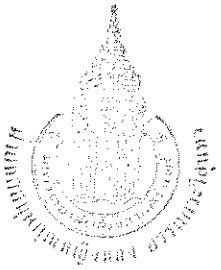

In Vitro Studies on Antioxidant and Immunomodulatory Activities of Thai Medicinal Plants in Longevity Formulas

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ชื่อวิทยานิพนธ์	การศึกษาฤทธิ์ต้านอนุมูลอิสระและผลต่อภูมิคุ้มกันในหลอดทดลองของสมุนไพรไทยในตำรับยาอายุวัฒนะ
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บทคัดย่อ

ศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต่อภูมิคุ้มกันในหลอดทดลองของสมุนไพรไทย 8 ชนิดในตำรับยาอายุวัฒนะที่หมอพื้นบ้านนำมาใช้ คือ เหงือกปลาหมอ กระชาย ขมิ้นอ้อย แห้วหมู ดีปลี ช้าพลู ม้ากระทืบโรง และว่านพังพอน สารสกัดชั้นน้ำและชั้นเมธานอลนำมาทดสอบฤทธิ์ต้านอนุมูลอิสระ ด้วยวิธี DPPH assay และการทดสอบโดยใช้เซลล์เพาะเลี้ยง ทดสอบฤทธิ์ต่อภูมิคุ้มกันในหลอดทดลองโดยหาค่าการแบ่งเซลล์ และการทำงานของเซลล์ลิมโฟซัยต์โดยวัดระดับสารสื่อกลางในกระบวนการตอบสนองของระบบภูมิคุ้มกันแบบอาศัยเซลล์คืออินเตอร์ลิวคินทู และอินเตอร์เฟอรอนแกมมา ผลการทดลองพบว่าพืชบางชนิดมีฤทธิ์ต้านอนุมูลอิสระ โดยสารสกัดม้ากระทืบโรง มีฤทธิ์ในการต้านอนุมูลอิสระดีที่สุดทั้ง 2 วิธี ผลการทดสอบฤทธิ์ต่อภูมิคุ้มกันพบว่า สารสกัดชั้นเมธานอลของม้ากระทืบโรงที่ความเข้มข้น 10 ไมโครกรัมต่อมิลลิลิตร กระตุ้นการแบ่งเซลล์ลิมโฟซัยต์ (ดัชนีการกระตุ้น 1.39 ± 0.43) และกระตุ้นการหลั่งอินเตอร์ลิวคินทู (33.97 ± 12.34 พิโคกรัมต่อมิลลิลิตร) อย่างมีนัยสำคัญทางสถิติ สารสกัดชั้นน้ำของดีปลี ความเข้มข้น 0.1 ไมโครกรัมต่อมิลลิลิตร กระตุ้นการแบ่งเซลล์ลิมโฟซัยต์ (ดัชนีการกระตุ้น 1.48 ± 0.31) และกระตุ้นการหลั่งอินเตอร์เฟอรอนแกมมา (19.03 ± 16.89 พิโคกรัมต่อมิลลิลิตร) อย่างมีนัยสำคัญทางสถิติ นอกจากนั้นสารสกัดชั้นน้ำของ ขมิ้นอ้อย ความเข้มข้น 0.5 ไมโครกรัมต่อมิลลิลิตรกระตุ้นการหลั่งอินเตอร์เฟอรอนแกมมา (49.45 ± 39.36 พิโคกรัมต่อมิลลิลิตร) อย่างมีนัยสำคัญทางสถิติ ผลการทดสอบแสดงว่าพืชดังกล่าวที่ใช้ในตำรับยาอายุวัฒนะมีฤทธิ์ต้านอนุมูลอิสระหรือมีฤทธิ์กระตุ้นภูมิคุ้มกันได้

การศึกษานี้ได้รายงานผลการต้านอนุมูลอิสระและผลในการกระตุ้นภูมิคุ้มกันของสารสกัดจากลำต้นม้ากระทืบโรง โดยแยกสารบริสุทธิ์และศึกษาสูตรโครงสร้าง พบสารสำคัญ 4 ชนิดคือ vitexin, 15α-hydroxyfriedelan-3-one, siphulitol และ mangiferin นำสารบริสุทธิ์ที่แยกได้มาทดสอบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต่อภูมิคุ้มกันในหลอดทดลอง พบว่าสาร mangiferin มีฤทธิ์กำจัดอนุมูลอิสระได้ดีที่สุด (EC_{50} 1.10 ± 0.18 ไมโครกรัมต่อมิลลิลิตร) เมื่อเทียบกับ quercetin (EC_{50} 1.35 ± 0.02 ไมโครกรัมต่อมิลลิลิตร) ส่วนสาร vitexin มีฤทธิ์ป้องกันเซลล์จากอนุมูลอิสระได้ดีที่สุด (IC_{50} 37.57 ± 1.15 ไมโครกรัมต่อมิลลิลิตร) เมื่อเทียบกับ quercetin ซึ่งใช้เป็นสารมาตรฐาน (IC_{50} 76.08 ± 1.03 ไมโครกรัมต่อมิลลิลิตร) สาร vitexin และ siphulitol ความเข้มข้น 0.5

ไมโครกรัมต่อมิลลิลิตรกระตุ้นการแบ่งเซลล์ลิมโฟซัยด์ (ดัชนีการกระตุ้น 1.06 ± 0.76 และ 1.07 ± 0.09 ตามลำดับ) อย่างมีนัยสำคัญทางสถิติ สาร mangiferin ความเข้มข้น 10 ไมโครกรัมต่อมิลลิลิตร กระตุ้นการแบ่งเซลล์ลิมโฟซัยด์ (ดัชนีการกระตุ้น 1.08 ± 0.09) และกระตุ้นการหลั่งอินเตอร์ลิวคิน ทู (23.54 ± 10.42 พิโคกรัมต่อมิลลิลิตร) อย่างมีนัยสำคัญทางสถิติ

การศึกษานี้แสดงว่าสารสกัดจากลำต้นม้ากระทืบโรงมีฤทธิ์ต้านอนุมูลอิสระและมีฤทธิ์กระตุ้นภูมิคุ้มกันได้ เป็นการรายงานสารสำคัญและฤทธิ์ทางชีวภาพครั้งแรกของสมุนไพรนี้ซึ่งเป็นข้อมูลทางวิทยาศาสตร์เพื่อสนับสนุนการใช้สมุนไพรพื้นบ้าน

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ABSTRACT

Eight herbal substances traditionally used in longevity recipes by traditional practitioners in Thailand as fowllow: *Acanthus ebracteatus*, *Boesenbergia pandurata*, *Curcuma zedoaria*, *Cyperus rotundus*, *Piper chaba*, *Piper sarmentosum*, *Salacia euphlebia* and *Tacca chantrieri* were tested for antioxidant and immunomodulatory activities. Crude water or methanol extracts were tested for antioxidants by using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method and a cell culture assay. In vitro immunomodulatory activity was determined by lymphocyte proliferation and cytokine assays. Some plant extracts showed free radical scavenging on DPPH and antioxidant activity on cell culture assay. *S. euphlebia* demonstrated high antioxidant activity in both assays. Result from immunomodulatory assay showed that at a concentration of 10 μg/ml, a methanolic extract of *S. euphlebia* significantly induced lymphocyte proliferation (Stimulation Index 1.39 ± 0.43) and interleukin 2 (IL-2) production (33.97 ± 12.34 pg/ml). A water extract of *P. chaba* at 0.1 μg/ml significantly induced lymphocyte proliferation (Stimulation Index 1.48 ± 0.31) and increased interferon gamma (IFN-γ) (19.03 ± 16.89 pg/ml). Moreover, a water extract of *C. zedoaria* at 0.5 μg/ml significantly increased IFN-γ (49.45 ± 39.36 pg/ml). The results suggest that some plants used for longevity have an antioxidative and immunostimulatory potential.

The present study reports antioxidant activity and immune-stimulting effects of *S. euphlebia* stem extract. To understand the antioxidant and the immune active compounds of this plant, four compounds were isolated and elucidated as vitexin, 15α-hydroxyfriedelan-3-one, siphulitol and mangiferin. In vitro studies of the four pure compounds were carried out. Mangiferin showed high free radical scavenging activity (EC_{50} 1.10 ± 0.18 μg/ml) comparing with quercetin (EC_{50} 1.35 ± 0.02 μg/ml) while vitexin (IC_{50} 37.57 ± 1.15 μg/ml) showed good result on cytoprotective effect compared with quercetin (IC_{50} 76.08 ± 1.03 μg/ml).

Vitexin and siphulitol at 0.5 μg/ml significantly induced human lymphocyte proliferation (Stimulation Index 1.06 ± 0.76 and 1.07 ± 0.09 respectively). Mangiferin at 10 μg/ml significantly enhanced lymphocyte proliferation (Stimulation Index 1.08 ± 0.09) and IL-2 secretion (23.54 ± 10.42 pg/ml). These observations suggest that the stem extract of *S. euphlebia* showed the effect on cell-mediated immune response. This is the first report of the activities and bioactive compounds of *S. euphlebia*. These results may scientifically explain the folk and alternative-medicine uses of some Thai medicinal plants in longevity formulas.

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Supreeya Yuenyongsawad

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LIST OF ABBREVIATIONS AND SYMBOLS

BHT	Butylated hydroxytoluene
CO_2	Carbon dioxide
°C	Degree Celsius
DNA	Deoxynucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration to give 50% response
ELISA	Enzyme-linked immunosorbent assay
g	Gram
h	Hours
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN-?	Interferon gamma
IL-2	Interleukin 2
IU	International Unit
LAK	Lymphokine activated killer
µg	Microgram
µl	Microliter
ml	Milliliter
Min	Minute
M	Molar
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt
nm	Nanometer
NK	Natural killer
pg	Picogram
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
SRB	Sulforhodamin B
SI	Stimulation index
tBHP	Tertiary butylhydroperoxide

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

Th-1	T helper cells Type 1
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

1. Background and rationale

Many plants have been used as therapeutic regimen for thousands of years, based on experience and folk remedies. Herbal formulas used for expanding life span are popular in Thailand in the forms of herbal pill, decoction or herb spirit. Several plant species have been claimed and used for longevity, tonic, lumbago, and aphrodisiac. Tonic herbs are the highest and most sought after herbal remedies in many traditional systems of healing, such as Traditional Chinese Medicine and Ayuravedic medicine. These herbs nourish specific cells, tissues, organs and the whole individual, and are often used for long periods of time. These remedies help focus and call forth the biogenetic potential or vitality of the individual for use such as supporting the immune system, nerve function or hormone system. Tonics are very gentle and slow stimulants, and they provide nutrients that the body can use, such as vitamins and many other constituents like flavonoids (1). Tonic herbs help the body to adjust to the effects of stress and to adapt in more positive way and maintain its equilibrium. However, most of the claimed medicinal usage in specific medicinal plants are lack of scientific evaluation and proven. The degenerative diseases associated with aging include cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts (2). Free radical and reactive oxygen species are an integral part of life and our metabolism. The active oxygen species are essential for production of energy, synthesis of biologically essential compounds, and phagocytosis, a critical process of immune system. They also play a vital role in signal transduction, on the other hand, they can be very damaging because they are extremely reactive and almost instantly attack other molecules. There is now increasing evidence which shows that these active oxygen species may play a causative role in a variety of diseases including heart disease, cancer, and aging (3), atherosclerosis, rheumatoid arthritis, immune system decline, brain dysfunction and other chronic diseases common among older adult (4). It is envisaged that drugs with multiple mechanisms of protective action, including antioxidant properties, may become available in attempts to minimize tissue injury in human diseases (5). Study of the possible immunomodulatory effects of medicinal plant on cell-mediated immunity is a matter of interest

for many investigators. The proliferation of T and B cells, natural killer cells and lymphokine-activated killer cells that is required to mount an effective defense against pathogens and tumors cells appears to be inhibited markedly with age and upon exposure to oxidants (2). The potential uses of immunomodulators in clinical medicine include the reconstitution of immune deficiency and the suppression of normal or excessive immune function. Immunomodulators are being used as immunotherapy in diseases such as cancer, infectious diseases, immunodeficiency disorders, autoimmune disease and inflammation. Some medicinal plants have been shown to be strong immunomodulators (6).

Elderly adults are less capable of producing lymphocytes to combat challenges to the immune system. The infection-fighting cells that are produced are less vigorous and less effective than those found in younger adults. When antibodies are produced, the duration of their response is shorter in older adults and fewer cells are produced than in younger adults. The immune system of younger adults including lymphocytes and other types of cells typically reacts more strongly and more rapidly to infection than does an older adult's. In addition, elderly adults, particularly after 70, are more likely to produce autoantibodies, which attack parts of the body itself instead of infections. Autoantibodies are factors in causing rheumatoid arthritis and atherosclerosis (7).

In this study, eight Thai medicinal plants frequently mentioned in the longevity recipes (8) and broadly used by traditional medicine practitioners in Southern Thailand, namely *Acanthus ebracteatus* Vahl (Acanthaceae), *Boesenbergia pandurata* Holtt., *Curcuma zedoaria* Roscoe (Zingiberaceae), *Cyperus rotundus* Linn. (Cyperaceae), *Piper chaba* Hunt, *Piper sarmentosum* Roxb. (Piperaceae), *Salacia euphlebia* Merr. (Celastraceae) and *Tacca integrifolia* Ker-Gawl. (Taccaceae), were selected. This study was designed to investigate whether antioxidant and/or modulation of immunological reaction could support the folkloric efficacy of some plants in longevity formulas.

2. Review of literature

2.1 Antioxidant and oxidative stress

Antioxidant status reflects the balance between the antioxidant defenses and oxidants in living organisms. Impairment of the antioxidant defenses and /or high levels of oxidants can lead to oxidative stress. Oxidative stress in humans has been implicated in the pathogenesis of major chronic diseases. Specifically, a role of oxidative stress has been demonstrated for

inflammation, carcinogenesis and atherosclerosis, and suggested for many other diseases and for aging as shown in table 1-1. For this reason, there has been strong interest in preventing oxidative stress and maintaining an antioxidant status that supports good health. The antioxidant system is composed of exogenous and endogenous components. The exogenous components derived from the diet include vitamins such as E and C, minerals such as selenium and other components such as carotenoids and phytochemicals. The endogenous components include the tripeptide glutathione, the iron-binding proteins transferring and ferritin, and the enzymes catalase and superoxide dismutase (9).

An antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. The term oxisizable substrate includes almost everything found in living cells, including proteins, lipids, carbohydrates and DNA. Mechanisms of antioxidant action can include: removal of O_2, scavenging reactive oxygen or nitrogen species (ROS/RNS), inhibiting ROS/RNS formation, binding metal ions needed for catalysis of ROS generation and upregulation of endogenous antioxidant defenses.

2.1.1 Free radicals and reactive oxygen species

Free radicals are chemical species, which have unpaired electrons as shown in table 1-2. Molecules are composed of atoms and electrons. Electrons are present generally in pairs. However, under certain conditions, molecules have unpaired electrons and as such they are called free radicals. Unpaired electrons usually seek other electrons to become paired. Thus, free radicals are in general reactive and attack other molecules, although some radicals are not reactive but stable enough to have long life. Reactive oxygen species donate oxygen-containing molecules, which are more active than the triplet oxygen molecule present in air. Free radicals and active oxygen species attack lipids, sugars, protein and DNA and induce their oxidation, which may result in oxidative damage such as deterioration of foods, membrane dysfunction, protein modification, enzyme inactivation, and break of DNA strands and modification of its bases. Most biological molecules are non radicals containing only paired electrons.

Table 1-1 Some clinical conditions in which oxygen free radicals are thought to be involved(5)

Category	Examples
Brain	Alzheimer's disease
	Parkinson's disease
	Neurotoxin
	Vitamin E deficiency
	Hyperbaric oxygen
	Hypertensive cerebrovascular injury
	Potentiation of traumatic injury
Eye	Cataractogenesis
	Ocular hemorrhage
	Photic retinopathy
	Degenerative retinal damage
	Retinopathy of prematurity
Heart cardiovascular system	Atherosclerosis
	Adriamycin cardiotoxicity
	Keshan disease (selenium deficiency)
Kidney	Aminoglycoside nephrotoxicity
	Autoimmune nephritic syndromes
Gastro-intestinal tract	Nonsteroidal antiinflamatory drug induced
	GI tract lesions
	Diabetogenic actions of alloxan
	Free fatty acid-induced pancreatitis
	Endotoxin liver injury
Inflammatory-immune injury	Rheumatoid arthritis
	Vasculitis (hepatitis B virus)
	Autoimmune diseases
	Glomerulonephritis
Iron overload	Nutritional deficiencies (Kwasiorkor)
	Thalassaemia and other chronic anaemias

Category	Examples
	treated with multiple blood transfusions
	Idiopathic haemochromatosis
Aging	Disorders of premature aging
	Age-related diseases
Red blood cell	Protoporphyrin photoxidation,
	Favism
	Sickle cell anaemia
Lung	Hypoxia
	Bronchopulmonary dysplasia
	Mineral dust pneumoconiosis
	Cigarette-smoke effect
	Emphysema
Ischaemia-reperfusion	Stroke/myocardial infarction
	Organ transplantation
Skin cancer	Radiation injury

Table 1-2 Reactive oxygen and nitrogen species (5)

Radicals		Nonradicals	
$O_2^{\cdot -}$	Superoxide	H_2O_2	hydrogen peroxide
$OH^{\cdot}$	Hydroxyl radical	$^1O_2$	singlet oxygen
$HO_2^{\cdot}$	Hydroperoxyl radical	LOOH	lipid hydroperoxide
L?	Lipid radical	Fe=O	iron-oxygen complexes
$LO_2^{\cdot}$	Lipid peroxyl radical	HOCl	hypochlorite
$LO^{\cdot}$	Lipid alkoxyl radical	O_3	ozone
$NO_2^{\cdot}$	Nitrogen dioxide	$ONOO^-$	peroxynitrite
$NO^{\cdot}$	Nitric oxide		

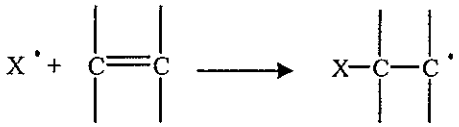
The important reactions of free radicals may be classified into the following categories:

1. Hydrogen Atom Transfer Reaction

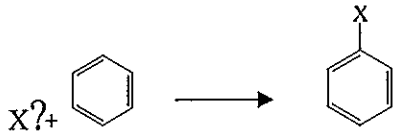
$$X^{\bullet} + RH \longrightarrow XH + R^{\bullet}$$

$$X^{\bullet} + RH \longrightarrow X^{-} + RH \longrightarrow XH + R^{\bullet}$$

2. Addition Reaction



3. Aromatic Substitution Reaction



4. ß-Scission Reaction

$$X^{\bullet} \longrightarrow Y^{\bullet} + \text{products}$$

5. Coupling Reactions

$$R^{\bullet} + R^{\bullet} \longrightarrow R\text{-}R$$

2.1.2 Antioxidant defences

Cells have a wide array of defences against oxidative damage. These can prevent damage due to free radical reactions by several mechanisms including:

1. Preventive antioxidants: suppress the formation of free radicals
   (a) Non-radical decomposition of hydroperoxides and hydrogen peroxide
   (b) Sequestration of metal by chelation
   (c) Quenching of active oxygen species

2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation

3. Repair and *de novo* enzymes: Repair the damage and reconstitute membranes

4. Adaptation: Generate appropriate antioxidant enzymes and transfer them to the right site at the right time and in the right concentration (3)

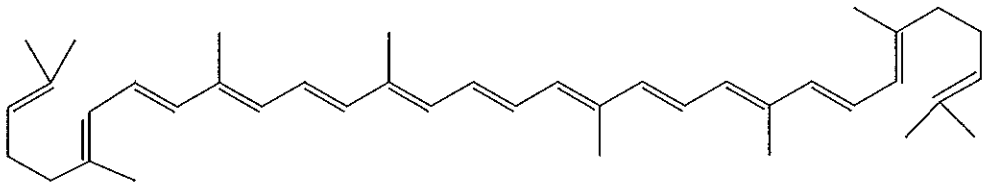
5. Prevention of mutations due to damaged molecules (4)

2.1.3 Phytochemical antioxidants

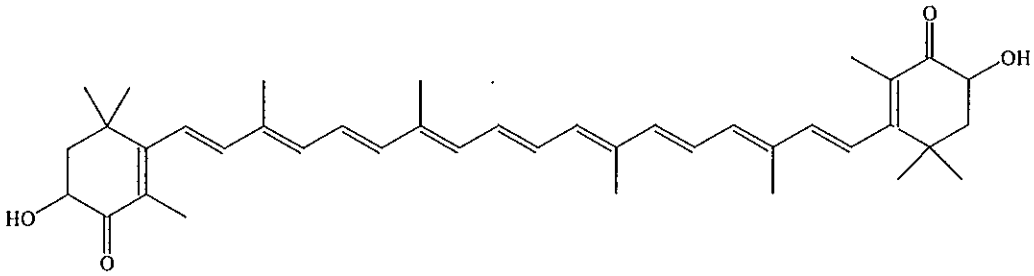
A large number of phytochemicals have attacked interest for their role in nutrition and health. Many of these phytochemicals shown in table1-3 affect the antioxidant status directly due to their function as antioxidants, indirectly by chelating prooxidant divalent metal such as Fe and Cu, or by sparing other antioxidants (9).

Table 1-3 Major classes of phytochemicals with antioxidant activity (10)

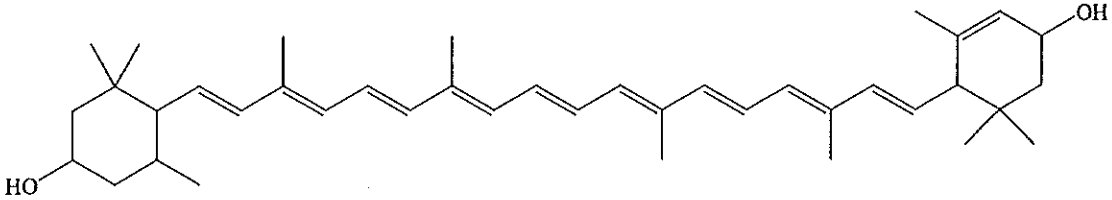
Class of Phytochemicals	Example Compounds
Carotenoids	lycopene, lutein, astaxanthin
Bioflavonoids	genistein, diadzein, quercetin
Phytosterols	sitosterol, stigmasterol, oryzanol
Tannins	catechins and other polyphenolic compounds
Chlorophylls	chlorophyll A and chlorophyllin
Terpenoids	limonin and limonene
Allylic compounds	diallyl sulfide and disulfide
Indoles	indole-3-carbinol



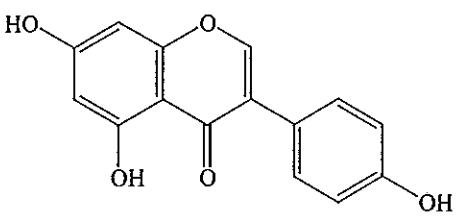
Lycopene



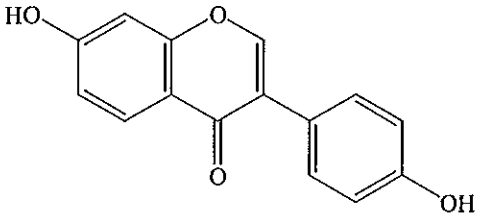
Astaxanthin



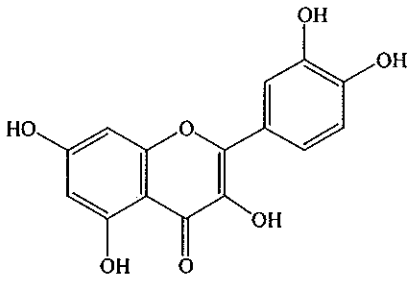
Lutein



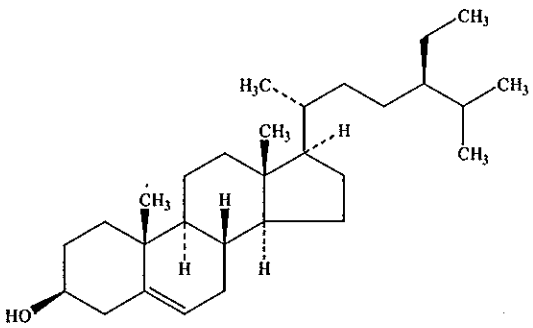
Genistein



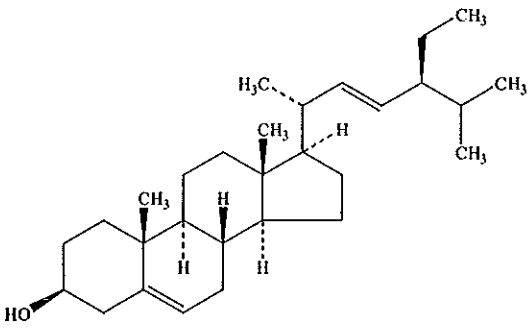
Diadzein



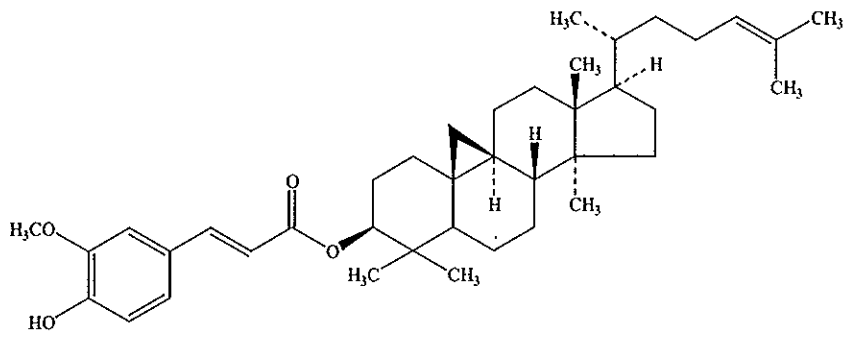
Quercetin



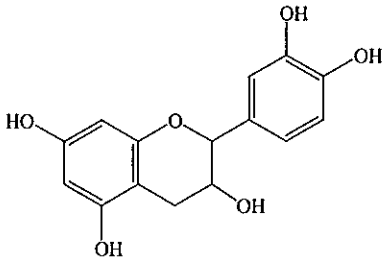
Sitosterol



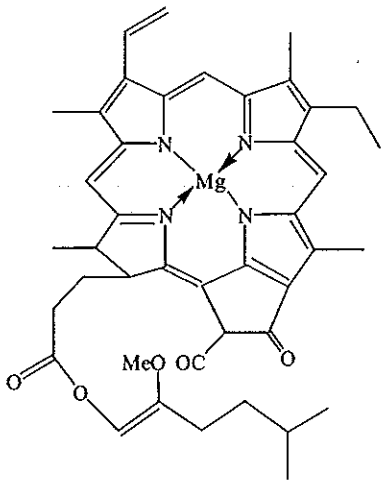
Stigmasterol



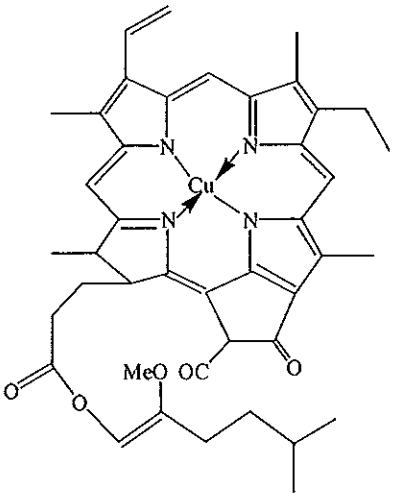
Oryzanol



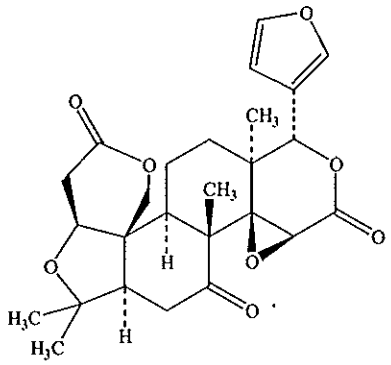
Catechin



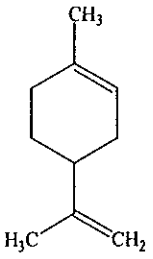
Chlorophyll A



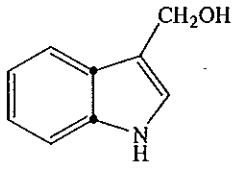
Chlorophyllin



Limonin



Limonene



Indole-3-carbinol

2.1.4 The free radical and aging

Many chemicals accumulate in the cells with age, including toxic and inert substances from the exterior and similar substances arising as byproducts of cellular metabolism. Fat-soluble substances (such as DDT and PCBs) are particularly slow to be eliminated. Iron tends to accumulate in cell nuclei with aging, as does aluminium. Aluminium transforms

metabolically active DNA into an inert state. Lead also accumulates in cells, and is neurotoxic. Cytochrome P-450 detoxification enzymes of the liver is also decline with age.

Aging due to free-radicals and glycation of macromolecules other than DNA would be expected more in non-diving cells than diving cells most notably in neurons. That lipofuscin is component of neuron aging due to free-radical damage is indicated by the high levels of metal (especially iron) in lipofuscin. Oxidative stress has been shown to promote lipofuscin formation, whereas antioxidants reduce lipofuscin formation. Although antioxidants cannot extend maximum lifespan of organisms as a whole, they may extend the maximum lifespan of neurons or even the entire brain. If so, antioxidants combined with organ replacement could be a mean of extending maximum lifespan (11).

2.1.5 Measurement of antioxidant activity

Methods to examine antioxidant activity of a sample can be divided in principle into two major categories: measuring its ability to donate electron to a specific ROS or to any electron acceptor and testing its ability to remove any sources of oxidative initiation e.g inhibition of enzyme, chelation of transition metal ions and absorption of UV radiation.

The ability of various antioxidants to determine radical chain processes was indirectly evaluated by different methods using various substrates. Many of these methods are often laborious and time-consuming, the results obtained depending on the substrate used and hydrophilic and lipophilic properties of the antioxidant tested (12).

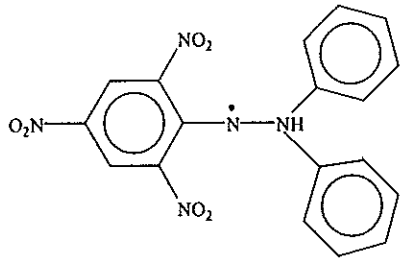
2.1.5.1 Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical

Several methods to determine free radical scavenging have been reported but the reaction with 1,1-diphenyl-2-picrylhydrazyl, DPPH?, has been one of the most studied.

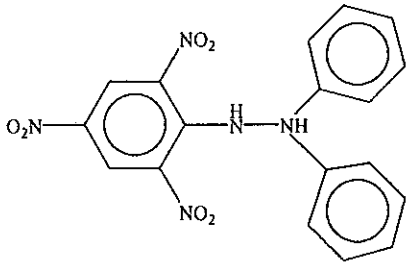
1,1-diphenyl-2-picrylhydrazyl hydrate, the violet color of which is modified to yellow, in presence of a free radical quencher, by appearance of reduced 1,1-diphenyl-2-picrylhydrazine. The reduced form characterized by an absorption band in ethanol solution at 520 nm (13). BHT, a synthetic phenolic antioxidant, terminates lipid peroxidation was used as positive control.

Percentage of inhibition was calculated using the following formula:

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



1,1-diphenyl-2-picrylhydrazyl
(violet)



1,1-diphenyl-2-picrylhydrazine
(yellow)

2.1.5.2 Cell culture assay

Excessive generation of ROS and reactive oxygen intermediates (ROI) can modify and damage DNA, carbohydrates, proteins, and polyunsaturated fatty acids in cells (14).

Endothelial cells as an element of the vascular system show a high risk for oxidant injury because of the close contact with flowing blood. Vascular endothelium is intimately involved in regulating coagulation and vasomotor activity. Normal endothelium facilitates anticoagulant processes, and synthesizes and secretes vasoactive substances implicated in the regulation of blood flow and peripheral vascular resistance. A growing body of evidence has revealed that free radical mediated reactions, such as lipid peroxidation, play a major role in atherogenesis. Oxidant injury of endothelial cells is considered an early event in the development of artheroslerosis (15).

The organic hydroperoxide, tertiary butylhydroperoxide (tBHP), is a useful model compound for the study of mechanisms of oxidative cell injury. Organic hydroperoxides form as a result of oxygen addition to alkyl radicals and/or by hydrogen atom abstraction from peroxyl radicals. tBHP may decompose to other alkyl and peroxyl radicals that accelerate lipid peroxidation chain reactions. This decomposition is aided by metal ions and their complexs. Proposed mechanisms of tBHP-induced toxicity include alteration in

intracellular calcium homeostasis following glutathione and protein thiol depletion, production of DNA strand break, onset of lipid peroxidation, and the production of tBHP radicals (16). Tertiary butylhydroperoxide was used because its miscibility with both water and lipids allows oxidation to occur in both cytosolic and membrane-delimited cellular compartment (17).
tBHP causes a number of changes in Retinal Pigment Epithelium cells including caspase activation, nuclear condensation, TUNEL-positive staining, and the phosphatidylserine on the cell surface. All of these findings are consistent with an antioxidant-induced apoptotic mechanism of cell death. Mitochondria recently have been found to play an important role in signaling apoptosis (18).

2.2 Immunomodulatory activity

The use of plant product as immunomodulator is still in a developing stage. There are several herbs used in the indigenous system of medicines that can modulate the body's immune system. A variety of plant derived materials such as polysaccharides, lectins, peptides, flavonoids and natural sulfur compounds have been reported to modulate the immune system (19).

The concept of immunomodulation has begun to find acceptance in medicine. The potential uses of immunomodulators in clinical medicine include the reconstitution of immune deficiency and the suppression of normal or excessive immune function. Immunomodulators are being used as immunotherapy in diseases as shown below(6).

1. Infectious condition : wounds, burns, abscess, fever, bacterial infection, viral infection
2. Tumor and cancers
3. Allergy : asthma, rash, acute dermatitis, insect bites
4. Inflammatory condition : rheumatism, pain, inflammation
5. Human immunodeficiency syndrome : AIDs
6. Autoimmune disease
7. Liver disease : hepatoma, hepatitis, hepatopathy, cirrhosis

Lymphocytes are the central cells of the immune system, responsible for adaptive immunity and the immunological attributes of diversity, specificity, memory, and self/nonself recognition. The other types of white blood cells play important roles, engulfing and destroying microorganisms, presenting antigens, and secreting cytokines (20).

Lymphocytes can be subclassified as B-lymphocytes (B-cells) or T-lymphocytes (T-cells) based on whether they mature in Bone marrow or the Thymus gland (all lymphocytes originate in bone marrow). Antigens are molecular portions of pathogens than act as identifiers. B-cells generate antibodies (humoral immunity) against antigens, whereas T-cells directly bind to antigens (cellular immunity).

The immune function that is required to mount an effective defense against pathogens and tumor cells appears to be inhibited markedly with age.

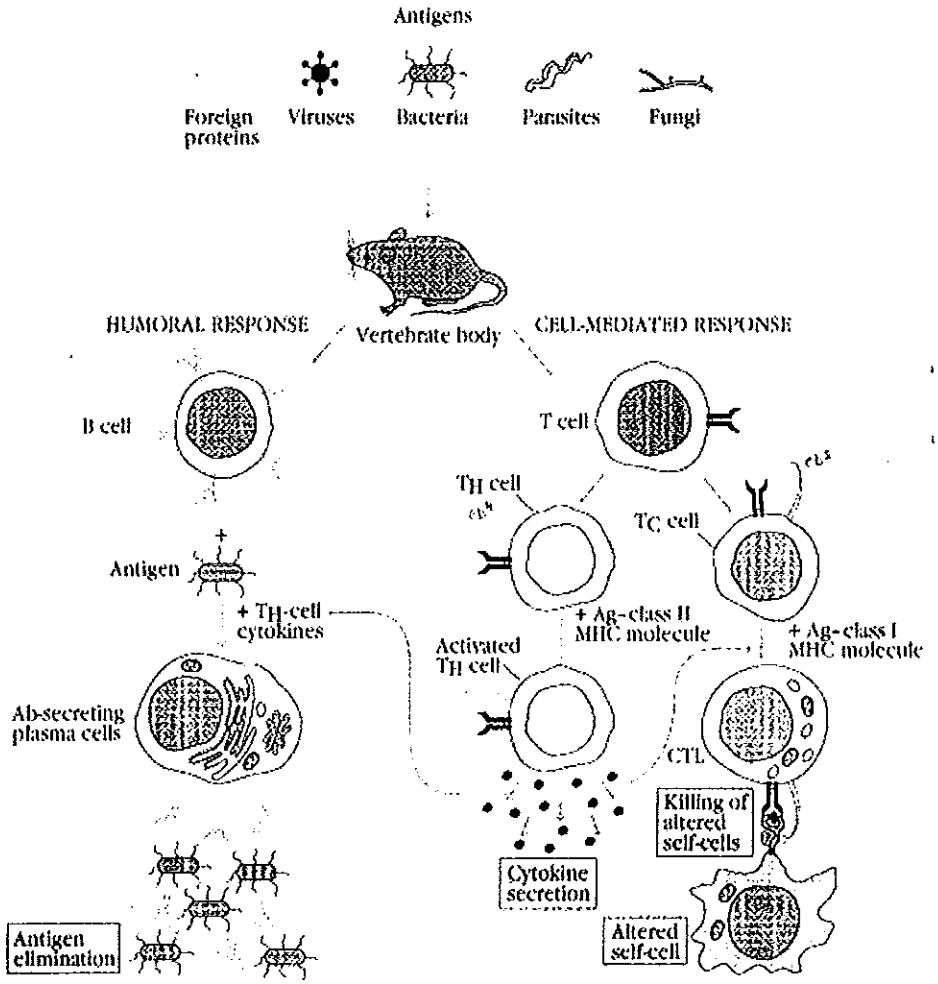


Figure 1 Overview of the humoral and cell-mediated branches of the immune system (20)

2.2.1 The immune system and aging

According to the immune system theory of aging, many aging effects are due to the declining ability of the immune system to differentiate foreign from self proteins. Not only does the immune system become less capable of resisting infection and cancer, but declining cell function could be due to attacks by the immune system against native tissues. Arthritis, psoriasis and other autoimmune diseases increase with age.

The thymus gland of the immune system reaches its greatest weight during puberty, and shrinks thereafter, with lymphoid tissue being replaced by fat. The shrinking of

the thymus gland proceeds far more rapidly than the progress of aging, at 50 the thymus of humans is typically only 5-10% of its original mass. Nonetheless, T-cells remain fairly constant over most of adult life due to peripheral proliferation although proliferation declines in the elderly.

T-lymphocytes that have not encountered an antigen since creation are called naive T-cells, whereas T-lymphocytes that have been clonally expanded to fight an invading antigen are called memory T-cells. T-cells of the elderly have a much higher ratio of memory T-cells to naive T-cells than younger people. Old memory T-cells have less CD28 surface protein than young memory T-cells and are thus less able to divide when presented with antigen CD28 ligation which is required for production of IL-2 cytokines. The elderly memory T-cells have short telomeres and are thought to accumulate because of increasingly defective apoptosis.

Two predominant forms of T-cells are cytotoxic T-cell (with CD8 surface receptors) and helper T-cells (with CD4 surface receptors). The cytotoxic T-cells attack bacteria or cancerous cells by punching holes in the cells and injecting them with toxic proteins. The helper T-cells secrete growth factors (cytokines) that foster the clonal expansion of other T-cell and/or of antibody-producing cells (the B-lymphocytes). Helper T-cells are more numerous in youth and maturity, but in the elderly the ratio of CD8 to CD4 cells increases. CD8 T-cells become more resistant to apoptosis with aging, whereas CD4 cells become more susceptible to apoptosis. There are two types of helper T-cells, designated Th1 (type 1) and Th2 (type 2). The Th1 cells promote growth of T-lymphocytes with the cytokine Interleukin-2 (IL-2), whereas the Th2 cells promote growth of B-lymphocytes with the cytokine Interleukin-4 (IL-4). Th1 cells are more prominent in autoimmune infections, whereas Th2 cells are more prominent in viral infections. In youth and maturity the Th1 cells predominate, but in the elderly the Th2 cells predominate. Moreover, aging is accompanied by a significant loss of IL-2 as well as of IL-2 receptors, a phenomenon thought to be responsible for the significant decline of proliferation (clonal expansion) in response to antigens seen with aging. The decline of T-cell activation due to reduced IL-2 production is at least partially due to oxidation-damaged proteosomes being less capable of inducing the gene transcription factor NF ?B.

Proliferation of T-cells in response to antigenic or mitogenic (cell-division stimulating) signals also declines with aging. Immune function is very important for the elderly because infection causes an increasing percentage of deaths for those over 80 years of age.

The aging immune system frequently produces increasing quantities of proinflammatory cytokines such as IL-1 and TNF. The increase in memory cells results in an increase in the cytokines IL-4 and IL-10 that are produced by the memory cells. Chronic inflammation is implicated in atherosclerosis, arthritis, Alzheimer's Diseases, cancer and numerous other afflictions affecting the elderly. Lifetime exposure to infectious disease reduces lifespan by accelerated immunosenescence and chronic inflammation (21).

2.2.2 Measurement of immune response

Immunocompetent cells require continued proliferation and differentiation for self renewal and protection of the host against pathogens. One way to evaluate the proliferation of leukocytes is the lymphoproliferative response of B and T cells to mitogens (22).

Measurement of immunity has often focused on measurement of humoral immunity to detect the presence of potentially protective antibodies to an infectious agent introduced by natural infection or by immunization. Cellular immune function is fundamentally more complex and less easy to measure.

Cellular immune assays measure current response in vitro, and sometimes in vivo, by elicitation of function response at the time of the test. Human studies have been based on observation of peripheral blood immune cells because the peripheral compartment is most accessible and readily measured (23).

In most cases, activation of T cells requires the presence of accessory cells or antigen presenting cells. Under appropriate condition, in vitro antigenic or mitogenic stimuli activate T cells via the T-cell receptor complex. This results in a number of biochemical and morphological changes that culminate in T-cell differentiation and proliferation, and expansion of memory cells (24).

Antigen is a molecule which elicits a specific immune response when introduced into an animal. More specifically, antigenic (immunogenic) substances are as follows (25):

1. Generally large molecules (>10,000 daltons in molecular weight)
2. Structurally complex (proteins are usually very antigenic)
3. Accessible (the immune system must be able to contact the molecule)
4. Foreign (not recognizable as "self")

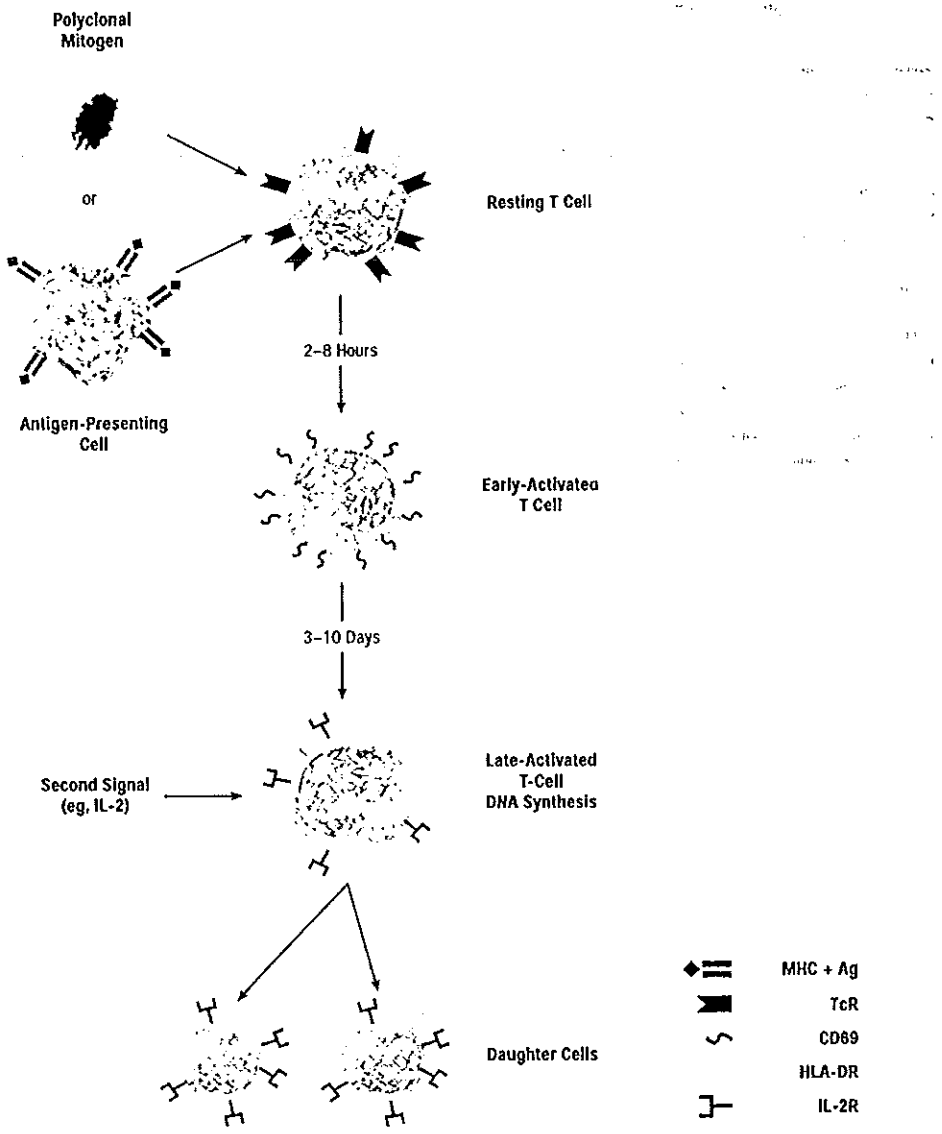


Figure 2 T cell activation under appropriate condition (24)

2.2.2.1 Cell proliferation

Measurement of immune response can be performed in microtiter tissue culture plates, typically in triplicate. Activation of T cells requires the accessory cells or antigen-presenting cells. Under appropriate condition, in vitro, antigenic, or mitogenic stimuli activate T cell via the T-cell receptor complex. This results in a number of biological and morphological changes that culminate in T-cell differentiation and proliferation (23).

2.2.2.2 Cytokine production

Cytokines are small molecules, proteins or glycoprotein less than 30 kDa that play an essential role in the immune response to infectious agent. Many cytokines, especially interleukins and interferons, are secreted by immune cells and are recognized by cytokine receptors on other immune cells.

They are secreted by T helper cells or macrophages but are sometimes produced by other types of cells as well. Cytokine control the immune response by influencing and changing the balance of T helper 1 (TH1) and T helper 2 (TH2) cells. Cytokines act to change the T helper cell balance in a number of ways. Most are involved in cell activation, proliferation or differentiation.

Cytokine of the TH1 subset, IFN-? activates macrophages, stimulating these cells to increase microbicidal activity, induces antibody-lass switching to IgG classes that support phagocytosis and fixation of complement. TH 1 cells produce Interleukin-2 (IL-2) and Interferon-gamma (IFN- ?) cytokines that promote the differentiation of fully cytotoxic Tc cells from CD8+ precursors. This pattern of cytokine production makes the TH1 subset particularly suited to respond to viral infections and intracellular pathogens. Interleukin-2 (IL-2) enhances host immunity by stimulating T cell, stimulating the production of lymphokine, activating killer cells (LAK), NK cell and cytotoxic T cell. Finally, IFN-? inhibits the expansion of TH 2 population (20).

2.3 Plant sources

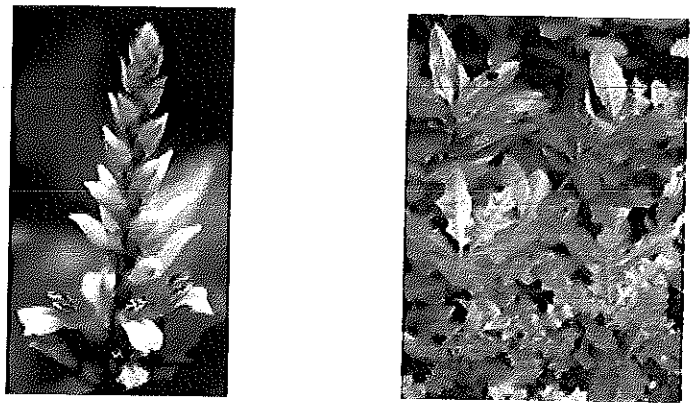


Figure 3 *Acanthus ebracteatus* Vahl (Acanthaceae)

A. ebracteatus is an erect, branching herb or undershrub which grows in brackish swamps near the sea. It has opposite, shiny leaves which are toothed with stiff spines. The white flowers are borne on an erect spike (26). Water extracted from the bark is used to treat colds and skin allergies. Ground fresh bark is used as an antiseptic. Tea brewed from the leaves relieves pain and purifies the blood. In Thai traditional medicine, the plant is widely used as a purgative and an anti-inflammatory, as well as the leaves dispensed with *Piper nigrum* as tonic pills for longevity (27).

Some biological activities of this plant have previously been reported: the organic extracts were reported to have anti-mutagenic (28) and anti-tumour promoting properties and the aqueous extract was reported to inhibit eicosanoid synthesis (29). The chemical constituents from this plant have also been investigated, but only the low-molecular weight constituents have been isolated and identified (27). Due to the important physiological role of the complement immune system, complement modulation, either inhibition or stimulation, is related to various diseases and considered as an interesting target for drug development. Several plant polysaccharides are known to possess complement-modulating activity (30).

Figure 4 *Boesenbergia pandurata* Holtt., *B. rotunda* (Linn.) Mansf. (Zingiberaceae)

B. pandurata is a perennial herb with a small, slightly branched dark brown rhizome. The freshy roots are cylindrical to spindle in shape, yellowish brown in color and are arranged at right angle to the rhizome. The leaves are fairly large, oval in outline with acute apex. The inflorescence is composed of 4-6 flowers, the flowers blooming gradually from the base towards the apex of the inflorescence. Individual flowers are reddish purple in color (31).

The aromatic tuberous roots are used as condiments in Thai food. The rhizomes and roots are reputed to increase physical efficiency. They are used as an antidysentary and carminative in traditional Thai medicine. The rhizomes contain of volatile oil, 1,8-cineole, boesenbergin A, camphor, chavicinic acid, 2,6-dihydroxy-4-methoxychalcone,dl-pinocembrin (2,3-dihydroxychrysin), dl-pinostrobin (5-hydroxy-7-methoxyflavanone), cardamonin, 2,4-dihyroxy-6-methoxychalcone (31).

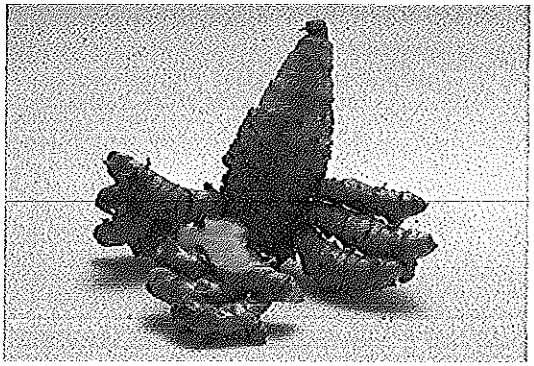


Figure 5 *Curcuma zedoaria* Roscoe (Zingiberaceae)

Zedoary grows in tropical and subtropical wet forest regions. The rhizome is large and tuberous with many branches. The leaf shoots are long and fragrant, reaching 1 m in height. The plant bears yellow flowers with red and green bracts. It is an antiseptic and a paste applied locally to cuts and wounds help healing. The rhizomes have been used as antiflatulence and anti-inflammatory in Thai traditional medicine. The aqueous acetone extract was found to inhibit release of β-hexosaminidase, as a marker of antigen IgE mediated degranulation, in RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice. The active compounds are curcumin and bisdemethoxycurcumin. Furthermore curcumin inhibited antigen-induced release of TNF-α and IL-4, both of which participate in the late phase of type I allergic reaction in RBL-2H3 (32).

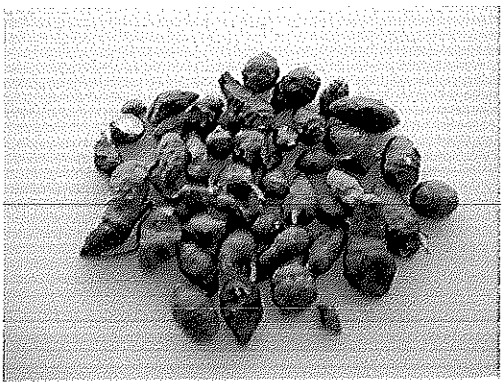


Figure 6 *Cyperus rotundus* Linn. (Cyperaceae)

C. rotundus is a perennial plant. Stems are tuberous at base, rising singly from a creeping, underground root-stock about 10-25 cm tall. Leaves are linear, dark green. Flowers are in small inflorescence with 2-4 bracts. The inflorescence consists of a few slender branches, spikes consisting of about 2-10 spikelets, glumes rather narrow, blunt, closely overlapping with 3 stamens: style 3-branched. Nut is oblong-obovate nearly half as long as glume, strongly 3-angled, yellow, turning black when ripe.

In Thai traditional textbooks, the rhizomes are used as diuretic, cardiotonic health promoter, anti-inflammatory, for longevity etc. A methanol extract exhibited anti-inflammatory effect in the formalin-induced rat pal edema model. Using the in vitro albumin stabilizing assay, a water extract had no anti-inflammatory activity (33). Several pharmacologically active substances have been identified as α-cyperone, β-selinene, cyperene, cyperotundone, patchoulenone, sugeonol, kobusone and isokobusone.

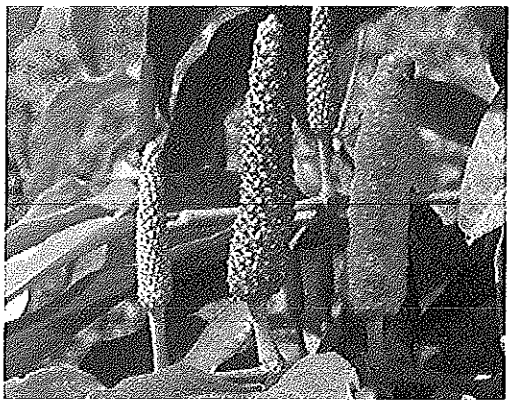


Figure 7 *Piper chaba* Hunt, *P. longum* Linn., *P. retrofractum* Vahl. (Piperaceae)

Pipers are climber with oblong, shining green leaves. The inflorescence is subcylindrical and composed of numerous greenish white flowers. The fruits are cylindrical in shape and reddish orange in color when ripe. Long pepper is the dried entire spike of immature fruits derived from either of the above species. It contains about 1 percent of volatile oil, 6 percent of piperine and a pungent resin, chavicin (31).

In traditional Thai medicine, fruits are used for treatment of bronchial asthma, bronchitis, muscle pain and incurable disease; as a fire element tonic, emmenagogue. A methanol extract when intraperitonially injected at a dose of 125 mg/kg to mice showed barbiturate potentiation, decreased the mortality rate of strychnine-treated mice, reduced concentrations of isolated rat iliums and inhibited the activity of acetylcholine (33).

Piperine was found to act as a hydroxyl radical scavenger at low concentrations, but at higher concentrations, it activated the fenton reaction resulting in increased generation of hydroxyl radicals. Whereas it acts as a powerful superoxide scavenger with IC_{50} of 1.82 mM, a 52% inhibition of lipid peroxidation was observed at a dose of 1400 μM with an IC_{50} of 1.23 mM (34). Alcoholic extract of the fruits of *P. longum* and piperine were studied for their immunomudulatory activity. Administration of *P. longum* extract and piperine increased the total WBC count to 142.8 and 138.9%, respectively, in Balc/c mice. The number of plaque forming cells also enhanced significantly by the administration of the extract 100.3% and piperine 71.4% on 5^{th} day after immunization. Bone marrow cellularity and α-esterase positive cells were also increased by the administration of *P. longum* extract and piperine (35).

Figure 8 *Piper sarmentosum* Roxb. (Piperaceae)

Piper sarmentosum is a glabrous, creeping, terrestrial herb with procumbent branches. Leaves are thin, lower leaves usually ovate-cordate, ovate to obliquely or rounded at base, 5-7 radiating nerves from base, petiole 2.5-5 cm long. Spike is short, dense, blunt, cylindric in procumbent branches; male flowers and female flower 0.7 cm long, fruit obovoid, sweet to taste (36). It is used for feverish diseases, for digestive disorders, and toothache. The extract may be applied externally to treat pain in the bones. When the root is chewed with betel nut, it is said to be helpful for the treatment of coughs and asthma.

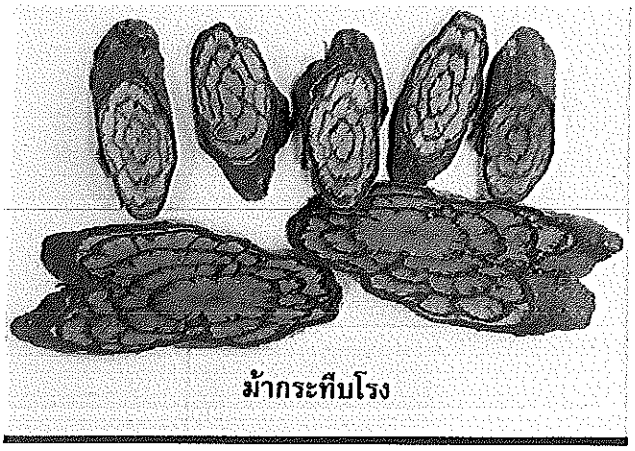


Figure 9 *Salacia euphlebia* Merr. (Celastraceae)

Salacia euphlebia is a scandent shrub. Stipule is triangular 1/3-1/2 mm long. Leaves are chartaceous, elliptic-oblong and lanceolate base cuneate obtuse or rounded; apex acuminate to acuminate-caudate; margin distinctly or rarely obscurely apiculate-crenuate, nerve 6-8 pairs; flower greenish yellow, in axillary fascicles. Ovary is 3-celled with 2 ovules in each cell, attached at the central part of the axis. Fruit is subglobose and smooth (37).

A number of *Salacia spp.* has been studied for their chemical constituents and their biological activities.

S. reticulate has been isolated gutta percha, sitosterol, pristimerin, mangiferin, epi-kokoondiol, salacenonal, salaciquinone, isoiguesterinol and 30-hydroxypristimerin from the stem bark. Sulfonium ion derivatives, kotalanol (from roots and stems) and salacinol (from roots), have been identified as the antidiabetic. Both are potent α-glucosidase inhibitors that have been shown to inhibit increases in serum glucose levels (38).

The crude chloroform bark extract from *S. liana* shows in vitro cytotoxic activity against Hep-G2, H-4IIE and SK-Mel-28 tumor cells. The compounds were isolated as follows: friedelin, 1-hydroxy-3,6-dimethoxy-8-methyl-9H-xanthen-9-one, friedelan-3-on-29-al, canophyllol, 29-hydroxyfriedelan-3-one, and the cytotoxic component, tingenone (39).

The crude dichloromethane bark extract of *S. penetenensis* shows antibacterial and cytotoxic activity. The biologically active materials are tingenone and netzahualcoyonol. Also isolated from the extract were 3-methoxyfriedel-2-en-1-one and 29-hydroxyfriedelan-3-one (40).

Three quinine methides, 28-nor-isoquesterin-17-carbaldehyde, 17-(methoxycarbonyl)-28-nor-isoiguesterin and 28-hydroxyisoiguesterin together with celastrol, pristimerin and isoiguesterol were isolated from the roots of *S. kraussii*. The isolates showed antimalarial activity (41).

Figure 10 *Tacca integrifolia* Ker-Gawl. (Taccaceae)

Tacca integrifolia is a robust plant with a cylindric rhizome. It has large leaf blades on a petiole up to 15 inches long. Flower scapes on well established plants can grow to 3 ft tall. Flowers are a purple color and seen hanging forward from in-between the hood. In Thai herbal medicine, rhizomes are used for controlling blood pressure and improving sexual function (42). Its rhizome contains ochratoxin A, amino acids, n-triacontanol, castanogenin, betulinic acid, quercetin-3-α-arabinoside and taccalin.

Table 1-4 Chemical constituents reported in eight genera from literatures

Chemical constituents	References
Acanthus ebracteatus (aerial part)	
Plucheoside B, 1a	(27)
Alangionoside C, 1b	(27)
Ebracteatoside A, 1c	(27)
Premnaionoside, 1d	(27)
Benzoxazinoid glucoside, 1e	(27)
Adenosine, 1f	(27)
Verbascoside, 1g	(27)
Isoverbascoside, 1h	(27)
Leucosceptoside A, 1i	(27)
Martynoside, 1j	(27)
ß-hydroxyacteoside, 1k	(27)
Schaftoside, 1l	(27)
Luteolin-7- *O*- ß-D-glucuronide, 1m	(27)
Apigenin-7- *O*- ß-D-glucuronide, 1n	(27)
Magnolenin C, 1o	(27)
Boesenbergia pandurata (root)	
Pinostrobin, 2a	(43)
Pinocembrin, 2b	(44)
Alpinetin, 2c	(43)
5-7-dimethoxyflavanone, 2d	(45)
Dimethoxyflavone, 2e	(45)
3',4',5,7-tetramethoxyflavone, 2f	(45)
2,6-dihydroxy-4-methoxychalcone, 2g	(46)
Cardamonin, 2h	(44)
Panduratin A, 2i	(47)
Panduratin B, 2j	(48)

Table 1-4 Chemical constituents reported in eight genera from literatures (continued)

Chemical constituents	References
Boesenbergin A, 2k	(46)
Boesenbergin B, 2l	(44)
Rubranine, 2m	(47)
Geranial, 2n	(49)
Neral, 2o	(49)
Primaric acid, 2p	(47)
Boesenboxide, 2q	(47)
Camphor, 2r	(47)
Chavicinic acid, 2s	(47)
1,8-cineole, 2t	(47)
Curcuma zedoaria (root)	
Curcumin, 3a	(50)
Bisdemethoxycurcumin, 3b	(50)
Demethoxycurcumin, 3c	(50)
3,7-dimethylindan-5-carboxylic acid, 3d	(50)
Curcolonol, 3e	(50)
Guaidiol, 3f	(50)
1,7-bis(4-hydroxyphenyl)-1,4,6 -heptatrien-3-one, 3g	(51)
Procurcumenol, 3h	(51)
Epiprocurcumenol, 3i	(51)
Cyperus rotundus (root)	
a-cyperone, 4a	(52)
ß-selinene, 4b	(52)
Cyperotundone, 4c	(52)
Cyperene, 4d	(53)

Table 1-4 Chemical constituents reported in eight genera from literatures (continued)

Chemical constituents	References
Patchoulenone, **4e**	(53)
Sugeonol, **4f**	(53)
Kobusone, **4g**	(54)
Isokobusone, **4h**	(54)
Piper chaba (fruit)	
Piperine, **5a**	(55)
Piperchabamide A, **5b**	(56)
Piperchabamide B, **5c**	(56)
Piperchabamide C, **5d**	(56)
Piperchabamide D, **5e**	(56)
Piperanine, **5f**	(56)
Pipernonaline, **5g**	(56)
Dehydropipernonaline, **5h**	(56)
Piperlonguminine, **5i**	(56)
Refractamide B, **5j**	(56)
Guineensine, **5k**	(56)
Piper sarmentosum (fruit)	
Pellitorine, **6a**	(57)
Guineensine, **5k**	(57)
Brachyamide B, **6b**	(57)
Sarmentine, **6c**	(57)
Sarmentosine, **6d**	(57)
(+)-asarinin, **6e**	(57)
Sesamin, **6f**	(57)
3′,4′,5′-trimethoxycinnamoyl pyrrolidine, **6g**	(57)
1-piperetty pyrrolidine, **6h**	(57)

Table 1-4 Chemical constituents reported in eight genera from literatures (continued)

Chemical constituents	References
Salacia beddomei (stem)	
15a-hydroxy-friedelan-3-one, **7a** (58)	
1ß-15a-dihydroxy-friedelan-3-one, **7b**	(58)
15a-hydroxy-friedelane-1-3-dione, **7c**	(58)
Betulin, **7d**	(58)
S. campestris (stem)	
Salacin, **7e**	(59)
Pristimerin, **7f**	(59)
Maytenin, **7g**	(59)
20a-hydroxymaytenin, **7h**	(59)
Netzahualcoyene, **7i**	(59)
S. chinensis (stem)	
Mangiferin, **7j**	(60)
S. kraussii (stem)	
28-nor-isoquesterin-17-carbaldehyde, **7k**	(61)
17-(methoxycarbonyl)-28-nor -isoiguesterin, **7l**	(61)
28-hydroxyisoiguesterin, **7m**	(61)
Celastrol, **7n**	(61)
Pristimerin, **7f**	(61)
Isoiguesterin, **7o**	(61)
Isoiguesterol, **7p**	(61)
S. liana (stem)	
Friedelin, **7q**	(62)
1-hydroxy-3,6-dimethoxy-8-methyl -9H-xanthen-9-one, **7r**	(62)
Friedelan-3-on-29-al, **7s**	(62)

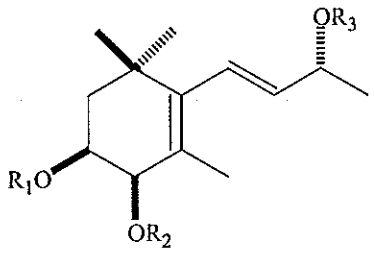
Table 1-4 Chemical constituents reported in eight genera from literatures (continued)

Chemical constituents	References
Canophyllol, **7t**	(62)
29-hydroxyfriedelan-3-one, **7u**	(62)
Tingenone, **7v**	(39)
S. macrosperma (root)	
Mangiferin, **7j**	(63)
Pristimerin, **7f**	(63)
Salaspermic acid, **7w**	(64)
Tingenone, **7v**	(65)
S. oblonga (stem)	
15a-hydroxy-24-nor-friedelene -5-ene-1,3-dione, **7x**	(66)
Mangiferin, **7j**	(60)
S. penetenensis (stem)	
Tingenone, **7v**	(62)
Netzahualcoyonol, **7y**	(62)
3-methoxyfriedel-2-en-1-one, **7z**	(62)
29-hydroxyfriedelan-3-one, **7u**	(62)
S. prinoides (root bark)	
Friedel-1-en-3-one, **7aa**	(67)
Isoiguesterinol, **7bb**	(67)
Friedelane-1-3-dione, **7cc**	(62)
Friedelin, **7q**	(62)
Mangiferin, **7j**	(60)
Pristimerin, **7f**	(68)
Salacenonal, **7dd**	(68)
Salaciquinone, **7ee**	(68)
S. reticulate (root bark)	
Sitosterol, **7ff**	(38)

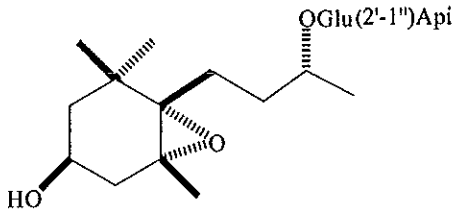
Table 1-4 Chemical constituents reported in eight genera from literatures (continued)

Chemical constituents	References
30-hydroxypristimerin, **7gg**	(68)
Pristimerin, **7f**	(69)
epi-kokoondiol, **7hh**	(70)
Isoiguesterinol, **7bb**	(68)
Hydroxyferruginol, **7ii**	(60)
Lambertic acid, **7jj**	(60)
Kotalagenin 16-acetate, **7kk**	(60)
26-hydroxy-1,3-friedelanedione, **7ll**	(60)
Mangiferin, **7j**	(71)
Maytenfolic acid, **7mm**	(60)
3ß,22ß-dihydroxyolean-12-en-29-oic acid, **7nn**	(60)
Kotalanol, **7oo**	(60)
Salacinol, **7pp**	(60)
Salacenonal, **7dd**	(59)
Salaciquinone, **7ee**	(59)
Tacca integrifolia (root)	
Ochratoxin A, **8a**	(72)
n-triacontanol, **8b**	(73)
Castanogenin, **8c**	(73)
Betulinic acid, **8d**	(73)
Quercetin-3-a-arabinoside, **8e**	(73)
Taccalin, **8f**	(73)

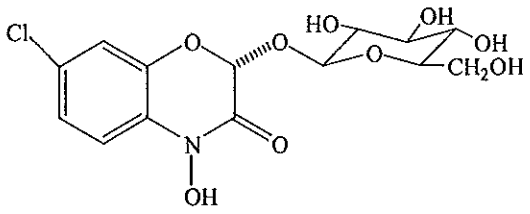
Chemical structures of the reported compounds are as follows:



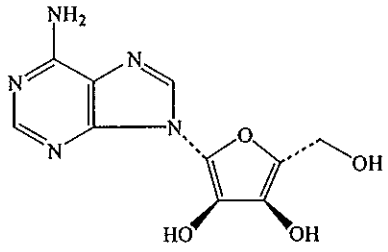
	R_1	R_2	R_3
Plucheoside B, **1a**	Glu	H	H
Alangionoside C, **1b**	H	Glu	H
Ebracteatoside A, **1c**	H	H	Glu (2′-1″) Api



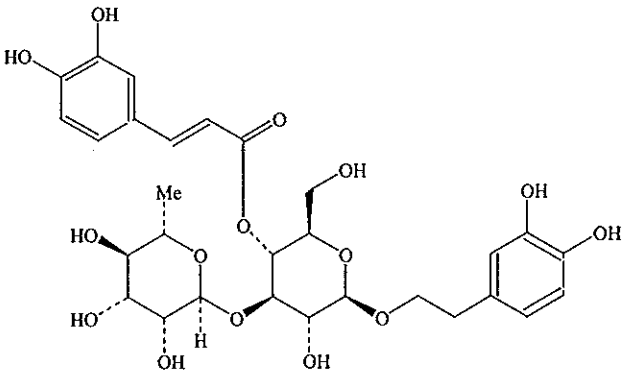
Premmaionoside, **1d**



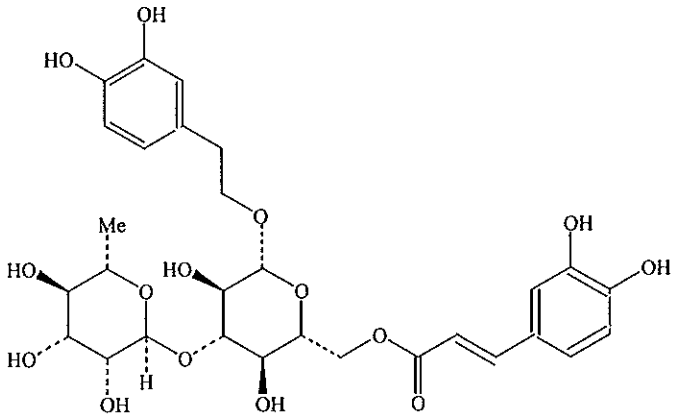
Benzoxazinoid glucoside, **1e**



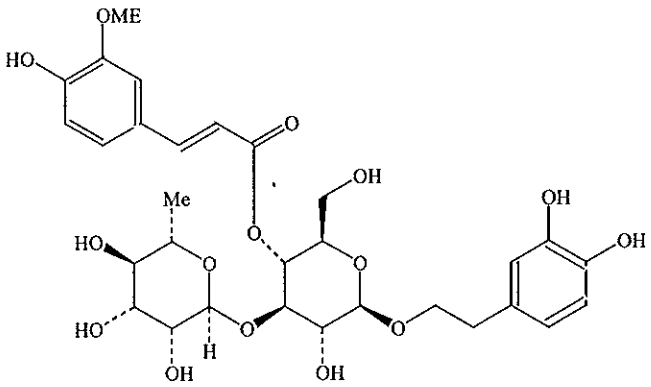
Adenoside, **1f**



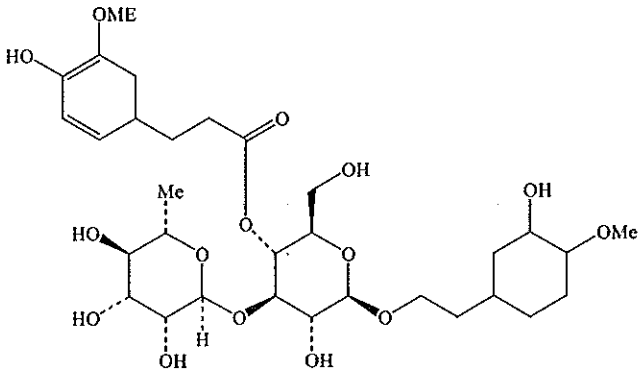
Verbascoside, **1g**



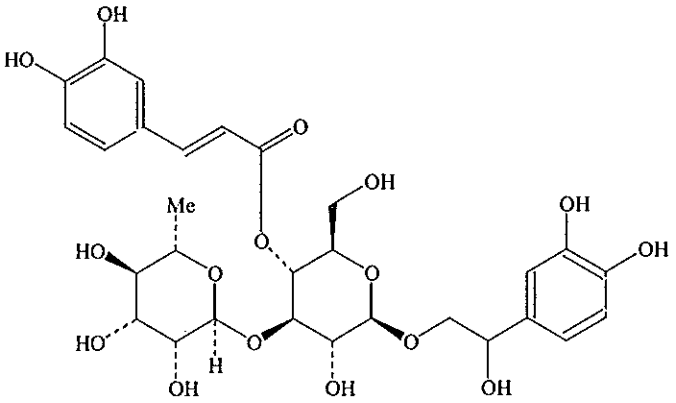
Isoverbascoside, **1h**



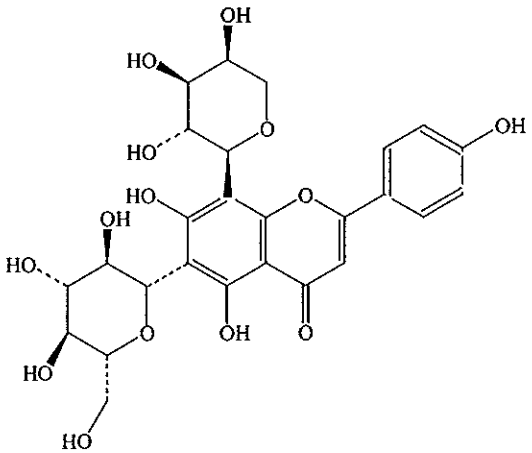
Leucosceptoside A, **1i**



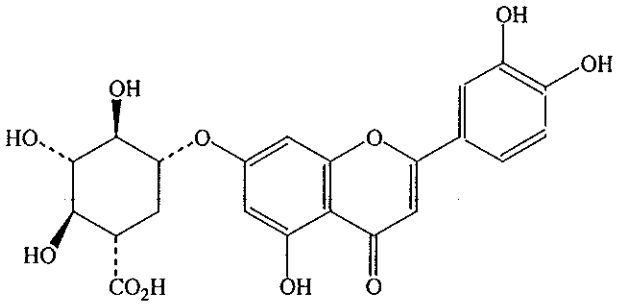
Martynoside, 1j



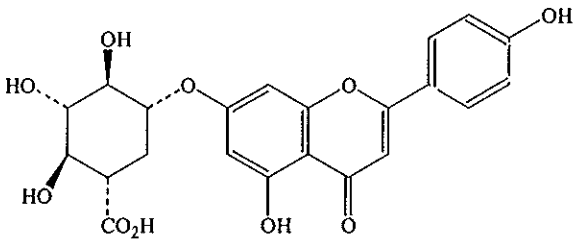
ß-hydroxyacteoside, 1k



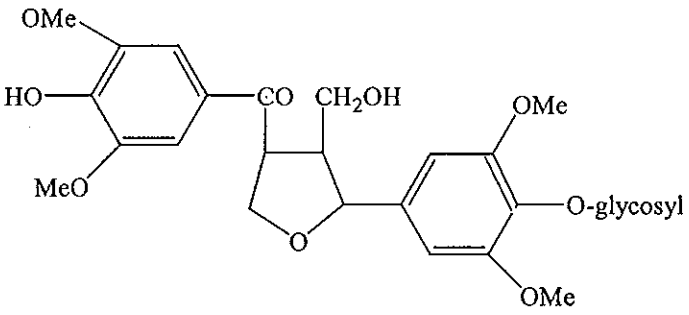
Schaftoside, 1l



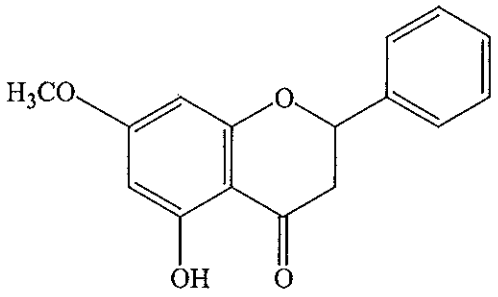
Luteolin-7- *O*- ß-D-glucuronide, **1m**



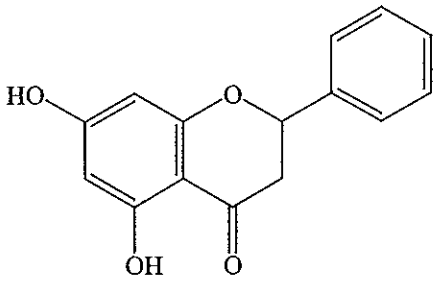
Apigenin-7- *O*- ß-D-glucuronide, **1n**



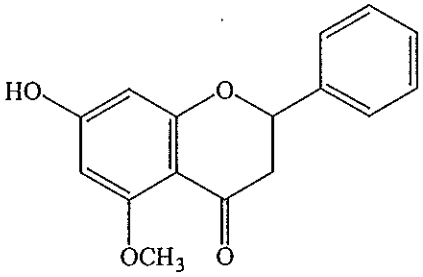
Magnolenin C, **1o**



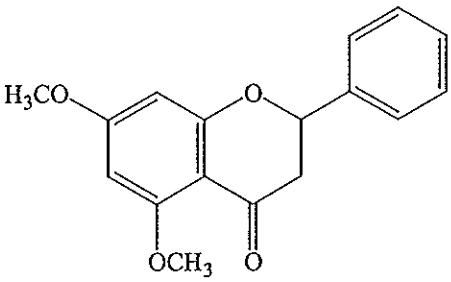
Pinostrobin, **2a**



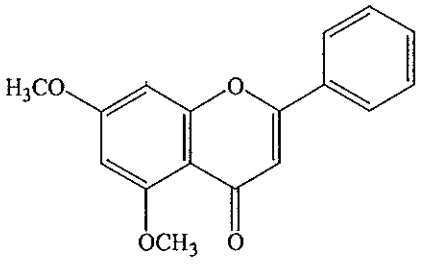
Pinocembrin, **2b**



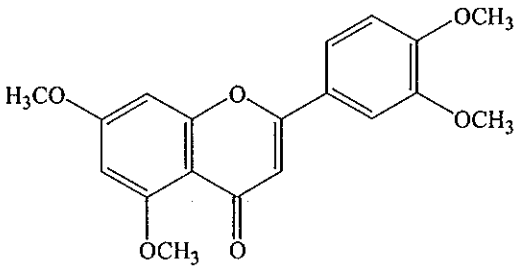
Alpinetin, **2c**



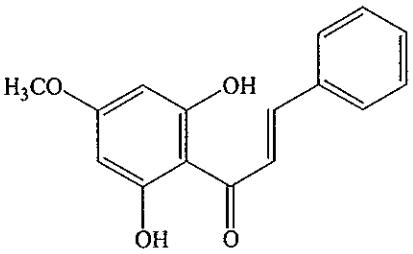
5,7-dimethoxyflavanone, **2d**



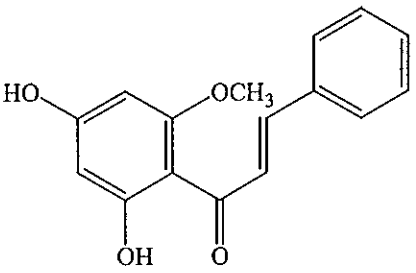
Dimethoxyflavone, **2e**



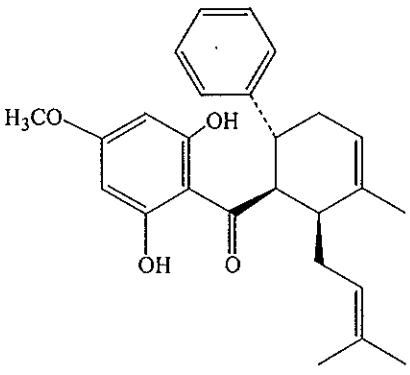
3′,4′,5,7-tetramethoxyflavone, **2f**



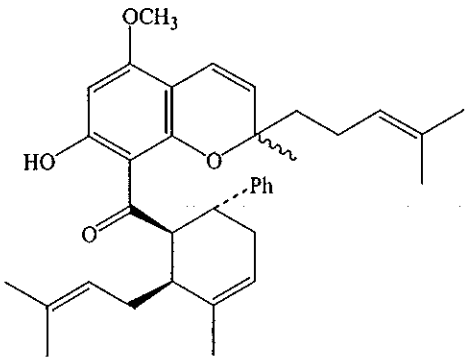
2,6-dihydroxy-4-methoxychalcone, **2g**



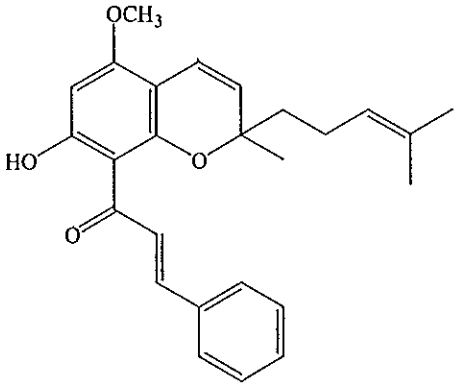
Cardamonin, **2h**



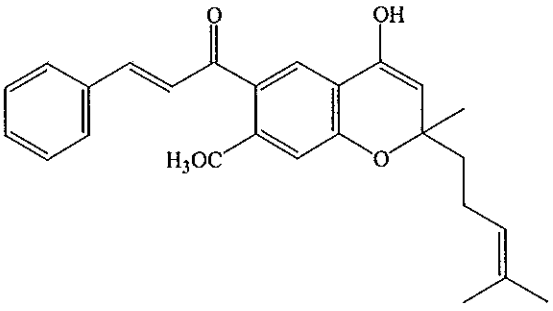
Panduratin A, **2i**



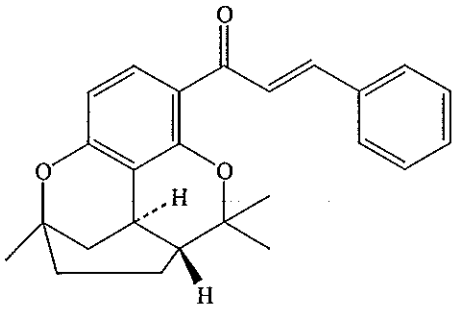
Panduratin B, **2j**



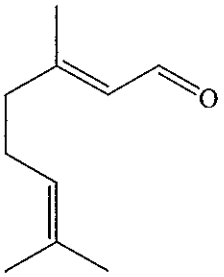
Boesenbergin A, **2k**



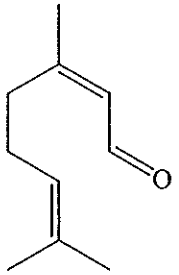
Boesenbergin B, **2l**



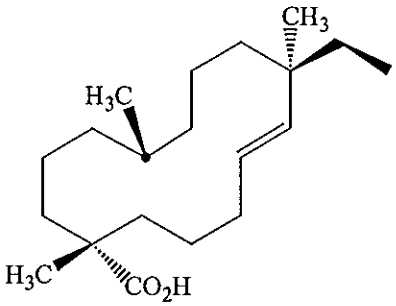
Rubranine, **2m**



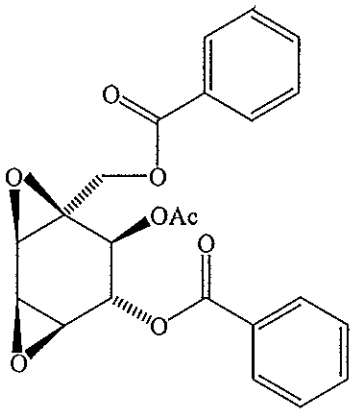
Geranial, **2n**



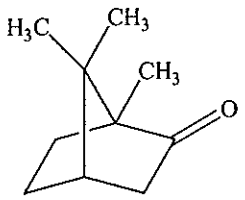
Neral, **2o**



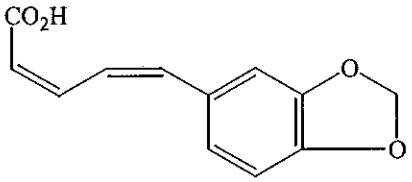
Primaric acid, **2p**



Boesenboxide, **2q**

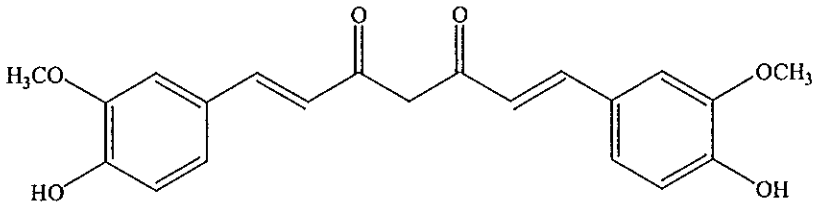


Camphor, **2r**

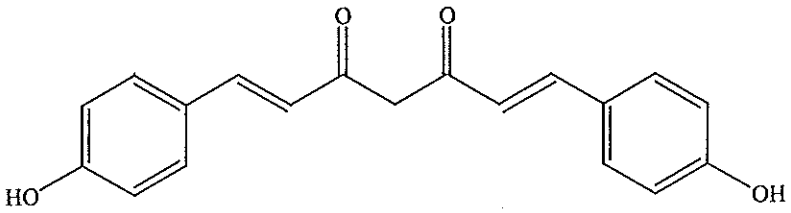


Chavicinic acid, **2s**

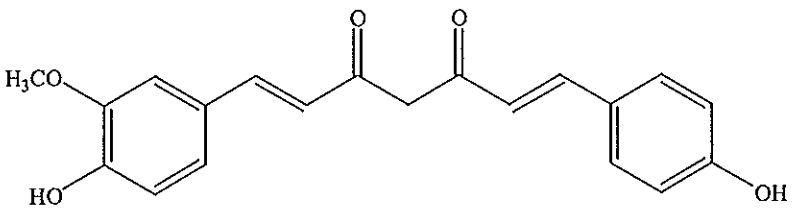
1,8-cineole, **2t**



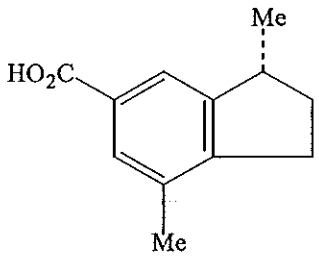
Curcumin, **3a**



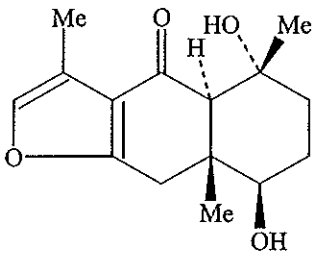
Bisdemethoxycurcumin, **3b**



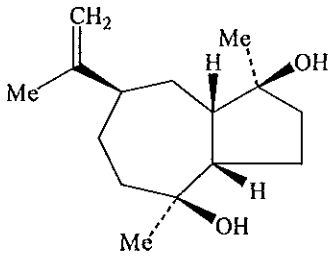
Demethoxycurcumin, **3c**



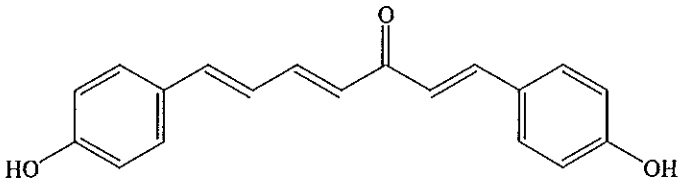
3,7-dimethylindane-5-carboxylic acid, **3d**



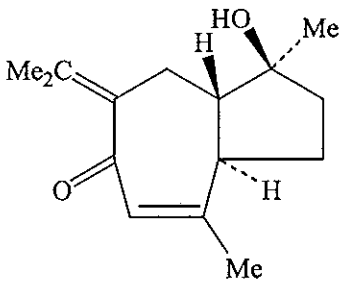
Curcolonol, **3e**



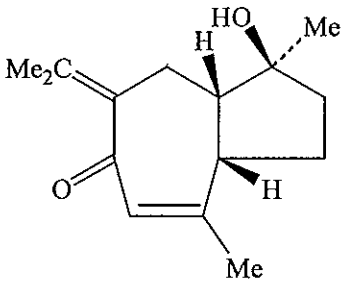
Guaidiol, **3f**



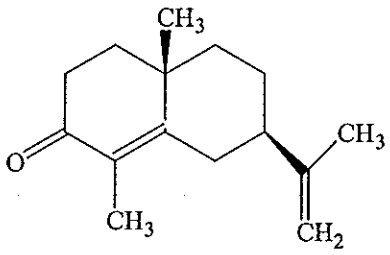
1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one, **3g**



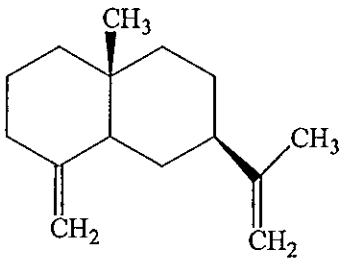
Procurcumenol, **3h**



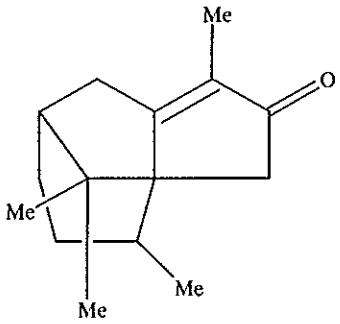
Epiprocurcumenol, **3i**



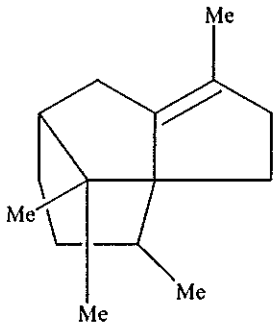
a-Cyperone, **4a**



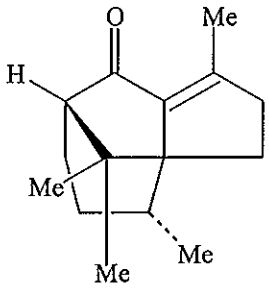
ß-Selinene, **4b**



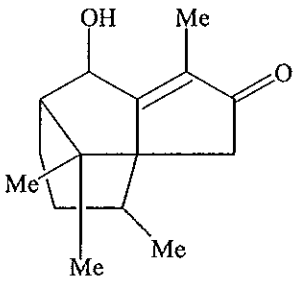
Cyperotundone, **4c**



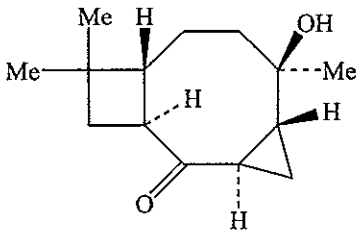
Cyperene, **4d**



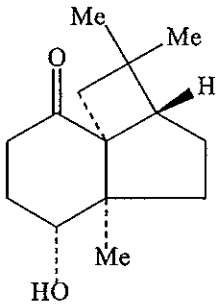
Patchoulenone, **4e**



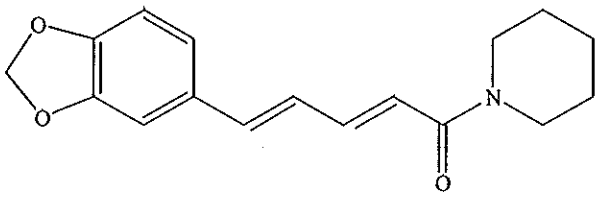
Sugeonol, **4f**



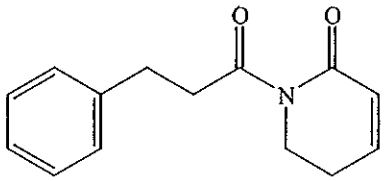
Kobusone, **4g**



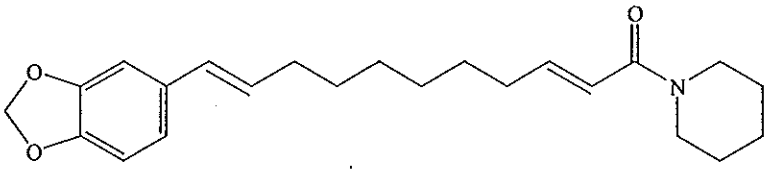
Isokobusone, **4h**



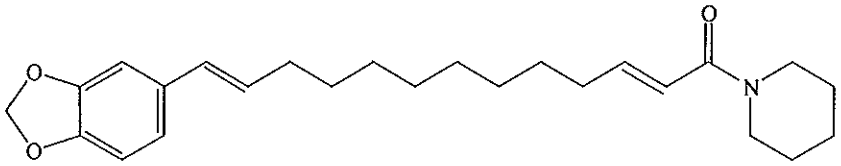
Piperine, **5a**



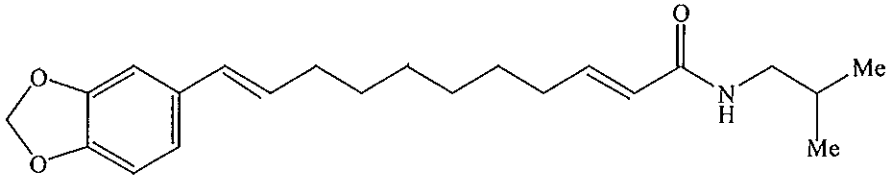
Piperchabamide A, **5b**



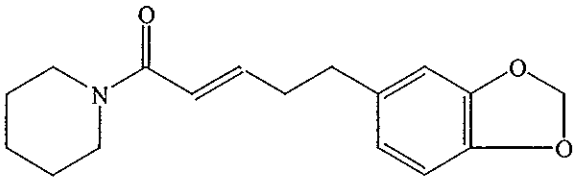
Piperchabamide B, **5c**



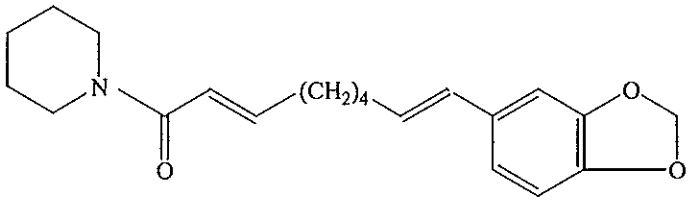
Piperchabamide C, **5d**



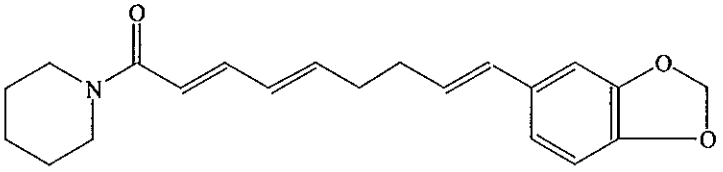
Piperchabamide D, **5e**



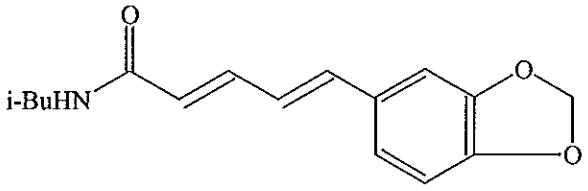
Piperanine, **5f**



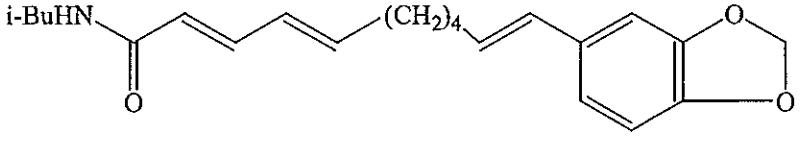
Piperononaline, **5g**



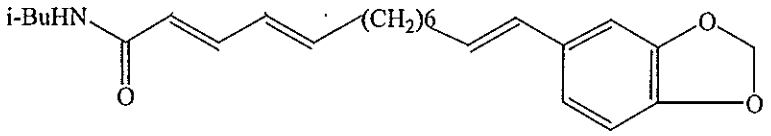
Dehydropipernonaline, **5h**



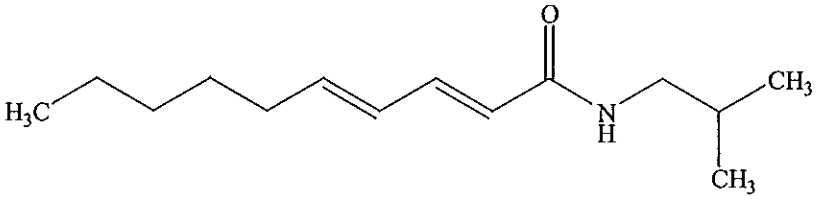
Piperlonguminine, **5i**



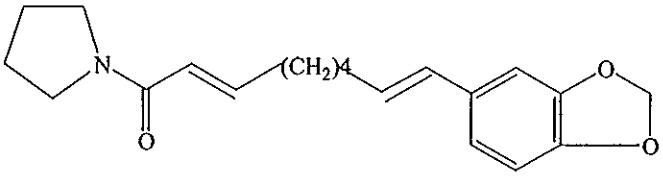
Refractamide B, **5j**



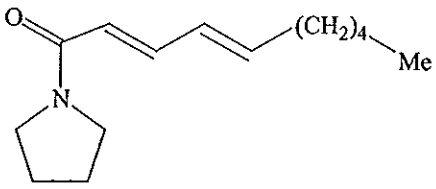
Guineensine, **5k**



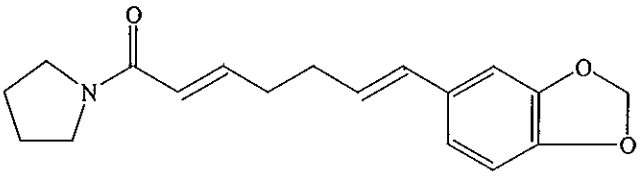
Pellitorine, **6a**



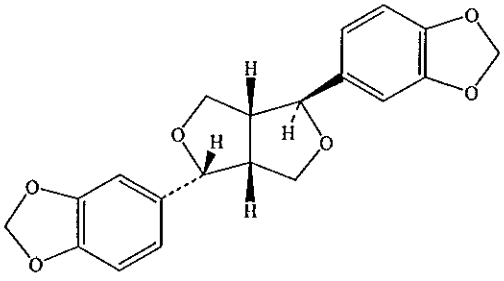
Brachyamide B, **6b**



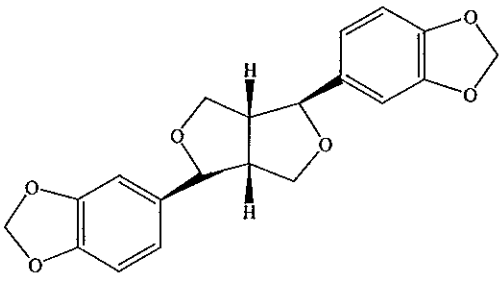
Sarmentine, **6c**



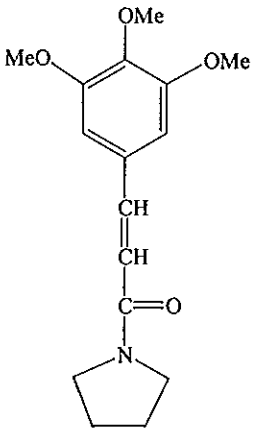
Sarmentosine, **6d**



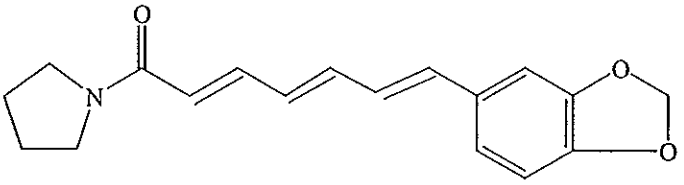
(+)-asarinin, **6e**



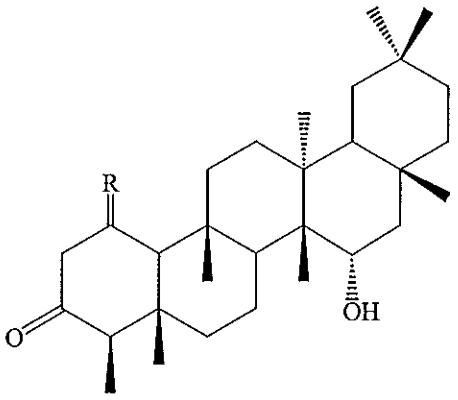
Sesamin, **6f**



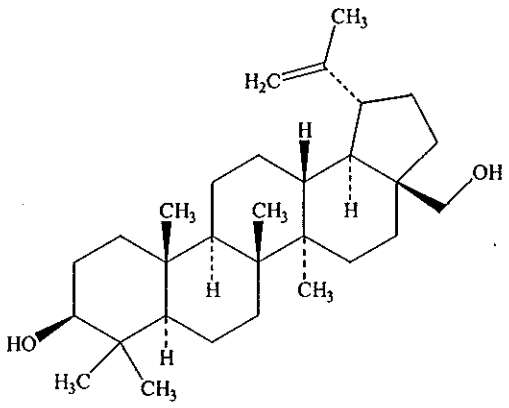
3′, 4, 5-trimethoxycinnamoyl pyrrolidine, **6g**



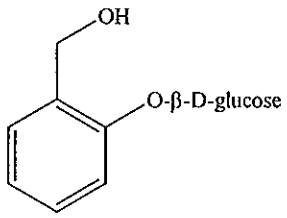
1- piperetty pyrrolidine, **6h**



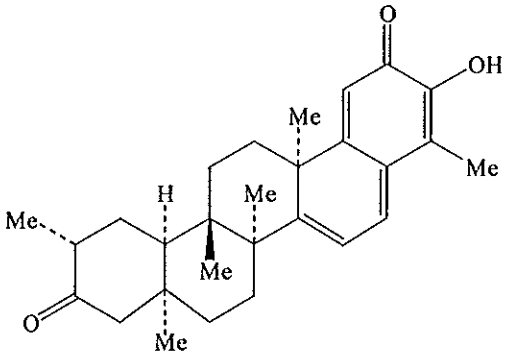
	R
15a-hydroxy-friedelan-3-one, **7a**	H_2
1ß-15a-dihydroxy-friedelan-3-one, **7b**	ß-OH, H
15a-hydroxy-friedelane-1-3-dione, **7c**	O



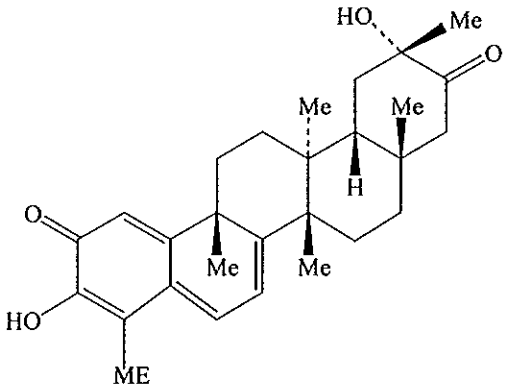
Betulin, **7d**



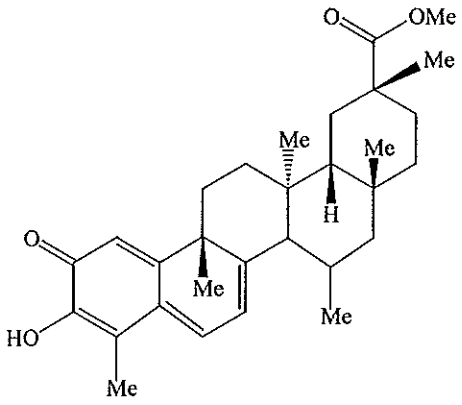
Salacin, **7e**



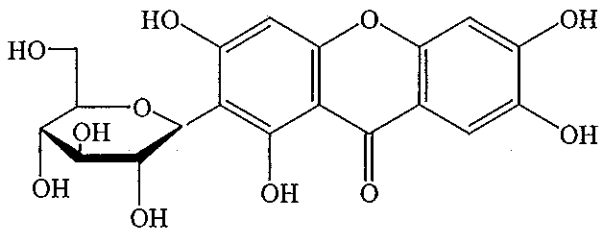
Maytenin, **7g**



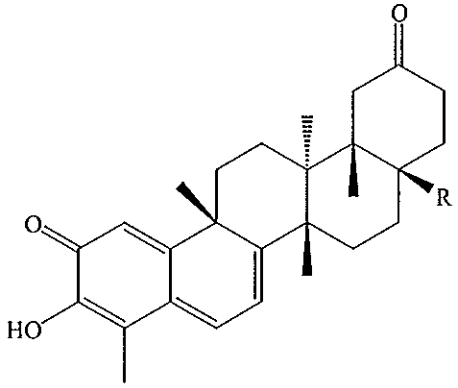
20a-hydroxymaytenin, **7h**



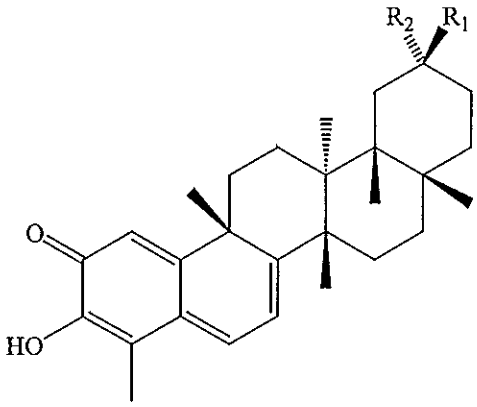
Netzahualcoyene, **7i**



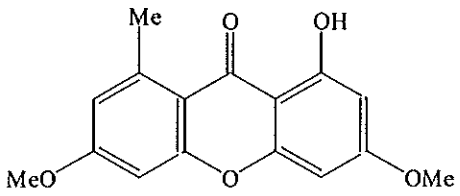
Mangiferin, **7j**



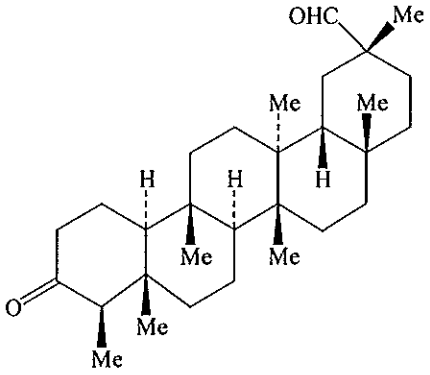
	R
28-nor-isoquesterin-17-carbaldehyde, **7k**	CHO
17-(methoxycarbonyl)-28-nor-isoiguesterin, **7l**	COOMe
28-hydroxyisoiguesterin, **7m**	CH_2OH
Isoiguesterin, **7o**	CH_3



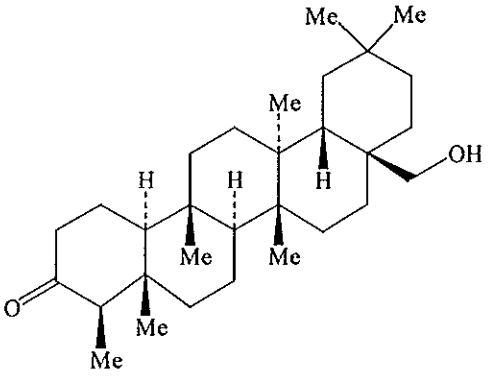
	R_1	R_2
Celastrol, **7n**	Me	COOH
Pristimerin, **7f**	Me	COOMe
Isoiguesterol, **7p**	H	CH_2OH



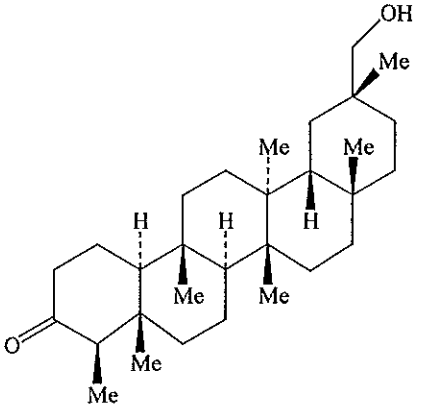
1-hydroxy-3,6-dimethoxy-8-methyl-9H-xanthen-9-one, **7r**



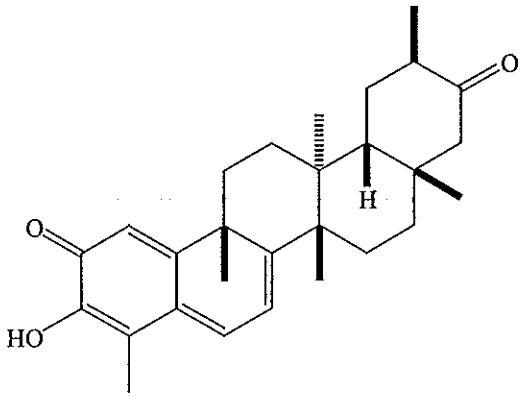
Friedelan-3-on-29-al, **7s**



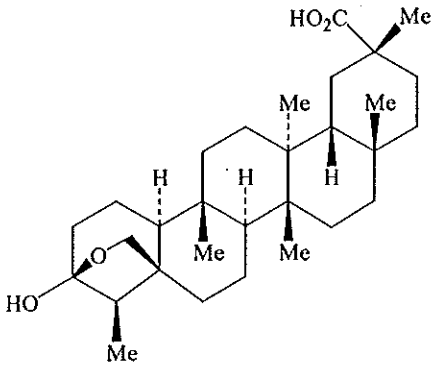
Canophyllol, **7t**



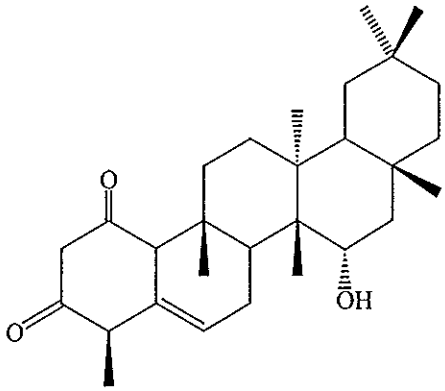
29-hydroxyfriedelan-3-one, **7u**



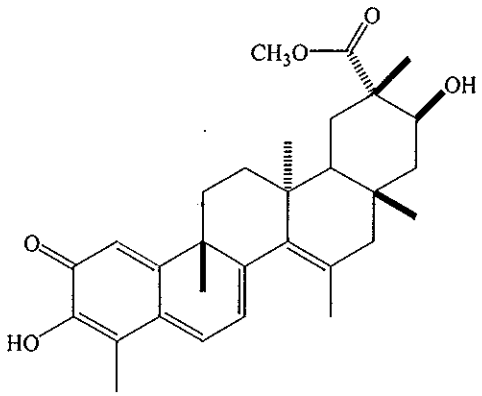
Tingenone, **7v**



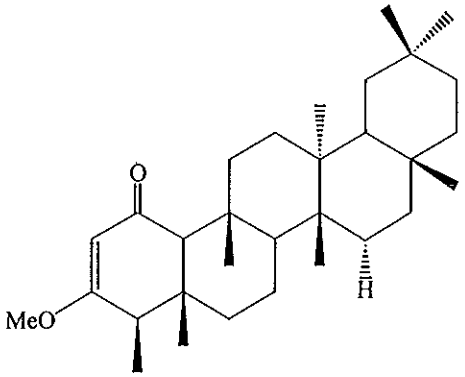
Salaspermic acid, **7w**



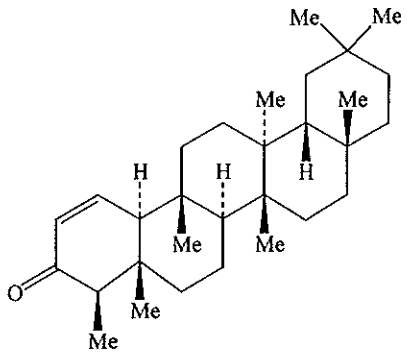
15a-hydroxy-24-nor-friedelene-5-ene-1,3-dione, **7x**



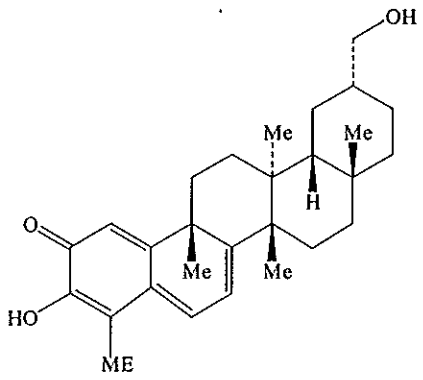
Netzahualcoyonol, **7y**



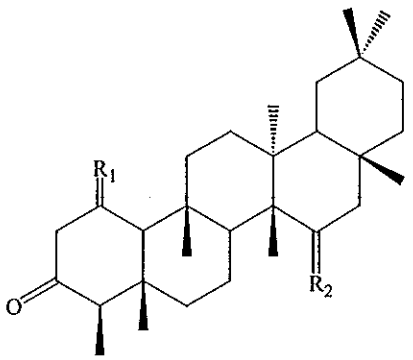
3-methoxyfriedel-2-en-1-one, **7z**



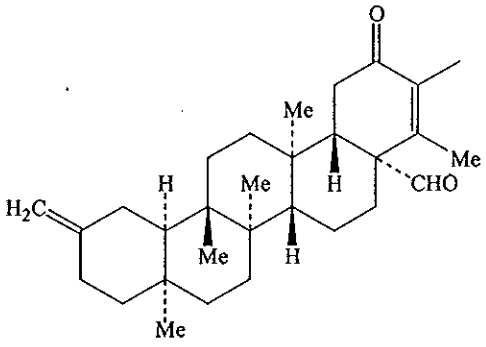
Friedel-1-en-3-one, **7aa**



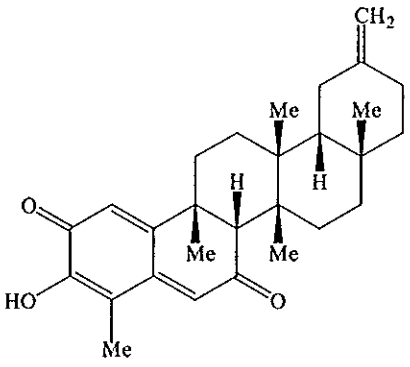
Isoiguesterinol, **7bb**



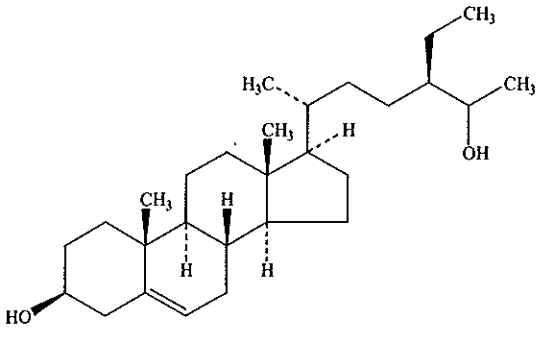
	R1	R2
Friedelin, 7q	H_2	H_2
Friedelane-1-3-dione, 7cc	O	H_2



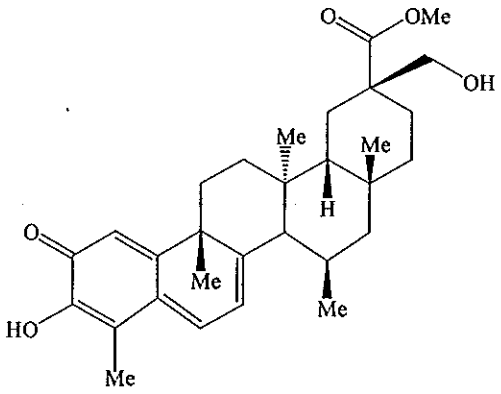
Salacenonal, 7dd



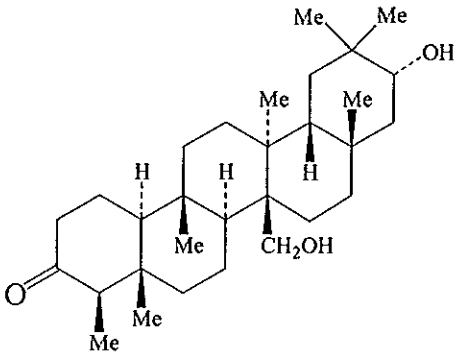
Salaciquinone, 7ee



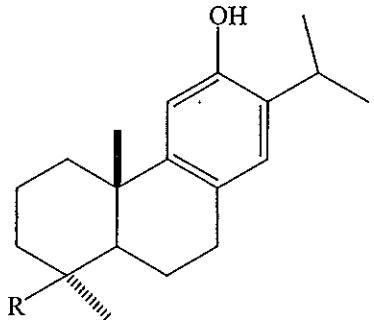
Sitosterol, 7ff



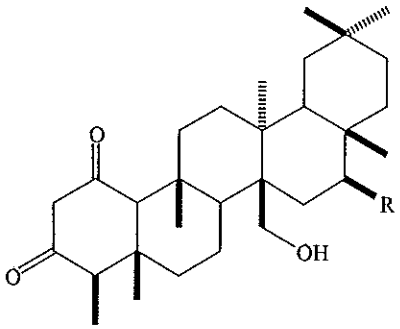
30-hydroxypristimerin, **7gg**



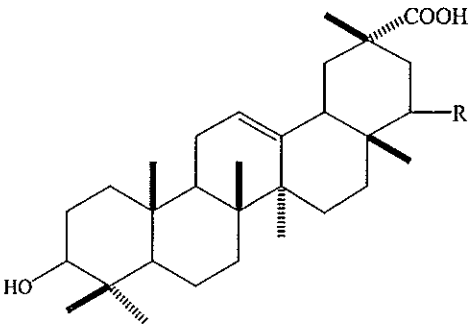
epi-kokoondiol, **7hh**



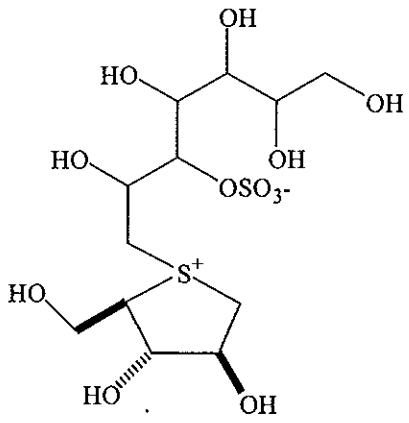
	R
Hydroxyferruginol, **7ii**	CH_2OH
Lambertic acid, **7jj**	COOH



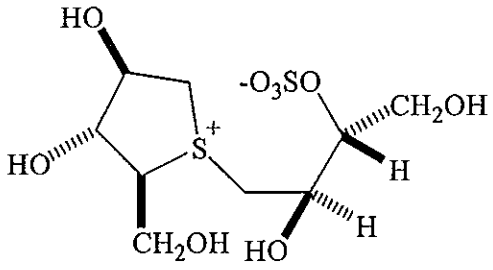
	R
Kotalagenin 16-acetate, **7kk**	$OCOCH_3$
26-hydroxy-1,3-friedelanedione, **7ll**	H



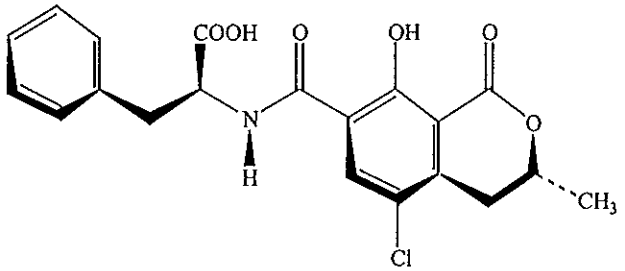
	R
Maytenfolic acid, **7mm**	a-OH
3ß,22ß-dihydroxyolean-12-en-29-oic acid, **7nn**	ß-OH



Kotalanol, **7oo**



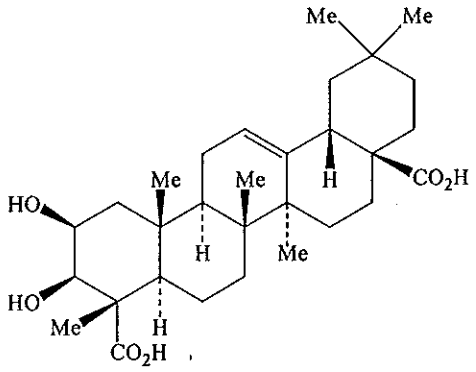
Salacinol, **7 pp**



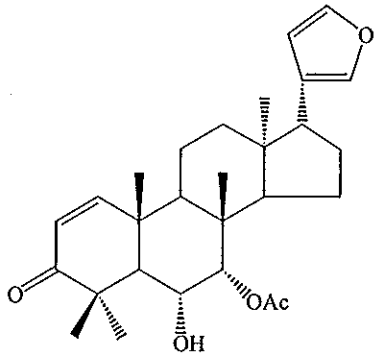
Ochratoxin A, **8a**

$CH_3(CH_2)_{28}CH_2OH$

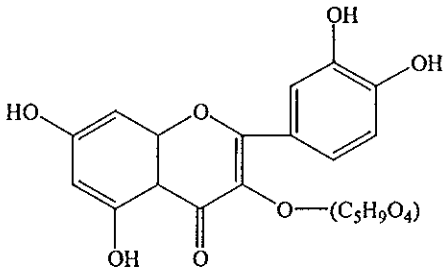
n-triacontanol, **8b**



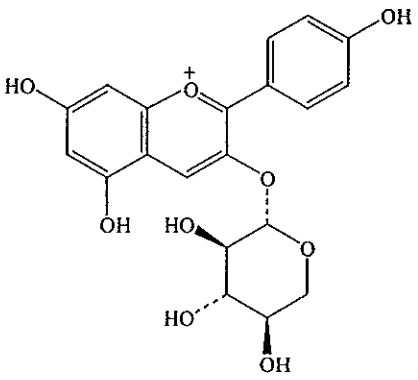
Castanogenin, 8c



Betulinic acid, 8d



Quercetin-3-a-arabinoside, 8e



Taccalin, 8f

3. Objectives

The objectives of this study are as follows:

3.1 To determine the antioxidant activity of some Thai medicinal plants used in longevity formulas.

3.2 To determine the immunomodulatory activity of some Thai medicinal plants used in longevity formulas.

3.3 To isolate and identify substances from the active plant extracts.

3.4 To determine antioxidant and immunomodulatory activities of isolated pure compounds.

CHAPTER 2

RESEARCH METHODOLOGY

1. Plant materials

Four Thai medicinal plants: *Boesenbergia pandurata* root, *Cyperus rotundus* root, *Curcuma zedoaria* root and *Piper chaba* fruit were purchased from herbal medicine shop in Hatyai, Songkhla Province. The four stems of *Acanthus ebracteatus*, *Piper sarmentosum*, *Salacia euphlebia* and *Tacca integrifolia* were collected in August 2001 from Chana, Songkhla Province. Authentification of plant materials were carried out at the Department of Botany, Faculty of Sciences, Prince of Songkla University and also by comparing with the authentic specimens at Department of Forestry, Ministry of Agriculture, Bangkok, Thailand. Voucher specimens were deposited in Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

2. Chemicals and reagents

2.1 Chemicals and reagents for extraction

95% Ethanol, commercial grade (L.B. Science®, Thailand) was predistilled before used.

Chloroform, AR grade (Merck®, Germany)

Methanol, AR grade (Merck®, Germany)

Ethyl acetate, AR grade (Merck®, Germany)

2.2 Chemicals and reagents for antioxidant activity assay

Absolute ethanol, AR grade (Merck®, Germany)

1,1-diphenyl-2-picrylhydrazyl, (Fluka®, Switzerland)

Butylated hydroxytoluene, AR grade (Sigma®, USA)

Phosphate buffer saline (Gibco BRL, USA)

Trypan blue stain (Gibco BRL, USA)

Tertiary butylhydroperoxide (Sigma®, USA)

2.3 Chemicals and reagents for cell culture

RPMI 1640 (Biochrom, Germany)

Fetal calf serum (Gibco BRL, USA)

Penicillin/streptomycin (Biochrom, Germany)

Tertiary butylhydroperoxide (Sigma®, USA)

Sulforhodamin B (Sigma®, USA)

2.4 Chemicals and reagents for immunomodulatory assay

Ficoll-plague (Isoprep®, Robbin, Norway)

Human IL-2 ELISA Kit (Pierce Endogen, USA)

Human IFN-? ELISA Kit (Pierce Endogen, USA)

RPMI 1640 (Biochrom, Germany)

Fetal calf serum (Gibco BRL, USA)

Penicillin/streptomycin (Biochrom, Germany)

Cell Titer 96 (Promega, USA)

2.5 Chemicals and reagents for chromatography

Methanol, AR grade (Merck®, Germany)

Ethyl acetate, AR grade (Merck®, Germany)

Sephadex LH 20 (Sigma®, USA)

Silica gel (Merck®, Germany)

Silica gel GF 254 (Merck®, Germany)

Dianion HP-20 (Supelco®, USA)

3. Instruments

3.1 Apparatus for extraction

Rotaevaporatory (Eyela, Japan)

Freeze Dryer (Dura Dry,USA)

3.2 Apparatus for antioxidant activity assay

Spectronic® Genesys 5 (Miltonroy, USA) UV spectrophotometer

PowerwaveX, (Bio-Tek Instruments, USA) microplate reader

3.3 Apparatus for cell culture assay and immunomodulatory assay

PowerwaveX , (Bio-Tek Instruments, USA) microplate reader

3.4 Apparatus for structure elucidation

Melting point was determined on Microscope Heating stage 350 (Leitz, Germany). Optical rotation was observed on Polarimeter P-1020 (JASCO). NMR spectra were recorded on a Unity Inova 500 NMR (Varian, Germany) spectrometer using TMS as an internal standard with ^{1}H and ^{13}C nuclei observed and were recorded as chemical shift parameter (d) value in ppm down field from tetramethylsilane (TMS d 0.00). MS were recorded on Mat 95 XL, Thermo Finnigan, Germany.

4. Methods

4.1 Extraction of crude extract

Each dried chopped plant (100 g) was extracted with methanol and boiling water for 3 times. The methanol extracts were evaporated under reduced pressure and water extracts were freeze dried. The yields of the extracts were recorded in Table 4-1.

4.2 Isolation of chemical constituents from *S. euphlebia*

4.2.1 Extraction

Air-dried ground stems of *S. euphlebia* (2.0 kg) were extracted three times with methanol (7 L). After filtration, the solvent was removed under reduced pressure to yield a residue 298 g.

4.2.2 Isolation and purification

Crude methanol extract (10 g) was chromatographed on silica gel column eluted with ethyl acetate with increasing amounts of methanol; 75 ml fractions being collected. Fraction 9 gave 74 mg white crystal (SE 2). Fractions 22-28 were rechromatographed on Sephadex LH-20 column, eluting with methanol to give 8 mg yellow crystal (SE 1). Fractions 49-56 were rechromatographed on Sephadex LH-20

column, eluting with methanol to give 131.2 mg white crystal (SE 3). Fractions 63-69 were rechromatographed on Sephadex LH-20 column, eluting with methanol to give 8.1 mg pale yellow amorphous (SE 4). Fractions 70-75 were chromatographed on Dianion and eluted with water to give 5 mg clear needle crystal (SE 5).

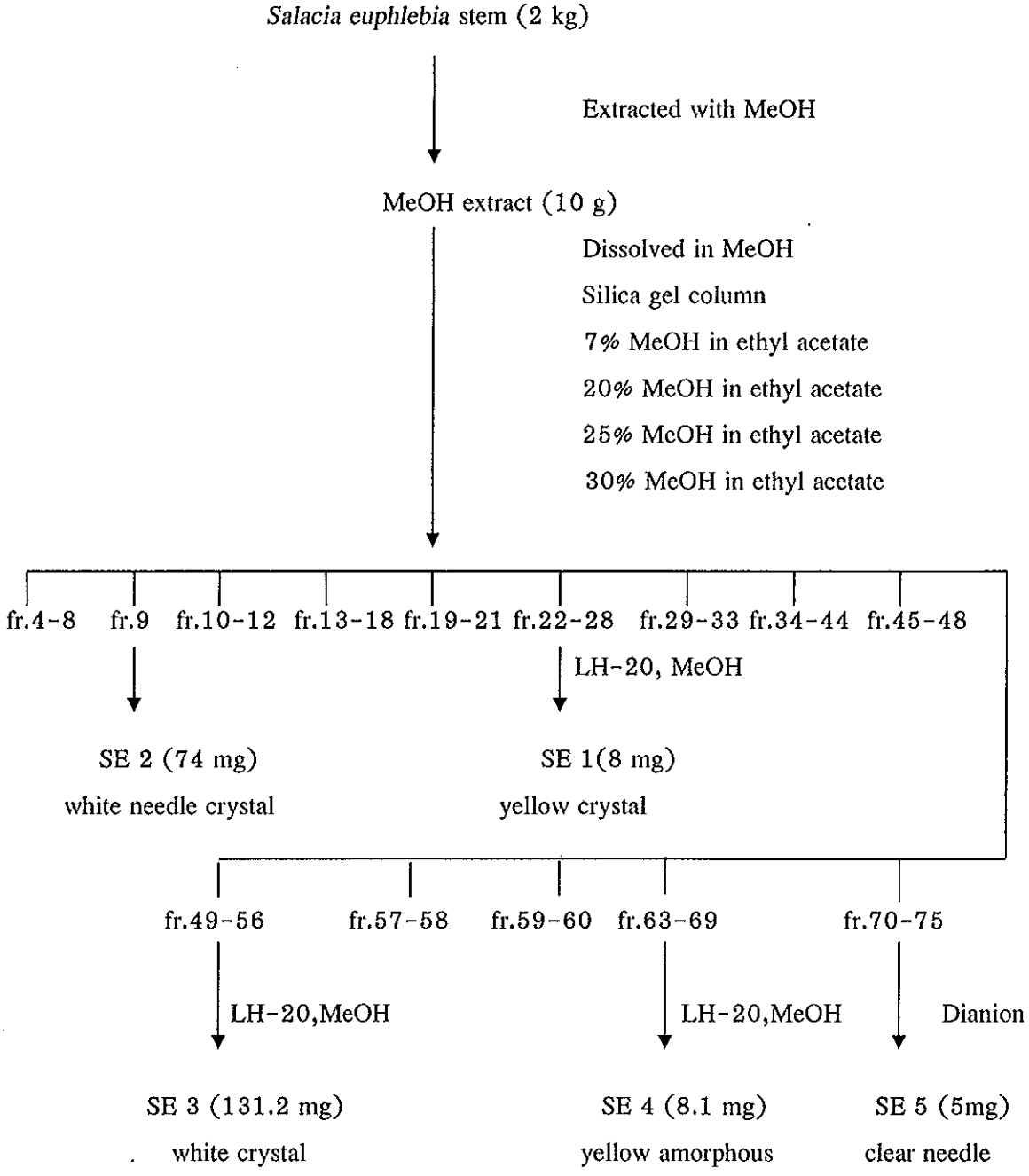


Chart 1 Extraction and isolation of compounds from *S. euphlebia* Merr.

The structures of the isolated compounds were identified by spectroscopic technique and compared their spectral data with the previously reported data.

4.2.3 Characterization of the isolated compounds

Compound SE 1 was isolated as yellow amorphous powder. The molecular formula was established.

mp (see CHAPTER 3)

MS (see CHAPTER 3)

^{1}H NMR see Table 3-2

^{13}C NMR see Table 3-3

Compound SE 2 was isolated as white powder. The molecular formula was established.

mp (see CHAPTER 3)

MS (see CHAPTER 3)

^{1}H NMR see Table 3-4

^{13}C NMR see Table 3-5

Compound SE 3 was isolated as white powder. The molecular formula was established.

mp (see CHAPTER 3)

MS (see CHAPTER 3)

Optical rotation (see CHAPTER 3)

^{1}H NMR see Table 3-6

^{13}C NMR see Table 3-7

Compound SE 4 was isolated as pale yellow needle. The molecular formula was established.

mp (see CHAPTER 3)

MS (see CHAPTER 3)

^{1}H NMR see Table 3-8

^{13}C NMR see Table 3-9

4.3 Antioxidant assay

4.3.1 Assay for DPPH radical scavenging

The free radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was carried out as described by Hatano *et al.* (74). The alcoholic or water-based solution of the sample and positive control, butylated hydroxytoluene (BHT), were prepared with a varied dilution 6.25, 12.5, 25, 50, 100 μg/ml and mixed with the same volume alcoholic solution of DPPH $6x10^{-5}$M (500 μl). The mixture was shaken and allowed to stand for 30 minutes. Its absorbency was then measured at 520 nm. The scavenging effect of the extract was expressed in term of effective concentration 50% (EC_{50}). Each concentration was tested in triplicate with Spectronic Genesys 5 spectrophotometer. Results are shown in Tables 3-12, 3-13.

Pure compounds were tested in 96-well plate. Results are shown in Table 3-14.

The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The result was expressed as the percent inhibition calculated as shown below:

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

EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50 %) was obtained by linear regression analysis of dose response curve plotting between % inhibition and concentrations.

4.3.2 Cell culture assay for antioxidant

We tested the decline of endothelial cells after oxidant injury with tBHP and determined protection provided by crude extract and pure compounds.

Endothelial cells (ECV-304 cell line), established by Paris University, was provided by Dr. Primchanien Moongkarndi, Faculty of Pharmacy, Mahidol University. Growth profile of cell injury by tertiary butylhydroperoxide (tBHP) was evaluated as shown in Figure 15.

Confluent cultures were incubated in the presence and absence of specific oxidants for concentrations of 5, 50, 100, 250, 400, 500, 600, 800, 1000, 2000 μM as well as different times of exposure 0.5, 1, 2, 3, 6, 12, 24 h. Suitable effect (Cytotoxicity of about 70%) was detected, using the following cell culture assay:

The assay was done according to Rong et al.(15) with some modifications. Endothelial cells (ECV-304 cell line) were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in 5% CO_2 atmosphere with 85% humidity for 3 days. Cells were harvested by trypsinization, counted, and seeded in 96-well plates with the density of $3x10^4$ cells/ml.

Crude extract was dissolved in culture medium at the concentrations of 5, 50, 100 µg/ml. Pure compounds and positive control, quercetin, were dissolved in medium to give final concentrations of 1, 5, 10, 50, 100 µg/ml, added in each 8 wells (100 µl/well).

After the cells grew to confluence (5 days), plant extracts were added. Plates were incubated in CO_2 incubator for 16 h, and then washed with fresh medium and induced oxidative stress by incubation with tertiary butylhydroperoxide (tBHP) 500 µM for 6 h. Cell viability was assessed by Sulforhodamin B (SRB) colorimetry. The absorbance was measured at 492 nm using a microplate reader (75). Percent tBHP damage was calculated by comparing with non-protected cells.

4.4 Assay for immunomodulatory activity

The protocol was ethically approved by institutional review board of Faculty of Medicine, Prince of Songkla University. The ages of healthy volunteers are 20-50 years old.

4.4.1 Isolation of human peripheral blood mononuclear cells

The method was followed Rowland-Jones and McMichael (76). Venous bloods from ten healthy volunteers were collected in heparin (10 UI/ml blood). Peripheral blood mononuclear cells were separated from other cells using Ficoll-plaque density gradient centrifugation and resuspended in RPMI 1640 medium supplemented with 3% fetal calf serum at a cell concentration of $2x10^6$cells/ml and tested immediately.

4.4.2 Preparation of herbal extracts

The dried plant preparations were homogenized in RPMI medium to enhance solubility, filtered through a 0.22 micron millipore membrane to remove particulate material and any bacterial contaminants, and used fresh the same day.

4.4.3 Lymphocyte proliferation (77) and (78)

One hundred µl of $1x10^6$cells/ml was added to each well of 96 tissue culture plate. 100 µl of media containing final concentrations 0.1, 0.5, 1, 10, 50, 100 µg/ml of the extracts were added and incubated at 37°C with 5% CO_2, humidified atmosphere for 3 and 5 days respectively to find out optimum incubation time. T-lymphocyte proliferation was evaluated with MTS/PMS (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, USA). 20 µl of MTS/PMS was added to each well. The plate was incubated for 16 h in 5% CO_2 incubator then absorbance at 490 nm was measured using microplate reader. The mean value of each of the ten independent experiments performed in triplicate and then stimulation index (SI) was calculated.

4.4.4 Cytokine assay (79)

Five hundreds µl of a suspension of lymphocyte cell ($2x10^6$cells/ml) in culture medium were cultured, with optimal concentration from cell proliferation of test samples, in 48 wells plate at 37°C in 5%CO_2 for 48, 72 and 96 h respectively. After each culture period, the supernatants were obtained by centrifugation. Cytokine, interleukine2 (IL-2) and interferon gamma (IFN-?) secretion were detected using commercially available ELISA kit following the manufacture's instruction. Results from the cytokine release assay were expressed as picogram (pg)/ml. The detection limit for IL-2 and IFN- ? were less than 6 and 2 pg/ml respectively.

4.4.5 Statistical analysis

Experimental data were analyzed with Probit analysis for antioxidant assay and Paired-t test for immune assay using SPSS for windows version 10.0. *P* value of 0.05 or less was considered statistically significant.

CHAPTER 3

RESULTS

1. Plant extracts

Crude methanol and water extracts of plant materials were measured and percent yields of dry weight were shown in Table 3-1.

Table 3-1 Percent yield of dry crude methanol (M) and water (W) extracts

Plant sources	Extract	%Yield(w/w)
Acanthus ebracteatus Vahl	methanol (AEM)	14.5
	water (AEW)	4.6
Boesenbergia pandurata Holtt.	methanol (BPM)	7.9
	water (BPW)	2.3
Curcuma zedoaria Roscoe	methanol (CZM)	14.7
	water (CZW)	2.4
Cyperus rotundus L.	methanol (CRM)	12.8
	water (CZW)	2.4
Piper chaba Hunter	methanol (PCM)	16.6
	water (PCW)	2.7
Piper sarmentosum Roxb.	methanol (PSM)	12.4
	water (PSW)	3.2
Salacia euphlebia Merr.	methanol (SEM)	16.1
	water (SEW)	7.4
Tacca integrifolia Ker-Gawl.	methanol (TIM)	15.1
	water (TIW)	3.1

2. Characterization of the isolated compounds

Structure elucidations of the isolated compounds were achieved using ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC and HMBC experiments while MS gave the molecular weight.

2.1 Identification of compound SE 1

SE 1 was obtained and recrystallized as a yellow amorphous with melting point 260-261° C. The EI mass spectrum of SE 1 showed the molecular ion peak at *m/z* 432 corresponding to the molecular formula $C_{21}H_{20}O_{10}$, which was in good agreement with the number of signals observed in the ^{13}C NMR spectrum and the number of protons registered in the ^{1}H NMR spectrum. Its ^{13}C NMR spectrum showed 19 resonances, sorted by DEPT experiments, including one methylene, nine methines and nine quaternary carbons. The presence of the flavonoid skeleton was evident by the typical AA BB proton doublets centered at d_H 8.02 and 6.89 (2H each), and the singlets at d_H 6.79 and 6.27 (1H each) for H-3 and one A-ring proton, respectively, in the ^{1}H NMR spectrum. These assignments were confirmed by the HMQC experiment, which showed the corresponding crosspeaks at d_C at 129.4, 116.3, 102.9 and 98.6 for C-2′ and C-6′, C-3′ and C-5′, C-3 and the corresponding carbon of the A ring, respectively. The second hydroxyl group could be located at C-7, as in the apigenin derivatives. The presence of a hexose was evident by the presence of six signals in the ^{13}C NMR spectrum between 60 and 85 ppm which correlated in the HMQC with the signal between 3.0 and 4.7 ppm in the ^{1}H NMR spectrum. COSY experiment established the connectivity for the hexopyranose which was determined as a ß-D-glucopyranoid system by the all trans diaxial coupling pattern within the pyranoid system, yielding only large coupling constants (10 Hz) for H-1″ through H-5″. The presence of the C-8 to C-1″ bond was determined by the upfield shift of H-1″ (d_H 4.68) and by the observed crosspeaks between H-1″ and C-8 (d_C 105.1), C-7 (d_C 163.1) and C-9 (d_C 156.5) in the HMBC spectrum. Complete ^{1}H and ^{13}C assignments are shown in Tables 3-2 and 3-3, respectively.

Table 3-2 ^{1}H NMR spectral Data for SE 1 compared with vitexin (500 MHz, DMSO-*d*6)

^{1}H-position	d (ppm) experiment	d (ppm) reference (80)
3	6.79s	6.62s
6	6.27s	6.27s
2′	8.02d	8.01
	(J=8.1)	(J=8.4)
3′	6.89d	6.89d
	(J=8.1)	(J=8.4)
5′	6.89d	6.89d
	(J=8.1)	(J=8.4)
6′	8.02d	8.01d
	(J=8.1)	(J=8.4)
1″	4.68d	4.68d
	(J=9.7)	(J=9.9)
2″	3.83 br-t	
	(J=9.0)	
3″	3.25 m	
4″	3.35 m	
5″	3.23 m	
6″	3.76, 3.52 m	
4′-OH	10.37 br s	10.26 br s
7-OH	10.83 br s	10.73 br s
5-OH	13.17 br s	13.14 br s

Table 3-3 ^{13}C NMR spectral data for SE 1 compared with vitexin (in DMSO-*d*6)

^{13}C-position	d (ppm) experiment (in DMSO-*d*6)	d (ppm) reference (80) (in DMSO-*d*6)
2	164.4	163.9
3	102.9	102.4
4	182.6	182.0
5	160.9	161.0
6	98.6	98.1
7	163.1	162.5
8	105.1	104.6
9	156.5	155.9
10	104.5	104.0
1′	122.1	121.5
2′	129.4	128.8
3′	116.3	115.7
4′	161.6	160.3
5′	116.3	115.7
6′	129.4	128.8
1?	73.8	73.3
2?	71.3	70.8
3?	79.1	78.8
4?	71.0	70.5
5?	82.3	81.7
6?	61.8	61.3

Therefore, this substance was established as 8-C-ß-D-glucopyranosyl apigenin (vitexin). Direct comparison of the ^{13}C NMR spectral assignments were in good agreement with those published previously (80).

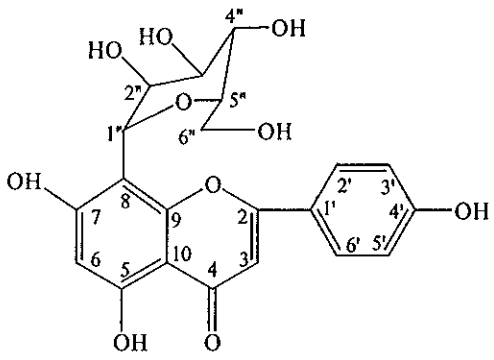


Figure 11 Structure of SE 1

2.2 Identification of compound SE 2

SE 2 was obtained as white powder with melting point 275-276 °C. The ^{13}C NMR spectrum showed 30 carbon atom signals. The EI mass spectrum of SE 2 showed molecular ions M^+ at *m/z* 412, 355, 274, 248, 230,177.

The ^{1}H NMR spectrum shows seven tertiary methyl singlets at H-28 (d 1.29), H-26 (d 1.06), H-29 (d 1.01), H-27 (d 0.99), H-30 (d 0.94), H-25 (d 0.87) and H-24 (d 0.73), a doublet methyl at H-23 (d 0.87, J=7.78 Hz) and a doublet methine signal at H-4 (d 2.24, J=6.89 Hz), and methylene protons at H-2 (d 2.50 and 2.24). ^{13}C NMR spectrum of the compounds showed totally 30 carbon signals, one carbonyl, eight methyls, ten methylenes, five methines and six quaternary carbon signals. A singlet at d 213.14 ppm, assigned to a C-3 ketone. From the NMR spectra indicated that SE 2 possesses friedela skeletal (62). The carbinol methine proton shift at d 3.73 and the hydroxylated carbon chemical shift at 74.63 have been directly assigned for a 15a-hydroxyl substitution by analogy to the identical shifts observed in literature (62). Moreover, the ^{13}C NMR shifts observed for the rings C, D and E are in good agreement with the corresponding shifts in literature (62) thus confirming a 15a-hydroxyl substitution. The remaining carbon appeared signals occuring between d 80 and 6.5 ppm. Complete ^{1}H and ^{13}C assignments are shown in Tables 3-4 and 3-5, respectively.

Table 3-4 ^{1}H NMR spectral data of SE 2 compared with 15a-hydroxyfriedelan-3-one (500 MHz, $CDCl_3$)

^{1}H -position	d (ppm) experiment (in $CDCl_3$)	d (ppm) reference (62) (in $CDCl_3$)
1	1.97(m), 1.68(m)	1.94, 1.68
2	2.50(m), 2.24(m)	2.40, 2.28
4	2.24(d, J= 6.89 Hz)	2.24
15	3.73(d, J=7.78Hz)	3.70
16	2.17(dd, J=7.7, 16.5 Hz),	2.16
	1.25(m)	1.25
23	0.87 (J=7.78 Hz)	0.87
24	0.73(s)	0.72
25	0.87(s)	0.88
26	1.06(s)	1.06
27	0.99(s)	0.99
28	1.29(s)	1.29
29	1.01(s)	1.01
30	0.94(s)	0.94

Table 3-5 ^{13}C NMR Spectral data for SE 2 compared with 15a-hydroxyfriedelan-3-one (in $CDCl_3$) (62)

^{13}C -position	d (ppm) experiment (in $CDCl_3$)	d (ppm) reference (62) (in $CDCl_3$)
1	22.36	22.35
2	41.51	41.50
3	213.14	213.07
4	58.17	58.18
5	42.03	42.03

^{13}C -position	d (ppm) experiment (in $CDCl_3$)	d (ppm) reference (62) (in $CDCl_3$)
6	41.27	41.29
7	19.99	19.99
8	53.45	53.46
9	37.80	37.81
10	59.39	59.40
11	35.75	35.76
12	31.19	31.19
13	40.57	40.58
14	44.10	44.10
15	74.63	74.60
16	48.36	48.38
17	30.21	30.22
18	41.59	41.60
19	35.59	35.59
20	28.15	28.16
21	31.90	31.91
22	38.85	38.85
23	6.82	6.82
24	14.50	14.50
25	17.99	17.99
26	14.07	14.06
27	18.76	18.75
28	32.61	32.61
29	30.92	30.92
30	35.67	35.67

Comparing with the reported data, the MS, ^{1}H NMR and ^{13}C NMR data were in good agreement with those reported in literatures (62). This compound was identified as 15a-hydroxyfriedelan-3-one.

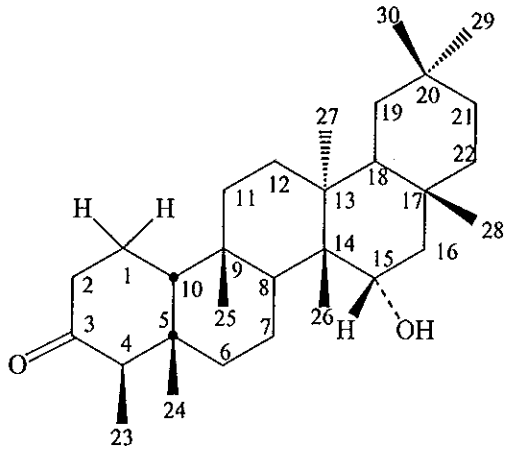


Figure 12 Structure of SE 2

2.3 Identification of compound SE 3

SE 3 was obtained and recrystallized as yellow amorphous with melting point of 122-123 °C. The ^{13}C NMR spectrum showed seven carbon atom signals. DEPT measurements indicated five methines, one methylene and one methyl carbon signals. The EI mass spectrum of SE 3 shows the molecular ion peak at *m/z* 196 corresponding to the formula $C_7H_{16}O_6$. $[\alpha]_D^{22}$ +3° (c. 1.5, in water)

The ^1H NMR spectrum of SE 3 contained an intense broad signal at d 4.0-4.5 ppm and several signals in the a-anomeric region and one intense signal in the high field region assigned to the protons of methyl groups. Two signals at d 3.6 and 3.38 ppm assigned to the methylene protons. The rest of the main signals could be assigned to the protons H-2, H-3, H-4, H-5 and H-6 of heptitol. The ^{13}C NMR spectrum signals between d 75-60 ppm can be assigned to sugar carbons while the group of signals at d 17.91 ppm should arise from methyl carbon. The HMQC spectrum showed that H-1 at d 3.6 and 3.33 ppm was correlated to carbons at d 64.01 ppm. In the HMBC spectrum H/C correlation was observed. All together, the spectroscopic data suggest that SE-3 is siphulitol. Assignments of the ^1H and ^{13}C NMR resonances are presented in Tables 3-6 and 3-7 respectively.

Table 3-6 ^{1}H NMR spectrum of SE 3 (500 MHz, DMSO- *d*6)

^{1}H -position	d (ppm) experiment (in DMSO-*d*6)
1	3.6 (dd, J=10.75), 3.33 (dd, J=10.76)
2	3.45 m
3	4.13 (dd, J=7.32)
4	3.54 (dd, J=7.32)
5	3.38 (dd, J=5.03)
6	3.76 (dq, J=5.03, 6.41)
7	1.03 (s, J=6.41)

Table 3-7 ^{13}C NMR spectral data for SE 3 (in DMSO-*d*6)

^{13}C -position	d (ppm) experiment (in DMSO-*d*6)
1	64.01
2	71.38
3	70.73
4	70.15
5	73.59
6	67.95
7	17.91

From these results it can be concluded that SE 3 is siphulitol.

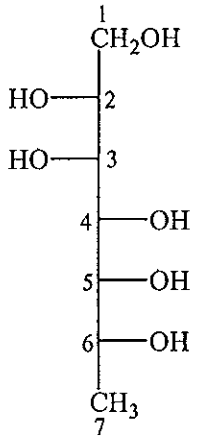


Figure 13 Structure of SE 3

2.4 Identification of compound SE 4

SE 4 was obtained as pale yellow needle with melting point of 278-280 °C. The ^{13}C NMR spectrum showed 19 carbon atom signals. SE 4 showed in ESIMS $[M+H]^+$ ion at m/z 423 corresponding to molecular formula $C_{19}H_{18}O_{11}$.

The ^{1}H NMR spectrum of SE 4 (Table 4-8), showed the chemical shifts at d 6.36, 6.85 7.36 and 13.75, were assigned as proton at C4, C5, C8 and 1-OH respectively. The sugar moiety was indicated two one proton doublets at d 4.59 (J= 9.83 Hz) and d 3.66 (J=10.71 Hz), two one proton doublet of doublets at d 4.05 (J= 9.38, 8.92 Hz) and d 3.42 (J=11.4, 5.7 Hz) and three proton triplets at d 3.2, 3.16 and 3.17. The 2D COSY experiment demonstrated the glycosidic chaining and the 2D HMQC map indicated that all sugar carbons were in the d 60-85 ppm range. The glucose was identified on the basis of its ^{1}H NMR and ^{13}C NMR data (81).

The ^{13}C NMR showed three shielded aromatic methane groups at d 93.7 (C-4); d 103.1 (C-5) and d 108.6 (C-8) ppm respectively. Among the ten quartenary carbon atom signals noted in the HMQC map, six corresponded to O-linked aromatic carbon atoms (145-165 ppm), while the peak at d 180.16 ppm indicated the presence of carbonyl function. The 2D NMR experiment showed that there was only one aromatic proton on the A-ring. The substitution pattern of B-ring was clearly indicated by the two singlets at d 6.85 (H-5) and d 7.36 (H-8). This was confirmed by the HMBC ^{2}J correlations noted between H-5 (d 6.85) and the carbon atom at 151.6 (C-6) and d 154.7 (C-4b), and between H-8 (d 7.36) and the signals at d 112.2 (C-8a) and d 144.5 (C-7). Moreover, the 6, 7-*ortho*-dihydroxylation pattern of B-ring was confirmed by reciprocal shielding of C-6 and C-7 at d

151.6 and d 144.5 respectively. Furthermore, ^{13}C chemical shifts of the genin were in accordance with those noted in the literature for tetrahydroxyxanthone. Finally the cross peaks noted in the HMBC experiment between H-1′ (d 4.62) and the carbon atoms C-2 (d 108.1), C-1 (d 162.68) and C-3 (d 164.7) indicated the linkage between the sugar moiety and the aglycone part.

Table 3-8 ^{1}H NMR spectral Data for SE 4 compared with mangiferin (500 MHz, DMSO-*d*6) (82)

^{1}H -position	d (ppm) experiment (in DMSO-*d*6)	d (ppm) reference(82) (in DMSO-*d*6)
1-OH	13.75 s	
4	6.36s	6.37s
5	6.85s	6.86s
8	7.36s	7.36s
1′	4.59 (d, 9.83)	4.64 (d, 8)
2′	4.05 (dd, 9.38, 8.92)	3.8-3.0
3′	3.2 (t, 8.4)	3.8-3.0
4′	3.16 (t, 9.15)	3.8-3.0
5′	3.17 (dd, 10.98, 5.04)	3.8-3.0
6′a	3.66 (d, 10.71)	3.8-3.0
6′b	3.42 (dd, 11.4, 5.7)	3.8-3.0
3-OH	10.44 br	
6-OH	4.80 br	
7-OH	4.40 br	

Table 3-9 ^{13}C NMR Spectral data for SE 4 compared with mangiferin (in DMSO-*d*6) (82)

^{13}C -position	d (ppm) experiment (in DMSO-*d*6)	d (ppm) reference(82) (in DMSO-*d*6)
1-OH	162.6	161.8
2	108.1	107.7
3	164.7	163.9
4	93.7	93.4
4a	157.0	156.3
4b	154.7	154.1
5	103.1	102.7
6	151.6	150.9
7	144.5	143.8
8	108.6	108.2
8a	112.2	111.8
8b	101.8	101.4
CO	180.1	179.2
1′	73.4	73.2
2′	70.5	70.4
3′	79.3	79.0
4′	70.9	70.7
5′	81.9	81.6
6′	61.5	61.6

Comparing with the reported MS, ^{1}H NMR and ^{13}C NMR data (82), SE 4 was identified to be mangiferin (1, 3, 6, 7-tetrahydroxyxanthone-C_2-ß-D-glucoside).

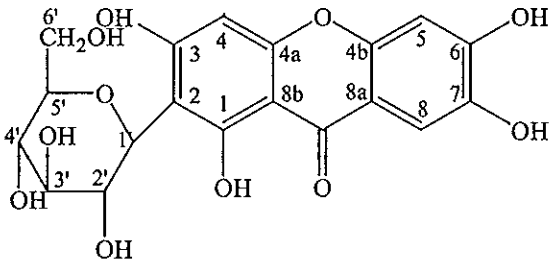


Figure 14 Structure of SE 4

3. Antioxidant activity

3.1 Free radical scavenging activity by DPPH assay

The scavenging activity of crude extract at 100 μg/ml on DPPH radical is shown in Table 3-10.

Table 3-10 Effect of crude methanol and water extracts on DPPH radical scavenging activity

Botanical name	Extract	% scavenging (100 μg/ml)
Acanthus ebracteatus	Methanol (AEM)	69.64 ± 0.16
	Water (AEW)	86.86 ± 0.67
Boesenbergia pandurata	Methanol (BPM)	36.57 ± 0.65
	Water (BPW)	74.90 ± 1.20
Cyperus rotundus	Methanol (CRM)	71.60 ± 0.16
	Water (CRW)	88.22 ± 0.67
Curcuma zedoaria	Methanol (CZM)	67.13 ± 0.16
	Water (CZW)	82.24 ± 0.88
Piper chaba	Methanol (PCM)	11.19 ± 0.66
	Water (PCW)	90.54 ± 0.33
Piper sarmentosum	Methanol (PSM)	30.86 ± 0.13
	Water (PSW)	52.89 ± 0.88
Salacia euphlebia	Methanol (SEM)	75.23 ± 1.64
	Water (SEW)	75.92 ± 0.46
Tacca integrifolia	Methanol (TIM)	32.79 ± 0.93
	Water (TIW)	73.74 ± 0.33

The calculated EC_{50} from the plot of concentration-dependent inhibition of DPPH radical by crude extracts and BHT as positive control is shown in Table 3-11. The EC_{50} values are the mean ± SD for triplicate determinations.

Table 3-11 EC_{50} values of crude extracts and standard in DPPH assay

Botanical name	Extract	EC_{50} (μg/ml)
Acanthus ebracteatus	methanol (AEM)	48.61 ± 0.17
	water (AEW)	40.19 ± 0.74
Boesenbergia pandurata	methanol (BPM)	>100
	water (BPW)	51.71 ± 4.43
Cyperus rotundus	methanol (CRM)	67.36 ± 1.96
	water (CRW)	25.47 ± 0.71
Curcuma zedoaria	methanol (CZM)	64.36 ± 3.12
	water (CZW)	41.27 ± 1.69
Piper chaba	methanol (PCM)	>100
	water (PCW)	35.69 ± 0.91
Piper sarmentosum	methanol (PSM)	>100
	water (PSW)	95.03 ± 4.51
Salacia euphlebia	methanol (SEM)	21.12 ± 0.88
	water (SEW)	9.05 ± 0.59
Tacca integrifolia	methanol (TIM)	>100
	water (TIW)	66.21 ± 0.14
BHT		17.36 ± 0.38

Crude water extracts showed higher activity than methanolic extracts except *Salacia euphlebia* which exhibited about 75% scavenging activity in both parts. The ability to inhibit DPPH free radical scavenging increased linearly with increasing concentration. Compare with BHT crude extract from *Salacia* demonstrated appreciable antioxidant activity.

3.2 Free radical scavenging activity of pure compounds

The EC_{50} of pure compounds isolated from *Salacia euphlebia* on DPPH radical is shown in Table 3-12.

Table 3-12 DPPH radical scavenging activity of compounds isolated from *Salacia euphlebia*

Compounds	DPPH radical scavenging EC_{50} (μg/ml)
vitexin	>100
15a-hydroxyfriedelan-3-one	>100
siphulitol	>100
mangiferin	1.10 ± 0.18
quercetin (positive standard)	1.35 ± 0.02

Mangiferin showed high free radical scavenging activity comparable with quercetin while others showed low activity.

3.3 Cell culture antioxidant activity

Confluent cultures were incubated in the presence and absence of specific oxidants for different concentrations 5, 50, 100, 250, 400, 500, 600, 800,1000, 2000 μM as well as different times of exposure 0.5, 1, 2, 3, 6,12, 24 h. Suitable effect (Cytotoxicity about 70%) was achieved at 6 h with 500 μM tBHP as shown in Figure 15.

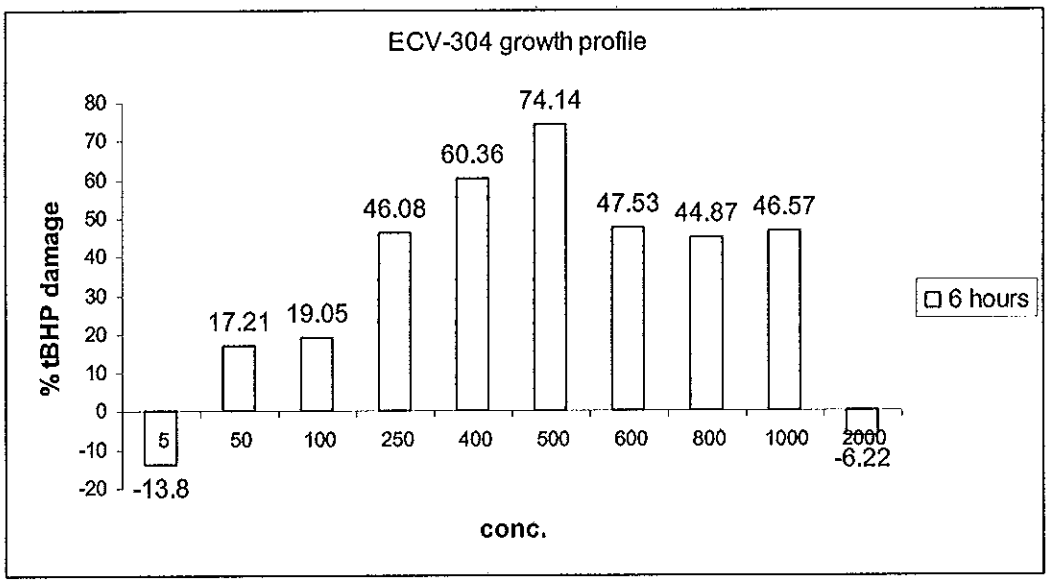


Figure 15 Growth profile of cell injury by tBHP

3.3.1 Cell culture antioxidant of crude extracts

The effect of crude methanol and water extracts on tBHP-induced cell damage is shown in Figures 16 and 17 respectively.

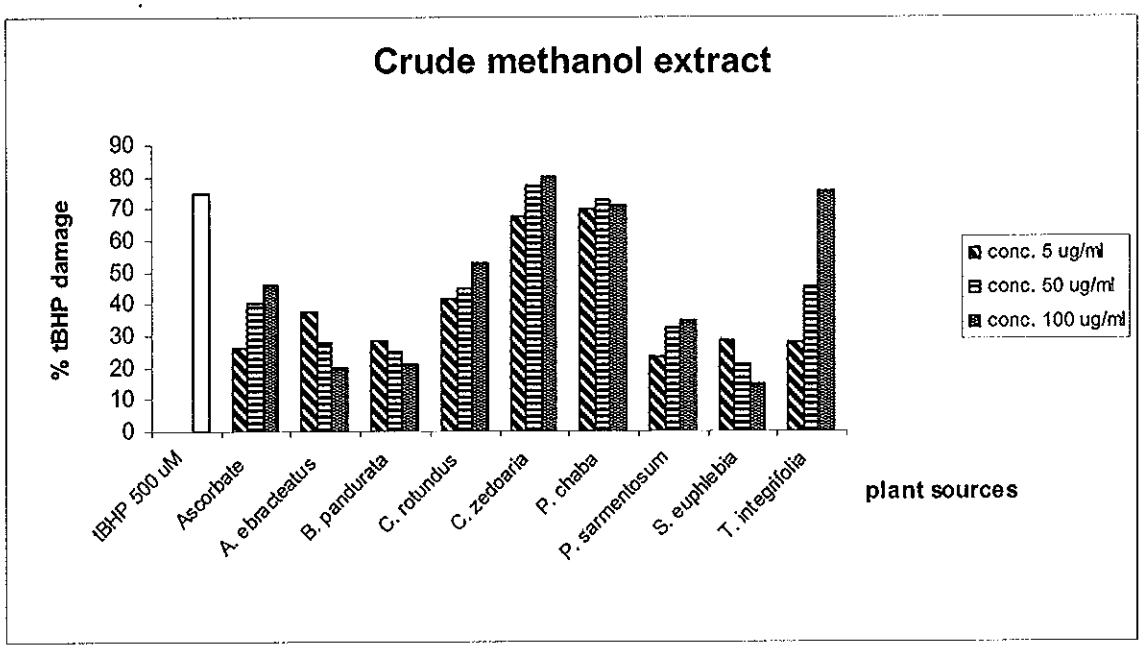


Figure 16 Cytoprotective effect of crude methanol extracts

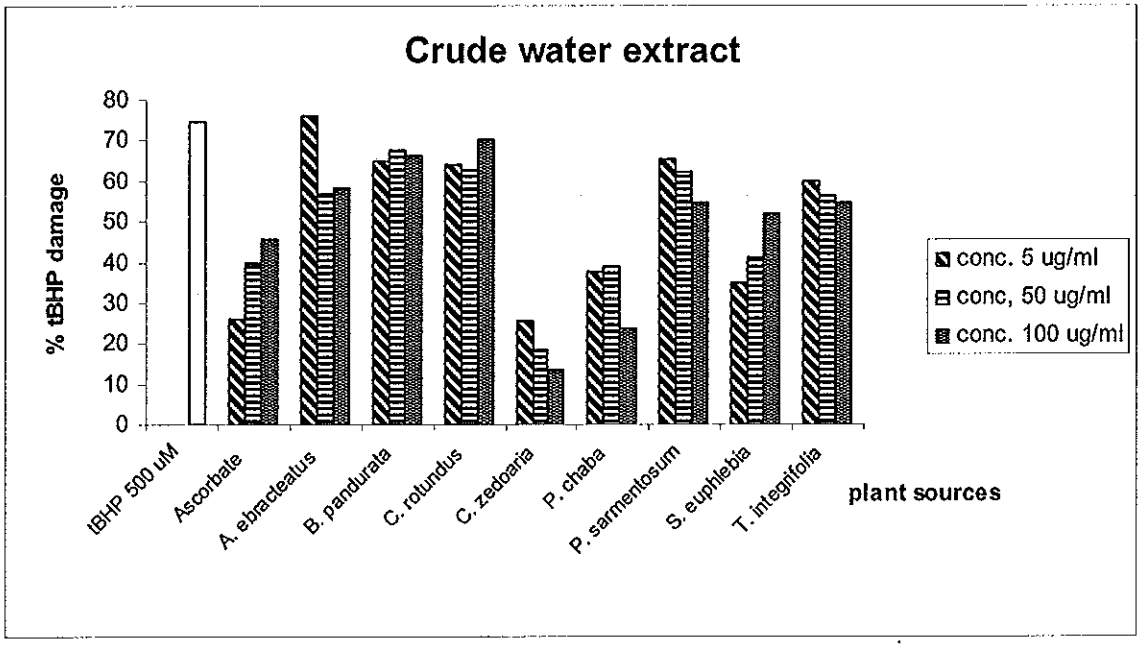


Figure 17 Cytoprotective effect of crude water extracts

For cell culture assay, the methanol extracts of *A. ebracteatus, B. pandurata, and S. euphlebia* showed high cytoprotective effect whereas water extracts of *C. zedoaria* and *P. chaba* showed high protection activity on tBHP induced cells damage in dose dependent manner as shown in Figures 16 and 17 respectively.

3.3.2 Cell culture antioxidant of pure compounds

The IC_{50} of pure compounds on tBHP-induced cell damage is shown in Table 3-13. Quercetin was used as positive control.

Table 3-13 Cytoprotective effect of pure compounds

Compound	% tBHP damage					IC_{50}
	1 μg/ml	5 μg/ml	10 μg/ml	50 μg/ml	100μg/ml	μg/ml
Vitexin	70.56 ± 1.29	68.81 ± 1.62	63.95 ± 0.83	47.05 ± 0.47	40.12 ± 1.18	37.57 ± 1.15
15α-hydroxyfriedelan-3-one	98.34 ± 0.96	95.02 ± 0.71	90.52 ± 0.42	87.92 ± 0.73	57.91 ± 0.58	> 100
Siphulitol	95.71 ± 0.19	90.65 ± 0.16	78.95 ± 0.57	63.96 ± 0.76	52.06 ± 2.30	> 100
Mangiferin	73.62 ± 0.15	70.01 ± 1.95	68.10 ± 0.75	56.53 ± 0.58	49.13 ± 0.83	90.57 ± 1.35
Quercetin	89.47 ± 1.85	76.19 ± 0.82	68.71 ± 0.60	58.85 ± 0.64	46.66 ± 0.98	76.08 ± 1.03

Vitexin showed good result on cytoprotective effect compared with quercetin.

3.4 Immunomodulatory activity

3.4.1 Cell proliferation of crude extracts

Crude methanol and water extracts of plant samples were examined for cell proliferation activity on human lymphocytes. As shown in Table 3-14, methanol extract of *S. euphlebia* at 10 μg/ml and water extract of *P. chaba* at 0.1 μg/ml significantly increased the lymphocyte proliferation (SI 1.39 ± 0.43 and 1.48 ± 0.31, $p<0.05$ respectively), whereas other plant extracts induced cell proliferation but not significant.

Table 3-14 Effect of crude methanol and water extract on cell proliferation

Extract	Stimulation index (mean±SD)
control	1.00 ± 0.00
AEM (10 μg/ml)	1.15 ± 0.21
BPM (10 μg/ml)	1.29 ± 0.43
CRM (10 μg/ml)	1.10 ± 0.27
CZW (0.5 μg/ml)	1.11 ± 0.26
PCW (0.1 μg/ml)	1.48 ± 0.31*
PSM (10 μg/ml)	1.16 ± 0.30
SEM (10 μg/ml)	1.39 ± 0.43*
TIM (10 μg/ml)	1.27 ± 0.41

Stimulation index; the value are mean±SD (N=10), *p<0.05 compared with control by Paired-Samples T test.

Cell activation was investigated by measure IL-2 and IFN-? production. The production of IL-2 and IFN-? was quantified in supernatants from PBMC that had been stimulated for 72 and 96 h. The optimum time for detect cytokine production of BPM, PCM and PSM was 72 h whereas AEM, CRM, CZW, SEM and TIM was 96 h. The result showed in Tables 3-15 and 3-16 respectively.

3.4.2 Cytokine IL-2 of crude extracts

Table 3-15 Effect of crude extract on IL-2 production

Extract	IL-2 production (pg/ml)
Control 72h	27.92 ± 15.26
BPM	22.56 ± 7.62
PCW	30.50 ± 15.33
PSM	22.09 ± 9.36
Control 96h	25.49 ± 12.20
AEM	28.59 ± 13.16
CRM	29.39 ± 21.29
CZW	31.68 ± 12.59
SEM	33.97 ± 12.34*
TIM	29.34 ± 12.68

Values are mean±SD, N=10 *p<0.05 compared with control by Paired-Samples T test.

3.4.3 Cytokine IFN-? of crude extracts

Table 3-16 Effect of crude extract on IFN-? production

Extract	IFN-? production(pg/ml)
Control 72h	2.96 ± 3.38
BPM	15.31 ± 27.69
PCW	19.03 ± 16.89*
PSM	4.20 ± 4.36
Control 96h	10.90 ± 8.76
AEM	24.97 ± 29.54
CRM	9.70 ± 8.58
CZW	49.45 ± 39.36*
SEM	18.52 ± 16.31
TIM	23.87 ± 27.21

Values are mean±SD, N=10 *p<0.05 compared with control by Paired-Samples T test.

For cytokine assay, as shown in Tables 3-15 and Table 3-16, mononuclear cells stimulated with methanol extract of *S. euphlebia* significantly increase production of IL-2 at 10 μg/ml of 96 h culture interval. Whereas, water extracts of *C. zedoaria* at 0.5 μg/ml and *P. chaba* at 0.1μg/ml significantly increased IFN-? secretion at 96 and 72 h culture intervals respectively when compared with the non-stimulate control.

3.4.4 Cell proliferation of pure compounds

Mononuclear cells were preincubated with pure compounds at various concentrations (0.5, 1.0 and 10 μg/ml) for 96 h and the highest stimulation index showed in Table 3-17. Phytohemagglutinin-P (PHA-P) was used as positive control.

Table 3-17 Effect of pure compounds on cell proliferation

Compound	Stimulation index
Control	1.00 ± 0.00
Vitexin (0.5 μg/ml)	1.06 ± 0.76*
15a-hydroxyfriedelan-3-one (10 μg/ml)	1.08 ± 0.13
Siphulitol (0.5 μg/ml)	1.07 ± 0.09*
Mangiferin (10 μg/ml)	1.08 ± 0.09*
PHA-P (10 μg/ml)	1.35 ± 0.16*

Values are mean± SD, N=10 *p<0.05 compared with control by Paired-Samples T test.

3.4.5 Cytokine production of pure compounds

Mononuclear cells were culture with pure compounds at appropriate concentration selected from cell proliferation outcome. Supernatants were measured by ELISA test kits for evaluate the effect of pure compounds on cytokine IL-2 and IFN-? expression. Results are showed in Tables 3-18 and 3-19 respectively.

Table 3-18 Effect of pure compounds on IL-2 production

Compound	IL-2 production (pg/ml)
Control	10.64 ± 12.91
Vitexin	13.40 ± 9.63
15 a-hydroxyfriedelan-3-one	15.05 ± 20.69
Siphulitol	12.37 ± 12.38
Mangiferin	23.54 ± 10.42*
PHA-P	327.80 ± 114.36*

Values are mean±SD, N=10 *p<0.05 compared with control by Paired-Samples T test.

Table 3-19 Effect of pure compounds on IFN-? production

Compound	IFN-? production(pg/ml)
Control	60.67 ± 26.48
Vitexin	75.18 ± 74.99
15 a-hydroxyfriedelan-3-one	52.42 ± 34.13
Siphulitol	49.39 ± 13.04
Mangiferin	58.79 ± 30.22
PHA-P	425.21 ± 179.73*

Values are mean±SD, N=10 *p<0.05 compared with control by Paired-Samples T test.

Proliferation assay and cytokine production were measured for immunomodulatory activity. Vitexin and siphulitol at 0.5 μg/ml significantly induced lymphocyte proliferation whereas mangiferin at 10 μg/ml significantly induced both cell proliferation and IL-2 production.

CHAPTER 4

DISCUSSION

Unlike other herbs that are used for specific symptoms, tonic herbs are nutritive and nourishing. Tonic herbs strengthen and improve specific organs, systems, weaknesses or the body as a whole. They are generally gentle herbs that are used to stimulate and increase the function of organs that are not operating at their highest level and to prevent a decline in the function of organs. Tonic herbs may be used to increase awareness of energetic, psychological and spiritual states in human beings.

Reactive oxygen species produced by sunlight, ultraviolet light, chemical reactions and metabolic process have a variety of pathological effects, such as carcinogenesis and cellular degeneration related to aging. Antioxidant activity is an important activity in view of the free radical theory of aging and associated diseases.

Antioxidant activities of crude extracts of the eight plants were evaluated using free radical scavenging and cell culture assay. *S. euphlebia* showed the highest scavenging effect on DPPH. The molecule of DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (non radical) (13). Positive DPPH test suggests that the sample is an electron donor and can react to convert them to more stable product and terminate radical chain reaction.

In cell culture, the organic hydroperoxide, tertiary butylhydroperoxide (tBHP) caused oxidative damage in a number of cell types (83). It is a useful model compound for the study of mechanism of oxidative cell injury (84). Excessive generation of reactive oxygen species can damage DNA, carbohydrate, proteins and polyunsaturated fatty acids in cells (85). Tertiary butylhydroperoxide cause a number of changes in retinal pigment epithelium cells which were characteristic of an oxidant-induced apoptotic mechanism of cell death (18). Mitochondria recently have been found to play an important role in signaling apoptosis (86). Using an in vitro model of oxidant injury induced by tBHP, it was found that tBHP caused cellular damage in endothelial cells by damaging the mitochondria (15). Furthermore, cytochrome C is released in associated with the loss of the mitochondria member potential, and caspase activation occurs over a time course consistent with cleavage of proteins which may

contribute to the morphological picture of apoptosis (87). tBHP induced DNA damage may be triggered by two mechanism, first iron metabolism plays a key role that leads to free radical formation and further radical mediated processes and second, thiol oxidation followed by activation of a calcium-dependent endonuclease, which can lead to DNA strand breaks. Althought vitamin C is a strong antioxidant, a high intake of vitamin c may lead to, in some situation, a pro-oxidant activity in the body when free transition metals are available at the same time (88). The results suggest that crude extracts may protect endothelial cells against oxidative stress by protecting DNA fragmentation, mitochondria damage or reduced caspase-3 activity.

Results in the present study were derived from aqueous extracts and methanolic extracts of the selected medicinal herbs. The same plant species might yield different results because of the different constituents contained in the aqueous and methanolic extracts.

The effects of crude extract on the cell proliferation and activation of lymphocyte in vitro assay were estimated through the measurement of cytokine IL-2 and IFN-?. The extracts of *S. euphlebia* and *P. chaba* showed stimulatory activity on human lymphocyte by enhancing T cell proliferation. The lymphocyte proliferation activity may be mediated through activated release of cytokine such as IL-2 and IFN-?. Measurement of proliferate response of lymphocytes is the most commonly used technique in evaluating cell-mediated immune response. Decreased proliferation may indicate impaired cell-mediated immune function and has been observed in chronic diseases, cancer, HIV infection, and with aging. Cytokines are involved in the regulation of cell activation, growth and differentiation, inflammation, and immunity (89). The extract of *S. euphlebia* induced IL-2 production. IL-2, previously known as T cell growth factor, secreted from activated helper T lymphocyte (Th-1) when stimulated by mitogen or antigen. IL-2 can promote natural killer (NK) cell activation and proliferation, B cell proliferation (20), T cell proliferation and differentiation, activation of cytotoxic lymphocytes and macrophage. IL-2 may be related to the activation of many cells that can produce a cytotoxic anticancer effect, e.g. lymphokine activated killer (LAK) cells (90). Another cytokine, interferon gamma, is secreted from activated T lymphocyte. In this study we found that the extracts of *C. zedoaria* and *P. chaba* induced IFN-? secretion. IFN-? showed antiviral activity both DNA and RNA virus, antibacterial by stimulate macrophage activities, anticancer by inhibit DNA synthesis, increase activity of cytotoxic T lymphocyte, stimulate expression of human leukocyte antigen (HLA) and tumor associated antigen on cancer cell, and induced tumor necrosis factor (TNF) secretion. Moreover IFN-? suppress the Th-2 subset

which cause allergy (20). According to the literatures (32), curcumin and bisdemethoxycurcumin isolated from *C. zedoaria* showed anti-allergy effect on the release of TNF-a and interleukin 4 (IL-4) in RBL-2H3 cells. Alcoholic extract of the fruits of *P. longum* and its component, piperine, increase the total white blood cell count in Balb/c mice (35).

From the results of antioxidant and immune assay we used methanol extract of *Salacia euphlebia* stem for further study on antioxidant and immunomodulatory activity. Upon chromatographic separation the crude methanolic extract yielded four compounds. The compounds were identified on the basis of spectroscopic method as vitexin, 15 a-hydroxyfriedelan-3-one, siphulitol and mangiferin.

Antioxidant activity of the isolated compounds with regard to the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was studied, as the concentration of mangiferin causing 50% inhibition of the free radical (IC_{50}) was assayed and found to be higher than the others. The mangiferin bioactivities observed have been related to some antioxidative as well as free radical captodative functions since its structure seems to be associated with certain flavonoid systems and their activities (91). Therefore, it is important to bear in mind that flavonoids and BHT possess various biological functions in addition to their antioxidative or antilipoperoxidative activities, which may affect the toxicity of certain compounds (16).

For the current study, vascular endothelial cells were exposed to concentrations of positive control, quercetin, or pure compounds. The cells were then exposed to tertiary-butylhydroperoxide to simulate oxidative cell damage. These results were then compared to cells similarly exposed, but not pretreated with antioxidants. Endothelial cells that were treated with quercetin and vitexin had significantly less damage.

Vitexin showed strong cytoprotective activity comparing with positive control quercetin. From the literature (91), vitexin, apigenin-8-C-ß-D-glucopyranoside, can be used effectively for the prevention of UV-induced adverse skin reactions such as free radical production and skin cell damage and demonstrated antiviral, antimicrobial, antioxidant and radioprotective activities.

The capacity of flavonoids to act as antioxidants depends upon their molecular structures. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity. Quercetin was capable of protecting against tBHP

induced DNA single strand breaks by scavenging ROS and chelating transition metal ions, such as ferrous, ferric, and cuprous ions (84). Quercetin, the predominant flavonoid in the human diet, has been unobtainable as a practical dietary supplement due to poor absorption. In fruits and vegetables, quercetin is bound to sugars that make it absorbable. Water-soluble quercetin concentrates the key flavonoid benefit of fruit and vegetable consumption into a convenient, absorbable form. Several studies have shown the flavonoids to act as scavengers of superoxide anions, singlet oxygen, hydroxyl radicals and lipid peroxidation chain reactions. Hence, the flavonoids may protect tissues against oxygen free radicals and lipid peroxidation events that may be involved in pathological diseases such as coronary heart disease and cancer.

Pure compounds except 15 a-hydroxyfriedelan-3-one increased response on immune system. Vitexin and siphulitol have significant effect on cell proliferation, whereas, mangiferin significantly induced cell proliferation and IL-2 production. Decreased proliferation may indicate an impaired cell-mediated immune function that has been observed in such chronic diseases as cancer, HIV infection, and aging. From literature mangiferin have been reported as antidiabetic, antiviral and antitumor activities (92).

Chemical data in taxonomy are important adjunct to morphological evidence as it reflects on relationships of plants at another level of structure organization. 15 a-hydroxyfriedelan-3-one including its derivative were found in *S. beddomei*, *S. liana* and *S. petenensis*. Xanthone, mangiferin seem to be the typical secondary metabolite of *Salacia*, which has previously been reported from a number of species e.g. *S. oblonga*, *S. chinensis*, *S. prinoides*, *S. reticulata* and *S. macrosperma*. Vitexin and siphulitol are the first report in this genus. There have no reference report on spectral data of siphulitol.

Some Thai medicinal plants in longevity formulas could protect the cells from oxidative stress that may be involved in pathological diseases and could stimulate lymphocyte proliferation or enhance production of IL-2 that helps strengthening the immune system to fight against diseases. To our knowledge, this is the first report about antioxidant and immune activities of *S. euphlebia*. Siphulitol has been isolated from the lichen *Siphula ceratites* and have no previous report on antioxidant and immune system.

CHAPTER 5

CONCLUSIONS

The plant materials were extracted with methanol and water. Antioxidant activities of the crude extracts were assessed by measuring ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and to protect endothelial induced cell damaged by tertiary butylhydroperoxide *in vitro*. Immunomodulatory activity was studied using lymphocyte proliferation assay for cell growth and cytokine production for cell function.

The plants which pronounced free radical scavenger, antioxidant and/or immunomodulatory activity are *S. euphlebia*, *P. chaba* and *C. zedoaria* may imply that antioxidant effect reduced cell injury and inflammation which are the causes of several chronic diseases. Increased production of IL-2 and IFN-? in respond to stimulation of plant extract implied the protective immune respond to viral and bacterial infection leading to healthy living. Other plants in this study might have different biological mechanisms which need further study to clarify other specific biological effect on immune response.

In the present study the effects of *Salacia* on the antioxidant and immunostimulatory activities were investigated. The extract containing potential bioactive compounds were estimated through the measurement of cytoprotective for antioxidant assay and the effects of compounds on the cell proliferation and activation of lymphocyte were estimated through the measurement of cytokine production. The present results propose that the *Salacia* extracts have antioxidant and immunostimulant activity, which indicates its effectiveness in diseases caused by overproduction of radicals. This is the first report of phytochemical studies and bioactive compounds of *S. euphlebia*. These results may scientifically explain the folk and alternative-medicine uses of these medicinal plants in longevity formulas.

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APPENDIX

DPPH scavenging activity of crude extracts

sample	conc	OD	OD	OD	%inh	%inh	%inh
EtOH		0.177	0.178	0.182	0.179	0.179	0.179
H_2O		0.174	0.172	0.173	0.173	0.173	0.173
AEW	5	0.150	0.154	0.152	13.295	10.983	12.139
	25	0.106	0.105	0.104	38.728	39.306	39.884
	50	0.027	0.046	0.037	84.393	73.410	78.613
	100	0.022	0.022	0.024	87.283	87.283	86.127
BPW	5	0.153	0.147	0.147	11.561	15.029	15.029
	25	0.118	0.121	0.117	31.792	30.058	32.369
	50	0.086	0.085	0.090	50.289	50.867	47.977
	100	0.046	0.042	0.043	73.410	75.722	75.144
CRW	5	0.145	0.146	0.145	16.185	15.607	16.185
	25	0.084	0.082	0.073	51.445	52.601	57.803
	50	0.026	0.023	0.026	84.971	86.705	84.971
	100	0.019	0.021	0.021	89.0173	87.861	87.861
CZW	5	0.145	0.142	0.145	16.185	17.919	16.185
	25	0.106	0.104	0.104	38.728	39.884	39.884
	50	0.075	0.079	0.073	56.647	54.335	57.803
	100	0.032	0.031	0.029	81.503	82.081	83.237
PCW	0.1	0.251	0.23	0.213	-45.087	-32.948	-23.121
	0.5	0.198	0.200	0.201	-14.451	-15.607	-16.185
	2.5	0.127	0.152	0.132	26.589	12.1387	23.699
	5	0.137	0.141	0.139	20.809	18.497	19.653
	25	0.107	0.106	0.107	38.150	38.728	38.150
	50	0.058	0.063	0.061	66.474	63.584	64.739
	100	0.017	0.016	0.016	90.173	90.751	90.751
PSW	5	0.156	0.150	0.155	9.826	13.295	10.405
	25	0.131	0.13	0.129	24.277	24.855	25.433
	50	0.093	0.09	0.109	46.243	47.977	36.994
	100	0.080	0.081	0.083	53.757	53.179	52.023

SEW	5	0.241	0.246	0.238	44.213	43.056	44.907
	25	0.115	0.117	0.113	73.379	72.916	73.842
	50	0.11	0.109	0.108	74.537	74.768	75.000
	100	0.106	0.102	0.104	75.463	76.389	75.926
TSW	5	0.165	0.165	0.164	4.624	4.624	5.202
	25	0.138	0.142	0.141	20.231	17.919	18.497
	50	0.105	0.104	0.104	39.306	39.884	39.884
	100	0.045	0.045	0.046	73.988	73.988	73.410
BHT	6.25	0.125	0.132	0.124	30.168	26.257	30.726
	12.5	0.105	0.105	0.103	41.341	41.341	42.458
	25	0.066	0.064	0.064	63.128	64.246	64.246
AEM	5	0.35	0.35	0.339	17.840	17.840	20.422
	25	0.275	0.266	0.266	35.446	37.559	37.559
	50	0.169	0.177	0.182	60.328	58.451	57.277
	100	0.109	0.108	0.109	69.553	69.832	69.553
BPM	100	0.276	0.272	0.272	36.111	37.037	37.037
CRM	5	0.395	0.391	0.394	-10.335	-9.218	-10.056
	25	0.304	0.304	0.314	15.084	15.084	12.290
	50	0.168	0.176	0.203	53.073	50.838	43.296
	100	0.102	0.102	0.101	71.508	71.508	71.788
CZM	5	0.386	0.386	0.384	-7.821	-7.821	-7.262
	25	0.269	0.26	0.274	24.860	27.374	23.464
	50	0.182	0.179	0.199	49.162	50.000	44.413
	100	0.118	0.117	0.118	67.039	67.318	67.039
SEM	5	0.352	0.347	0.343	1.676	3.073	4.189
	25	0.146	0.143	0.126	59.218	60.056	64.804
	50	0.123	0.113	0.102	65.642	68.436	71.508
	100	0.082	0.091	0.093	77.095	74.581	74.022
PCM	100	0.382	0.387	0.382	11.574	10.417	11.574
PSM	100	0.299	0.299	0.298	30.787	30.787	31.018
TIM	100	0.291	0.294	0.286	32.639	31.944	33.796

DPPH scavenging activity of pure compounds

sample	conc	OD	OD	OD	%inh	%inh	%inh
EtOH		0.253	0.254	0.254	0.254	0.254	0.254
H_2O		0.231	0.234	0.236	0.233	0.236	0.238
SE 1	12.5	0.230	0.230	0.235	9.329	9.329	7.359
	25	0.239	0.232	0.235	5.782	8.541	7.359
	50	0.238	0.234	0.238	6.176	7.753	6.176
	100	0.227	0.233	0.235	10.512	8.147	7.359
SE 2	12.5	0.264	0.260	0.265	-4.073	-2.496	-4.468
	25	0.253	0.257	0.254	0.262	-1.314	-0.1314
	50	0.245	0.250	0.243	3.416	1.445	4.205
	100	0.248	0.243	0.238	2.234	4.205	6.176
SE 3	12.5	0.252	0.267	0.256	0.657	-5.256	-0.919
	25	0.253	0.246	0.251	0.263	3.022	1.051
	50	0.242	0.243	0.240	4.599	4.205	5.388
	100	0.240	0.242	0.234	5.388	4.599	7.753
SE 4	0.1	0.198	0.200	0.201	21.944	21.156	20.762
	0.5	0.127	0.152	0.132	49.934	40.0788	47.963
	2.5	0.073	0.074	0.068	71.222	70.828	73.193
	25	0.066	0.066	0.065	73.981	73.981	74.376
	50	0.065	0.066	0.074	74.376	73.982	70.828
	100	0.063	0.064	0.061	75.164	74.770	75.953
Quercetin	0.1	0.219	0.209	0.215	13.666	17.608	15.243
	0.5	0.150	0.169	0.160	40.867	33.377	36.925
	2.5	0.074	0.069	0.070	70.828	72.799	72.405

Lymphocyte proliferation of crude extracts

ctrl	AEM	BPM	CRM	CZW	PCW	PSM	SEM	TIM
1	1.18	1.23	1.31	1.17	1.30	1.10	1.17	1.30
1	1.07	1.17	1.15	1.52	1.43	1.05	1.14	1.00
1	0.87	0.69	0.64	0.88	0.91	0.72	0.88	0.82
1	1.02	0.83	1.05	0.67	1.39	1.30	1.2	1.01
1	1.56	1.66	1.13	1.25	1.54	1.04	1.74	1.07
1	1.24	2.05	1.41	1.42	2.06	1.43	2.26	2.03
1	1.44	1.78	0.85	0.99	1.53	1.76	1.78	1.37
1	1.04	1.39	0.93	0.89	1.23	1.36	1.57	1.10
1	1.03	1.17	1.55	0.96	1.78	0.99	1.16	1.95
1	1.01	0.99	1.02	1.10	1.64	0.87	0.98	1.09
Max.	1.56	2.05	1.55	1.52	2.06	1.76	2.26	2.03
Min.	0.87	0.69	0.64	0.67	0.91	0.72	0.88	0.82
Median.	1.055	1.20	1.09	1.04	1.48	1.07	1.18	1.09
Ave.	1.14	1.29	1.10	1.08	1.48	1.16	1.39	1.27
SD	0.21	0.43	0.27	0.26	0.31	0.30	0.43	0.41

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	CTRL - AEM	-.1460	.2131	6.740E-02	-.2985	6.468E-03	-2.166	9	.058
Pair 2	CTRL - BPM	-.2960	.4292	.1357	-.6031	1.106E-02	-2.181	9	.057
Pair 3	CTRL - CRM	-.1040	.2704	8.549E-02	-.2974	8.940E-02	-1.216	9	.255
Pair 4	CTRL - CZW	-8.50E-02	.2610	8.253E-02	-.2717	.1017	-1.030	9	.330
Pair 5	CTRL - PCW	-.4810	.3142	9.936E-02	-.7058	-.2562	-4.841	9	.001
Pair 6	CTRL - PSM	-.1620	.3035	9.598E-02	-.3791	5.513E-02	-1.688	9	.126
Pair 7	CTRL - SEM	-.3880	.4336	.1371	-.6982	-7.78E-02	-2.830	9	.020
Pair 8	CTRL - TIM	-.2740	.4076	.1289	-.5656	1.756E-02	-2.126	9	.062

Lymohocye proliferation of pure compounds

ctrl	SE 1	SE 2	SE 3	SE 4
1	1.01	1.08	1.01	1.01
1	1.15	1.05	1.04	1.04
1	1.03	0.97	1.00	0.99
1	1.01	0.88	0.95	1.12
1	1.15	1.07	1.05	0.99
1	1.11	1.12	1.18	1.15
1	0.98	1.20	1.10	1.08
1	0.99	1.29	1.14	1.28
1	1.00	0.96	1.01	1.02
1	1.17	1.22	1.28	1.2
Min.	0.98	0.88	0.95	0.99
Max.	1.17	1.29	1.28	1.28
Ave.	1.06	1.08	1.08	1.09
Stdv.	0.07	0.13	0.09	0.09

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	CTRL - SE1	-6.00E-02	7.572E-02	2.394E-02	-.1142	-5.83E-03	-2.506	9	.034
Pair 2	CTRL - SE2	-8.40E-02	.1278	4.042E-02	-.1754	7.436E-03	-2.078	9	.067
Pair 3	CTRL - SE3	-7.60E-02	9.969E-02	3.152E-02	-.1473	-4.69E-03	-2.411	9	.039
Pair 4	CTRL - SE4	-8.80E-02	9.807E-02	3.101E-02	-.1582	-1.78E-02	-2.838	9	.019
Pair 5	CTRL - SE5	-.1070	8.341E-02	2.638E-02	-.1667	-4.73E-02	-4.057	9	.003

IL-2 production of crude extracts

	ctrl	BPM	PCW	PSM	ctrl2	AEM	CRM	CZW	SEM	TIM
1	35.88	25.86	30.08	14.26	27.97	37.46	12.68	33.24	55.38	46.42
2	16.73	21.80	37.01	18.63	6.58	17.36	30.67	33.21	29.41	22.43
3	9.75	15.10	18.63	28.77	19.26	19.10	11.22	34.48	29.41	15.46
4	22.06	13.02	18.25	9.68	17.30	12.07	16.83	25.87	23.01	23.01
5	39.79	24.03	28.14	23.34	46.65	40.48	65.15	24.71	54.87	48.02
6	57.62	41.16	70.64	39.79	44.19	50.07	67.21	63.10	41.16	45.96
7	41.85	23.34	26.77	31.57	29.51	42.53	38.42	29.50	29.51	28.14
8	20.63	19.68	21.59	17.30	21.59	16.83	10.16	29.20	23.01	16.83
9	22.54	21.59	22.06	13.02	21.50	21.11	22.06	13.49	23.49	27.30
10	12.34	20.04	31.83	24.56	20.43	28.97	19.56	30.03	30.42	19.88
Min.	9.75	13.02	18.25	9.68	6.58	12.07	10.16	13.49	23.01	15.46
Max.	57.62	41.16	70.64	39.79	46.65	50.07	67.21	63.10	55.38	48.02
Median	22.30	21.69	27.45	20.98	21.54	25.04	20.81	29.76	29.46	25.15
Ave.	27.92	22.56	30.50	22.09	25.49	28.59	29.396	31.68	33.97	29.34
Stdv.	15.26	7.62	15.33	9.36	12.20	13.16	21.29055	12.59	12.34	12.69

IL-2 Production of pure compounds

	ctrl	SE 1	SE 2	SE 3	SE 4
1	0.346	16.355	16.355	11.553	22.759
2	8.351	0.346	6.749	18.308	9.952
3	6.749	17.956	13.154	13.154	11.553
4	40.369	32.369	70.787	41.970	45.172
5	14.768	0	9.952	19.557	23.548
6	3.548	16.355	0	0	18.630
7	0	13.154	5.149	7.561	17.956
8	0	19.557	0	0	33.966
9	24.360	6.749	21.158	5.149	27.562
10	7.962	11.163	7.252	6.453	24.332
Min.	0	0	0	0	9.952
Max.	40.369	32.369	70.787	41.970	45.172
Ave.	10.645	13.400	15.055	12.370	23.543
Stdv.	12.907	9.634	20.698	12.386	10.422

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	CTRL - SE1	-2.7550	13.7115	4.3360	-12.5637	7.0536	-.635	9	.541
Pair 2	CTRL - SE2	-4.4102	11.0738	3.5019	-12.3320	3.5115	-1.259	9	.240
Pair 3	CTRL - SE3	-1.7252	8.8354	2.7940	-8.0457	4.5953	-.617	9	.552
Pair 4	CTRL - SE4	-12.8977	10.2548	3.2429	-20.2335	-5.5618	-3.977	9	.003
Pair 5	CTRL - SE5	-2.2707	7.0687	2.2353	-7.3274	2.7859	-1.016	9	.336

IFN production of crude extracts

	ctrl	BPM	PCW	PSM	ctrl	AEM	CRM	CZW	SEM	TIM
1	10.76	4.12	13.35	4.12	28.47	18.88	9.27	35.48	55.38	47.28
2	2.65	11.13	19.62	7.81	8.18	101.1	8.55	116.99	24.04	12.98
3	4.86	8.18	15.93	6.34	15.93	3.02	5.6	124.73	21.46	4.13
4	1.91	2.28	3.39	0.07	2.28	0	1.18	24.04	23.01	0.07
5	5.23	3.39	24.41	0	9.65	24.41	26.63	35.48	5.23	90.06
6	0	91.9	62.03	0	5.97	14.08	3.73	31.42	4.49	15.93
7	0	0	1.92	0	0.07	0.07	0	8.96	0.44	0.44
8	0	21.59	22.06	13.2	21.59	21.11	22.06	23.49	16.35	27.30
9	3.56	10.48	18.66	4.80	6.75	30.44	11.25	50.55	5.76	22.78
10	0.67	0	8.89	5.67	10.14	36.55	8.77	43.38	29.01	17.79
Min.	0	0	1.92	0	0.07	0	0	8.96	0.44	0.07
Max.	10.76	91.9	62.03	13.2	28.47	101.1	26.63	124.73	55.38	90.06
Median	2.28	6.15	17.29	4.46	8.91	19.99	8.66	35.48	18.90	16.86
Ave.	2.96	15.31	19.03	4.20	10.90	24.96	9.70	49.452	18.52	23.87
Stdv.	3.38	27.69	16.89	4.36	8.76	29.54	8.58	39.357	16.31	27.21

IFN production of pure compounds

	ctrl	SE 1	SE 2	SE 3	SE 4
1	66.35	273.21	32.24	46.92	52.53
2	36.56	59.44	30.94	26.62	31.81
3	33.53	5.59	11.94	39.58	52.53
4	90.10	48.65	88.38	65.06	66.35
5	114.72	109.97	127.67	63.33	121.2
6	50.37	55.56	58.58	56.85	65.06
7	68.94	74.56	58.58	58.15	79.99
8	66.35	59.88	52.10	59.88	74.56
9	35.65	24.3	38.87	37.55	20.23
10	42.14	40.67	24.86	40.02	23.65
Min.	33.53	5.59	11.94	26.62	20.23
Max.	114.72	273.21	127.67	65.06	121.2
Ave.	60.47	75.18	52.42	49.39	58.79
Stdv.	26.48	74.99	34.13	13.04	30.23

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	CTRL - SE1	-19.9894	78.1389	27.6263	-85.3152	45.3364	-.724	7	.493
Pair 2	CTRL - SE2	8.3136	15.3862	5.4398	-4.5496	21.1768	1.528	7	.170
Pair 3	CTRL - SE3	13.8190	18.7235	6.6197	-1.8342	29.4722	2.088	7	.075
Pair 4	CTRL - SE4	-2.1368	14.8762	5.2595	-14.5736	10.3001	-.406	7	.697
Pair 5	CTRL - SE5	-2.3754	15.8771	5.6134	-15.6490	10.8982	-.423	7	.685

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (B.Sc.)	Kasetsart University	1980
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List of Publication and Proceeding

1. Supreeya Yuenyongsawad, Niwat Keawpradub, Sanan Subhadhirasakul and Charuporn Promwong. Bioactive compounds from *Salacia euphlebia* Merr. XI International Congress "Phytopharm 2007" Russia St.petersburg 27-29 June 2006 : 546-547
2. Supreeya Yuenyongsawad, Charuporn Promwong, Niwat Keawpradub and Sanan Subhadhirasakul. In vitro study of immunomodulatory activity of some Thai medicinal plants in longevity formulas The Fourth Indochina Conference on Pharmaceutical Sciences University of Medicine and Pharmacy at Ho Chi Minh City Vietnam 10-13 Nov.2005 505-507