on the polyphenol content and antioxidant activity. Higher content and stronger antioxidant activity was reported with polar solvents (Siddhuraju et al., 2002). Consequently, methanolic extracts of various plant parts indicate higher TPC and TFC values than other solvent extracts.

Methanol has a high polarity index (Cowan, 1999) in comparison with the other solvents, and hence it is able to extract more flavonoid and phenolic compounds. Phenolic compounds are usually associated with other biomolecules (such as proteins, polysaccharides, terpenes, inorganic compounds, and chlorophyll) and hence methanol was the most suitable for the extraction of these compounds.

HPLC is a broadly utilized chromatographic method used in the separation of molecules in a solution based on their hydrophobicity. Consequently, a reduction in the polarity of the mobile phase will result in a reduction in solute retention (McMaster, 1994; Weston and Brown, 1997). In the present study RP-HPLC was used to investigate flavonoid and phenolic profiling of *Veitchia merrillii* fruit extracts. The findings of the HPLC analysis indicate that solvent polarity influenced the types of flavonoid and phenolic compounds extracted from *Veitchia merrillii* fruits.

The present results showed that gallic acid and pyrogallol were the major phenolic compounds in the methanolic extracts with values of 101.6 ±0.19 and 913.1 ±0.79 µg/g dry wt, respectively. The values were very much higher than gallic acid values reported in fresh Mauritian black tea leaves (0.006 µg/g dry wt of tissue) (Ramma et al., 2005) and in two other medical plants: *Valeriana wallachi* and *Tectona grandis* (with 17.48, 20.76 and 17.80 µg/ g dry wt., respectively) (Shalini and Srivastava, 2009).

The results also demonstrated that naringenin was the main flavonoid compound available in methanolic extracts of *Veitchia merrillii* fruits, with a value of 895.4 ±0.36 µg/g dry sample. The secondary metabolites derived from plants including phenolic compounds, flavonoids compounds and essential oils have been shown to have biological activity. Compounds including quercetin, naringin, gallic acid and pyrogallol have been reported to possess anti-inflammatory activity as well as antioxidant characteristics (Manpong et al., 2009; Nicolis et al., 2008).

Antioxidants have the ability of reducing free radicals and are able to decrease the rate of lipid production and peroxidation in the human body which cause a variety of human diseases and aging, and antioxidants are responsible for preventing oxidative damage of cellular components.

Overall two basic categories of antioxidants are known, ie. natural and synthetic antioxidants. A very rich source of natural antioxidants is provided by plants (Karimi et al., 2011). Antioxidants are responsible for the prevention of oxidative damage caused to cellular components as a result of biochemical reactions. Some flavonoids and phenolics appear to be more active than vitamins, but their activity is dependent on the structure and total number of hydroxyl groups (Oskoueian et al., 2011).

The antioxidant activity of *Veitchia merrillii* fruits was determined in three solvents of different polarities including water, ethyl acetate and methanol. The findings using the three different methods clearly demonstrated different levels of low to moderate antioxidant activity in *Veitchia merrillii* fruit extracts. This radical scavenging activity of the extracts is attributed to the natural properties of flavonoids and phenolic compounds present which contribute to the electron transfer/hydrogen donating activity (Gulcin et al. 2004). The flavonoids and phenolic compounds are two important groups secondary metabolites with abilities and properties to serve as antioxidants (Karimi et

al., 2010). Many flavonoids and phenolic compounds have been frequently reported to possess potent anti-cancer, antioxidant, anti-bacterial, anti-carcinogenic, anti-inflammatory or anti-viral activity to a lesser or greater extent (Tapiero et al., 2002; Larson, 1998; Karimi et al., 2010). Amic et al. (2003) investigated 29 flavonoids and their findings clearly demonstrated that highly active flavonoids possess a 3'4' – dihydroxy occupied B ring and/or 3-OH group. Flavonoids serve as a source of readily available ''H" atoms such that subsequent radicals generated may be delocalized over the flavonoid structure (Burda and Oleszek, 2001; Majo et al., 2005). Rapisarda et al. (1999) reported the antioxidant capacity of different varieties of pigmented oranges, but their antioxidant capacity seemed to be influenced by concentrations of anthocyanins in the pigmented orange juices.

The radical scavenging activity of *Veitchia merrillii* fruit extracts are attributed to the various bioactive compounds comprising of flavonoids and phenolic compounds. The antioxidant capacity was dependent on total amount of phenols and the ability to interact with the biomembrane.

Cancer comprises of a group of diseases characterized by cells that grow out of control, and in a majority of cases, they form masses of cells or tumors, that might destroy, crowd out or infiltrate normal tissues (Karimi et al., 2012). There are a variety of plant products which have been applied to cure or prevent such diseases throughout history. Natural compounds in some medicinal plants have provided lead structures which have been frequently utilized to plan and design new drugs (Rates, 2001). The last few decades has led to the development of a great deal of novel anticancer drugs, drug combinations, and chemotherapy strategies through scientific exploration of the enormous pool of natural, biological, and synthetic products . Edible plants are increasingly being considered as valuable sources of anticancer drugs. There is a large

amount of scientific evidence indicating medicinal plants as a main source of novel healthcare products (Aziz et al., 2003).

Previous investigations have indicated that polyphenols and various flavonoid compounds from a variety of plants have remarkable anticancr activity against variant cell lines (Ramos, 2007; Mavundza et al., 2010). Growth inhibition of HL–60 leukemia cells with citrus flavonoids were reported by Manthey et al. (2001). Luo et al. (2009) reported that kaempferol was able to inhibit the growth of ovarian cancer cell lines (91%) and A2780/CP70 (94%) at concentrations of 20 and 40 μM, respectively. Quercetin was also reported to be able to inhibit prostate cancer growth (Verschoyle et al., 2007). Inhibition of breast cancer cell lines (MCF–7 and MDA–MB–231) by quercetin was reported by Gibellini et al. (2010). Arts et al. (2005) reported catechin had the ability to control postmenopausal cancer and help protect against rectal cancer.

A survey of the literature indicated that no investigations had been reported on cytotoxic effects of *Veitchia merrillii* fruit extracts. The findings of the present study on cytotoxic effects of *Veitchia merrillii* fruit extracts indicated moderate activity and all extracts were able to inhibit NIH/3T3 (Fibroblast cells) and Human hepatocytes (Chang liver cells). Cytotoxic cell type specificity of plant extracts is likely to be due to the presence of specific classes of compounds in the extract (Kamuhabwa et al. 2000). Mavundza et al. (2010) and Oskoueian et al. (2011) reported the capability of phenolic and flavonoid compounds to serve as anticancer agents. Zhang et al.(2008) indicated that quercetin, kaempferol, coumaric acid, ellagic acid, and anthocyanins isolated from strawberry could inhibit growth of human cancer cell lines including oral (KB, CAL-27), breast (MCF-7), prostate (LNCaP, DU-145), and colon (HT-29, HCT-116) cancer. Polyphenols like quercetin, resveratrol, epicatechin and catechin isolated from wine

extract (Kampa et al., 2000) and green tea polyphenols like epicatechin and epigallocatechin (Weisburg et al., 2004) play indispensable roles as anticancer agents. Thus the results of the present study suggest that the effects observed can be attributed to the presence of specific flavonoids and phenolic compounds in the fruit extracts.

CHAPTER 6

CONCLUSION

Natural phytochemicals including phenolic and flavonoid compounds are the major bioactive compounds that are known to be absolutely beneficial against numerous ailments and they have also been frequently reported to possess a great deal of various biological effects, including anticancer and antioxidant activities. The food products obtained from plants are the key sources of these compounds for humans. Supplementation of the secondary metabolites as food additives can contribute in retaining the necessary health-beneficial amount of the compounds in the diet, and the extraction is a significant stage in the preparation and production of these food additives.

The present research was carried out to evaluate the phytochemicals and their biological activity including cytotoxic effects and antioxidant potential of *Veitchia merrillii* fruits. Water, ethyl acetate and methanol were utilized to extract the *Veitchia merrillii* fruits. From the amounts of organic extracts present it was concluded that methanol was the most effective solvent in comparison to water and ethyl acetate. The study on total flavonoid and phenolic contents demonstrated that dried fruit extracts of *Veitchia merrillii* particularly with methanol and ethyl acetate as solvents exhibited a higher content of total flavonoid and phenolic componuds in comparison with the hot water extract. ABTS scavenging assay, NO scavenging activity, and DPPH radical scavenging assay demonstrated that the methanolic extracts exhibited the highest level of antioxidant activity in comparison with hot water and ethyl acetate extract.

From the analysis of bioactive compounds using RP-HPLC), it can be concluded that pyrogallol, gallic acid, vanillic acid, caffeic acid, syringic acid, rutin and naringin

existed as the major flavonoid and phenolic compounds in the extracts of *Veitchia merrillii* fruits. The results also demonstrated that methanolic extracts exhibited a higher presence of flavonoid and phenolic compounds in comparison with ethyl acetate and water extracts.

The study on *in vitro* cytotoxicity against NIH/3T3 (Fibroblasts cells) and Human hepatocytes (Chang liver cells indicated that there was a weak to moderate cytotoxic activity exhibited by the different extracts and that the compounds which were present in the extracts were non-toxic to humans. This suggests that these compounds can be considered as suitable agents as potential therapeutics. The application of *Veitchia merrillii* fruits as a potential natural medicine is therefore justifiable. Future investigations need to focus on the isolation, purification and characterization of the individual bioactive compounds for the development of potential phytomedical and botanical drugs. Evaluation of anti diabetic, antiviral and anti-tumor activity of the plant *in vitro* and *in vivo* models would also be of interest. It is also strongly recommended that studies to establish an effective metabolomics platform be carried out using proton nuclear magnetic resonance (H NMR) techniques that may lead to the development of rigorous quality control and product standardization practices in the natural health products industry. Overall the findings from the biological assays suggest that *Veitchia merrillii* fruits are a useful source of bioactive compounds endowed with interesting biological activities, possibly as strong antioxidant agents. The presence of flavonoids and phenolics with antioxidative and cytotoxicity action, suggests the *Veitchia merrillii* fruits as novel potential sources of natural antioxidants in the future. The present study provides novel scientific data on *Veitchia merrillii* fruits, including information on flavonoid and phenolic compounds which has never been reported in previous investigations.

REFERENCES

Ahmad, R., Ali, A.M., Israf, D.A., Ismail, N.H., Shaari, K. and Lajis, N.H. (2005). Antioxidant, radical-scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some *Hedyotis* species. *Life Science,* 76, 1953-1964.

Akinmoladun, A., Ibukun, E., Afor, E., Obuotor, E. and Farombi, E. (2007). Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum*. *Scientific Research Essay*, 2, 163-166.

Amic, D., Davidovic-Amic, D., Beslo, D., and Trinajstic, N. (2003). Structure radical scavenging activity relationships of flavonoids. *Croatica Chemical Acta*, 76, 55-61.

Ao, C., Li, A., Elzaawely, A.A., Xuan, T.D., and Tawata, S. (2008). Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. extract. *Food Control*, 19, 940–948.

Arts, I.C. and Hollman, P.C. (2005). Polyphenols and disease risk in epidemiologic studies. *American Journal of Clinical Nutrition*, 81 (1 supp1), 317S-325S.

Aruoma, OI. 1999. Free Radicals, Antioxidants and International Nutrition. Asia Pacific: *Clinical Nutrition Journals*, 8:53-63.

Aziz, K.A., Till, K.J., Chen, H., Slupsky, J.R., Campbell, F., Cawley JC, Zuzel M.(2003). The role of autocrine FGF-2 in the distinctive bone marrow fibrosis of hairy-cell leukemia (HCL). *Blood*;1:102(3):1051-6.

Balu, M., Sangeetha, P., Haripriya, D., and Panneerselvam, C. (2005). Rejuvenation of antioxidant system in central nervous system of aged rats by grape seed extract. *Neuroscience Letters*, 383(3): 295-300.

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

Bonnely, S., Davis, A.L., Lewis, J.R. and Astill, C. (2003). A model oxidation system to study oxidized phenolic compounds present in black tea. *Food Chemistry,* 83: 485-492.

Boudet, A.M. (2007). Evolution and current status of research in phenolic compounds. *Phytochemistry*, 68(21), 2635-2648.

Burda, S. and Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agriculture and Food Chemistry*, 49, 2774-2779.

Carper, J. (1988). *The Food Pharmacy*. New York: Bantam Books.

Casmir, C. A., and David, B. (2002). *Chemistry, Nutrition, and Biotechnology of Lipid Foods*, New York: Marcel Dekker Incorporated.

Castelluccio, C., Paganga, G., Melikian, N., Bolwell, G.P., Pridham, J., Sampson, J. and Rice-Evans, C. (1995). Antioxidant potential of intermediates in phenylpropanoid metabolism in higher palnts. *FEBS Letters,* 368: 188-192.

Castelucci, S., Rogerio, A.D.P., Ambrosio, S.R., Arakawa, N.S., Lira, S.P., Faccioli, L.H. and Costa, F.B.D. (2007). Anti-inflammatory activity of *Dasyphyllum brasiliensis* (Asteraceae) on acute peritonitis induced by β-glucan from *Histoplasma capsulatum*. *Journal of Ethnopharmacology* 112: 192-198.

Chan, PH., Longar, S., and Fishman, RA. (1987) Protective effects of liposome-entrapped superoxide dismutase on posttraumatic brain edema. *Ann Neurol,* 21: 540−547.

Chang, W.S., Lee, Y.J., Lu, F.J. and Chiang, H.C. (1993). Inhibitory effects of flavonoids on xanthine oxidase. *Anticancer Research,* 13, 2165–2170.

Cherng, J. M., Shieh, D. E., Chiang, W., Chang, M. Y. and Chiang, L.C. (2007). Chemopreventive effects of minor dietary constituents in common foods on human cancer cells. *Bioscience, Biotechnology, and Biochemistry,* 71, 1500–1504.

Cheung, L.M., Cheung, C.K.P. and Ooi, E.C.V. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, 81: 249-255.

Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. *The American Journal of Clinical Nutrition,* 81(Suppl), 223S–229S.

Coleman MP, Quaresma M, Berrino F, Lutz JM, De Angelis R, Capocaccia R, Baili P, Rachet B, Gatta G, Hakulinen T, Micheli A, Sant M, Weir HK, Elwood JM, Tsukuma H, Koifman S, E Silva GA, Francisci S, Santaquilani M, Verdecchia A, Storm HH, Young JL (2008). Cancer survival in five continents: a worldwide population-based study (CONCORD). Lancet Oncology. 9:730-756.

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews,* 12 (4), 564-582.

Crozier, A., Jensen, E., Lean, M.E.J. and Mcdonald, M.S. (1997). Quantitative analysis of flavonoids by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A,* 761, 315-321.

Cushnie, T. and Lamb, A.J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents,* 26(5), 343-356.

Dahanukar, S.A., R.A. Kulkarni and N.N. Rege (2000). Pharmacology of medicinal plants and natural products. Indian Journal of Pharmacology. 32: S81-S118.

Denisov ET, Denisova TG, Trepalin SV, Drozdova TI. Database: Oxidation and Antioxidants in Organic Chemistry and Biology. New York: CRC Press, 2005.

De Rijke, E., Out, P., Niessen, W. M. A., Ariese, F., Gooijer, C., & Brinkman, U. A. T. (2006). Analytical separation and detection methods for flavonoids. Journal of Chromatography A, 1112, 31–63.

Economou, K.D., Oreopoulou, V. and Thomopoulos, C.D. 1991. Antioxidant activity of some plant extracts of the family Labiatae. *Journal of the American Oil Chemist Society* 68: 109-113.

Edeoga, H.O., Okwu, D.E., Mbaebie, B.O. 2005. Phytochemical constiuents of some Nigerian medicinal plants. *African Journal of Biotechnology* 4 (7): 685-688

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria, Planta Medica. 64, 711–713.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, naringenin. Dietary sources, bioactivities, bioavailability and epidemiology. *Nutrition Research, 24*, 851-874.

Fabbi, P., Ghigliotti, G., Brunelli, C., Balbi, M., Spallarossa, P., Rossettin, P., Barsotti, A., Odetti, P., and Garibaldi, S. (2004). Intense lipid peroxidation in premature clinical coronary atherosclerosis is associated with metabolic abnormalities. *Laboratory and Clinical Medicine*, 143(2): 99-105.

Falleh, H., Ksouri, R., Chaieb, K., Karray-Bouraoui, N., Trabelsi, N., Boulaaba, M. and Abdelly, C. (2008). Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. *Comptes Rendus Biologies* 331: 372–379

Fang, Z., Zhang, Y., Lu, Y., Mab, G., Chen, J., Liu, D. & Ye, X. (2009). Phenolic compounds and antioxidant capacities of bayberry juices. *Food Chemistry*, 113, 884–888.

Farah, I. O., and Begum, R. A. (2003). Effect of *Nigella sativa* and oxidative Stress on the survival pattern of MCF-7 breast cancer cells. *Biomed Sci Instrum.* 39: 359-364.

Faried, A., Kurnia, D., Faried, L., Usman, N., Miyazaki, T., Kato, H. and Kuwano, H., (2007). Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines. *International Journal of Oncology*, 30, 605-613.

Francis, F.J. (Ed.). (2000). Encyclopedia of food science and technology (2th ed.). New York: Wiley.

Galindo, M.F., Ikuta, I., Zhu, X., Casadesus, G. and Jordán, J. (2010). Mitochondrial biology in Alzheimer's disease pathogenesis. *Journal of Neurochemistry*, 114, 933–945.

García-Alonso, M., de Pascual-Teresa, S., Santos-Buelga, C. and Rivas-Gronzalo, J.C. (2004). Evaluation of the antioxidant properties of fruits. *Food Chemistry*, 84: 13-18.

Giao, M. S., Gonzalez-Sanjose, M. L., Rivero-Perez, M. D., Pereira, C. I., Pintado, M. E., and Malcata, F. X. (2007). Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenolic content on extraction features. *Journal of the Science of Food and Agriculture*, 87, 2638–2647.

Gibellini L, Pinti M, Nasi M, De BS, Roat E, Bertoncelli L, Cossarizza A (2010). Interfering with ROS Metabolism in Cancer Cells: ThePotential Role of Quercetin. Cancers, 2: 1288-1311.

Gorinstein, S., Caspi, A., Zemser, M. and Trakhtenberg, S. (2000). Comparative contents of some phenolics in beer, red and white wines. *Nutrition Research,* 20(1): 131-139.

Grice, H.C. (1986). Safety evaluation of butylated hydroxytoluene (BHT) in the liver, lung and the gastrointestinal tract. *Food and Chemical Toxicology,* 24: 1127-1130.

Gulcin, I., Beydemir, I.G.S.S., Elmastaş, M., and Küfrevioğlu, O.I. (2004). Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.). *Food Chemistry,* 87, 393-400.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of medicine, 27*, 1-93.

Halberstein, R.A. (2005). Medicinal plants: Historical and cross-cultural usage patterns. *Annals of Epidemiology, 15*, 686-699.

Halicia, M., Odabasoglua, F., Suleymanb, H., Cakirc, A., Asland, A., and Bayir, Y. (2005). Effects of water extract of *Usnea longissima* on antioxidant enzyme activity and mucosal damage caused by indomethacin in rats. *Phytomedicine*, 12, 656–662.

Halliwell, B. (2002). Hypothesis: Proteasomal Dysfunction. A Primary Event in Neurogeneration that Leads to Nitrative and Oxidative Stress and Subsequent Cell Death. *Annuals of the New York Academy of Sciences*, 962(1):182–194.

Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* (2000);100:57-70.

Harborne, J.B. (1994). *The Flavonoids, Advance in Research Since* (*1986*). Chapman and Hall, London.

Harborne, J.B. and Williams, C. A. (2000). Advances in flavonoid research since (1992). *Phytochemistry*, 55, 481–504.

Harvey, A. (2000). Strategies for discovering drugs from previously un explored natural products. *Drug Discovery Today, 5*, 294-300.

Hasty, P., Campisi, J., Hoeijmakers, J., van Steeg, H., Vijg, J. (2003). Aging and genome maintenance: lessons from the mouse? Science 299, 1355–1359.

Havsteen, B.H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics*, 96, 67- 202.

Hayouni, A., Abedrabba,M., Bouix,M. and Hamdi, M. (2007). The effects of solvents and extraction method on the phenolic contents and biological activities in vitro of Tunisian *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts. *Food Chemistry*, 105, 1126–1134.

Hertog, M.G.L., Hollman, P.C.H. and Venema, D.P. (1993). Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry* 40: 1591-1598.

Ismail, H.I., Chan, K.W., Mariod, A.A. and Ismail, M. (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chem*istry, 119(2), 643-647.

Jakobek, L., Seruga, M., Seruga, B., Novak, I. and Medvidovic- Kosanovic, M. (2009). Phenolic compound composition and antioxidant activity of fruits of Rubus and

Prunus species from Croatia. *International Journal of Food Science and Technology*, 44, 860–868.

Jing, Q.Y., Su, F.Y., Ming, F.Z. and Wen, H.H. (2003). Effects of root exudates and aqueous root extracts of cucumber (*Cucumis sativus*) and allelochemicals, on photosynthesis and antioxidant enzymes in cucumber. *Biochemical Systematics and Ecology*, 31: 129-139.

Jonville, M.C., Kodja, H., Humeau, L., Fournel, J., De Mol, P., Cao, M., Angenot, L., Frederich, M. (2008). Screening of medicinal plants from Reunion Island for antimalarial and cytotoxic activity. Journal of Ethnopharmacology 120, 382-386.

Kaiserová, H., Šimůnek, T., Wim, J.F., Vijgh, V.D., Bast, A., and Kvasničková, E. (2007). Flavonoids as protectors against doxorubicin cardiotoxicity: Role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochimica et Biophysica Acta* 1772 : 1065–1074.

Kampa, M., Hatzoglou, A., Notas, G., Damianaki, A., Bakogeorgou, E., Gemetzi, C., Kouroumalis, E., Martin, P.M. and Castanas, E. (2000). Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutrition and Cancer*, 37, 223-233.

Kamuhabwa A, Nshimo C, de Witte P (2000). Cytotoxicity of some medicinal plant extracts used in Tanzanian traditional medicine. Journal of Ethnopharmacology. 70:143-149.

Karimi E., Oskoueian E., Hendra R., Oskoueian A and Jaafar H.Z.E. Phenolic Compounds Characterization and Biological Activities of *Citrus aurantium* Bloom.. Molecules. (2012), 17, 1203-1218.

Karimi, E. Jaafar, H and Ahmad, S. Phytochemical Analysis and Antimicrobial Activities of Methanolic Extracts of Leaf, Stem and Root from Different Varieties of Labisa pumila Benth. Molecules. (2011), 16, 4438-4450.

Karimi, E., Oskoueian, E., Hendra, R. and Jaafar, H.Z.E. (2010). Evaluation of *Crocus sativus* L. stigma phenolic and flavonoid compounds and its antioxidant activity. *Molecules*, 15, 6244-6256.

Katsouyanni K., Trichopoulos, D. and Boyle, P. (1986). Diet and breast cancer: A case control study of patients with breast cancer,benign epithelial and fiberocystic disease of the breast. *Journal of Cancer*, 45:825-828.

Kawanishi, S. Hiraku, Y. Oikawa, S. (2001). Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging, Mutat Res 488, 65–76.

Khan, M.A. Baseer, A. (2000). Increased malondialdehyde levels in coronary heart disease, J Pak Med Assoc. 50, 261–264.

Kim, H.K., Jeong, T-S., Lee, M-K., Park, Y.B. and Choi, M-S. (2003). Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. *Clinica Chimica Acta*, 327: 129-137.

Klein, P.J., Van Vleet, T.R., Hall, J.O. and Coulombe, R.A.Jr. (2003). Effects of dietary butylated hydroxytoluene on aflatoxin B_1-relevent metabolic enzymes in turkeys. *Food and Chemical Toxicology,* 41: 671-678.

Korkina, L.G and Afans'ev, I.B. (1997). Antioxidant and chelating properties of Flavonoids. *Adv Pharmacol*. 38, 151–163.

Križková, L., Nagy, M., Polónyi, J., Dobias, J., Belicová, A., Grančai, D. and Krajčovič, J. (2000). Phenolic acids inhibit chloroplast mutagenesis in *Euglena gracilis. Mutation Research,* 469: 107-114.

Kuti, J.O. (2004). Antioxidant compounds from four *Opuntia* cactus pear fruits varieties. *Food Chemistry,* 85: 527-533.

Larson, R.A. (1998). The antioxidants of higher plants. *Photochemistry,* 27, 969-978.

Leonarduzzi, G., Arkan, M., Hüveyda, B., Chiarpotto, E., Sevanian, A. and Poli, G . (2000). Lipid oxidation products in cell signaling. *Free Radical Biology and Medicine,* 28 (9): 1370-1378.

Li, W., Chen, S., Fabricant, D., Angerhofer, C.K., Fong, H.H.S., Farnsworth, N.R. and Fitzloff, J.F. (2002). High-performance liquid chromatography analysis of black Cohosh (*Cimicifuga racemosa*) constituents with in-line evaporative light scattering and photodiode array detection. *Analysis Chimica Acta,* 471: 61-75.

Lim, G.C.C., (2003) *Cancer in Malaysia - There is Light at the End of the Tunnel.*Medical Journal of Malaysia, 58 (5). pp. 632-635.

Lim, K-T., Hu, C. and Kitts, D.D. (2001). Antioxidant activity of a *Rhus verniciflua* Stokes ethanol extract. *Food and Chemical Toxicology,* 39: 229-237.

Lin JK and Tsai SH. (1999). Chemoprevention of Cancer and Cardiovascular Disease by Resveratrol. Proc. Natl. Sci. Counc. ROC(B). 23(3), 99-106.

Loft, S. and Poulson, H.E. (1996). Cancer risk and oxidative DNA damage in man. *Molecular Medicine,* 74:297-312.

Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC. (2009). Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer. 61:554–563.

Majo, D.D., Giammanco, M., Guardia, L.M., Tripoli, E., Giammanco, S. and Finotti, E. (2005). Flavanones in Citrus fruit: Structure antioxidant activity relationships. *Food Research International*, 38, 1161–1166.

Manach, C., Scalbert, A., Morand, C., Remesy and C., Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition,* 79, 727-747.

Manian, R., Anusuya, N., Siddhuraju, P. and Manian, S. (2008). The antioxidant activity and free radical scavenging potential of two different solvent extracts of *Camellia sinensis* (L.) O. Kuntz, *Ficus hengalensis* L. and *Ficus racemosa* L. *Food Chemistry,* 107, 1000-1007.

Manpong, P., Douglas, S., Douglas, P.L., Pongamphai, S. and Teppaitoon, W. (2009). Response surface methodology applied to the extraction of phenolic compounds from Jatropha curcas Linn. Leaves using supercritical CO_2 with a methanol co-solvent. *Journal of Food Process Engineering*, 15, 1-20.

Manthey JA, Grohmann K, Guthrie N. Biological properties of citrus flavonoids pertaining to cancer and inflammation. (2001). Curr Med Chem. 8:135–153.

Marinova, D., Ribarova, F. and Atanassova, M. (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *Journal of The University of Chemical Technology and Metallurgy* 40: 255-260.

Mattila, P. and Hellstrom, J. (2007). Phenolic acids in potatoes, vegetables, and some of their products. *Journal of Food Composition and Analysis*, 20, 152-160.

Mau, J.L., Lai, E.Y.C., Wang, N.P., Chen, C.C., Chang, C.H. and Chyau, C.C. (2003). Composition and antioxidant activity of the essential oil from *Curcuma zedoaria*. *Food Chemistry,* 82: 583-591.

Mavundza, E.J., Tshikalange, T.E., Lall, N., Hussein, A.A., Mudau, F.N. and Meyer, J.J.M. (2010). Antioxidant activity and cytotoxicity effect of flavonoids isolated from *Athrixia phylicoides. Journal of Medicinal Plant Research*, 4, 2584-2587.

May MJ and Ghosh S. (1997). Rel/NF-kappa B and I kappa B proteins: an overview. Seminars in Cancer Biology. 8, 63-73.

McCune LM, Johns T (2007). Antioxidant activity relates to plant part, life form and growing condition in some diabetes remedies. Journal of Ethnopharmacology. 112: 461-469.

McDonald, S., Prenzler, P.D., Antolovich, M. and Robards, K. (2001). Phenolic content and antioxidant activity of olive extracts. *Food Chemistry,* 73: 73-84.

McMaster, M. 1994. HPLC. A Practical User's Guide, pp 3-23. New York: VCH Publishers.

Middleton, E., Kandaswami, J.RC. and Theoharides, T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacological Reviews* 52: 673-751.

Miguel, M.G. (2010). Antioxidant activity of medicinal and aromatic plants. A review. *Flavour and Fragrance Journal*, 25, 291–312.

Morsy, M.A. and Khaled, M.M. (2002). Novel EPR characterization of the antioxidant activity of tea leaves. *Spectrochimica Acta Part A,* 58: 271-1277.

Naczk, M. and Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, 1054, 95-111.

Namiki, M. (1990). Antioxidants/antimutagens in food. *Critical Reviews in food science and Nutrition*, 29:273-300.

Narayana, K.R., Reddy, M.S., Chaluvadi, M.R., & Krishna, D.R. (2001). Bioflavonoids classification, pharmacological, biochemical, effects and therapeutic potential. *Indian Journal of Pharmacology*, 33, 2–16.

Negro, C., Tommasi, L. and Miceli, A. (2003). Phenolic compounds and antioxidant activity from red grape marc extracts. *Bioresource Technology*, 87: 41-44.

Netzel, G., Tian, Q., Schwartz, S. and Konczak, I. (2007). Native Australian fruits , a novel source of antioxidants for food. *Innov. Food Sci. Emerg. Technol.* 8 (3): 339-346.

Nichenametla, S.N, Taruscio, T.G., Barney, D.L. and Exon, J.H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition*, 46(2), 161-183.

Nicolis, E., Lampronti, I., Dechecchi, M.C., Borgatti, M., Tamanini, A., Bianchi, N., Bezzerri, V., Mancini, I., Giri, M.G., Rizzotti, P., Gambari, R. and Cabrini, G. (2008). Pyrogallol, an active compound from the medicinal plant *Emblica officinalis*, regulates expression of pro-inflammatory genes in bronchial epithelial cells. *International Journal of Immunopharmacology*, 8, 1672-1680.

Nijveldt, R.J., Nood, E., Hoorn, D.E.C., Boelens, P.G., Norren, K. and Leeuwen, P.A.M. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition*, 74, 418–425.

Nuutila, A.M., Puupponen-Pimiä, R., Aarni, M. and Oksman-Caldentey, K.M. (2003). Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. *Food Chem*istry, 81(4), 485-493.

Oskoueian, E., Abdullah, N., Zuhainis, S.W., Omar, A.R., Ahmad, S., Kuan, W.B., Zolkifli, N.A., Hendra, R. and Ho, Y.W. (2011). Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Jatropha curcas* Linn. *Journal of Medicinal Plant Research,* 5(1), 49-57.

Osman, H., Nasarudin, R. and Lee, S.L. (2004). Extracts of cocoa (*Theobroma cacao* L.) leaves and their antioxidation potential. *Food Chemistry,* 86: 41-46.

Ou, B., D. Huang, M. Hampsch-Woodill, J.A. Flanagan and E.K. Deemer (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. Journal of Agriculture and Food Chemistry, 50: 3122-3128.

Palma, M. and Taylor, L. (1999). Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. *Journal of Chromatography A,* 849, 117-124.

Perucka, I. and Materska, M. (2001). Phenylalanine ammonia-lyase and antioxidant activities of lipophilic fraction of fresh pepper fruits *Capsicum annum* L. *Innovative Food Science and Emerging Technologies*, 2: 189-192.

Pokorny, J. and Korczak J. (2001). *Preparation of Natural Antioxidants. In Antioxidants in Food.* pp. 311-330. Boca Raton, Fla.: CRC Press.

Ponce, A.G., del Valle, C.E. and Roura, S.I. (2004). Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. *Swiss Society of Food Science and Technology,* 37: 199-204.

Pourcel, L., Routaboul, J-M., Cheynier, V., Lepiniec, L. and Debeaujon, I. (2006). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends In Plant Science* 12(1): 29-36.

Ramma, A.L., Bahorun, T., Crozier, A., Zbarsky, V., Datla, K.P., Dexter, D.T. and Aruoma, O.I. (2005). Characterization of the antioxidant functions of flavonoids and proanthocyanidins in Mauritian black teas. *Food Research International* 38: 357-367.

Ramos, S. (2007). Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *Journal of Nutritional Biochemistry,* 18, 427–442.

Rapisarda, P., Tomaino, A., Cascio, L.R., Bonina, F., Pasquale, D.A. and Saija, A. (1999). Effectiveness as influenced by phenolic content of fresh orange juices. *Journal of Agriculture and Food Chemistry,* 47, 4718–4723.

Rates, S.M.K. (2001). Plants as source of drugs. *Toxicon* 39: 603-613.

Rice-Evans, C. (2001). Flavonoid antioxidants. *Current Medicinal Chemistry,* 8, 797–809.

Rice-Evans, C.A. and Burdon, R H. (1994). *Free radical damage and its control,* New York : Elsevier.

Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M. and Bruni, R. (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chemistry*, 91, 621-632.

Sanchez-Moreno, C. (2002). Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. *International Journal of Food Science and Technology,* 8(3): 121-137.

Sasaki, Y.F., Satomi, K., Asako, K., Miyuki, O., Kazumi, K., Kayoko, I., Kazuyuki, T. and Shuji, T. (2002). The comet assay with 8 mouse organs: results with 39 currently used food additives. *Mutation Research,* 519: 103-119.

Sastre, J. Pallardo, F.V. Vina, J. (2000). Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life, 49: 427–435.

Scalbert A, Manach C, Morand C and Remesy, C (2005). Dietary polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition. 45:287-306.

Schieber, A., Keller, P. and Carie, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A,* 910: 265-273.

Shahidi, F. and Naczk, M. (2003). *Phenolics in food and nutraceuticals*. Boca Raton, Florida: CRC.

Shalini and Srivastava R. (2009). Antifungal activity screening and HPLC analysis of crude extracts from *Tectona grandis, Shilajit, Valeriana wallachi*. *Journal of Environmental, Agricultural and Food Chemistry*, 8, 218-229.

Shalini and Srivastava R. (2009). Antifungal activity screening and HPLC analysis of crude extracts from *Tectona grandis, Shilajit, Valeriana wallachi*. *Journal of Environmental, Agricultural and Food Chemistry*, 8, 218-229.

Siddhuraju, P., Mohan, P.S. and Becker, K. (2002). Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry*, 79: 61-67.

Simoes, J.M., Arthur, G., and Liebman, J.F.(1996). Energetics of organic free radicals, London : Blackie Academic and Professiona.

Singh, A. (2006). *Compendia of world's medicinal flora.* Enfield, New Hampshire, United States of America: Science Publishers.

Singh, N. and Rajini, P.S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry*, 85: 611-616.

Stahl, W. and Sies H. Protection against solar radiation – protective properties of antioxidants; in Gacomoni PU (ed): Sun Protection in Man. Amsterdam, Elsevier, (2002), pp 561–572.

Stewart B, Kleihues PE. World Cancer Report. Lyon, France: *IARC Press*, (2003).

Stewart B, Kleihues PE. World Cancer Report.Lyon, France: *IARC Press*, (2008).

Sun, T. and Ho, H. (2005). Antioxidant activities of buckwheat extracts. *Food Chemistry*, 90, 743–749.

Surai, P.F. (2005). Minerals and Antioxidants. In L. Tucker and J. Taylor-Pickard (Eds.), Redefining Mineral Nutrition (pp. 147–177). Nottingham: Nottingham University Press.

Tai, C., Jin-Feng, P., Liu, J., Jiang, G. and Zou, H. (2004). Determination of hydroxyl radicals in advanced oxidation processes with dimethyl sulfoxide trapping and liquid chromatography. *Analytica Chimica Acta*, 527 (1): 73-80.

Takabe, W. Niki, E. Uchida, K. Yamada, S. Satoh, K. Noguchi, N. (2001). Oxidative stress promotes the development of transformation: involvement of a potent mutagenic lipid peroxidation product, acrolein, Carcinogenesis. 22, 935–941.

Tapiero, H., Tew, K.D., Nguyen, B.G. and Mathé, G. (2002). Polyphenols: Do they play a role in the prevention of human pathologies? *Biomedicine and Pharmacotherapy*, 56, 200-207.

Tapiero, H., Tew, K.D., Nguyen, B.G. and Mathé, G. (2002). Polyphenols: Do they play a role in the prevention of human pathologies? *Biomedicine and Pharmacotherapy*, 56, 200-207.

Treutter, D. (2005). Significance of flavonoids in plant resistance and enhancement of their biosynthesis, *Plant Biology*, 7(6), 581-591.

Tsai, H.T., Tsai, Y.M., Yang, S.F., Wu, K.Y., Chuang, H.Y., Wu, T.N., Ho, C.K., Lin, C.C., Kuo, Y.S. and Wu, M.T. (2007). Lifetime cigarette smoke and second-hand smoke and cervical intraepithelial neoplasm--a community-based case-control study. *Gynecologic Oncology* 105(1):181-8.

Tsao, R., and Deng, Z. (2004). Separation procedures for naturally occurring antioxidant photochemicals. Journal of Chromtography B, 812, 85–99.

Turkmen, N., Sari, F. and Velioglu, Y.S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chemistry*, 99, 835–841.

Van Acker, S.A., Tromp, M.N., Haenen, G.R., van der Vijgh W.J. and Bast, A. (1995). Flavonoids as scavengers of nitric oxide radical. *Biochemical and Biophysical Research Communications*, 214, 755–759.

Verschoyle RD, Steward WP, Gescher AJ (2007). Putative cancerchemopreventive agents of dietary origin-how safe are they?. Nutr.Cancer, 59(2): 152-162.

Weisburg, J.H., Weissman, D.B., Sedaghat, T. and Babich, H. (2004). *In vitro* anti-cancer of epigallocatechin gallate and tea extracts to cancerous and normal cells

from the human oral cavity. *Basic and Clinical Pharmacology and Toxicology*, 95, 191-200.

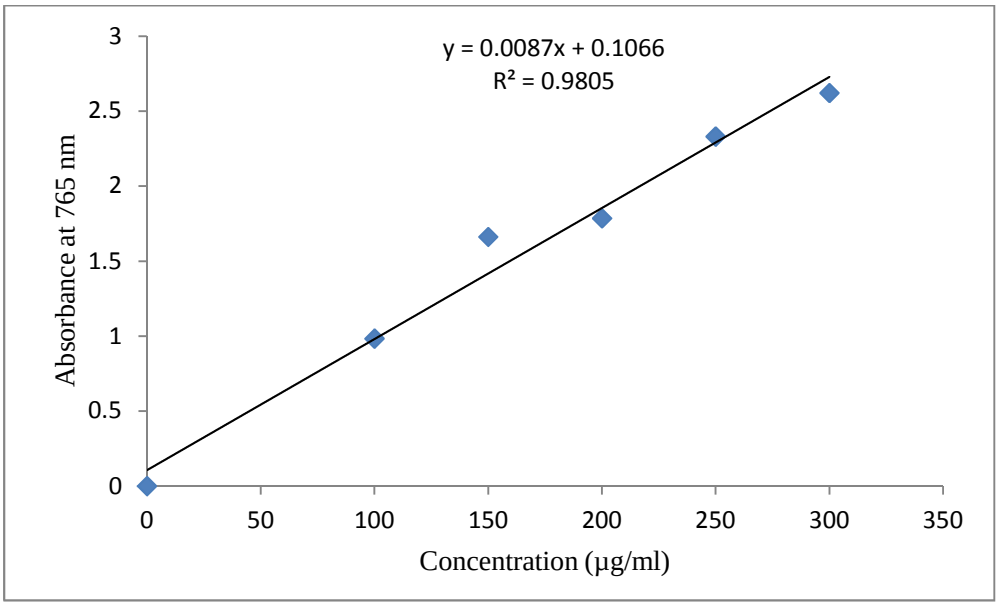
Weston, A. and Brown, P.R. 1997. HPLC and CE. *Principles and Practice*, pp 29-37. London-New York: Academic Press.

Wichi, H.P. (1988). Enhance tumor development by butylated hydroxyanisole (BHA) from the perspective of effect on forest-stomach and oesophageal squamous epithelium. *Food and Chemical Toxicology,* 26: 717-723.

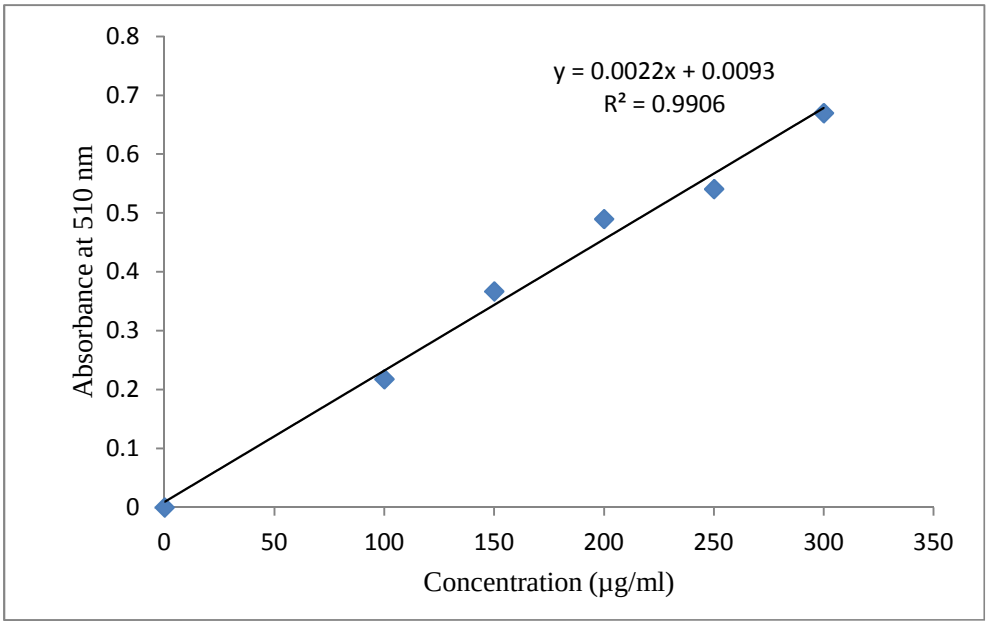
William, G.M., Iatropoulos, M.J. and Whysner, J. (1999). Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food and Chemical Toxicology,* 37: 1027-1038.

Zhang, Q. and Ye, M. (2008). Chemical analysis of the Chinese herbal medicine Gan-Cao 403 (licorice). *Journal of Chromatography A*, 1216, 1954-1969.

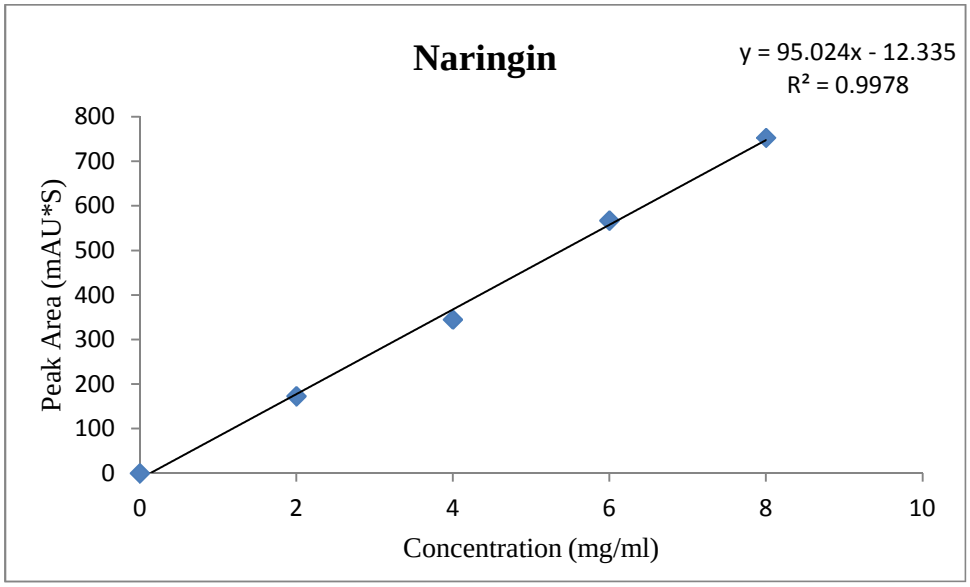
APPENDICES



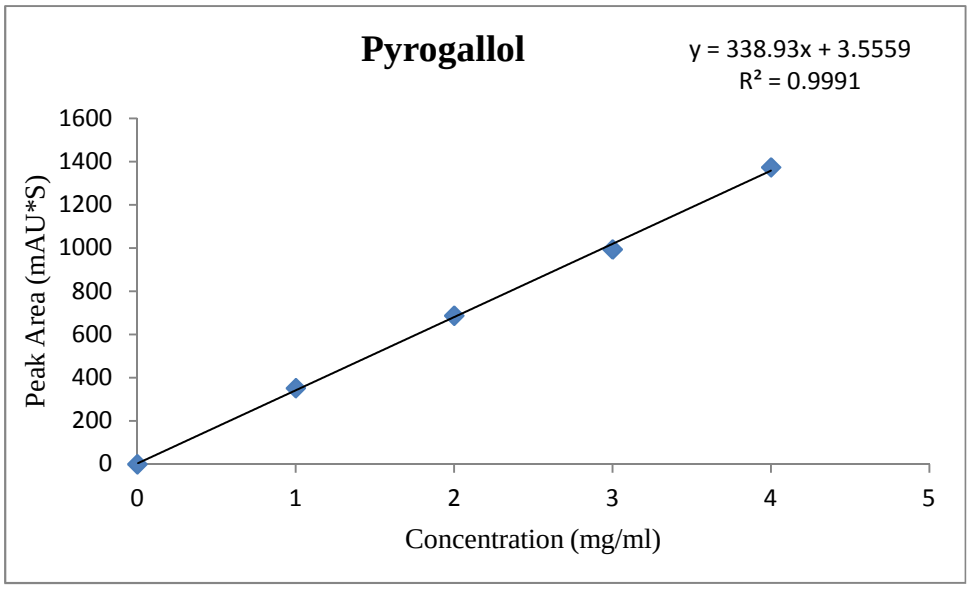
AP 1: A standard curve of total phenolic concentration (µg GAE/ml) .



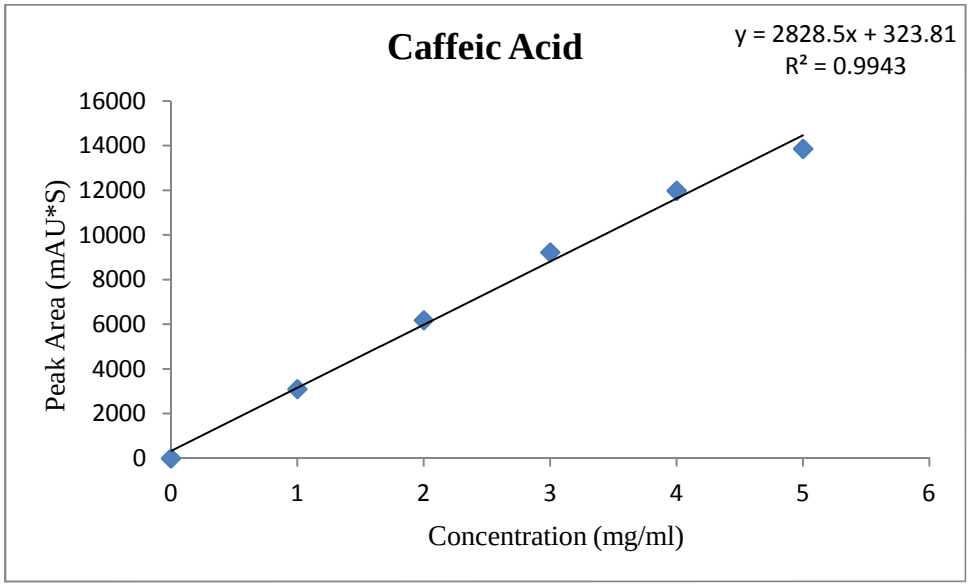
AP 2: A standard curve of total flavonoids concentration (µg Rutin/ml).



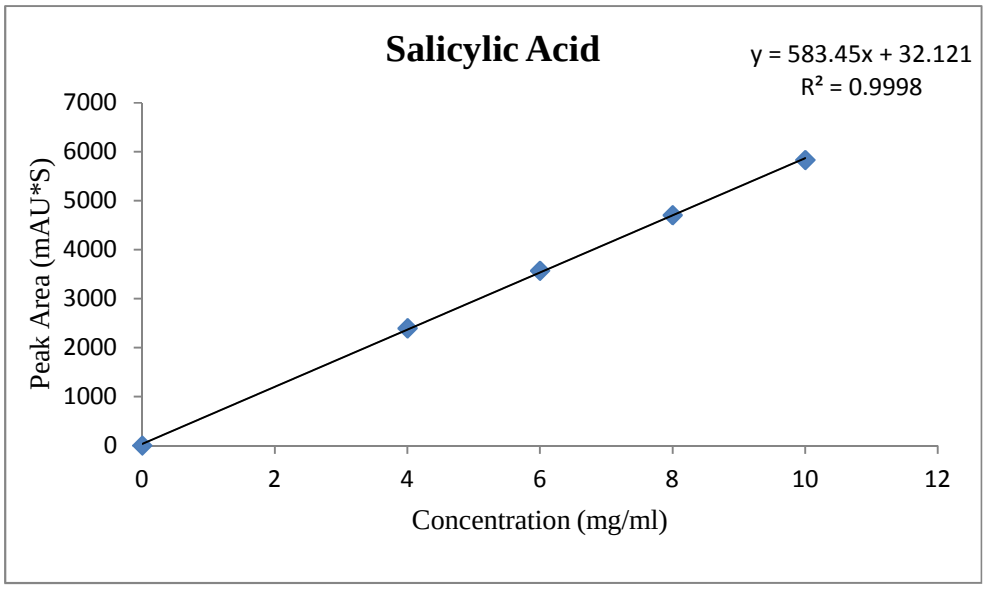
AP 3: A standard curve for estimation of naringin concentration (mg/ml).



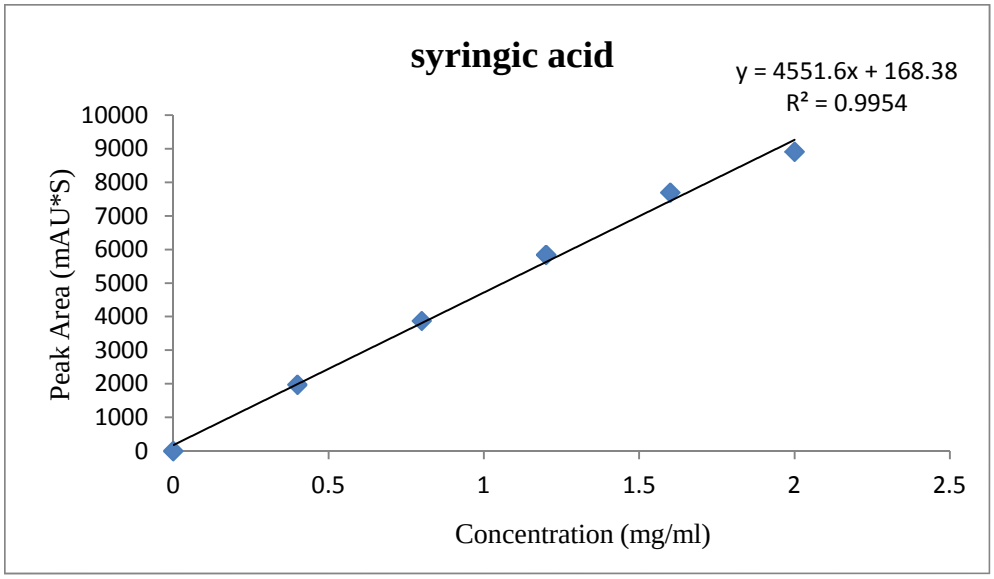
AP 4: A standard curve for estimation of pyrogallol concentration (mg/ml) .



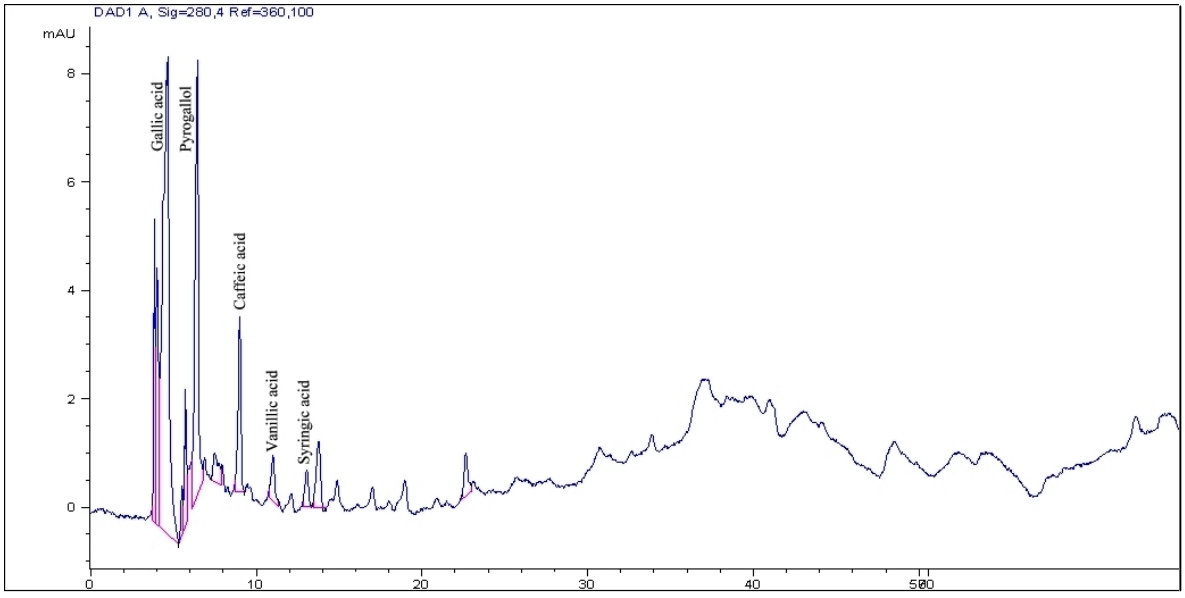
AP 5: A standard curve for estimation of caffeic acid concentration (mg/ml).



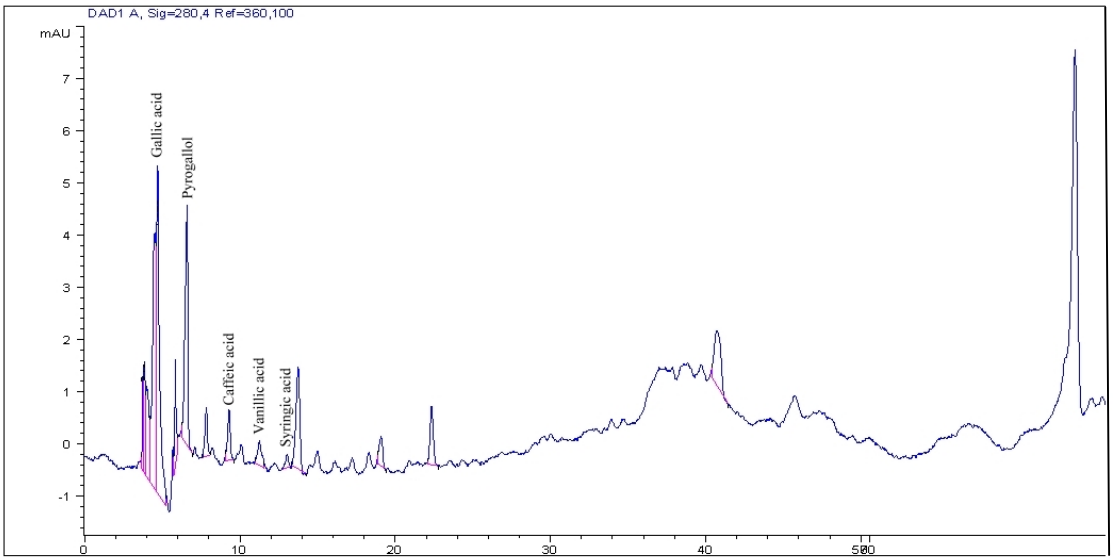
AP 6: A standard curve for estimation of salicylic acid concentration (mg/ml).



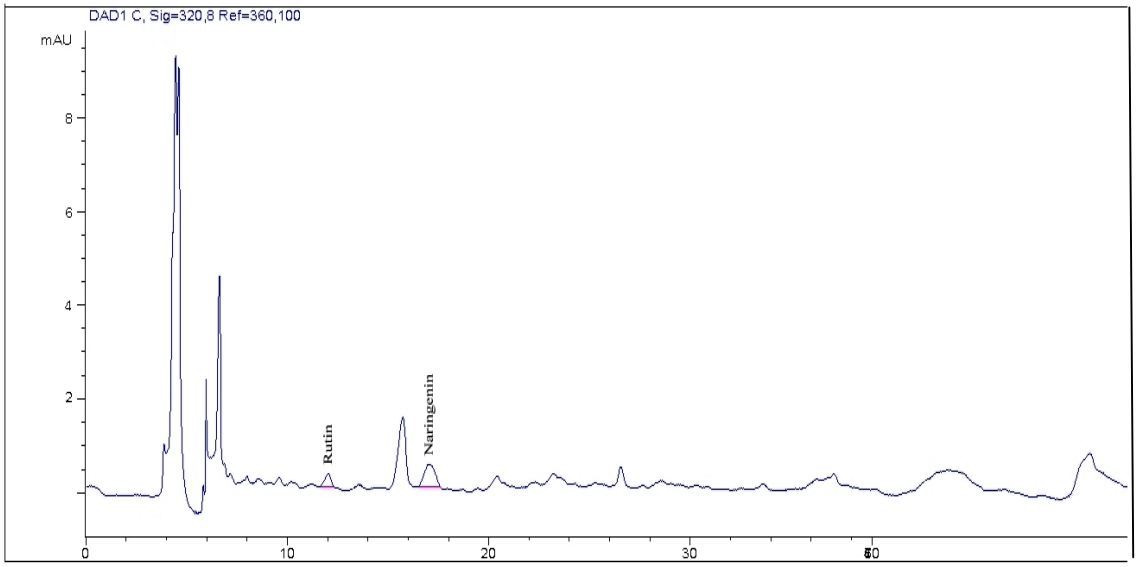
AP 7: A standard curve for estimation of syringic acid concentration (mg/ml).



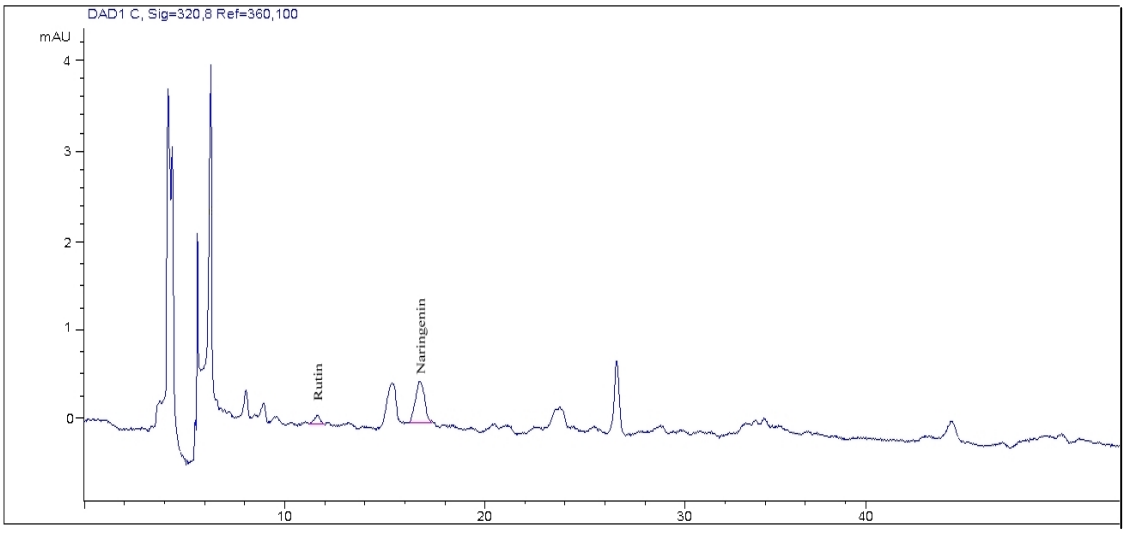
AP 8: The RP-HPLC chromatogram of phenolic compounds in ethyl acetate extracts of *Veitchia merrillii* fruits. Identification of compounds: Gallic acid, Pyrogallol, Caffeic acid, Vanilic acid and syringic acid.



AP 9: The RP-HPLC chromatogram of phenolic compounds in water extracts of *Veitchia merrillii* fruits. Identification of compounds: Gallic acid, Pyrogallol, Caffeic acid, Vanilic acid and syringic acid.



AP 10: The RP-HPLC chromatogram of flavonoid compounds in methanolic extract of *Veitchia merrillii* fruits. Identification of compounds: Rutin and Naringenin.



AP 10: The RP-HPLC chromatogram of flavonoid compounds in water extract of *Veitchia merrillii* fruits. Identification of compounds: Rutin and Naringenin.